Structure et fonction des hélicases de la famille RecQ : rôle du doigt de zinc dans la régulation des activités enzymatiques

Jie Lin Liu

N° ENSC-(n° d'ordre)

THESE DE DOCTORAT

DE L'ECOLE NORMALE SUPERIEURE DE CACHAN

Présentée par

Monsieur Jie Lin LIU

pour obtenir le grade de

DOCTEUR DE L'ECOLE NORMALE SUPERIEURE DE CACHAN

Domaine :
SCIENCES DE LA VIE ET DE LA SANTE

Sujet de la thèse:

Structure et fonction des hélicases de la famille RecQ: Rôle du doigt de zinc dans la régulation des activités enzymatiques

Thèse présentée et soutenue à Cachan le 31 / 05/ 2006 devant le jury composé de :

M. Christian AUCLAIR	**Président**
M. Eric LE CAM	**Rapporteur**
Mme Christiane ELIE	**Rapporteur**
Mme Mounira AMOR-GUERET	**Examinateur**
M. Xu Guang XI	**Directeur de thèse**

Nom du Laboratoire :LBPA
ENS CACHAN/CNRS/UMR 8113
61, avenue du Président Wilson, 94235 CACHAN CEDEX (France)

En souvenir de mon père

A ma mère

A Xiaoli et Yixin

REMERCIEMENTS

Ce travail a été réalisé dans le Laboratoire de Biotechnologie et Pharmacologie Génétique Appliquée, CNRS UMR 8113, Ecole Normale Supérieure de Cachan, et dirigé par Monsieur Xu Guang XI.

Je tiens à exprimer mes plus sincères remerciements à mon directeur de thèse Monsieur Xu Guang XI, qui m'a confié cette étude, pour l'accueil qu'il m'a fait dans son équipe, pour ses conseils avisés, pour ses encouragements et compréhension, et sa gentillesse.

Je remercie très sincèrement Monsieur le Directeur Christian AUCLAIR, de m'avoir accueilli dans son laboratoire, ainsi que pour son soutien tout au long de ce travail.

Je voudrais aussi remercier Mesdames Christiane ELIE et Mounira AMOR-GUERET, Monsieur Eric LE CAM qui ont accepté de juger ce travail.

Toute la partie française de cette thèse a été en partie corrigée par Madame Marie-Alix POUL et Monsieur Nicolas BAZEILLE, que je remercie pour ses aides précieuses.

Je remercie Messieurs Linsheng XIAO, Liang DENG et Yu LI pour ses corrections de mes manuscrits anglais.

Je tiens à exprimer toute ma reconnaissance à mes collègues, Mesdames Suzanne BARREY, Catherine BONNEMAISON, Christine LEGAY, Françoise CHAMINADE, Martine LEFORT, Françoise SIMON, Aihua ZHANG, Shuang LU, Rongbing GUO, Yan HE ; Mademoiselle Hua RENG ; Messieurs Jean-Claude BROCHON, Pascal Rigolet, Philippe FOSSE, Patrick TAUC, Eric DEPREZ et Houqiang XU. Merci à tous pour vos conseils, votre soutien, votre gentillesse.

Enfin, je tiens spécialement à adresser mes remerciements au Ministère de l'éducation de Chine qui a financé ce travail de thèse, ainsi qu'à l'Université Médicale de Guiyang en Chine où je travaillais comme enseignant et qui m'a permis de prolonger l'absence de mon poste afin de pouvoir terminer ma thèse.

SOMMAIRE

LISTE DES FIGURES

INTRODUCTION GENERALE

CHAPITRE 1 : INTRODUCTION

LISTE DES TABLEAUX

CHAPITRE 1 : INTRODUCTION

CHAPITRE 2 : RESULTATS

Article № 1

Article № 2

Article № 3

Article № 4

ABREVIATIONS ET NOTATIONS

Aa :	Acide aminé
ADN:	Acide désoxyribonucléique
AMPPNP:	Adénosine-5-(β, γ-imido) triphosphate
ARN:	Acide ribonucléique
ARNm:	ARN messager
ARNt:	ARN de transfert
ATM:	Ataxie-télangiectasie-mutée
ATP:	Adénosine triphosphate
ATPase:	Adénosine triphosphatase
ATP-γ-S:	Adénosine 5-O-(thiotriphosphate)
ATR:	ATM and Rad3 related
BLM:	Protéine dont les mutations sont à l'origine du syndrome de Bloom.
bp:	Paire de bases
BASC:	Complexe de surveillance de génome associé à BRCA1
BS:	Syndrome de Bloom
CD:	Dichroïsme circulaire
CDB:	Cassure double-brin
CSB:	Cassure simple-brin
EDTA:	Ethylène diamine tétra-acétate
FRET:	Transfert d'énergie de résonance de fluorescence résolue en temps
K_{cat}:	Constante catalytique
LOH:	Perte d'hétérozygote
NHEJ:	Non Homologous End Joining
NTP:	nucléoside triphosphate
RecQ:	Produit du gène *recQ* d'*Escherichia coli.* La protéine est une hélicase
RecQ4:	Protéine dont les mutations sont à l'origine du syndrome de

Rothmund-Thomson (RTS).

RECQ5 : Produit du gène *RECQ5* humain. La protéine est une hélicase

RH: Recombinaison homologue

RPA: Protéine A de réplication

RTS: Syndrome de Rothmund-Thomson

SSB: Protéine fixant l'ADN simple brin

WRN: Protéine multifonctionnelle dont les mutations sont à l'origine du syndrome de Werner.

WS: Syndrome de Werner

Résumé

La famille RecQ des ADN hélicases est impliquée dans la maintenance de la stabilité génomique. Chez l'homme, l'altération de l'une des trois hélicases homologues RecQ mène respectivement au syndrome de Bloom, de Werner et de Rothmund-Thomson, qui sont associés à une prédisposition au cancer et/ou au vieillissement prématuré. En général, les membres de la famille RecQ contiennent les domaines hélicase, RecQ C-terminal (RecQ-Ct) et Hélicase ARNase D C-terminal (HRDC). Nous nous sommes intéressés, au cours de ce travail, au rôle du doigt de zinc du domaine RecQ-Ct de ces hélicases.

Dans un premier temps, nous avons étudié la fonction du doigt de zinc de l'hélicase RecQ d'*E. coli* en introduisant des mutations ponctuelles par mutagenèse dirigée et en caractérisant la protéine mutante par diverses techniques biochimiques et biophysiques. Les résultats indiquent que le motif à doigt de zinc est impliqué dans la fixation à l'ADN et le repliement correct de la protéine RecQ. Ces données contribuent à expliquer la perte de la fonction hélicase et la perte de l'activité ATPase, car il n'y a plus de fixation à l'ADN

Ensuite, trois isoformes d'hélicase RECQ5 humaine ont été utilisées comme modèle pour caractériser la modulation fonctionnelle du domaine hélicase par le motif à doigt de zinc. Nous avons montré pour la première fois que le domaine hélicase de RECQ5 possède une activité intrinsèque élevée qui favorise l'hybridation de l'ADN et que RECQ5β possède deux motifs séparés impliqués dans le mécanisme d'hybridation de l'ADN. Basé sur la caractérisation quantitative des multiples activités enzymatiques des protéines RECQ5, nous avons conclut que le motif à doigt de zinc de RECQ5β régule la fixation à l'ADN, l'activité ATPase, le déroulement de l'ADN, l'hybridation de l'ADN et l'échange des brins d'ADN.

Enfin, nous avons exprimé, purifié et analysé les homologues SubL et SubS de l'hélicase RecQ chez *Bacillus subtilis* qui contiennent les domaines hélicase et RecQ-Ct. Nos données démontrent que ces deux protéines possèdent des activités ATPase ADN dépendante et hélicase de polarité 3'-5'. Leur spécificité de substrat

d’ADN est différente. Nous avons montré que SubL est capable de favoriser l’hybridation de l’ADN, à l’inverse de SubS. Ces résultats sont utiles pour comprendre les rôles des hélicases SubL et SubS dans le métabolisme de l’ADN.

Mots-clés : Hélicase, ATPase, Doigt de zinc, RecQ, RECQ5, SubL, SubS, humain, *Escherichia coli*, *Bacillus subtilis*, Hybridation d'ADN, Echange des brins d’ADN

Summary

The RecQ family of the DNA helicases is involved in the maintenance of genomic stability. The alteration of one of the three human RecQ homologue helicases leads to the Bloom's syndrome, the Werner's syndrome and the Rothmund-Thomson's syndrome, respectively, which are associated with the predisposition to cancer and/or premature ageing. In general, the members of the RecQ family contain three domains: helicase, RecQ C-terminal (RecQ-Ct) and Helicase RNaseD C-terminal (HRDC). During this work, we analyzed the function of the zinc finger of the RecQ-Ct domain of the RecQ family helicases.

In a first part, the function of the zinc finger of the RecQ helicase of *E. coli* has been investigated by using site-directed mutagenesis and various biochemical and biophysical techniques. We showed that the zinc finger is involved in DNA binding and in the correct folding of the RecQ protein. These findings explain the loss of the hélicase activity and we also observed the loss of ATPase activity.

Subsequently, three isoforms of human RECQ5 have been used as a model system to characterize the functional modulation of a RecQ helicase core by a zinc finger motif. It has been shown for the first time that the RECQ5 helicase core has an intrinsically high ssDNA annealing activity, and RECQ5β has two separate motifs that mediate ssDNA annealing. Based upon quantitative characterization of the multiple enzymatic activities of the RECQ5 proteins, we conclude that the zinc finger motif of RECQ5 regulates the DNA binding, ATPase, unwinding, strand-annealing and strand-exchange activities of the RECQ5 helicase core.

Finally, the SubL and SubS homologues of the RecQ helicase in *Bacillus subtilis* which contain the hélicase and RecQ-Ct domain have been expressed, purified and analyzed by using biochemical approaches. The two proteins have been shown to be a DNA dependent ATPase and a DNA helicase of the 3'-5' polarity. They possess the specificity of the DNA substrate and SubL is able to promote DNA annealing, but not SubS. These results could be useful to comprehend the roles of the two helicases in DNA metabolism.

Keywords: Helicase, ATPase, Zinc finger, RecQ, RECQ5, SubL, SubS, human, *Escherichia coli*, *Bacillus subtilis*, DNA annealing, DNA exchange

INTRODUCTION GENERALE

De nombreux mécanismes impliqués dans le métabolisme de l'ADN nécessitent l'ouverture de la double hélice de l'ADN. Ces réactions, qui nécessitent la rupture des liaisons hydrogènes formées entre les bases complémentaires de l'ADN (Watson *et al.*, 1953), sont réalisées par une vaste famille d'enzymes : les hélicases. Cette activité d'ouverture de l'ADN nécessite l'hydrolyse de l'ATP. La classification des différentes familles d'hélicases est essentiellement basée sur la structure primaire de leurs domaines hélicases et la caractérisation de différents motifs spécifiques (Soultanas et Wigley, 2001).

La découverte de la première hélicase remonte à 1976 (Abdel-Monem *et al.,* 1976). Depuis, de nombreuses hélicases ont été isolées à partir de procaryotes, eucaryotes, bactériophages et virus (Tuteja et Tuteja, 2004). Toutes les hélicases connues catalysent le déroulement de l'ADN. Ces enzymes partagent toutes les mêmes propriétés biochimiques à savoir la fixation de l'ADN et l'hydrolyse de nucléoside triphosphate (NTP) stimulée par la fixation des acides nucléiques. D'autres caractéristiques définissent les hélicases telles que, la polarité de leur activité du déroulement ATP dépendante ou encore leur structure quaternaire (Tuteja et Tuteja, 2004). Les hélicases se fixent sur l'ADN simple brin et se déplacent de façon unidirectionnelle. En général, l'affinité des hélicases pour l'ADN simple brin, est bien plus élevée que pour l'ADN double brin. Par ailleurs, centaines hélicases nécessitent une structure de type « fourche de réplication » pour un meilleur déroulement (Bachrati et Hickson, 2003).

Un groupe particulier de ces hélicases, la famille RecQ, est fortement conservé de la bactérie à l'homme. La famille RecQ des hélicases a été nommée par référence à l'hélicase RecQ d'*E. coli* (Opresko *et al.,* 2004). L'intérêt de l'étude de cette famille a notamment été influencé par la découverte des maladies génétiques humaines liées à l'altération de trois des membres de cette famille. La déficience de ces enzymes (BLM, WRN, RecQ4) chez l'homme est associée à une prédisposition au cancer et/ou à un vieillissement prématuré qui sont caractéristiques des syndromes de Bloom, de Werner et de Rothmund-Thomson. Les hélicases de la famille RecQ participent à de nombreux processus associés au maintien de l'intégrité du génome et l'altération des

hélicases BLM, WRN et RecQ4, est associée à une grande instabilité du génome, et à des dysfonctionnements durant les différentes étapes du métabolisme de l'ADN.

Les hélicases de la famille RecQ telles que RecQ, BLM et WRN possèdent une fonction particulière qui consiste en la reconnaissance et la migration des jonctions de Holliday (intermédiaire des phases de recombinaison)(Mohaghegh *et al.*, 2001b). Elles sont également impliquées dans la résolution de structures particulières, telles que les D-loop(van Brabant *et al.,* 2000), ou les G-quadruplexes (Mohaghegh *et al.*, 2001b ; Sun *et al.*, 1998 ;1999 ; Fry et Loeb, 1999; Wu et Maizels, 2001; Han et Hurley, 2000), formées respectivement durant la recombinaison et au niveau des télomères.

Toutes les hélicases RecQ possèdent une activité ATPase qui est nécessaire au déroulement de la double hélice. En général, cette activité est stimulée par la présence de l'ADN simple brin (Bennett *et al.*, 1998).

Les hélicases possèdent une polarité spécifique qui est définie par la direction de leur progression le long de l'ADN simple brin. La polarité de l'activité hélicase s'explique par son chargement au niveau de l'ADN. Lorsqu'elle se fixe au niveau de l'extrémité 3' sortante de l'ADN sa polarité est de type 3' - 5'. A l'inverse sa polarité est de type 5' - 3' lorsqu'elle est liée à l'extrémité 5' sortante de l'ADN. Des études ont mis en évidence que la plupart des hélicases RecQ possédaient une polarité de type 3' - 5' (Nakayama, 2002).

Les hélicases RecQ existent sous formes monomériques ou oligomériques (Opresko *et al.,* 2004). La propriété particulière des hélicases oligomériques est qu'elles possèdent des sites multiples de fixation à l'ADN aussi bien sur le simple brin que sur le double brin.

Les hélicases de la famille RecQ sont hautement conservées des procaryotes aux eucaryotes et possèdent un certain nombre de domaines (Fig. 1) bien définis du point de vue biochimique et structurale.

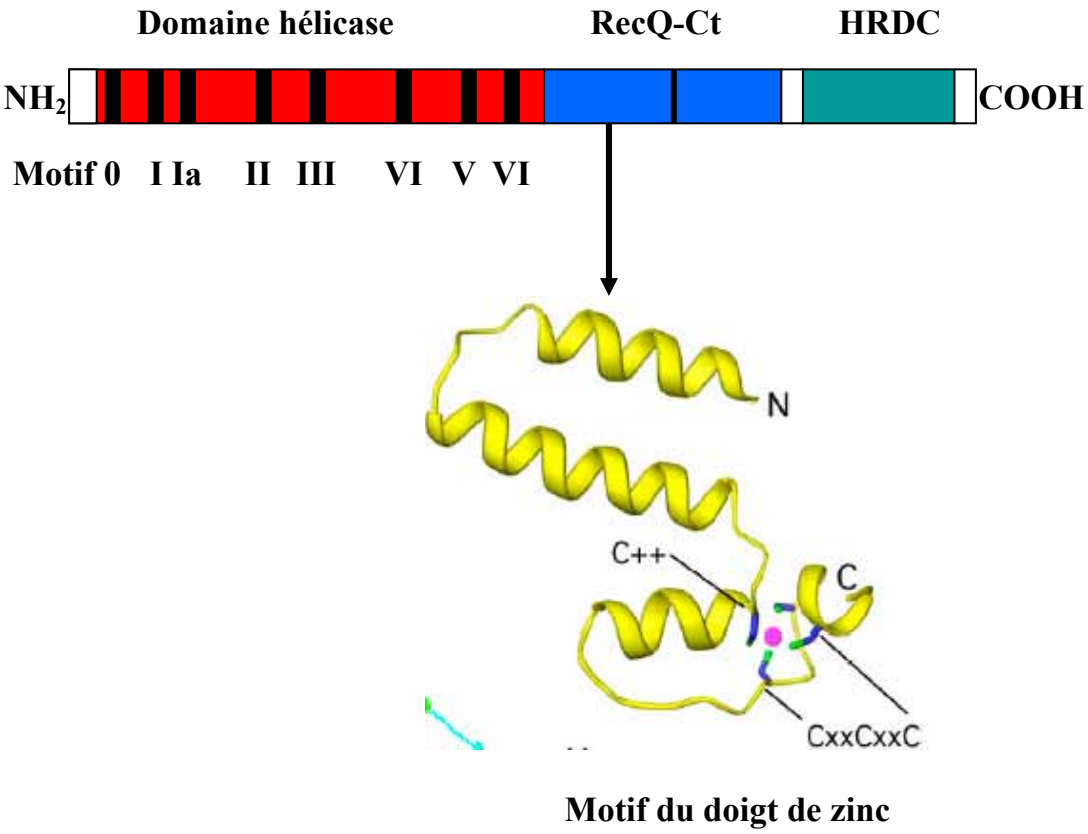


Fig. 1 Structure de l'hélicase RecQ d'*E. coli*

Diagramme schématique de l'hélicase RecQ d'*E. coli.* Les trois domaines conservés, hélicase, RecQ C-terminal conservé (RecQ-Ct), et Hélicase et Rnase D C-terminal (HRDC) , sont représentés en différents couleurs. La structure du motif à doigt de zinc dans le domaine RecQ-Ct de l'hélicase RecQ d'*E. coli* est représentée en jaune. Les chaînes latérales des cystéines sont définies, et représentées en vert. L'ion Zn^{2+} est représenté par une sphère rose (modifié d'après Bernstein *et al.*, 2003).

Le domaine le plus conservé est le domaine hélicase. Ce domaine catalyse le déroulement de l'ADN double brin. Pour la plupart des hélicases RecQ, le domaine hélicase est suivi par le domaine RecQ C-terminal conservé (RecQ-Ct domain), lui-même suivi par le domaine " Hélicase et RNase D C-terminal" (HRDC domain). Ces deux derniers domaines sont impliqués dans les interactions ADN-protéines et protéines-protéines. Des études récentes, ont permis de définir les frontières de ces différents domaines et de révéler leur structure par cristallographie aux rayons X (Bernstein *et al.,* 2003).

L'activité ATPase, et la conversion de l'énergie résultante permettant le déroulement de l'ADN, sont coordonnées par le domaine hélicase composé d'une série de 8 motifs (motifs 0, I, Ia, II, III, IV, V et VI). (Fig. 1)

La suppression partielle du motif 0 conduit à une importante réduction de l'activité ATPase. La séquence LxxxFGxxxFRxxQ de ce motif est particulièrement conservée de la bactérie jusqu'à l'homme. La perte d'activité ATPase après délétion du motif 0 et la présence conservée de résidus aromatiques et glutamiques au niveau de cette séquence, suggèrent que ce motif joue un rôle majeur dans la fixation de l'ATP, rôle qui est par ailleurs commun à toutes les hélicases RecQ (Bernstein et Keck., 2003). La mutation d'un résidu GLU en ARG au niveau de ce motif est suffisante pour causer le syndrome de Bloom chez l'homme (Ellis *et al.*, 1995,1996).

Le motif I contient une sérine (ou une thréonine), qui participe à la fixation de l'ion Mg^{2+} indispensable à l'hydrolyse de l'ATP (Bernstein *et al.*, 2003). Le motif II est une séquence acide, de type ASP-GLU-X-X où le glutamate joue le rôle de base catalytique (Gorbalenya *et al.,*1993). Les analyses sur des mutants ont révélé que le motif I est nécessaire à l'activité des hélicases RecQ. Une mutation dans ce motif conduit au syndrome de Werner (Gray *et al.* 1997) et induit une perte des activités ATPase et hélicase.

D'autres motifs du domaine hélicase semblent posséder un rôle important pour le déroulement de l'ADN. Il est probable que ces motifs participent à la fixation de l'ADN. Les motifs Ia, III et V contiennent des résidus qui établissent le contact direct avec l'ADN simple brin (Rong *et al.,* 2000).

La plupart des membres de la famille RecQ possède un second domaine très conservé, située à l'extrémité C-terminale de leur domaine hélicase : le domaine RecQ C-terminal conservé (RecQ-Ct). Ce domaine RecQ-Ct contient une région particulièrement conservée. Cette région est formée de 4 hélices α qui permettent la formation d'une structure en doigt de zinc et la fixation d'ion Zn^{2+} par l'intermédiaire de 4 Cystéines. Deux de ces résidus cystéine sont situés au niveau des extrémités N-terminales des hélice α (Fig. 1) (Bernstein *et al*., 2003).

Le doigt de zinc possède de nombreuses fonctions essentielles à l'hélicase. Des études sur des mutants ont révélé qu'une modification au niveau du site de fixation de l'ion Zn^{2+} induit une perte de solubilité de l'enzyme et une dégradation plus rapide par les protéases (Janscak *et al.,* 2003).

Dans cette thèse, nous avons tout d'abord étudié l'importance du doigt de zinc de l'hélicase RecQ d'*E. coli*. Cette structure semble jouer à la fois un rôle de médiateur au niveau de la fixation à l'ADN mais aussi un rôle important dans le repliement de la protéine. Ensuite nous avons réalisé l'étude de la régulation des activités enzymatiques des hélicases RECQ5 humaines par son doigt de zinc. Nous avons aussi étudié la fonction biochimique et la spécificité de substrat de l'hélicase homologue RecQ de *Bacillus subtilis*. Ce travail a été conduit au Laboratoire de Biotechnologie et Pharmacologie Génétique Appliquée, UMR 8113 CNRS, Ecole Normale Supérieure de Cachan, en collaboration avec le laboratoire de Physique de Matière Mollette, Institut de Physique, Académie chinoise des Sciences.

Chapitre 1 : Introduction

1. L’ADN et les ADN hélicases

1.1. L’ADN

L'ADN, sigle d’acide désoxyribonucléique, est une longue molécule que l'on retrouve dans tous les organismes. L'information génétique est stockée dans les deux brins de l’ADN. Les règles de Watson et Crick montrent que les deux brins d’ADN sont complémentaires : les paires de bases Adénine et Thymine ainsi que Guanine et Cytosine s’apparient par des liaisons hydrogènes (Watson et Crick, 1953). Les expériences de Meselson et Stahl démontrent que la réplication de l'ADN est semi-conservative : chaque brin d’ADN sert de matrice pour la formation d’un nouveau brin complémentaire. La réplication de l’ADN commence en une seule origine chez les procaryotes, mais en des sites multiples, situés à l’intérieur de la molécule. Dès le démarrage du processus de synthèse, l’ADN évolue dans les deux directions, 3’ et 5’, chaque brin antiparallèle servant de matrice. Cette croissance est assurée par des ADN polymérases responsables de la synthèse de l’ADN. La synthèse ne peut procéder que dans la direction 5’- 3’, en prenant le brin parental 3’- 5’ comme modèle. (Hennen, 2001)

Chacune des deux molécules d'ADN fille hérite d'un brin de l'ADN parental tandis que l'autre est synthétisé à partir de nucléotides libres. Cette réplication de l’ADN de type semi-conservatif et assure la transmission de l’information génétique.

L’ADN est le support de l'hérédité et est transmis aux descendants lors des processus de reproduction des organismes vivants. Il est à la base des processus biologiques aboutissant à la synthèse des protéines. Ainsi la connaissance de la structure de l’ADN et de son métabolisme est essentielle à la compréhension de la génétique en général et plus particulièrement des maladies génétiques (Murray *et al.*, 2000).

1.2. Contexte d'étude des ADN hélicases

L'ADN double brin renferme l'information génétique. Pour y accéder, lors de nombreux processus biologiques importants, les duplexes d'ADN doivent être transitoirement déroulés. Pour ce faire, il existe une classe d'enzymes connues sous le nom d'hélicase. Elles catalysent le déroulement de l'ADN double brin et jouent ainsi un rôle essentiel dans de nombreux processus cellulaires, tels que la réplication, la réparation, la recombinaison et la transcription de l'ADN (Matson, 1991 ; Lohman, 1992 ; Tuteja, 2003). La majorité des hélicases déroulent des duplexes d'ADN ou des duplexes d'ARN ; certaines peuvent dérouler l'ADN double brin aussi bien que l'ARN double brin et les hybrides ADN/ARN. Le nombre d'hélicases au sein des organismes supérieurs est remarquablement élevé ; approximativement 1% à 2% des gènes du génome eucaryote codent pour des ARN hélicases ou ADN hélicases. De multiples ADN hélicases ont déjà été isolées de divers organismes (Matson, 1991 ; Lohman, 1992 ; Tuteja, 2003 ; Tuteja et Tuteja, 1996). Ces protéines fonctionnent comme des moteurs moléculaires se déplaçant le long de l'ADN en utilisant l'énergie libérée par l'hydrolyse de nucléosides triphosphates (NTP) (West, 1996). La plupart des ADN hélicases contiennent de courtes séquences conservées d'acides aminés, définissant des motifs sur leur domaine hélicase.

Les ADN hélicases sont classées de deux manières. Une première classification se fait principalement suivant leur mode d'action. La plupart des hélicases progressent le long de l'ADN avec une polarité définie, qui est 3'-5 'ou 5'-3'. Cependant, cette classification n'est vraiment applicable qu'aux hélicases qui se fixent une extrémité de l'ADN simple brin et progressent le long de cette ADN simple brin avant d'initier le déroulement du duplexe au niveau de la jonction ADN simple brin /ADN double brin. Il est beaucoup plus difficile de classer sur cette base, les hélicases qui peuvent dérouler les bouts francs de l'ADN. La deuxième méthode de classification, fondée sur la conservation de séquences, indique que les hélicases comportent cinq superfamilles. Les superfamilles 1 et 2 (SF1 et SF2) contiennent la plupart des membres (Gorbalenya et Koonin, 1993)(Fig. 2). Ces derniers contiennent sept motifs,

à savoir, I, Ia, II, III, IV, V et VI. Les hélicases contenant l'ensemble de ces sept motifs s'appellent également les hélicases DEAD/H (Gorbalenya *et al.*, 1988 ; Linder *et al.* , 1989 ; Tanner *et al.* , 2003).

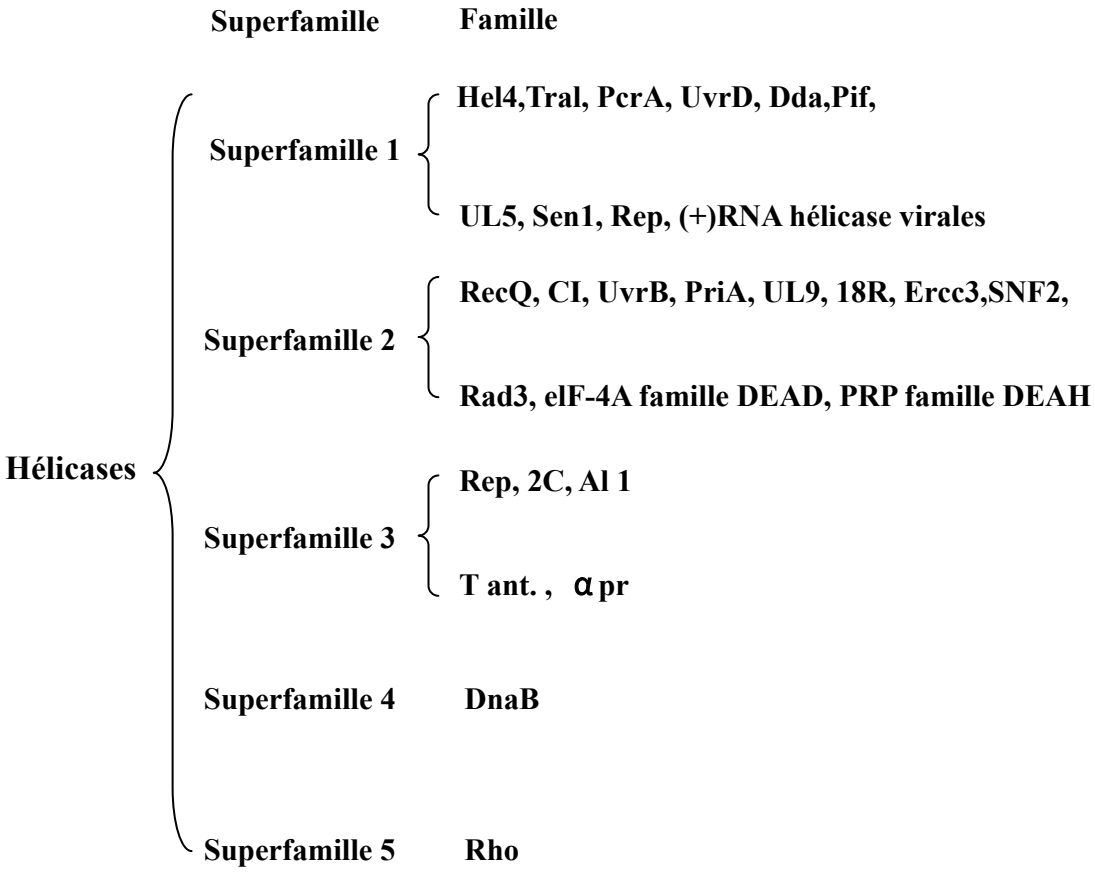


Fig. 2. Les superfamilles des hélicases (modifié d'après Gorbalenya et Koonin, 1993).

Etant donnée la nécessité de la séparation des brins de l'ADN pour effectuer les principales étapes de sa réplication ou de sa réparation, il n'est pas étonnant que des mutations dans les gènes codant des hélicases conduisent souvent à de graves conséquences. En effet, de nombreux gènes d'hélicases sont essentiels pour la survie des cellules, bien que ce ne soit pas une caractéristique universelle. Il est possible que la redondance existe dans quelques familles d'hélicase (comme dans la famille RecQ), ce qui limite la sévérité du phénotype dans le cas de la perte fonctionnelle de l'un des

membres de la famille (Bachrati et Hickson, 2003). Ces mutations sont d'un grand intérêt pour l'étude des hélicases humaines qui provoquent des désordres héréditaires identifiés.

L'étude des ADN hélicases a débuté il y a plusieurs décennies. Voici un rappel de l'historique de la recherche sur les ADN hélicases.

1.2.1. Les étapes de la découverte des ADN hélicases

La première ADN hélicase a été découverte chez *E. coli* en 1976 et a été classifiée comme enzyme capable de dérouler l'ADN (Abdel-Monem *et al.* , 1976). Depuis, de nombreuses ADN hélicases ont été isolées à partir d'organismes procaryotes et eucaryotes. En ce qui concerne les hélicases de la famille RecQ, les deux dernières découvertes sont :

1). La découverte d'un nouveau motif hélicase, appelé « le motif 0 », identifié dans l'hélicase RecQ d'*E. coli* (Bernstein et Keck, 2003).

2). La caractérisation d'une cinquième hélicase de type RecQ chez l'homme, RecQ5β qui possède en plus de son activité hélicase une activité permettant l'hybridation de l'ADN (Garcia *et al.*, 2004).

Les étapes importantes de la découverte des ADN hélicases sont récapitulées dans le tableau 1.

Tableau 1. Historique des ADN hélicases. (modifié d'après Tuteja *et al*., 2004)

Année.	Découverte.
1953-76 :	Années précédant la découverte des hélicases
1953 :	La structure duplex de l'ADN est résolue (Watson et Crick, 1953).
1967 :	La protéine Rep est la première hélicase identifiée chez *E. coli* par des critères génétiques (Denhardt *et al.*, 1967).
1976 :	Hoffman-Berling isole la première ADN hélicase (hélicase I produit de gène de traI) d'*E. coli* (Abdel-Monem *et al.*, 1976).
1978 :	L'existence de la première ADN hélicase eucaryote est démontrée chez le lis (Hotta et Stern, 1978).
1979 :	La protéine Rep d'*E. coli* contient une activité d'ADN hélicase (Yarranton et Gefter 1979).
1982 :	La première protéine de bactériophage caractérisée comme ADN hélicase est la protéine du gène 41 du bactériophage T4 (Venkatesan *et al.,* 1982).
1982-83 :	Une analyse biochimique directe est mise au point pour mesurer l'activité d'hélicase (analyse de déplacement de brin) - (Venkatesan *et al.,* 1982 ; Matson *et al.,* 1983).
1985 :	La première ADN hélicase de mammifère est identifiée dans le thymus de veau (Hubscher et Stalder, 1985).
1986 :	La première ADN hélicase virale identifiée est l'antigène T de SV40 (Stahl *et al.*, 1986). La première ADN hélicase de levure est l'ATPase III (Sugino *et al.*, 1986).
1988 :	Hodgeman et Gorbalenya découvrent les motifs hélicase des superfamilles I et II (Sept motifs d'acide aminé conservés) (Hodgeman, 1988).
1989 :	Deux superfamilles d'hélicase (SF1 et SF2) sont identifiées (Gorbalenya *et al.*, 1989) dont la famille DEAD-box (Linder *et al.*, 1989).
1990 :	La première ADN hélicase humaine (HDH) est purifiée (Tuteja *et al.,* 1990). La protéine RecQ d'*E. coli* est une ADN hélicase (Umezu *et al.,* 1990).
1992 :	La première ADN hélicase mitochondriale est isolée dans le cerveau bovin (Hehman et Hauswirth, 1992).
1996 :	La première ADN hélicase de chloroplaste est purifiée à partir du pois (Tuteja *et al.*, 1996). Cristallisation de la première ADN hélicase (PcrA de bactérie thermophile) (Subramanya *et al.,* 1996).
1997 :	Le complexe Mcm4/6/7 des cellules HeLa est une ADN hélicase (Ishimi, 1997). La protéine du syndrome de Werner (WRN) est une ADN hélicase (Gray *et al.,* 1997).

	Le produit de gène du syndrome de Bloom(BLM) est une ADN hélicase (Karow *et al.,* 1997).
1998 :	Sgs1 (slow growth suppressor -inhibiteur de croissance lent), hélicase RecQ de levure, est une ADN hélicase (Bennett *et al.,* 1998).
2000 :	Le premier gène d'ADN hélicase de plante (PDH45) codant l'enzyme biochimiquement active est cloné (Pham *et al.*, 2000). Le premier eIF-4A (du pois) est une ADN hélicase (Pham *et al.,* 2000).
2002 :	La première ADN hélicase biochimiquement active chez un parasite de la Malaria est mise en évidence (Tuteja *et al.*, 2002).
2003 :	Un nouveau motif hélicase (motif Q) est identifié dans des hélicases DEAD-box (Tanner *et al.,* 2003). L'importance d'une ADN hélicase dans l'insertion d'élément transposable est démontré dans le génome de maïs (Lal *et al.,*2003). Un nouveau motif d'hélicase, appelé « le motif 0 », est identifié dans l'hélicase RecQ d'*E. coli* (Bernstein et Keck, 2003).
2004	La protéine RecQ5β chez l'homme contient une activité du déroulement et de l'hybridation de l'ADN (Garcia *et al.,* 2004)

1.2.2. Analyse de l'activité ADN hélicase

Afin d'identifier et d'étudier la fonction enzymatique des ADN hélicases, des analyses biochimiques ont été établies. L'hélicase déroule le substrat de l'ADN partiellement duplex (oligonucléotide avec une extrémité marquée au ^{32}P complexé à une plus longue molécule d'ADN simple brin) libérant les deux brins de l'ADN (Venkatesan *et al.* , 1982; Matson *et al.* , 1983). Le simple brin radiomarqué et le substrat sont identifiés par électrophorèse suivie d'une autoradiographie (Fig. 3). L'étiquette radioactive dans l'ADN permet la visualisation directe (Fig. 3b) et la quantification rapide des résultats. En outre, les chercheurs ont développé plusieurs autres analyses : par exemple, la méthode rapide dite « quench-flow » (Amaratunga et Lohman, 1993), les analyses fondées sur l'utilisation de la fluorescence (Raney *et al.* , 1994), l'analyse par filtration (Sivaraja *et al.* , 1998), l'analyse par scintillation de proximité(Kyono *et al.* , 1998), l'analyse par FRET (transfert d'énergie de résonance de fluorescence résolue en temps) (Earnshaw *et al.* , 1999), l'analyse basée sur la technologie de « flash plate »(Hicham Alaoui-Ismaili *et al.*, 2000), enfin l'analyse d'extinction de fluorescence résolue en temps et l'analyse basée sur l'électrochimioluminescence (Zhang *et al.* , 2001). Les analyses ci-dessus ont été peu utilisées. Xu *et al.* (2003b) ont récemment mis au point une nouvelle méthode pour la détection simultanée de la fixation à l'ADN et du déroulement de l'ADN catalysé par l'hélicase, par polarisation de fluorescence.

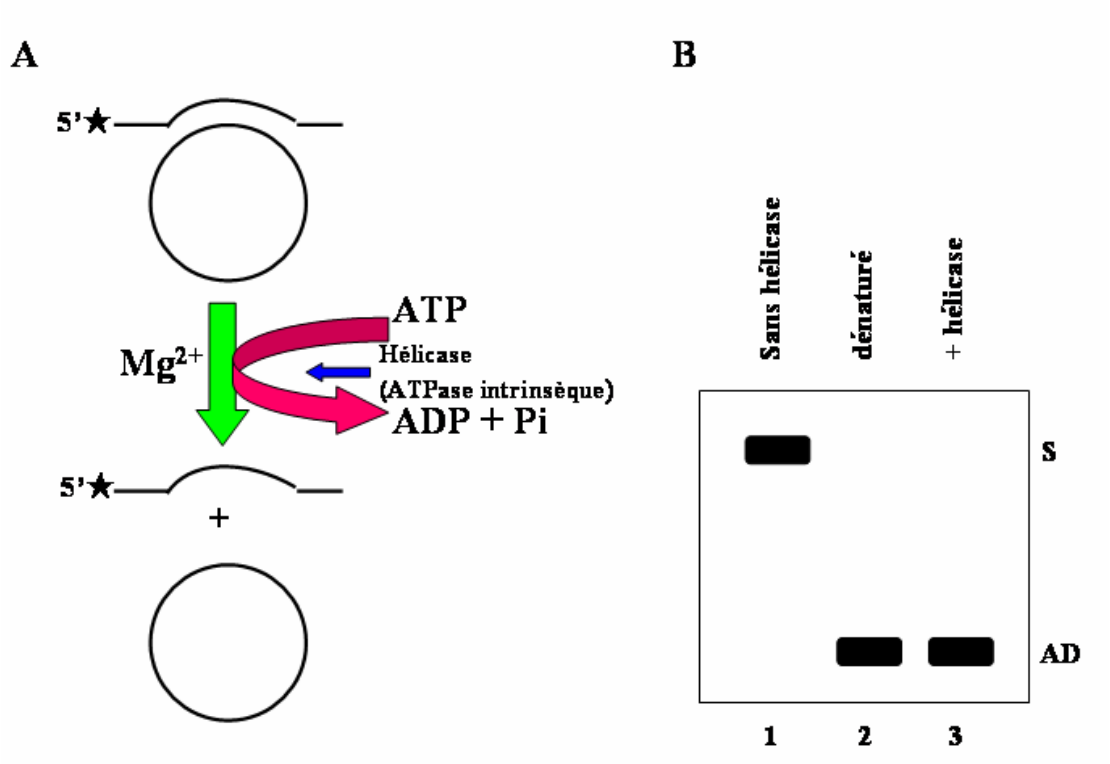


Fig. 3. Schéma de l'analyse biochimique pour mesurer l'activité de déroulement de l'ADN associée aux ADN hélicase (a) et l'autoradiographie du gel (b).

(a) Les astérisques indiquent l'extrémité marquée au ^{32}P de l'ADN. Le substrat d'ADN duplex a été préparé en hybridant l'oligonucléotide radioactif à un ADN simple brin M13 (circulaire) comme décrit précédemment (Tuteja *et al.*, 1996). (b) Voie 1, réaction sans enzyme ; Voie 2, substrat dénaturé par la chaleur ; Voie 3, réaction en présence d'ADN hélicase. S, Substrat; AD, oligonucléotide radiomarqué.

1.2.3.Caractéristiques enzymatiques des ADN hélicases.

L'activité du déroulement des acides nucléiques est commune à toutes les ADN hélicases. Elles partagent toutes les même propriétés biochimiques à savoir :

– la fixation de nucléosides triphosphates (NTP)

– une hydrolyse de NTP stimulée par les acides nucléiques

– la fixation aux acides nucléiques

– un déroulement des acides nucléiques duplexes NTP-dépendant avec une polarité spécifique.

1.2.3.1. Fixation du NTP et son hydrolyse

Toutes les ADN hélicases fixent le NTP et possèdent une activité NTPase dépendante stimulée par la présence d'acides nucléiques. Cette activité est nécessaire pour le déroulement des duplexes d'ADN. L'hélicase RecQ monomérique d'*E. coli* ne peut pas hydrolyser l'ATP en absence d'ADN. En général, les hélicases hexamèriques impliquées dans la réplication, fixent fortement l'ADN simple brin en présence de NTP. A l'inverse, elles le fixent faiblement en présence de NDP. Cette fixation a pour conséquence de changer la conformation des hélicases homo-oligomériques. Elle peut aussi affecter l'assemblage des sous-unités de la protéine. Le NTP ne se fixe pas avec une même affinité sur tous les sites de fixation des hélicases hexamèriques. Cela dépend des hélicases. En général, les ADN hélicases possèdent une très faible activité NTPase en l'absence d'ADN simple brin. Leur activité NTPase est stimulée par la présence d'ADN simple brin (Bennett *et al.*, 1998).

1.2.3.2. Les ADN hélicases fixent l'ADN.

Des ADN hélicases ont besoin d'ADN simple brin comme zone de fixation. Elles se fixent indépendamment de la nature de la séquence de l'ADN et elles progressent de façon unidirectionnelle. Ces enzymes fixent l'ADN simple brin avec une affinité plus élevée que l'ADN double brin. D'autres hélicases telles que RecBCD, l'antigène T du virus SV40, et RuvB se fixent de préférence à l'ADN double brin. De nombreuses hélicases ont besoin d'une structure type « fourche de réplication » pour un déroulement optimal. Certaines ADN hélicases telles que RecBCD, UvrD, Rep et RecQ d'*E. coli* et l'antigène T de SV40 peuvent également initier le déroulement aux niveau des extrémités franches de l'ADN double brin (Lohman, 1992). Les hélicases RecG, RuvA et RuvB d'*E. coli* se fixent spécifiquement aux jonctions de Holliday (Tsaneva *et al.,* 1993 ; Whitby *et al.,* 1994).

Suivant leur structure quaternaire, les ADN hélicases peuvent être regroupées en hélicases monomériques ou multimériques.

Les hélicases monomériques peuvent contenir deux domaines différents, un pour l'ADN simple brin et l'autre pour la fixation de l'ADN double brin. Les hélicases II, IV, UvrD et PriA bactériennes (Matson, 1991 ; Mechanic *et al.*, 1999), l'hélicase Dda du bactériophage T4 (Morris *et al.,* 2001), les hélicases IV, V et VI humaines (Tuteja et Tuteja, 1996), l'hélicase 45 (PDH45) de pois et PDH65 (Pham *et al.,* 2000 ; Tuteja *et al.,* 2001) sont les quelques exemples des formes monomériques.

Certaines hélicases fonctionnent sous forme dimérique. L'hélicase GP41 du bactériophage T4 (Delagoutte et von Hippel, 2005), l'hélicase du virus hépatite C (Khu *et al.,* 2001) et l'hélicase RecQ1 humaine (Cui *et al.,* 2004) sont les enzymes actives à l'état de dimère.

L'oligomérisation de nombreuses ADN hélicases peut être modulée par les interactions avec d'autres ligands (ATP, ADN). Par exemple, l'hélicase Rep d'*E. coli* est un monomère jusqu'aux concentrations de 12 mM en l'absence de l'ADN mais forme un dimère en fixant à l'ADN (Matson, 1991). L'hélicase UvrD peut aussi former une complexe protéine-ADN dimérique active (Maluf *et al.*, 2003).

Les formes actives de nombreux ADN hélicases sont oligomériques: DnaB, RuvB, MCM et Rho d'*E. coli*. De même, le gène 4 du bactériophage T7 code pour une hélicase/primase oligomérique tout comme le gène 41 du bactériophage T4 (Singleton *et al.,* 2000). Enfin, l'exemple de l'antigène T de SV40, confirme l'existence d'hélicase oligomérique chez les virus. Toutes ces enzymes se replient et s'associent pour former une structure quaternaire en forme d'anneau hexamèrique (Matson, 1991 ; Lohman, 1992 ; Whitby *et al.,* 1994 ; Bujalowski et Jezewska, 1995 ; Dillingham *et al.*, 2003 ; Delagoutte et von Hippel, 2002) (Fig. 4D). Une hélicase oligomérique possède de multiples sites fixant l'ADN, qui lui permettent de fixer à la fois l'ADN simple brin et l'ADN duplexe. La figure 4 montre les différentes formes d'interaction entre les ADN hélicases et une fourche de réplication. Dans tous ces modèles, au moins une sous-unité fixe l'ADN simple brin le long duquel elle se déplace (Delagoutte et von Hippel, 2002).

Les études de microscopie électronique ont confirmé que l'ADN simple brin traverse le centre de l'anneau formé par les hélicases hexamèriques. Ces résultats prouvent que l'ADN hélicase hexamèrique de G40P encercle le brin 5', interagit avec l'ADN duplexe à la jonction de l'ADN simple brin/ l'ADN double brins, et exclut le brin 3' (Fig. 4D) (Ayora *et al.* , 2002).

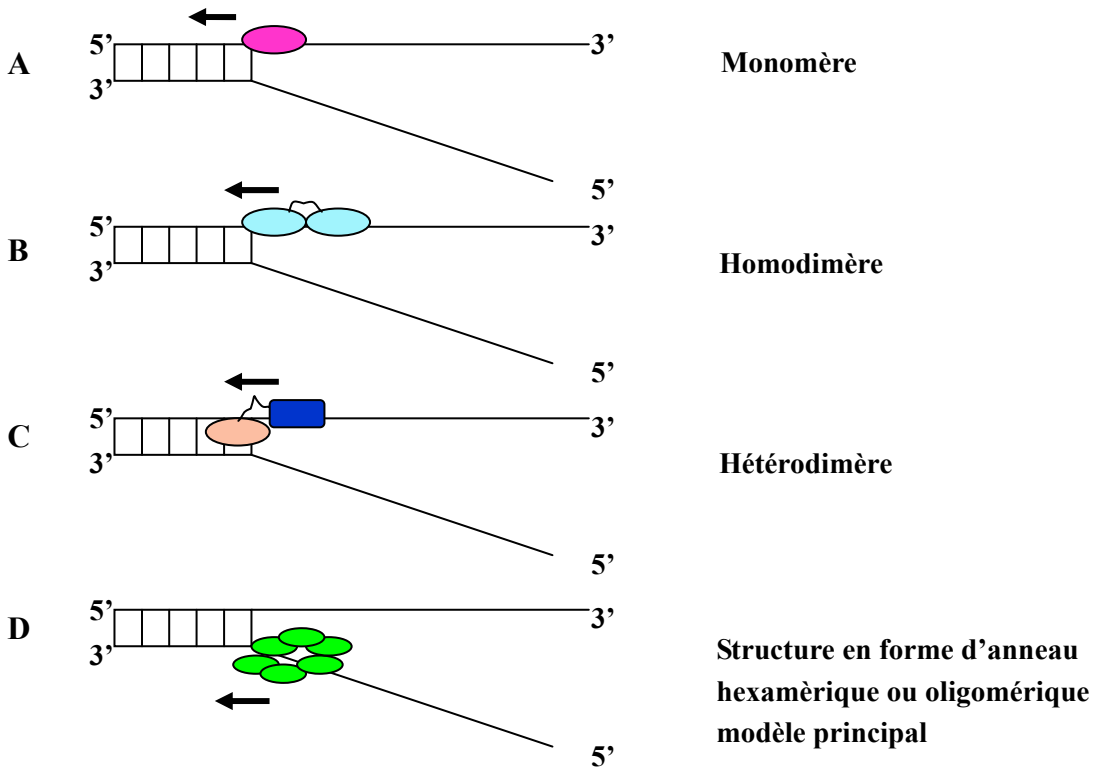


Fig. 4. Interaction d'ADN hélicases monomériques ou oligomériques avec l'ADN au niveau d'un ADN de type fourche. (a) L'hélicase monomérique fixe à l'ADN simple brin et à l'ADN double brins. (b) Dans des hélicases homodimériques, une sous-unité fixe toujours l'ADN simple brin le long de laquelle elle se déplace. (c) L'hélicase hétérodimérique contient deux domaines séparés : une sous-unité fixe et interagit avec l'ADN double brin et ancre l'hélicase au niveau de la jonction ADN simple brin/ADN double brin et l'autre sous-unité interagit avec l'ADN simple brin et transloque le long de ce brin.(d) Les hélicases hexamèriques présentent une structure en forme d'anneau qui permet aux protéines d'encercler l'ADN simple brin et d'exclure l'autre brin. Dans ce cas, l'hélicase se fixe à la jonction de l'ADN simple brin/double brin. (d'après Tuteja *et al*., 2004)

1.2.3.3. Polarité des ADN hélicases.

La plupart des ADN hélicases possèdent une polarité spécifique, qui se définit suivant la direction du mouvement de l'hélicase le long de l'ADN simple brin sur lequel elles se sont fixées (3'- 5' ou 5'- 3'). La polarité du déroulement des hélicases se détermine habituellement en utilisant un substrat d'ADN se composant d'un ADN simple brin partiellement duplexé sur l'extrémité 5' ou 3'. La structure du substrat employé pour l'étude de la polarité *in vitro* est illustrée dans la figure 5. Lorsque l'on parvient à déterminer la polarité des hélicases impliquées dans la réplication d'ADN, cela permet d'estimer la position relative de l'enzyme sur les brins de tête « leading strand » (polarité 3' - 5') ou de queue « lagging strand » (polarité 5' - 3'). Dillingham *et al.* (2003) ont montré par le passé que RecBCD possède deux hélicases RecB et RecD qui déroulent l'ADN respectivement dans les directions 3' - 5' et 5' - 3'. Il existe également des hélicases « bipolaire ». Les études sur l'hélicase PcrA de *Bacillus anthracis* ont montré que cette dernière possède à la fois les activités hélicases 3' - 5' et 5' - 3'. Ces études ont été réalisé à partir de substrats simple brin de poly(dT) contenant une région duplex en 3' ou en 5' (Naqvi *et al.,* 2003). Récemment, Constantinesco et al. (2004) ont montré que l'ADN hélicase HerA des *archaea* hyperthermophiles peut se fixer sur les extrémités 3' ou 5' d'un ADN et dérouler l'ADN duplex dans les deux polarités.

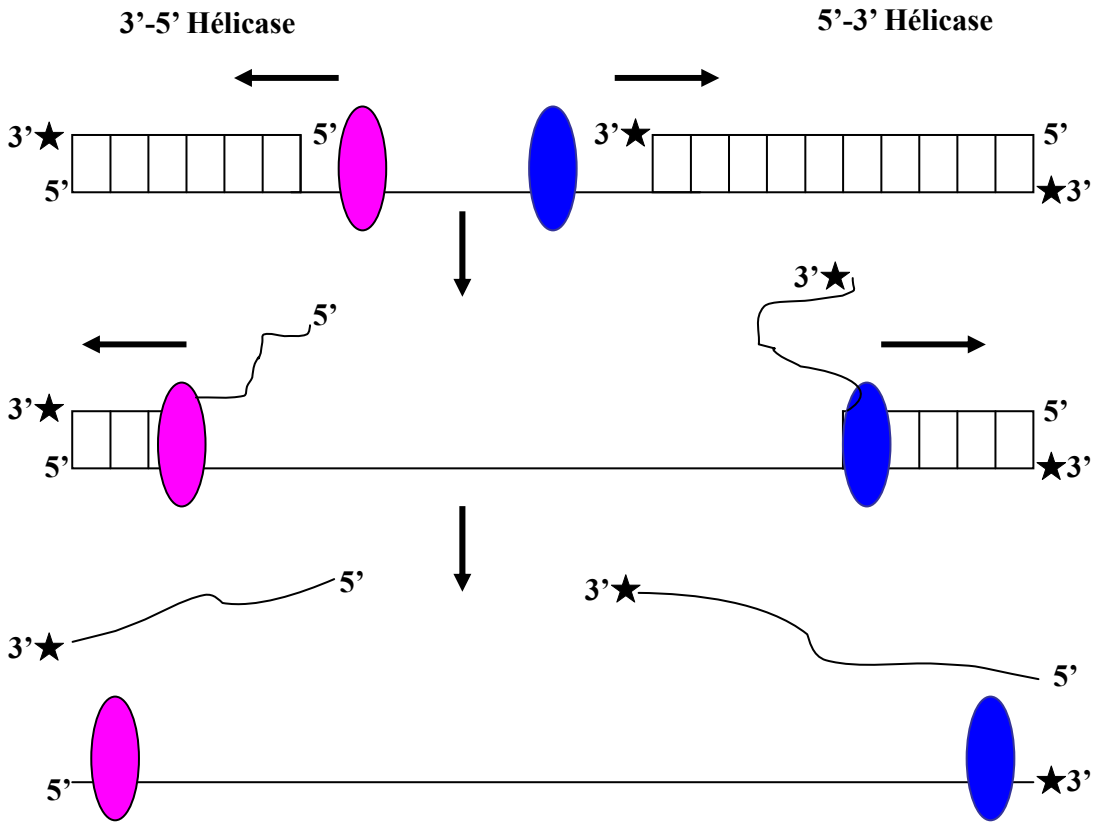


Fig. 5. Structure du substrat généralement utilisé pour déterminer la polarité des hélicases. L'hélicases 3' - 5' est à gauche et l'hélicase 5' - 3' est à droite. Les astérisques indiquent l'extrémité marquée au ^{32}P. (modifié d'après Soultanas et Wigley, 2001)

1.2.4. Mécanismes d'action des ADN hélicases

L'activité de déroulement de l'ADN effectué par les hélicases a été classé en deux mécanismes « actif ou passif » (Figure 6). Dans le mécanisme passif, l'hélicase ne participe pas directement à la déstabilisation de l'ADN double brin. Elle exclue l'ADN simple brin par sa translocation sur l'autre brin. Une implication importante du mécanisme passif l'enzyme n'est pas capable de dérouler le duplex d'ADN à un taux plus élevé que celui des fluctuations thermiques. En revanche, le mécanisme actif exige que l'hélicase déstabilise activement le duplex d'ADN. Dans ce cas, le taux de déroulement de l'ADN catalysé par l'enzyme est plus rapide que celui des fluctuations thermiques (Lohman et Bjornoson, 1996 ; Soultanas et Wigley, 2000).

Les mécanismes actifs exigent aussi que l'hélicase soit capable de fixer l'ADN double brin et l'ADN simple brin et tous les deux simultanément durant l'étape intermédiaire. Les premiers types de mécanisme actif sont le modèle dit « inchworm model » (Yarranton et Gefter, 1979 ; Hill et Tsuchiya, 1981) et le modèle dit « rolling model » (Wang et Lohman, 1992 ; Lohman, 1992). Ces deux modèles de mécanisme de déroulement de l'ADN ont été proposés pour les hélicases Rep/PcrA. Un autre modèle de mécanisme actif est un modèle de torsion dans lequel l'hélicase n'interagit pas directement avec le duplex d'ADN mais se fixe simultanément aux deux simple-brins au niveau de la jonction d'ADN simple brin/ADN double brin et déroule le duplex d'ADN par un mécanisme de détorsion.

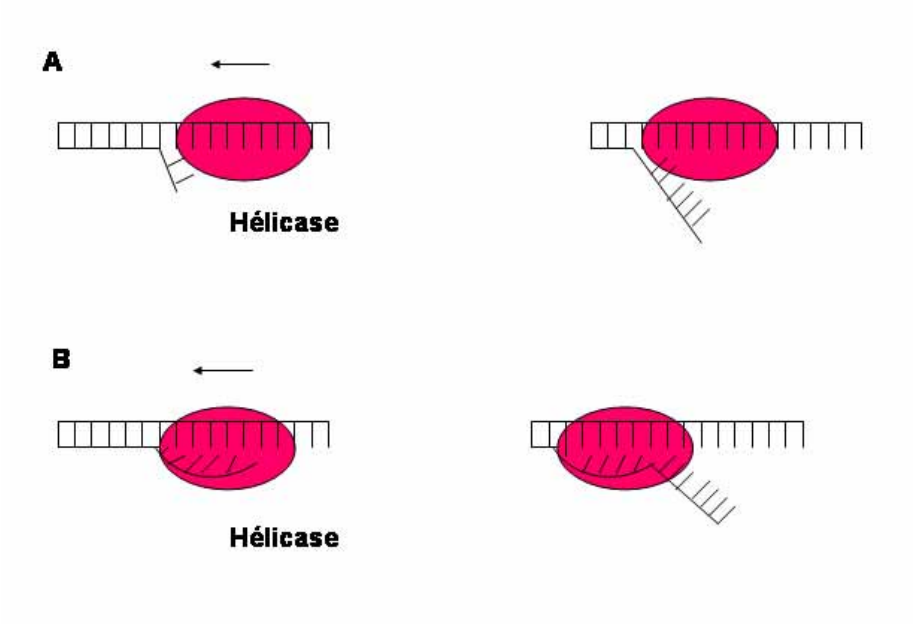


Fig. 6 Mécanismes passif et actif d'action d'hélicase

A. Dans le mécanisme passif, l'hélicase ne contacte pas le duplex d'ADN. Elle exclue l'ADN simple brin par sa translocation sur l'autre brin. **B.** Dans le mécanisme actif, l'hélicase interagit directement avec l'ADN double brin et déstabilise le duplex, déroulant donc activement les brins (d'après Soultanas et Wigley, 2001).

2. Famille RecQ des ADN hélicases.

La famille RecQ est nommée par référence au nom du gène recQ d'*Escherichia coli*, gène qui a été identifié il y a plus de 30 ans lors d'un criblage pour des mutations conférant une résistance à la privation en thymine (Nakayama et al., 1984,1985). Ce gène a été nommé RecQ par H. Nakayama de l'université de Kyushu, Japon (le « Q » provient de Kyushu) (Nakayama *et al.,* 1984,1985). Plus récemment, d'autres membres de la famille ont été identifiés chez les eucaryotes inférieurs et supérieurs par diverses approches génétiques et biochimiques (Chakraverty et Hickson, 1999; Karow et al., 2000; van Brabant et al., 2000; Mohaghegh et Hickson, 2001a). En général, les organismes unicellulaires possèdent une seule enzyme RecQ, tandis que les organismes plus complexes en possèdent deux ou plus. Chez *Escherichia coli* le seul membre de la famille est l'hélicase RecQ, tout comme Sgs1 chez la levure bourgeonnante, *Saccharomyces cerevisiae*, et Rqh1 chez la levure fissipare, *Schizosaccharomyces pombe*. Chez l'homme on trouve 5 membres de la famille RecQ. L'altération de l'une de trois de ces hélicases RecQ humaines provoquent des désordres cliniques définis associés à la prédisposition au cancer et au vieillissement prématuré : Les hélicases WRN, BLM, et RECQ4 responsables respectivement du syndrome de Werner (WS), de Bloom(BS), et de Rothmund-Thomson (RTS) (Ellis *et al.,* 1995,1996; Yu *et al.*, 1996; Kitao *et al.*, 1999), et dont les caractéristiques cliniques sont résumées dans le tableau 2 (Hickson, 2003). Les patients atteints du syndrome de Bloom (BS) sont prédisposés à de nombreux types de cancer à partir d'un âge moyen de 24 ans. Les patients atteints du syndrome de Werner (WS) sont particulièrement prédisposés aux sarcomes, au vieillissement prématuré et aux maladies associées au vieillissement. Les patients atteints du syndrome de Rothmund-Thomson (RTS) ont une poïkilodermie caractéristique et sont prédisposés aux ostéosarcomes et au vieillissement prématuré. Une quatrième maladie, le syndrome de RAPADILINO, qui partage certaines caractéristiques cliniques avec le RTS, a également été récemment liée à des mutations de RECQ4 (Siitonen *et al.,* 2003). Ainsi, les activités des protéines RecQ chez l'homme peuvent avoir un effet

important sur la santé. Les bases moléculaires de l'instabilité génomique et du vieillissement prématuré ne sont pas bien connues. Au niveau cellulaire, tous les mutants déficients en hélicase RecQ montrent une instabilité génomique, bien que les caractéristiques détaillées de cette instabilité puissent différer chez différents mutants et/ou espèces.

Tableau 2. Caractéristiques cliniques du syndrome de Bloom, du syndrome de Werner et du syndrome de Rothmund-Thomson. (d'après Hickson, I.D. 2003)

Syndrome	Caractéristiques cliniques principales	Prédisposition au Cancer
Le Syndrome de Bloom :	Nanisme proportionnel; induction d'érythème (en particulier sur le visage) lors d'exposition au soleil, diabète de type II; visage étroit et oreilles proéminentes; infertilité masculine et sous fertilité féminine; infections fréquentes.	La plupart des types ; en particulier le lymphome de non-Hodgkin, leucémies et carcinomes du sein, de l'intestin et de la peau
Le syndrome de Werner :	1. Caractéristiques du vieillissement physiologique, vieillissement prématuré, cheveux gris et amincissement des cheveux, perte de graisse sous-cutanée ; rides de la peau ; cataractes, ostéoporose; diabète de type II, atrophie des membres. athéroscléroses 2. quelques caractéristiques non spécifiques du vieillissement physiologique, stature petite, ulcères des membres inférieurs, calcification des tissus mous	Tumeurs d'origine mésenchymateuse; sarcomes des tissus mous et ostéosarcomes, quelques mélanomes et cancers de la thyroïde
Le Syndrome de Rothmund-Thomson :	Stature petite, anomalies du squelette (pas toujours), alopécie (scalp/sourcil, etc.), anomalies de pigmentation de la peau, atrophie de la peau.	Divers; principalement ostéosarcomes

2.1. Structure des hélicases RecQ.

La famille RecQ est définie par un domaine hélicase fortement conservé qui comporte environ 400 acides aminés et inclut sept motifs (I, Ia, II, III, IV, V, VI) qui sont caractéristiques d'une grande variété d'ADN hélicases et d'ARN hélicases de la superfamille II (SFII). Un huitième motif (motif 0) situé en position N- terminale du motif I est également conservé parmi certains des membres de la famille RecQ (Bernstein et Keck, 2003).

La structure tridimensionnelle du domaine hélicase de la protéine BLM a été modélisée à partir de la structure cristalline du domaine hélicase de l'hélicase PcrA (Rong *et al.,* 2000). A partir de ce modèle structural, Rong *et al.* (2000) ont suggéré que les motifs Ia, III et V contiennent des résidus qui établissent le contact direct avec l'ADN simple brin, les motifs I, II-IV et VI formant une poche pour fixer l'ATP (Fig. 7). Plusieurs mutations ponctuelles décrites chez des patients atteints du syndrome de Bloom sont situées dans ces motifs hélicases conservés, et ces mutations conduisent à la perte des activités ATPase et hélicase de l'enzyme. Les hélicases RecQ caractérisées chez les procaryotes et les eucaryotes (Fig. 8) peuvent être distinguées d'autres hélicases par les séquences particulières qui flanquent le domaine hélicase (Fig. 8).

Les hélicases RecQ peuvent être divisées en deux groupes : des formes courtes qui ne comportent que le domaine hélicase conservé, le domaine RecQ C-terminal (RecQ-Ct) et le domaine Hélicase et Rnase D C-terminal (HRDC) et des formes longues qui contiennent des domaines N et C – terminaux flanquant ces domaines centraux (Fig. 8). Exceptés certains motifs de séquence, ces domaines additionnels sont faiblement conservés. Dans les organismes qui expriment plusieurs enzymes RecQ, comme chez l'homme, il semble que ces domaines additionnels soient importants pour la différenciation fonctionnelle de ces 'isoformes' d'hélicase. Deux domaines - le domaine RecQ C-terminal (RecQ-Ct) et le domaine Hélicase et Rnase D C-terminal (HRDC) (Fig. 8), sont situés en C- terminal du domaine hélicase. Le domaine RecQ-Ct est unique aux hélicases de la famille RecQ, et a probablement un rôle dans les interactions avec d'autres protéines. Une publication récente de la

structure cristalline aux rayons X du noyau catalytique de l'hélicase RecQ d'*E. coli* indique que le domaine RecQ-Ct contient des motifs de fixation à l'ADN et aux protéines (Bernstein *et al.,* 2003). Le domaine RecQ-Ct de l'hélicase WRN humane permet également la fixation à l'ADN et à des protéines impliquées dans le métabolisme de l'ADN (von Kobbe *et al.,* 2003). Le domaine HRDC est trouvé dans certaines nucléases qui digèrent l'ARN, et pourrait être impliqué dans la fixation à l'ADN (Morozov *et al.,* 1997; Liu *et al.,* 1999). Les domaines HRDC de l'hélicase RecQ d'*E. coli* et de l'hélicase WRN humaine sont nécessaires pour la fixation stable à l'ADN mais pas pour l'activité hélicase (Bernstein et Keck, 2003 ; von Kobbe *et al.,* 2003). La structure tridimensionnelle du domaine HRDC de l'hélicase Sgs1 de *Sacchanomyces cerevisiae* a été déterminée(Morozov *et al.,* 1997; Liu *et al.,* 1999), et a été employée comme base pour établir un modèle structural général du domaine HRDC (Fig. 9). Le domaine HRDC montre une similitude structurale avec certains domaines d'autres hélicases, de recombinases et de polymérases, dans lesquels il permet un contact additionnel au substrat d'ADN, mais n'est pas impliqué dans l'activité enzymatique. Le domaine HRDC de l'hélicase Sgs1 se fixe à l'ADN simple brin *in vitro*, bien qu'avec une affinité faible, ce qui suggère une fonction auxiliaire dans l'identification de substrat.

Chez certaines hélicases RecQ, on trouve une séquence de signal de localisation nucléaire près de l'extrémité C- terminale (Kaneko *et al.,* 1997; Matsumoto *et al.,* 1997). Le domaine N- terminal des hélicases RecQ de forme longue ne contient pas de motifs fonctionnels évidents. Une exception à cette règle générale est la protéine WRN humaine et ses homologues, tels que le FFA-1 de *Xenopus laevis*, dans lesquels le domaine N- terminal contient un motif retrouvé dans certaines exonucléases. Ce domaine peut fonctionner indépendamment comme une 3'-5' exonucléase quand il est exprimé comme un fragment de protéine recombinante (Huang *et al.,* 1998 ; Kamath-Loeb *et al.,* 1998; Shen *et al.,* 1998a). Il a également été mis en évidence que, dans la protéine WRN intacte, les fonctions hélicase et exonucléase seraient coordonnées (Opresko *et al.,* 2001). Quelques membres de la famille RecQ – en particulier RECQ5 humain et ses homologues - existent sous 2 formes différentes,

courte et longue, qui sont produites par épissage alternatif de l'ARN messager (Sekelsky *et al.,* 1999 ; Shimamoto *et al.,* 2000).

Différentes conformations ont été observées pour les hélicases de la famille RecQ. L'hélicase RecQ d'*E. coli* forme des monomères en solution et déroule l'ADN et hydrolyse l'ATP en tant que monomère (Xu *et al.*, 2003a). Par contre, par chromatographie d'exclusion et/ou microscopie électronique, il a été montré que l'hélicase BLM forme des hexamères et l'hélicase WRN des trimères. Janscak *et al.* (2003) ont récemment étudié une forme tronquée en C terminal de l'hélicase BLM contenu des domaines RecQ-Ct et HRDC. Ce fragment forme des monomères en solution capables de fixer et dérouler l'ADN avec la même spécificité de substrat. Ces résultats indiquent que les domaines RecQ-Ct et HRDC sont indispensables à l'activité et la spécificité de cette hélicase mais qu'ils ne sont pas certainement impliqués dans l'oligomérisation. De plus, la configuration en hexamère n'ont pas indispensable pour l'activité.

Pour définir mieux les rôles précis des hélicases RecQ *in vivo*, un effort de recherche significatif a été fait pour caractériser les propriétés biochimiques des hélicases RecQ et identifier les interactions importantes entre les hélicases RecQ et d'autres protéines.

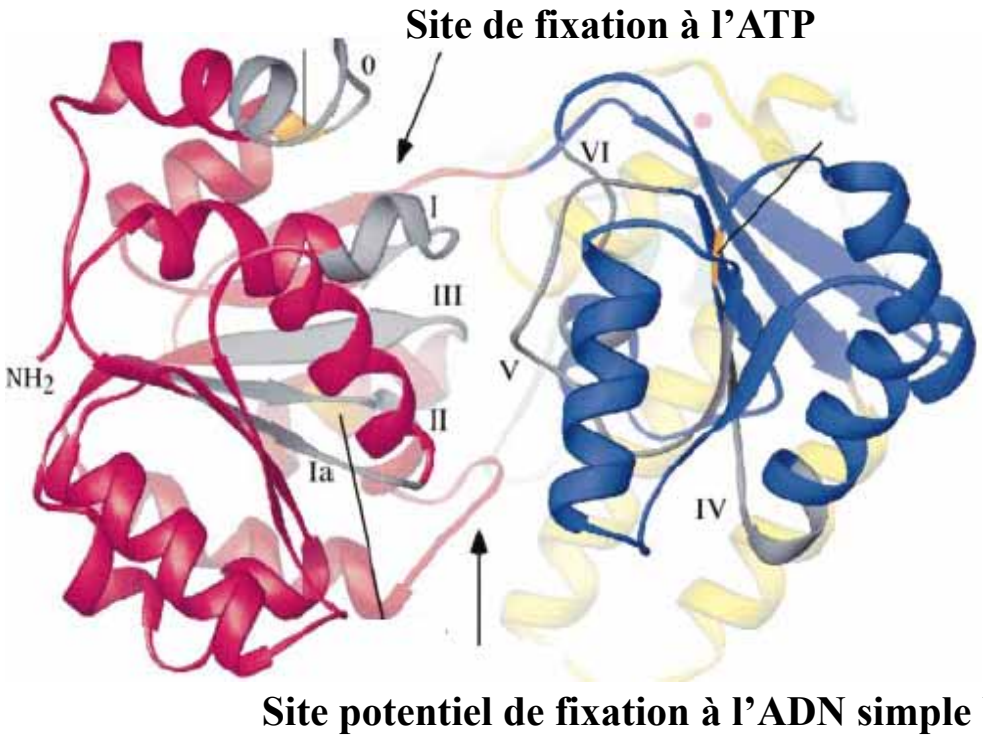


Fig. 7. Structure du domaine hélicase de l'hélicase RecQ chez *E. coli*

Vue dans la cavité formée par les deux sous-domaines hélicase. Les motifs hélicase sont colorés en gris. Les sites où on a observé la fixation du nucléotide et de l'ADN simple brin dans d'autres structures hélicase sont indiqués par des flèches (d'après Bernstein *et al.*, 2003).

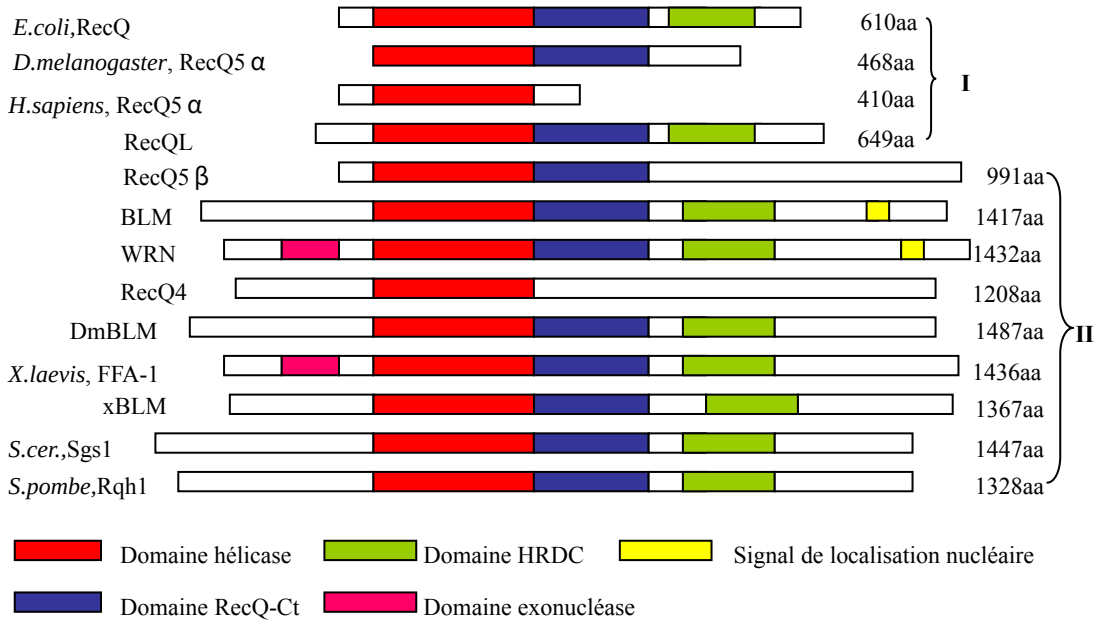


Fig. 8. Représentation schématique de certains membres de la famille RecQ. RecQ-Ct, le domaine RecQ conservé C-terminal ; HRDC, le domaine Hélicase et Rnase D C-terminal ; NLS, signal de localisation nucléaire. aa, acides aminés. (modifié d'après Bjergbaek *et al.*, 2002)

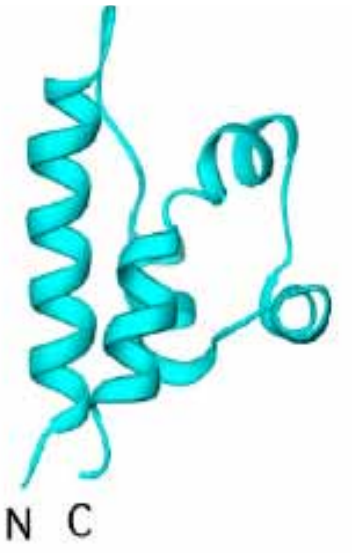


Fig. 9 Structure du domaine HRDC de l'hélicase Sgs1 chez *S. Cerevisiae* (d'après Liu *et al.*, 1999)

2.2. Caractéristiques biochimiques des RecQ hélicases.

Tous les membres de la famille des hélicases RecQ ont une activité ATPase Mg^{2+} et ADN dépendante. L'ADN simple brin active plus l'activité ATPase que l'ADN double brin sauf dans le cas de l'hélicase Sgs1 (Bennett *et al.,* 1998). Des molécules plus longues d'ADN simple brin sont des stimulateurs significativement meilleurs de l'activité ATPase que des molécules courtes, ce qui suggère que les hélicases RecQ se déplacent le long de l'ADN simple brin (Brosh *et al.,* 2000a; Karow *et al.*, 1997; Brosh *et al.,* 2000b). L'activité hélicase dépend de la présence de cations divalents (2-5 mM). Le cofacteur Mg^{2+} peut être remplacé par le Ca^{2+} ou le Mn^{2+}, alors que les ions Zn^{2+} sont inhibiteurs. L'activité ATPase des hélicases RecQ n'est pas particulièrement sélective. Tous les membres de la famille RecQ étudiés jusqu'ici acceptent et hydrolysent l'ATP et le désoxyadénosine 5'-triphosphate (dATP). Les enzymes dmRecQ5a de *Drosophila melanogaster* (Özsoy *et al.,* 2001) et BLM humain (Brosh *et al.,* 2001b), sont moins sélectifs puisque le GTP et le désoxyguanosine 5'-triphosphate (dGTP) peut être utilisé.

Les ADN hélicases de la famille RecQ déroulent l'ADN avec la polarité 3'-5' . Bien que cette polarité de la réaction ait été jusqu'ici démontrée seulement pour les hélicases RecQ d'*E coli* (Umezu *et al.*, 1990), Sgs1p (Lu *et al.,* 1996 ; Bennett *et al.,* 1998), hRecQ1/L (Seki *et al.*, 1994a-b), BLM (Karow *et al.*, 1997) et WRN (Shen *et al.,* 1998b), elle est probablement représentative de toute la famille.

Les activités catalytiques des hélicases de la famille RecQ présentent certaines caractéristiques, qui devraient refléter leurs fonctions *in vivo*. Tout d'abord, leurs activités de déroulement sont nettement stimulées par les protéines qui stabilisent l'ADN simple brin (SSB) comme c'est le cas pour d'autres hélicases. Ainsi, en présence de la protéine SSB d'*E coli*, l'hélicase RecQ d'*E coli* est non seulement capable de dérouler des duplexes partiels d'ADN avec une efficacité élevée mais aussi de dérouler des duplexes d'ADN à bouts francs (Fig. 10), qui sont des substrats peu efficaces en l'absence de SSB (Umezu et Nakayama, 1993). L'interprétation de la stimulation de l'activité hélicase par des protéines SSB est que la fixation de celles-ci

à l'ADN simple brin déroulé stabiliserait l'ADN, empêchant ainsi un cycle entre le déroulement et le rembobinage (Umezu et Nakayama, 1993; Harmon et Kowalczykowski, 2001) et la fixation inutile de l'hélicase à cet ADN (Harmon et Kowalczykowski, 2001). Bien qu'il ait été mis en évidence qu' *E coli* SSB stimulait le déroulement d'un duplexe d'ADN par Sgs1p (Bennett *et al.*, 1998) et WRN (Gray *et al.,* 1997; Shen *et al.,* 1998b; Brosh *et al.*, 1999), l'effet semble être limité à des substrats plus courts (< 100 bp). A l'inverse, la protéine A de réplication (RPA), la protéine SSB humaine, est une meilleure stimulatrice pour les activités de déroulement de WRN et BLM. Leurs activités sur un ADN duplexe plus long (>200 bp) sont nettement augmentées par RPA (Shen *et al.*, 1998b; Brosh *et al.*, 1999, 2000a). De plus, des interactions physiques directes entre RPA et ces hélicases humains ont été démontrées (Brosh *et al.,* 1999, 2000a).

Il y a des différences dans la spécificité de substrat parmi les ADN hélicases de la famille RecQ. Les préférences de substrat sont déterminées en comparant la quantité de produit, la cinétique de réaction, et/ou l'affinité de l'hélicase pour ces substrats.

Outre les ADN partiellement duplexes et les duplexes d'ADN à bouts francs déjà mentionnés, l'hélicase RecQ d'*E coli* est également capable de dérouler une variété étonnamment grande de substrats d'ADN avec des efficacités comparables en présence de SSB (Harmon et Kowalczykowski, 1998, 2001; Harmon *et al.*, 1999). L'hélicase RecQ d'*E coli* peut agir sur des structures d'ADN en D-loop, et des structures à 3 ou 4-extrémités de type jonction de Holliday (Figure 10).

En absence de protéines SSB, les longs duplexes d'ADN semblent être de médiocres substrats pour les homologues eucaryotes de RecQ. Les hélicases Sgs1p, BLM et WRN ne déroulent pas les duplexes d'ADN à bouts francs (Bennett *et al.,* 1999; Mohaghegh *et al.*, 2001b). En revanche, ils sont tout à fait efficaces dans le déroulement de structures de type jonctions de Holliday (Karow *et al.,* 2000; Constantinou *et al.,* 2000; Mohaghegh *et al.,* 2001b). C'est une caractéristique qui peut être associée à leur rôle hyper recombinogène observée dans les cellules des patients atteints du syndrome de Bloom ou de Werner. Les hélicases WRN et BLM se

fixent spécifiquement aux sites de jonction et ont une affinité relative plus élevée pour les substrats présentant des jonctions (Shen *et al*., 2000; Orren *et al.,* 2002; van Brabant *et al.,* 2000b; Brosh *et al.*, 2002). La capacité de dérouler des intermédiaires de recombinaison formés pendant la réplication semble être une fonction principale des hélicases RecQ.

Les hélicases WRN et BLM sont capables de dérouler un duplexe d'ADN à extrémités franches interrompu par une bulle interne (due à un mésappariement par exemple) (Figure 10B), mais les bulles inférieures à 4 nucléotides ne sont pas déroulées (Mohaghegh *et al.,* 2001b). L'hélicase WRN fixe préférentiellement les structures d'ADN présentant une D-loop (Figure 10C), comparé à un duplexe d'ADN présentant une simple bulle, et libère le brin entrant de l'ADN de la D-loop, qu'il s'agisse d'une queue simple brin 3'- ou 5' sortante ou qu'il n'y ait pas de queue du tout (Orren *et al.,* 2002). L'hélicase BLM est plus efficace sur les structures D-loop à queue simple brin 3' sortante, mais toutes les formes de D-loop étudiées sont déroulées plus efficacement que les duplexes d'ADN à bulle interne simple (van Brabant *et al.,* 2000b). Par conséquent, les hélicases RecQ semblent préférer les substrats qui imitent les intermédiaires de recombinaison et de réplication.

Pour déterminer si toutes les hélicases RecQ eucaryotes partagent des spécificités de substrat, la caractérisation des autres membres de la famille RecQ est nécessaire. Tout comme les hélicases WRN, BLM et Sgs1, la petite isoforme de l'hélicase DmRecQ5 de *Drosophile melanogaster* (Ozsoy *et al.,* 2003) et l'hélicase RecQ1 humaine (RecQL) (Cui *et al*., 2003) sont inactives sur les duplexes d'ADN à queue 5' sortante ou à bouts francs (Figure 10A) mais sont actives sur les substrats contenant des jonctions. En revanche, l'hélicase DmRecQ5 déroule préférentiellement les duplexes ADN présentant une fourche et est moins active sur les substrats type jonction de Holliday et bulle simple (Ozsoy *et al.*, 2003). Par conséquent, les hélicases RecQ ont probablement certains rôles complémentaires et/ou distincts *in vivo*.

Les hétéroduplexes ADN-ARN peuvent aussi être déroulés au moins par les hélicases WRN (Suzuki *et al.*, 1997) et Sgs1p (Bennett *et al.*, 1998, 1999). Ceci peut

concerner le traitement des fragments d'Okazaki par l'hélicase RecQ au niveau des fourches de réplication arrêtées observé chez *E. coli*, (Courcelle et Hanawalt, 1999).

Il y a certaines différences dans la spécificité de substrat entre les enzymes RecQ humaines, eucaryotes inférieurs et bactériens. Une propriété commune est leur capacité de dérouler des structures d'ADN autres que le duplexe standard d'ADN de forme B (Figure 10D). En particulier, un substrat préféré par les hélicases RecQ est l'ADN G-quadruplexe (Mohaghegh *et al.*, 2001b ; Sun *et al.*, 1998.1999 ; Fry et Loeb, 1999; Wu et Maizels, 2001; Han et Hurley, 2000). Les hélicases BLM, WRN et Sgs1 déroulent les structures G-quadruplexes avec un ADN simple brin à queue 3' sortante (Sun *et al.,* 1998; Fry et Loeb, 1999). Huber *et al.* (2002) ont constaté que les hélicases BLM et Sgs1 fixaient et déroulaient plus efficacement des G-quadruplexes que des jonctions de Holliday construites avec des oligonucléotides. De même, les hélicases BLM et WRN déroulent des triplexes d'ADN avec une queue d'ADN simple brin 3' sortante plus efficacement qu'un duplexe d'ADN avec une extrémité d'ADN simple brin 3' sortante (Brosh *et al.,* 2001b). Des structures intermédiaires d'ADN G-quadruplexe et triplexe ont été impliquées durant les réarrangements de l'ADN (délétions, échange de chromatide soeur, recombinaison homologue et non homologue) (Brosh *et al.* , 2001b). En fait, l'hélicase WRN, tout comme l'hélicase RecQ d'*E coli*, peut diminuer la pause de l'ADN polymérase δ induite par un ADN G-quadruplexe *in vitro* (Kamath-Loeb *et al.*, 2000, 2001). Ceci peut expliquer, au moins en partie, des défauts observés dans la synthèse d'ADN dans les cellules RecQ déficientes chez la levure et l'homme.

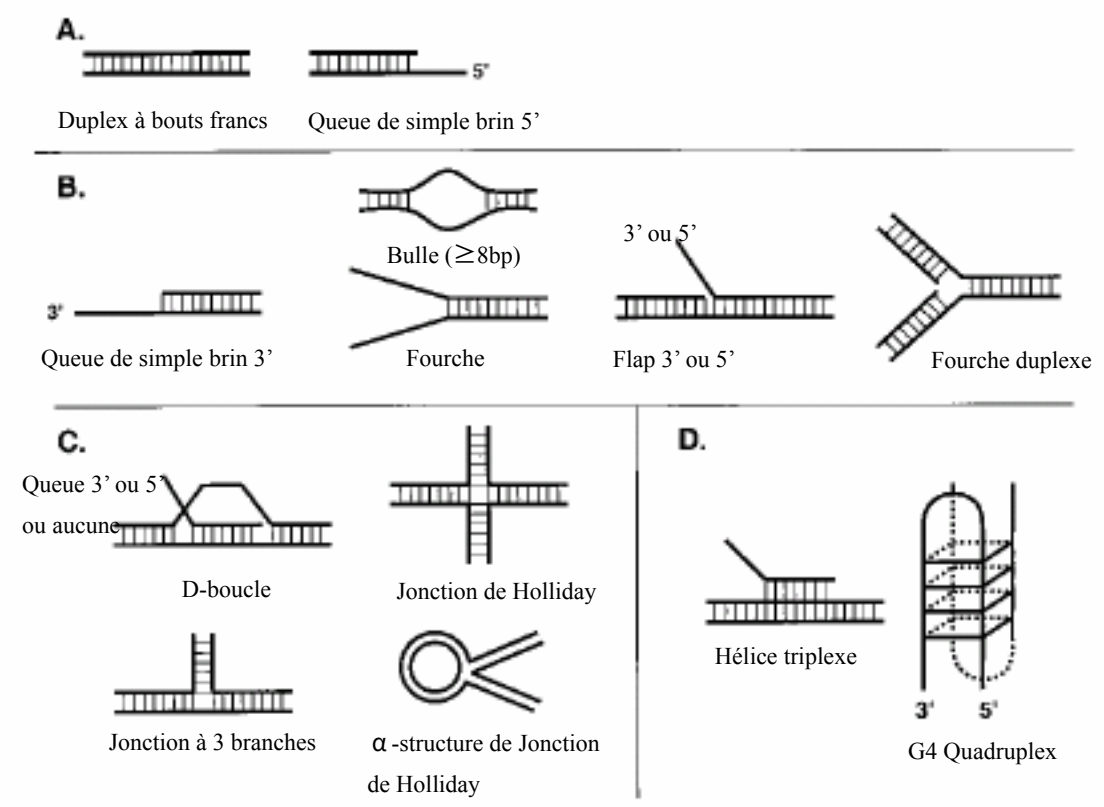


Fig. 10. Spécificité de substrat des hélicases RecQ. A, Substrats de l'hélicase RecQ d'*E. coli* en présence de SSB mais pas des hélicases RecQ eucaryotes. B, Duplexes d'ADN intermédiaires de forme B dans la réplication et la réparation. Ces substrats sont plus spécifiques des hélicases WRN, BLM et Sgs1. C, Intermédiaires d'ADN observés dans la recombinaison. Les hélicases WRN et BLM interagissent préférentiellement avec ces substrats relativement à ceux présentés en B. D, Structures alternatives d'ADN en forme de triple hélice ou de G-quadruplex (ADN télomérique). L'ADN G-quadruplexe est un substrat préférentiel pour WRN, BLM, et Sgs1 (d'après Opresko *et al,* 2004).

2.3. Fonctions des hélicases RecQ dans le métabolisme de l'ADN.

Les hélicases de la famille RecQ agissent préférentiellement *in vitro* sur des substrats correspondant à des intermédiaires de réplication et de recombinaison et interagissent avec des protéines impliquées dans ces processus. Ces résultats

indiquent que ces hélicases sont impliquées in vivo dans les mécanismes de réparation–recombinaison de l'ADN. Les fonctions de ces hélicases *in vivo* sont complexes et multiples. La connaissance de ces fonctions est essentielle pour comprendre la prédisposition au cancer et le vieillissement prématuré observés chez les patients déficients pour ces hélicases.

2.3.1 Rôles dans la réplication de l'ADN.

Il a été proposé que les hélicases RecQ interviennent lors de la réplication de l'ADN lorsque la fourche de réplication est cassée ou bloquée à l'encontre de lésions sur l'ADN. Chez *E. coli*, les hélicases RecQ et RecJ dénaturent le brin naissant aux fourches de réplication bloquées par des lésions induites par les UV (Courcelle *et al.*, 2003). Chez la levure, l'hélicase Sgs1 est nécessaire pour la stabilisation des fourches de réplication bloquées par des lésions induites par l'hydroxyurée (Cobb *et al.*, 2003). Effectivement, les cellules de patients atteints du syndrome de Bloom (BS) ou du syndrome de Werner (WS) sont caractérisées par des défauts dans la réplication de l'ADN (Hickson, 2003).

Un rôle possible des hélicases RecQ serait donc de déplacer les blacages de fourches durant la réplication de l'ADN. Les blocages potentiels qui bloquent la fourche de réplication peuvent être des structures secondaires de l'ADN telles que les « épingles à chevaux » et les G-quadruplex (Fig. 11a). L'hélicase WRN agit sur ces structures et permet à la polymérase δ de poursuivre la réplication de l'ADN (Kamath-Loeb *et al.*, 2001).

La réplication de l'ADN bloquée sur le brin de tête « leading strand » peut mener à la régression de la fourche et à la formation d'une « structure à 4 voies » de type jonction de Holliday ou d'une structure « pied de poulet » « chicken foot » (Fig. 11b). Dans ce dernier cas, la synthèse du brin de tête s'effectue en prenant pour matrice le brin de queue « lagging strand ». Le déroulement de la structure à 4-voies de type jonction de Holliday par l'hélicase RecQ permet à la polymérase de traverser la lésion et permet d'aboutir à une synthèse d'ADN normale.

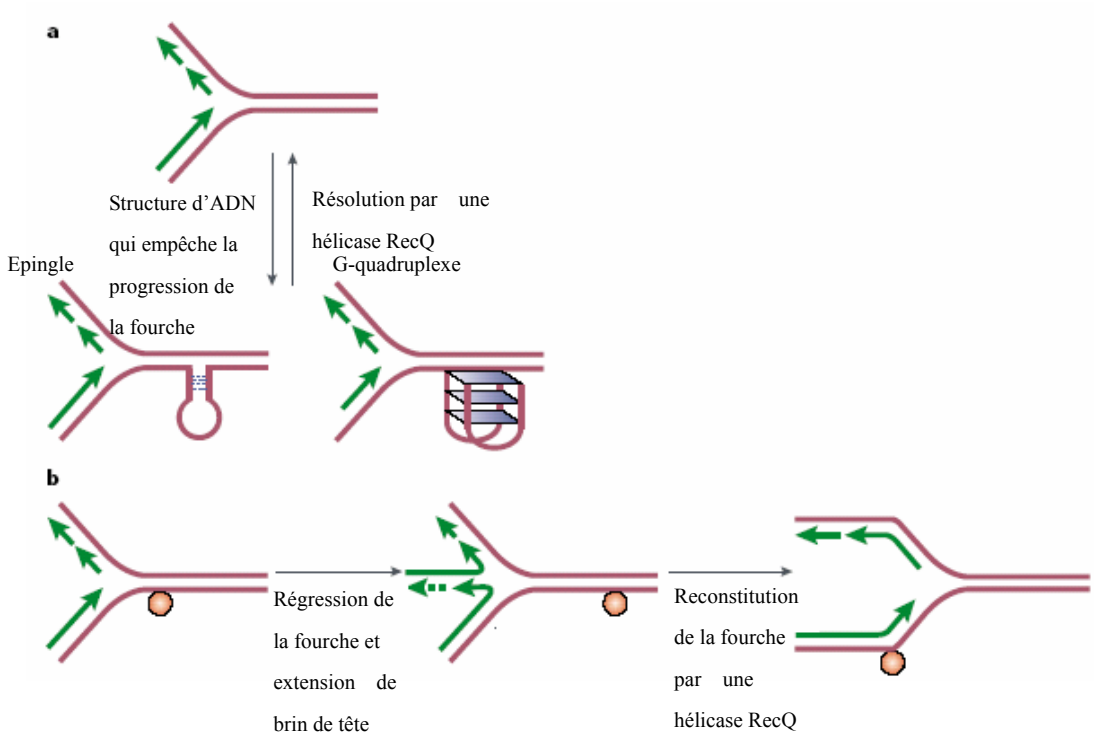


Fig. 11. Rôles potentiels des hélicases RecQ dans la maintenance de l'intégrité de génome durant la réplication de l'ADN.

a. La progression d'une fourche de réplication peut être bloquée par les structures secondaires dans l'ADN, telles que des épingles à cheveux ou des G-quadruplexe. Les hélicases RecQ peuvent résoudre ces structures et ainsi permettre la progression de la fourche de réplication.

b. Si une fourche rencontre une lésion (cercle orange) sur la matrice du brin de tête, la fourche peut régresser et les brins naissants d'ADN (vert) peuvent s'hybrider pour former une jonction à 4 branches. Le brin de tête est alors synthétisé en utilisant le brin de queue comme matrice. Une hélicase RecQ peut résoudre la jonction à 4 branches et permettre un « by pass » de la lésion. (modifié d'après Hickson, I.D. 2003)

2.3.2. Rôles dans la recombinaison et la réparation.

Une caractéristique des cellules eucaryotes déficientes en protéine RecQ est l'augmentation de la recombinaison, correspondant à un phénotype d'hyper recombinaison (Salk *et al.,* 1981; Groden et German, 1992 ;Watt *et al.*, 1996; Stewart *et al.*,1997 ; Lindor *et al.,* 2000 ; Onoda *et al.*, 2000, 2001 ; Myung *et al.*, 2001). Cela montre un rôle dans la régulation négative de la recombinaison par les hélicases de la famille RecQ. En outre, il y a plusieurs preuves pour suggérer la participation des hélicases de la famille RecQ dans la réparation des cassures double-brin (CDB) de l'ADN. La réparation des CDBs est effectuée principalement par recombinaison homologue (RH) chez la bactérie et la levure. Dans les cellules de mammifères, les cassures double-brin de l'ADN (CDB) sont réparées par deux mécanismes principaux : la jonction précise ou imprécise des extrémités (NHEJ : Non Homologous End Joining) et la recombinaison homologue (RH) (Thompson et Schild, 1999 ; Friedberg *et al.,* 1995).

Les hélicases BLM et WRN, comme l'hélicase RecQ chez *E. coli*, sont actives *in vitro* sur les structures d'ADN associées aux intermédiaires de recombinaison (Karow *et al.* , 2000; Constantinou *et al.* , 2000; Mohaghegh *et al.* , 2001a). *In vivo*, la déficience en hélicase BLM chez le poulet aboutit à une augmentation des échanges entre chromatides soeurs par recombinaison homologue (Wang *et al.*, 2000a). Puisque des échanges entre chromatides soeurs sont modifiées par recombinaison homologue (Sonoda et *al.,* 1999 ; Wang *et al.,* 2000a), BLM peut réguler au moins la recombinaison homologue. En revanche, les hélicases WRN et RTS régulent principalement la recombinaison illégitime (Prince *et al.* , 2001 ; Oshima *et al.*, 2002).

2.3.3. Rôles dans la maintenance des télomères.

Le vieillissement prématuré est une des manifestations cliniques principales du syndrome de Werner (Goto, 2000) et les cellules déficientes en protéine WRN montrent une augmentation de sénescence (Faragher *et al.,* 1993). La relation entre le vieillissement et les télomères est importante. En outre, des télomères altérés conduisent à une instabilité génomique et prédisposent à la transformation maligne

des cellules (Kim *et al.,* 2002). Des études récentes suggèrent que les hélicases de la famille RecQ incluant WRN sont impliquées dans la maintenance des télomères. Les cellules déficientes en protéine WRN ou Sgs1 montrent un défaut dans un mécanisme de prolongement alternatif du télomère qui fonctionne en l'absence d'une télomérase. L'hélicase Sgs1 ou WRN chez la souris est capable de supprimer la sénescence accélérée et la diminution de la taille des télomères dans des cellules de levure déficientes en Sgs1 et télomérase (Cohen et Sinclair, 2001; Huang *et al.,* 2001 ; Johnson *et al.,* 2001).

Les hélicases WRN, BLM et Sgs1 peuvent permettre le prolongement des séquences télomériques en dissociant les G-quadruplexes qui se forment au niveau de ces séquences. Elles peuvent avoir un effet catalytique direct dans le prolongement des télomères par recombinaison.

2.3.4. Rôles dans d'autres voies de réparation de l'ADN.

Le rôle des hélicases RecQ ne se limite pas à la réplication de l'ADN et à la recombinaison. Un rôle dans la réparation des cassures simple-brin de l'ADN (CSB) a également été proposé. Plusieurs enzymes qui interagissent avec l'hélicase WRN, y compris RPA, FEN1 et l'ADN polymérase δ, fonctionnent dans la réparation base par excision (BER). Ce processus est important pour la réparation des bases modifiées et des CSBs. L'hélicase WRN se fixe à l'ADN polymérase β impliquée dans le BER, et stimule la synthèse de l'ADN (Harrigan *et al.*, 2003). Une autre enzyme importante impliquée dans le BER, PARP-1 (« Poly(ADP-ribose) polymerase-1 »), se fixe fortement au domaine RecQ-Ct de WRN (von Kobbe *et al.,* 2003). Ces résultats suggèrent que l'hélicase WRN de la famille RecQ interviendrait dans la réparation des CSBs.

2.3.5. Rôles dans la réponse cellulaire aux dommages de l'ADN.

L'hélicase Sgs1 chez la levure est impliquée dans les processus de signalisation cellulaire de la présence de dommages de l'ADN en phase S (Khakhar *et al.*, 2003).

L'hélicase BLM fait partie du complexe BASC (complexe de surveillance du génome associé à BRCA1), qui inclut les kinases ATM et ATR et la protéine Nbs1 (Wang *et al.*, 2000). Les hélicases BLM et WRN co-localisent avec p53, une protéine impliquée dans la reconnaissance de toute une série d'états cellulaires, de carences (nutriments), de stress (après traitement génotoxique) et même probablement certains défauts de développement (Brosh *et al.*, 2001; Sengupta *et al.*, 2003). Elles sont phosphorylées de manière ATM et ATR dépendante en réponse au blocage de la fourche de réplication (Hickson, 2003 ; Franchitto et Pichierri, 2002). En outre, les complexes comprenant p53-BLM ou p53-WRN inhibent la résolution des substrats de type Jonction de Holliday et l'activité exonucléase de WRN (Bachrati et Hickson, 2003). Ainsi, les hélicases RecQ jouent probablement un rôle important dans la réponse cellulaire aux dommages de l'ADN, en phase S.

2.4. Rôles des hélicases RecQ dans le point de contrôle et dans l'apoptose.

De nombreuses données ont montré que les hélicases RecQ participent à deux processus qui sont associés à la prédisposition au cancer :

(1) le point de contrôle de cycle cellulaire qui surveille l'intégrité de l'ADN,

(2) l'apoptose qui permet d'éliminer des cellules irréversiblement endommagées.

L'hélicase Sgs1 de levure bourgeonnante co-localise avec la protéine kinase Rad53p aux « foci » nucléaires spécifiques durant la phase S et fonctionne dans la transmission de signal menant au point de contrôle (Frei et Gasser, 2000a ; Frei et Gasser, 2000b ; Oakley et Hickson, 2002). L'hélicase Rqh1 (homologue de RecQ de levure fissipare) a été également impliquée dans le point de contrôle de la phase S (Murray *et al.*, 1997 ; Davey *et al.*, 1998).

Des chercheurs ont étudié la relation entre les hélicases RecQ et le suppresseur de tumeur p53, dans son rôle apoptotique (Levine, 1997 ; Hakem et Mak, 2001). Les interactions entre p53 et les hélicases BLM et WRN sont ici aussi probablement impliqués. Une déficience en BLM ou WRN aboutit à une atténuation des processus d'apoptose régulés par p53 (Spillare *et al.*, 1999 ; Wang *et al.,* 2001). Cette atténuation

contribuerait ainsi à la prédisposition au cancer dans les patients atteints du syndrome de Bloom et du syndrome de Werner et probablement de ceux du syndrome de Rothmund-Thomson.

3. Le motif à doigt de zinc et sa fonction.

Le premier motif à doigt de zinc a été identifié il y a 15 ans comme le motif répétitif fixant l'ion Zn^{2+}, par l'intermédiaire de cystéine et d'histidine conservées, dans le facteur de transcription TFIIIA chez *Xenopus* (Millers *et al.*, 1985). Depuis, de nombreux autres motifs fixant l'ion Zn^{2+} ont été identifiés comme doigts de zinc. Un doigt de zinc est défini comme un petit domaine fonctionnel indépendamment replié, qui exige la coordination d'un ou plusieurs ions Zn^{2+} pour stabiliser sa structure.

3.1. Rôle du doigt de zinc dans les protéines en général.

Les protéines contenant un doigt de zinc font partie des protéines les plus abondantes des eucaryotes. Ils sont fortement impliqués dans mécanismes de reconnaissance de l'ADN et de l'ARN et dans les interactions protéines-protéines.

Les structures des doigts de zinc sont très diverses. Selon le nombre et le type d'acides aminés impliqués dans la coordination de l'ion Zn^{2+}, les doigts de zinc ont été divisés en plusieurs classes : Cys2His2, Cys2HisCys, Cys4-ribbon, Cys4 GATA, Cys6 et Cys8 (Leon et Roth, 2000).

Des doigts de zinc de type Cys2His2 sont trouvés dans 2% des gènes humains et sont des domaines fixant l'ADN dans de nombreux facteurs de la transcription humain ou de réparation (Tupler *et al.,* 2001). Ils contribuent à l'identification des séquences spécifiques de l'ADN.

Les interactions ARN-protéine sont essentielles dans de nombreux processus cellulaires, tels que la synthèse de l'ARN, l'épissage et la traduction. Des domaines à doigts de zinc sont connus pour interagir avec l'ARN, tel que la protéine Ncp7 du rétrovirus VIH. Les détails de l'identification de nombreux motifs à doigt de zinc dans d'autres protéines restent à étudier (Joho *et al.,* 1990; Theunissen *et al.,* 1992; Clemens *et al.,* 1993; Friesen et Darby 1997, 1998).

Les doigts de zinc sont aussi impliqués dans les interactions protéine-protéine (Crossley *et al.,* 1995; Merika et Orkin, 1995; Tsang *et al.*, 1998; Feng *et al.*, 1998; Sun et al., 1996; Borden, 2000). Ces interactions protéine-protéine peuvent être homo-typiques, comme dans la multimérisation de protéine, ou hétéro-typique, impliquant l'interaction coopérative avec une grande variété de facteurs protéiques au sein de complexes dont ceux impliqués dans le métabolisme de l'ADN (transcription, réplication, recombinaison ou réparation).

3.2. Rôle du doigt de zinc dans l'hélicase.

Des hélicases contenant le doigt de zinc de type Cys4 sont trouvées parmi les enzymes impliquées dans la réplication et la transcription de l'ADN. En outre, les analyses de ces motifs mutés dans plusieurs hélicases virales ont montré que ces motifs sont importants pour l'activité hélicase (van Dinten *et al.,* 2000 ; Biswas et Weller, 1999). Le motif à doigt de zinc est impliqué dans la réplication et la transcription des « nidovirus » par la régulation des activités enzymatiques de l'ATPase/hélicase nsp10 (Seybert *et al.,* 2005). La mutation dans le motif doigt de zinc de la protéine homologue de MCM d'archaebactérie *Methanobacterium thermoautotrophicum* aboutit à une perte des activités ATPase, hélicase et de fixation à l'ADN simple brin (Poplawski *et al.*, 2001). En plus de ceux-là, la protéine Mer3 de *Saccharomyces cerevisiae* possède le domaine hélicase et le domaine à doigt de zinc putatif qui sont importants pour la transition de CDBs (cassure de double brin) (Nakagawa et Ogawa, 1999). Récemment, la structure tridimensionnelle de l'hélicase RecQ tronquée à l'extrémité C-terminale a montré que l'enzyme se replie en quatre sous-domaines, dont deux se combinent pour former le domaine hélicase, tandis que les autres forment les motifs de fixation du zinc (Fig. 11) et l'hélice de type WH (Bernstein *et al.*, 2003a). La séquence du motif fixant le zinc dans la protéine RecQ bactérienne est C++x13-17 CxxCxxC, où les résidus"+" sont fréquemment électropositifs. Dans la protéine RecQ d'*E coli*, l'atome de zinc est fixé par quatre cystéines conservées situées au niveau d'une plateforme formée par les α-hélices 17 et 18. Le résidu Cys^{380} (indiquée C1) est situé au début de l'α-hélice 17. Cys^{400}

(indiquée C3) et Cys^{403} (indiquée C4) se trouvent respectivement au début et au milieu de la α-hélice 18, tandis que Cys^{397} (indiquée C2) est situé dans la boucle liant les deux hélices (Fig. 11). En outre, le motif doigt de zinc est stabilisé par trois liaisons hydrogène entre les résidus conservés phénylalanine (Phe^{374}), arginine (Arg^{381}) et asparagine (Asp^{401}) (Fig. 11).

Plusieurs études de mutants de l'hélicase RecQ dans lesquels les résidus de cystéine conservés ont été modifiés indiquent l'importance de doigt de zinc sur l'activité enzymatique. Deux des mutations cliniques connues du gène codant l'hélicase BLM qui mènent au syndrome de Bloom, portent une modification dans les codons des cystéines du domaine RecQ-Ct (Ellis *et al.,* 1995,1996 ; Foucault *et al.,* 1997). Le mutant C1055S de la protéine BLM humaine code pour une protéine inactive *in vitro* (activités ATPase et hélicase inactives) et ce mutant ne peut pas réduire le phénotype d'hyper recombinaison des cellules déficientes en protéine BLM dans des expériences de transfection (Neff *et al.,* 1999), indiquant un défaut grave de la fonction de la protéine. Des mutations ponctuelles analogues de l'hélicase BLM chez la souris aboutissent également à la perte des activités ATPase et hélicase *in vitro* (Bahr et al., 1998). Ces observations suggèrent que le motif fixant l'ion Zn^{2+} du domaine RecQ C-terminal (RecQ-Ct) joue un rôle important dans les réactions catalysées par les hélicases de la famille RecQ.

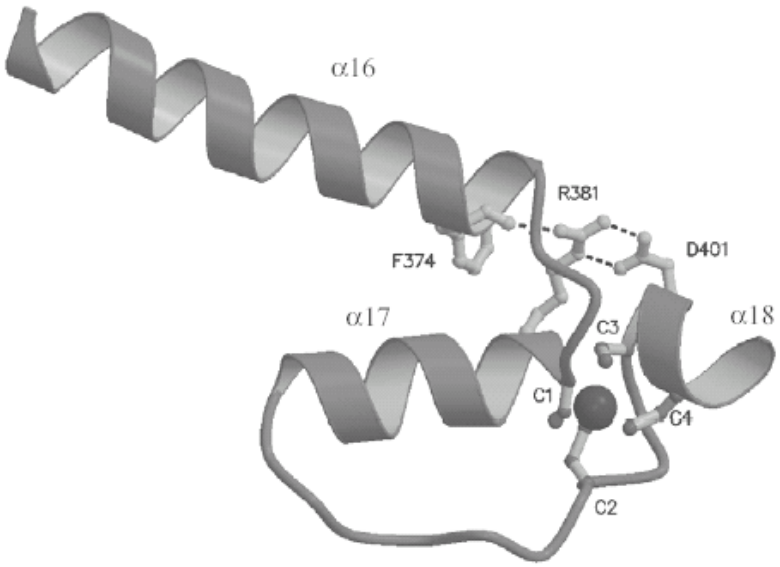


Fig. 12 Structure et Résidus conservés dans le motif à doigt de zinc de l'hélicase RecQ d'*E. coli*

Les quatre résidus conservés de cystéine, Cys^{380} (indiquée C1), Cys^{397} (indiquée C2), Cys^{400} (indiquée C3) et Cys^{403} (indiquée C4), sont colorés en gris. L'atome de zinc est coloré en gris foncé. Des labels des structures secondaires sont également indiqués. La conformation du motif doigt de zinc est stabilisée par trois liaisons d'hydrogène entre NH2 Arg^{381} et OD2 Asp^{401}, entre NE Arg^{381} et OD1 Asp^{401} et entre NH1 Arg^{381} et l'atome O de la chaîne principale de Phe^{374} (NH2, OD2, OD1, NE, et NH1 représentent les positions des atomes dans la molécule ; NH, heta de N ; OD, delta de O ; NE, Epsilon de N). Ces trois résidus colorés en gris clair contribuent au positionnement relatif des hélices 16, 17 et 18. (modifié d'après Bernstein *et al.*, 2003a)

4. Présentation du travail

Comme nous l'avons vu précédemment, la famille des hélicases RecQ est impliquée dans le maintien de la stabilité génomique chez les procaryotes et les eucaryotes (Chakraverty *et al.,* 1999). Des mutations des gènes correspondants peuvent mener à l'instabilité génomique et à plusieurs maladies humaines comprenant

les syndromes de Bloom et de Werner (Furuichi, Y. 2001). Généralement, les hélicases de la famille RecQ contiennent les domaines hélicase, HRDC et RecQ C-terminal (RecQ-Ct), ce dernier incluant le motif à doigt de zinc. La fonction du domaine hélicase a été caractérisée, mais celle du domaine RecQ-Ct reste à déterminer. Des études ont établi que les motifs à doigt de zinc sont impliqués dans des fonctions diverses comprenant les interactions protéine-ADN, le repliement de la protéine et les interactions protéine-protéine (Berg et Shi, 1996).

Donc, dans un premier temps, nous avons étudié la structure primaire et la fonction du motif à doigt de zinc dans RecQ-Ct. Plusieurs hélicases de la famille RecQ contiennent un motif doigt de zinc putatif du type C4 à l'extrémité C-terminale qui a été identifié dans la structure cristalline de l'hélicase RecQ d'*Escherichia coli*. Pour mieux comprendre le rôle de ce motif dans l'hélicase RecQ d'*E. coli*, nous avons construit une série de mutations ponctuelles changeant les cystéines (Cys) conservées aussi bien que d'autres résidus fortement conservés. Toutes les protéines mutantes, exprimées chez *E. coli* , ont montré un niveau élevé de susceptibilité à la protéolyse, rendant impossible l'analyse fonctionnelle. Par contre, une protéine double mutante a pu être purifiée. Dans ce mutant, les résidus Cys^{397} et Cys^{400} du motif à doigt de zinc ont été remplacés par des résidus asparagine (Asn). De légers changements locaux de conformation ont été détectés par dichroïsme circulaire, mais le reste de la protéine mutante présente une structure tertiaire correcte. En outre, l'enzyme mutante a une affinité pour l'ATP similaire à celle de l'enzyme sauvage mais est sévèrement altérée dans sa fonction de fixation à l'ADN, et ses activités ATPase et hélicase. Ces résultats indiquent que le motif à doigt de zinc est impliqué dans la fixation de l'ADN et peut-être dans le maintien de l'intégrité de la protéine entière. Nous avons également prouvé qu'après la formation du motif à doigt de zinc, l'atome de zinc n'est pas essentiel à l'activité enzymatique.

Ensuite, nous avons utilisé les trois isoformes RECQ5α, RECQ5β et RECQ5γ de l'hélicase RECQ5 humaine pour étudier le rôle du doigt de zinc dans la régulation des activités enzymatiques. Ces isoformes résultent de l'épissage alternatif au niveau de la transcription de gène RECQ5. Les trois protéines sont identiques dans les régions

N-terminales avec 410 résidus et toutes contiennent les sept motifs conservés du domaine hélicase. En plus du domaine hélicase, RECQ5β contient un domaine RecQ-Ct (RQC) putatif, tandis que RECQ5γ a une extension C-terminale qui n'est pas présente dans RECQ5β. Les trois protéines fixent l'adénosine triphosphate (ATP) avec une affinité semblable, mais l'ADN avec des affinités sensiblement différentes. RECQ5α et RECQ5γ sont déficient ou faible au niveau des activités ATPase et hélicase, tandis que RECQ5β est une ATPase et hélicase active. Remarquablement, RECQ5α possède une forte activité à favoriser l'hybridation de l'ADN, et c'est la première démonstration que le domaine hélicase de RecQ favorise l'hybridation de l'ADN. Notre étude montre que RECQ5β possède un doigt de zinc qui régule les activités enzymatiques multiples du domaine hélicase.

En fin, nous avons étudié les caractéristiques biochimiques des protéines SubL et SubS de la famille RecQ chez *Bacillus subtilis*. La protéine SubL contient un domaine hélicase, un motif fixant l'ion zinc et un domaine HRDC, mais la protéine SubS ne possède que un domaine hélicase et un motif fixant l'ion zinc. Les protéines SubL et SubS montrent l'activité ATPase en présence d'ADN. D'ailleurs, ils possèdent une activité hélicase de polarité 3'-5'. Les efficacités du déroulement et de l'activité ATPase de SubS sont environ 15 fois plus faibles que celles de SubL. Nous avons également montré que SubL possède une activité intrinsèque à favoriser l'hybridation de l'ADN et déroule deux intermédiaires importantes de la réplication et de la réparation de l'ADN, un ADN duplex avec un flap 5' et une structure de type fourche de réplication, à l'inverse SubS. Ces résultats indiquent que les hélicases SubL et SubS présentent des caractéristiques différentes dans leur mode d'action sur l'ADN.

Chapitre 2 : Résultats

1. Le motif à doigt de zinc de l'hélicase RecQ d'*Escherichia coli* est impliqué dans la fixation d'ADN et le repliement de la protéine

1.1. Présentation

Les hélicases de la famille RecQ sont nécessaires pour le maintien de l'intégrité de génome (Chakraverty et Hickson, 1999; Karow *et al.,* 2000b; van Brabant *et al.,* 2000a; Mohaghegh et Hickson, 2001a). L'hélicase RecQ d'*Escherichia coli* est le prototype de cette famille (Umezu *et al.*, 1990) et a été caractérisée à la fois comme initiateur de la recombinaison homologue et comme suppresseur de la recombinaison illégitime (Hanada *et al.*, 2000, Harmon *et al.,* 1998).

La structure cristalline aux rayons X du noyau catalytique de l'hélicase RecQ d'*E. coli* indique que cette enzyme contient les domaines hélicase et RecQ-Ct (Bernstein *et al.,* 2003a). RecQ-Ct contient un motif fixant l'ion zinc (Bernstein *et al.,* 2003a). La fonction du domaine hélicase a été étudiée, mais celle du domaine RecQ-Ct reste à déterminer. L'objectif de ce travail a été l'étude de la structure primaire et la fonction du motif à doigt de zinc du domaine RecQ-Ct de l'hélicase RecQ d'*E. coli.*

Six résidus (Cys^{380}, Arg^{381}, Cys^{397}, Cys^{400}, Asp^{401} et Cys^{403}) du motif doigt de zinc des hélicases de la famille RecQ sont fortement conservés (Fig.1 de l'article 1). Pour élucider les rôles joués par l'atome de zinc et le motif doigt de zinc de l'hélicase RecQ d'*E. coli*, nous avons muté ces résidus par mutagenèse dirigée. Quarte résidus (Cys^{380}, Cys^{397}, Cys^{400} et Cys^{403}) sont respectivement remplacés par Ala et deux résidus (Arg^{381} et Asp^{401}) sont respectivement remplacés par Asn. Ces mutants n'ont pas pu être étudié car les protéines correspondantes semblent totalement instables chez *E. coli* (protéolyse). Afin de modifier de façon ménagée le motif à doigt de zinc, nous avons remplacé simultanément les Cys^{397}et Cys^{400} par Asn. Une protéine portant cette double mutation a pu être purifiée et étudiée. Nous avons tout d'abord analysé cette protéine par dichroïsme circulaire pour déterminer l'effet de ces mutations sur la structure de la protéine. Ceci a permis de montrer que ces mutations ne conduisent qu'à une modification très localisée de la structure. De plus, les protéines sauvages et mutantes présentent un pattern de protéolyse ménagée identique et présentent un profil similaire par chromatographie d'exclusion. En résumé, les protéines sauvages et mutantes possèdent une structure tridimensionnelle similaire. Nous avons aussi étudié

la fixation de l'ATP par la technique de FRET (Transfert d'énergie de résonance de fluorescence). La protéine double mutante fixe l'ATP mais ne peut pas l'hydrolyser. La mutation double entraîne une perte des activités ATPase et hélicase. Par EMSA et anisotropie de fluorescence nous avons montré que la protéine mutée ne fixe plus l'ADN. Ces résultats démontrent que le motif à doigt de zinc joue un rôle essentiel dans la fixation de l'ADN.

En conclusion, le motif à doigt de zinc dans l'hélicase RecQ joue des rôles importants dans repliement de l'enzyme entière, dans la fixation de l'ADN et par conséquent dans les activités ATPase et hélicase. L'atome de zinc n'est pas essentiel à l'activité enzymatique après la formation du doigt de zinc.

L'ensemble de ces résultats est présenté dans l'article № 1.

1.2. Article № 1:

The Zinc Finger Motif of *Escherichia coli* RecQ Is Implicated in Both DNA Binding and Protein Folding

Jie Lin Liu, Pascal Rigolet, Shuo-Xing Dou, Peng-Ye Wang, and Xu Guang Xi

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 41, Issue of October 8, pp. 42794–42802, 2004
Printed in U.S.A.

The Zinc Finger Motif of *Escherichia coli* RecQ Is Implicated in Both DNA Binding and Protein Folding*

Received for publication, May 5, 2004, and in revised form, July 26, 2004
Published, JBC Papers in Press, July 29, 2004, DOI 10.1074/jbc.M405008200

Jie Lin Liu, Pascal Rigolet, Shuo-Xing Dou‡, Peng-Ye Wang‡, and Xu Guang Xi§

From the Laboratoire de Biotechnologies et Pharmacologie Génétique Appliquée CNRS UMR 8113, Ecole Normale Supérieure (ENS) Cachan, 61 avenue du Président Wilson, 94235 Cachan cedex, France and ‡Laboratory of Soft Matter Physics, Beijing National Laboratory for Condensed Matter Physics, Institute of Physics, Chinese Academy of Sciences, Beijing 100080, China

The RecQ family of DNA helicases has been shown to be important for the maintenance of genomic integrity. Mutations in human RecQ genes lead to genomic instability and cancer. Several RecQ family of helicases contain a putative zinc finger motif of the C_4 type at the C terminus that has been identified in the crystalline structure of RecQ helicase from *Escherichia coli*. To better understand the role of this motif in helicase from *E. coli*, we constructed a series of single mutations altering the conserved cysteines as well as other highly conserved residues. All of the resulting mutant proteins exhibited a high level of susceptibility to degradation, making functional analysis impossible. In contrast, a double mutant protein in which both cysteine residues Cys^{397} and Cys^{400} in the zinc finger motif were replaced by asparagine residues was purified to homogeneity. Slight local conformational changes were detected, but the rest of the mutant protein has a well defined tertiary structure. Furthermore, the mutant enzyme displayed ATP binding affinity similar to the wild-type enzyme but was severely impaired in DNA binding and in subsequent ATPase and helicase activities. These results revealed that the zinc finger binding motif is involved in maintaining the integrity of the whole protein as well as DNA binding. We also showed that the zinc atom is not essential to enzymatic activity.

The transient formation of single-stranded DNA (ssDNA)[1] intermediate is essential to all aspects of DNA metabolism including DNA replication, recombination, and repair. The unwinding and separation of the individual strands of double-stranded DNA (dsDNA) is catalyzed by a class of specialized enzymes known as DNA helicases (1, 2). These enzymes function as molecular motors that use the energy released from the hydrolysis of ATP to unwind and translocate along DNA in a sequential fashion (3–5). These ubiquitous enzymes have been identified in all living organisms from virus to human. It appears that they evolved from a common ancestor (6).

The RecQ helicase family is critical to the maintenance of genomic stability in prokaryotes and eukaryotes (7). Mutations of RecQ genes can lead to genomic instability and several human diseases including the Bloom and Werner syndromes (8). Recently, it has been shown that the tumor suppressor BRCA1-associated protein, BACH1, which shares homologies with other members of the RecQ family, possesses ATPase and helicase activities (9). The mutant BACH1 participates directly in breast and ovarian cancer development (9). The RecQ helicase from *Escherichia coli* is the prototype helicase of this family (10) and has been shown to initiate homologous recombination as well as suppress illegitimate recombination (11, 12).

The RecQ helicase family members contain a helicase domain characterized by the presence of seven so-called "helicase" motifs necessary for using energy derived from ATP binding and hydrolysis to unwind DNA (13, 14). Sequence analyses revealed that all of the RecQ helicases contain a C-terminal extension that can be further divided into two domains (Fig. 1*A*): the HRDC domain (helicase and RNase D C-terminal), which functions as an auxiliary DNA-binding domain (15, 16), and the RecQ C-terminal domain that contains a conserved CX_nCX_nCDXC motif (in which *X* is any amino acid) among the RecQ family of helicases (Fig. 1*A*) of which the function is still not clear. Recently, the three-dimensional structure of a C-terminal truncated form of RecQ helicase has revealed that the enzyme folds into four subdomains, two of which combine to form the helicase region, whereas the others form zinc binding (Fig. 1*B*) and winged-helix motifs (17). The zinc atom is bound by four conserved cysteine residues located at a platform composed of α-helices17 and 18. The cysteine residue Cys^{380} (labeled as C1) is located at the beginning of the α-helix 17. Cys^{400} (labeled as C3) and Cys^{403} (labeled as C4) are at the beginning and the middle of the α-helix 18, respectively, whereas Cys^{397} (labeled as C2) is located in the loop linking the two helices (Fig. 1*B*). In addition, the zinc finger motif may be further stabilized by three hydrogen bonds formed among the conserved residues of phenylalanine (Phe^{374}), arginine (Arg^{381}), and asparagine (Asp^{401}) (Fig. 1*B*). Previous studies have established that the zinc finger domains and other metal-binding protein domains are involved in diverse functions including protein-DNA interactions, protein folding, and protein-protein interactions (18). To elucidate the roles played by the zinc atom and the zinc finger motif in RecQ helicase, mutant RecQ molecules were engineered by site-directed mutagenesis within the zinc finger motif. Biochemical characterizations of these mutants showed that the zinc binding motif is essential to efficient

* This work was supported by the Centre National de la Recherche Scientifique (CNRS), the National Natural Science Foundation of China, and the Innovation Project of the Chinese Academy of Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 33-1-47-40-68-92; Fax: 33-1-47-40-76-71; E-mail: xi@lbpa.ens-cachan.fr.

[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; HRDC, helicase and RNase D C-terminal; FRET, fluorescence resonance energy transfer; PAR, 4-(2-pyridylazo)resorcinol disodium salt; mantATP, 2′(3′)-*O*-(*N*-methlanthraniloyl)adenosine 5′-triphosphate; DTT, dithiothreitol; CD, circular dichroism.

DNA binding and stabilization of the three-dimensional structure of RecQ molecules.

MATERIALS AND METHODS

[α-^{32}P]ATP was obtained from Amersham Biosciences. 4-(2-Pyridylazo)resorcinol disodium salt (PAR), EDTA, 2-mercaptoethanol, ATP, and α-chymotrypsin were obtained from Sigma. Chelex® 100 resin was purchased from Bio-Rad. The *N*-methylanthraniloyl derivatives of adenine nucleotides were synthesized according to Hiratsuka (19) and purified on DEAE-cellulose using a gradient of triethylammonium bicarbonate.

Expression of Wild-type Enzyme and Construction of the Zinc Finger Mutant of RecQ Helicase—The *E. coli* RecQ helicase containing a N-terminal His tag was expressed in *E. coli* and purified by nickel-chelating and anion-exchange chromatography as described previously (20). Mutations were made by a two-step polymerase chain reaction method (21). The outside primers were QFN (5′-GGAATTCCATATG-GTGAATGTGGCGCAGGCGGAAGTGTTG-3′) and QRX (5′-CCGCTC-GAGCTACTCTTCGTCATCGCCATCAACATG-3′). The primers used to introduce mutations are QMF1 (5′-CAGGAGCCGAACGGGAACAAC-GATATCTGC-3′) and QMF2 (5′-GCAGATATCGTTGTTCCCGTTCG-GCTCCTG-3′). The mutations resulting in coding changes are *underlined*. The mutant polymerase chain reaction products were digested with NdeI and XhoI, and the 1.85-kb fragment was subcloned into pET-15b. Following the mutagenesis, the entire gene was sequenced to ensure that no additional mutations were created. The primers used to construct other mutants that could not be purified to homogeneity are not shown.

Quantification of Zinc Ion Bound to E. coli RecQ Helicase—The zinc content of the wild-type and the zinc finger mutant of RecQ helicase was measured by the PAR assay as described by Hunt *et al.* (22). PAR has a low absorbance at 500 nm in the absence of zinc ion. However, in the presence of zinc ion, the absorbance at 500 nm increases dramatically due to the formation of the $PAR_2{\cdot}Zn^{2+}$ complex. To more precisely quantify the zinc content of both wild-type and mutant helicases, all of the buffers were treated with Chelex 100 resin. The enzymes were dialyzed against the EDTA-free Chelex-treated buffer passed over a 10-cm column of Chelex-100 and reconcentrated. To facilitate zinc release, the enzymes (1 nmol in a volume of 20 μl) were first denatured with Chelex-treated 7 M guanidine HCl and then transferred to a 1-ml cuvette and the volume was adjusted to 0.9 ml with buffer A (20 mM Tris-HCl at pH 8.0, 150 mM NaCl). PAR was added into the cuvette for a final concentration of 100 μM. The absorbance at 500 nm was measured. The quantity of zinc ion was determined from a standard curve of $ZnCl_2$ samples in a range of concentrations using the sample preparation procedure as described above with the RecQ helicase omitted. The zinc ion concentration was also determined using the absorbance coefficient for the $(PAR)_2{\cdot}Zn^{2+}$ complex ($\epsilon_{500\ nm} = 6.6 \times 10^4\ M^{-1}\ cm^{-1}$). The zinc-demetalated RecQ helicase was obtained by dialysis of purified RecQ helicase against buffer B (10% glycerol, 300 mM NaCl, 20 mM Tris-HCl at pH 8.0, 10 mM EDTA, 1 mM DTT) overnight at 4 °C (17).

Fluorescence Measurements—Fluorescence spectra were determined using Fluoro Max-2 spectrofluorimeter (Jobin Yvon, Spex Instruments S.A., Inc.) as described by Levin *et al.* (23). Some results were further confirmed by using PiStar-180 spectrometer (Applied Photophysics). In a $10 \times 10 \times 40\text{-mm}^3$ quartz cuvette, 0.5 μM RecQ protein in 1 ml of reaction buffer was excited at 280 nm and the fluorescence emission was monitored at 350 nm. mantATP binding to protein was measured by exciting the RecQ protein at 280 nm and measuring the fluorescence of mantATP at 440 nm because of FRET.

The observed fluorescence intensity, F_{obs}, was corrected for inner filter and sample dilution effect according to Equation 1,

$$F = F_{obs} \times 10^{[(A_{EX}+A_{EM})/2]} \times \frac{V_0 + V_i}{V_0} \quad \text{(Eq. 1)}$$

where F is the corrected fluorescence intensity, V_0 is the initial sample volume, V_i is the total volume of titrant added, and A_{EX} and A_{EM} are the absorbance values of the solution at excitation and emission wavelengths, respectively. The statistical thermodynamic model used to describe the ATP-RecQ complex formation is based on the observation that RecQ helicase has one ATP-binding site (17). Therefore, the one-to-one binding model was used to establish the mantATP binding to RecQ. We have chosen the macroscopic interaction constant defined in Equation 2,

$$K_d = [E_f][D_f]/[C] \quad \text{(Eq. 2)}$$

where E_f represents the concentration of free enzyme, D_f represents the concentration of free mantATP, K_d is the equilibrium interaction constant, and C is the concentration of the ATP-RecQ complex formed.

The concentrations of total enzyme, E_T, and total mantATP, D_T, can be written in terms of E_f and D_f as shown in Equations 3 and 4.

$$E_T = E_f + C \quad \text{(Eq. 3)}$$

$$D_T = D_f + C \quad \text{(Eq. 4)}$$

From Equations 2–4, the expression of C is obtained as shown in Equation 5.

$$C = \frac{(D_T + E_T + K_d) - \sqrt{(D_T + E_T + K_d)^2 - 4E_TD_T}}{2} \quad \text{(Eq. 5)}$$

The value of K_d can be obtained by fitting the fluorescence intensity values to Equation 6,

$$F = F_S + f_DD_T + f_CC \quad \text{(Eq. 6)}$$

where F_S is the starting fluorescence of the reaction mixture, f_D is the fluorescence coefficient of free mantATP, and f_C is the fluorescence coefficient of complex formed.

Limited Proteolytic Digestion—The wild-type, mutant, and Zn^{2+}-extracted RecQ helicases at a concentration of 15 μM and in a total volume of 30 μl were digested with α-chymotrypsin (Sigma) for 2 and 10 min at room temperature. The ratio between the protease and RecQ was 1:100 for each helicase. Aliquots (15 μl corresponding to 1 μg of protein) from the reaction were quenched with 15 μl of gel-loading buffer (250 mM Tris-HCl at pH 6.8, 3.4% SDS, 1.1 M 2-mercaptoethanol, 20% glycerol, and 0.01% bromphenol blue). The samples were boiled for 2 min and then analyzed by SDS-PAGE gel.

Agarose Gel Mobility Shift Assay—Gel mobility shift assays were performed as described previously (24). The reaction mixture (25 μl) contained 20 mM Tris-HCl at pH 7.6, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 2 mM $MgCl_2$, and 1 pmol (molecules) of the linearized DNA substrate (3 kb). The reaction was allowed to proceed for 10 min on ice and was terminated by the addition of 10 μl of loading buffer (80% glycerol, 0.1% bromphenol blue). The complexes were separated by electrophoresis through 0.8% agarose gel in TAE (Tris-acetate-EDTA) buffer at 100 V for 1.5 h and were visualized by ethidium bromide staining.

DNA Binding Assay under Equilibrium Condition—The binding of RecQ helicase to DNA was analyzed by fluorescence anisotropy using a Beacon 2000 fluorescence polarization spectrophotometer (PanVera) as described previously (24). An appropriate quantity of fluorescein-labeled ssDNA or dsDNA was added to a standard titration buffer (150 μl of total volume) in a temperature-controlled cuvette at 25 °C. The anisotropy of the fluorescein-labeled DNA was measured successively until it stabilized. An appropriate quantity of RecQ helicase then was added. The anisotropy then was measured continuously until it reached a stable plateau. To determine the concentration of the helicase-DNA complex, fluorescence signals observed in these RecQ helicase titrations were subtracted by those observed in the absence of enzyme. The increase in sample volume during the titration was taken into account in the analysis of the data. The reported values represent the averages of two to three measurements. The ssDNA used in this assay is a 36-mer 5′-fluorescein-labeled synthetic oligonucleotide (5′-F-AGACCC-TTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′). The dsDNA was generated with its complementary sequence (5′-ACTGCTA-GAGATTTTCCACACTGACTAAAAGGGTCT-3′).

Circular Dichroism Analysis—All of the spectra were collected in buffer C (20 mM Tris-HCl, 25 mM NaCl, 0.1 mM EDTA) in a 0.2-mm cuvette at 20 °C on a Jobin-Yvon V dichrograph spectrophotometer. The protein samples were rigorously dialyzed against buffer C before measurement. All of the spectra were recorded with a 0.2-nm step as follows. Buffer C only was added to a single chamber, and the spectrum was recorded. The protein sample then was added to the same chamber, and the spectrum was recorded. The protein-only spectrum then was obtained by subtracting the free protein spectrum from the mixture spectrum. The measurement results are reported as mean residue ellipticity (θ) (degrees per square centimeter per decimole). The ATPase activity, helicase activity, and protein concentration were determined before and after each measurement.

ATPase Assay and Determination of K_i for mantATP—The ATPase activity was determined in an assay by measuring the radioactive $^{32}P_i$ liberated during hydrolysis (25). The measurement was carried out at 37 °C in a reaction mixture containing 1.5 μM (nucleotide) of heat-

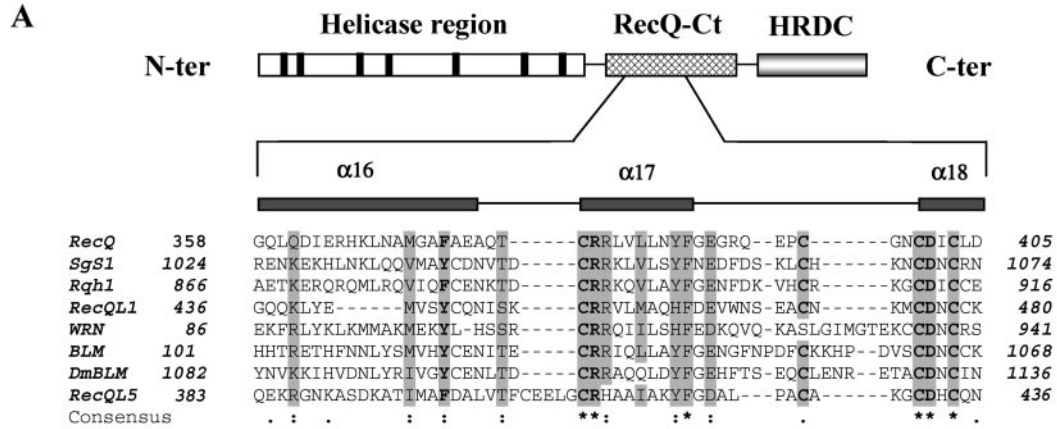


FIG. 1. *A*, schematic diagram of *E. coli* RecQ conserved regions and amino acid sequence alignment of the conserved putative C_4-type zinc finger motif among the RecQ family helicases. The multiple alignments were performed with the program ClustalW and refined manually. The numbers at the beginning and end of each sequence correspond, respectively, to positions of the first and the last amino acid residues. Highly conserved amino acid residues are *shadowed* in *gray*. In *boldface* are the four conserved cysteine residues and the fully conserved arginine, aspartic acid, and aromatic residues involved in three very important hydrogen bonds. The consensus sequence was generated by ClustalW. The protein accession numbers used by the NCBI are as follows: RecQ, P15043 (*E. coli*); SgS1, P35187 (*Saccharomyces cerevisiae*); Rqh1, Q09811 (*Schizosaccharomyces pombe*); RecQL1, NP_002898/P46063 (*Homo sapiens*); WRN, NP_000544/Q14191 (*H. sapiens*); BLM, A57570/P54132 (*H. sapiens*); DmBLM, Q9VGI8 (*H. sapiens*); and RECQL5, NP_004250 (*H. sapiens*). Secondary structure elements of RecQ appear with *boxes* designing the α-helices. *B*, conserved residues in the zinc finger motif. The positions of the four conserved cysteine residues, Cys^{380} (labeled as *C1*), Cys^{397} (labeled as *C2*), Cys^{400} (labeled as *C3*), and Cys^{403} (labeled as *C4*), are drawn in *gray* as they appear in the structure of the *E. coli* RecQ core. The zinc atom is in *dark gray*. Labels for the secondary structures are also indicated. The conformation of the zinc finger motif is obviously stabilized through three hydrogen bonds between NH2 Arg^{381} and OD2 Asp^{401}, between NE Arg^{381} and OD1 Asp^{401} and between NH1 Arg^{381} and the main chain atom O of Phe^{374}, involving highly conserved residues in the RecQ family (NH2, OD2, OD1, NE, and NH1 represent the positions of the atoms in the molecule; NH, N heta; OD, O delta; NE, N epsilon). These three residues drawn in *light gray* could contribute to the relative positioning of the helices α16, α17, and α18.

denatured HindIII-cut pGEM-7Zf linear DNA (3 kb) or ssDNA (60-mer) at the indicated concentration of ATP. The reactions were initiated by the addition of RecQ helicase into 100 μl of reaction mixture and stopped by pipetting 80 μl of aliquots from the reaction mixture every 30 s into a hydrochloric solution of ammonium molybdate. The liberated radioactive $^{32}P_i$ was extracted with a solution of 2-butanol-benzene-acetoneammonium molybdate (750:750:15:1) saturated with water. An aliquot of the organic phase was counted in 6 ml of Aquasol.

The competitive inhibition constant, K_i, for mantATP was determined by measuring the ATPase rate as a function of mantATP concentration and fit to Equation 7,

$$v = \frac{[\mathrm{ATP}]k_{\mathrm{cat}}}{[\mathrm{ATP}] + K_m^{\mathrm{ATP}}\left(1 + \frac{[\mathrm{mantATP}]}{K_i}\right)} \qquad \text{(Eq. 7)}$$

where v is the initial ATPase rate, k_{cat} is the catalytic constant, and K_m^{ATP} is the K_m for ATP.

Helicase Assay—An unwinding assay was performed using the Beacon 2000 fluorescence polarization instrument (26). An appropriate quantity of fluorescein-labeled duplex oligonucleotide was added to the helicase-unwinding buffer (150 μl of total volumes) in a temperature-controlled cuvette. The anisotropy was measured successively until it stabilized. Helicase then was added. When the higher anisotropy value became stable, the unwinding reaction was initiated by the rapid addition of ATP solution to give a final ATP concentration of 1 mM. The decrease of the anisotropy was recorded every 8 s until it became stable. The unwinding buffer contained 25 mM Tris-HCl, pH 8, 30 mM sodium chloride, 3 mM magnesium acetate, and 0.1 mM DTT. The data were fit to the exponential equation: $A = A_0\exp(-k_{obs}t)$, where A is the anisot-

TABLE I
Comparison of the basic properties of wild-type, double mutant, and zinc-demetalated helicases

Helicase	[Zn^{2+}]/[protein]	Stokes radius	Catalysis[a]		DNA binding[b]		ATP binding[c]
			k_{cat}(ATPase)	k_{cat}(Helicase)	K_d (ssDNA)	K_d (dsDNA)	K_d (mantATP)
			s^{-1}		nM		μM
Wild type	0.98 ± 0.11	35.2	36.1 ± 1.1	$2 \pm 0.058 \times 10^{-2}$	52 ± 1.2	81 ± 1.1	43.6 ± 1.1
Mutant (C397N/C400N)	0.02 ± 0.015	35.6	0	0	ND[b]	ND[b]	56.8 ± 1.3
Zinc-demetalated	0.28 ± 0.05	34.5	34.6 ± 1.2	$1.8 \pm 0.089 \times 10^{-2}$	65 ± 2.1	98 ± 1.3	47.6 ± 0.9

[a] Determined from Fig. 4.
[b] Determined from Fig. 5*B*. ND, non detectable, *i*.e., no DNA binding detected up to 3 μM protein.
[c] Determined from Fig. 3*D*.

ropy amplitude at time t, A_0 is a constant, and k_{obs} is the observed rate constant.

Size Exclusion Chromatography—Size exclusion chromatography was performed at 18 °C using an fast protein liquid chromatography system (ΔKTA, Amersham Biosciences) on a Superdex 200 (analytical grade) column equilibrated with elution buffer. Fractions of 0.5 ml were collected at a flow rate of 0.4 ml/min, and the absorbance was measured at 280 and 260 nm. The proteins used to prepare a calibration curve were as follows: thyroglobulin (bovine), 670 kDa; γ-globulin (bovine), 443 kDa; apoferritin, 158 kDa; bovine serum albumin, 66 kDa; ovalbumin, 44 kDa; and myoglobin, 17 kDa. Gel filtration chromatography was performed using a standard elution buffer (50 mM Tris-Cl at pH 7.5, 300 mM NaCl, 0.1 mM EDTA) with 1 mM ATP and 1 mM $Mg(OAc)_2$. 5 μM RecQ protein was incubated in the elution buffer with ATP and $MgCl_2$ for 2 min prior to injection onto the column.

RESULTS

Rationale for Site-directed Mutagenesis of the Zinc Finger Motif of RecQ Helicase—Site-directed mutagenesis was used to explore the functional significance of the putative zinc finger motif of the RecQ helicase. As shown in Fig. 1, six residues (Cys^{380}, Arg^{381}, Cys^{397}, Cys^{400}, Asp^{401}, and Cys^{403}) within this region are totally conserved among RecQ family members. Mutant RecQ enzymes were first engineered with single alanine or serine substitution at the position of each of the four cysteines. In addition, a careful analysis of the three-dimensional structure of the enzyme revealed that the conformation of the zinc finger motif is obviously stabilized through three hydrogen bonds among the highly conserved residues, Arg^{381}, Asp^{401}, and Phe^{374} (Fig. 1). These interactions may contribute to the relative positioning of the helices α_{16}, α_{17}, and α_{18} in the zinc finger motif (Fig. 1*B*). The residues Arg^{381} and Asp^{401} were thus replaced by asparagine and alanine, respectively. We found that all of these mutants displayed a high level of proteolysis and could not be purified to homogeneity, making functional studies impossible.

To disturb mildly the zinc finger motif, we decided to simultaneously substitute C2 and C3 with asparagine. This residue was chosen, because its side chain could conceivably act as ligand to zinc ion (27). Moreover, the single mutation with asparagine may have only a modest effect, whereas a double mutation should lead to a zinc finger motif that no longer binds to zinc ion. The expression analysis of these mutants shows that both single point mutants (C397N and C400N) became the inclusion body and could not be purified. However, ~50% of the expressed double mutant RecQ helicase (C397N/C400N) is soluble and is purified to homogeneity. Based on Sypro Orange-stained SDS-PAGE and electrospray mass spectrometry analyses, the purity of this modified RecQ helicase was determined to be >92%.

The ability of the purified double mutant enzyme to bind zinc was determined using the PAR assay. Although 0.98 mol of zinc were bound to 1 mol of the wild-type protein, only 0.02 mol of zinc was bound to 1 mol of the double mutant enzyme (Table I). These results are consistent with both the prediction from the primary amino acid sequence and the study of the three-dimensional structure of RecQ helicase, indicating that the enzyme contains a functional zinc-binding domain.

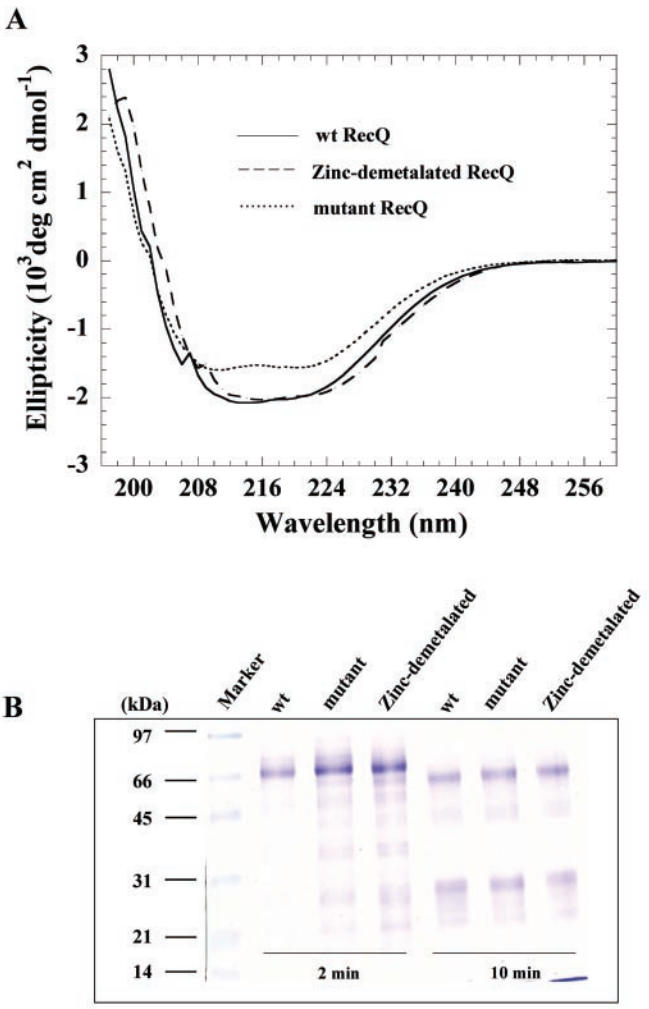


FIG. 2. *A*, normalized CD spectra of wild-type, mutant and zinc-demetalated helicases. The experiments were performed as described under "Materials and Methods." Protein concentrations were 5 μM for wild-type (*wt*) and zinc-demetalated helicases and 3 μM for mutant helicase. All of the spectra are given in units of mean residue ellipticity. *B*, results of limited proteolysis for wild-type, mutant, and demetalated RecQ helicases. In these experiments, 15 μM of enzyme was digested with chymotrypsin (0.15 μM) as described under "Materials and Methods." Samples (1 mg for each sample) were quenched at the indicated times, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. The molecular masses of standards (in kDa) are shown on the *left*.

To assess whether zinc ion is required for RecQ helicase function, zinc ion was extracted from wild-type enzyme by extensive dialysis against the dialysis solution (20 mM Tris-HCl at pH 7.9, 150 mM NaCl, 1 mM DTT, 5% glycerol) containing 15 mM EDTA. The obtained zinc-extracted wild-type helicase was termed zinc-demetalated RecQ helicase. Therefore, three preparations of enzymes were used for the following studies: the wild-type helicase; the double mutant helicase; and the zinc-demetalated helicases.

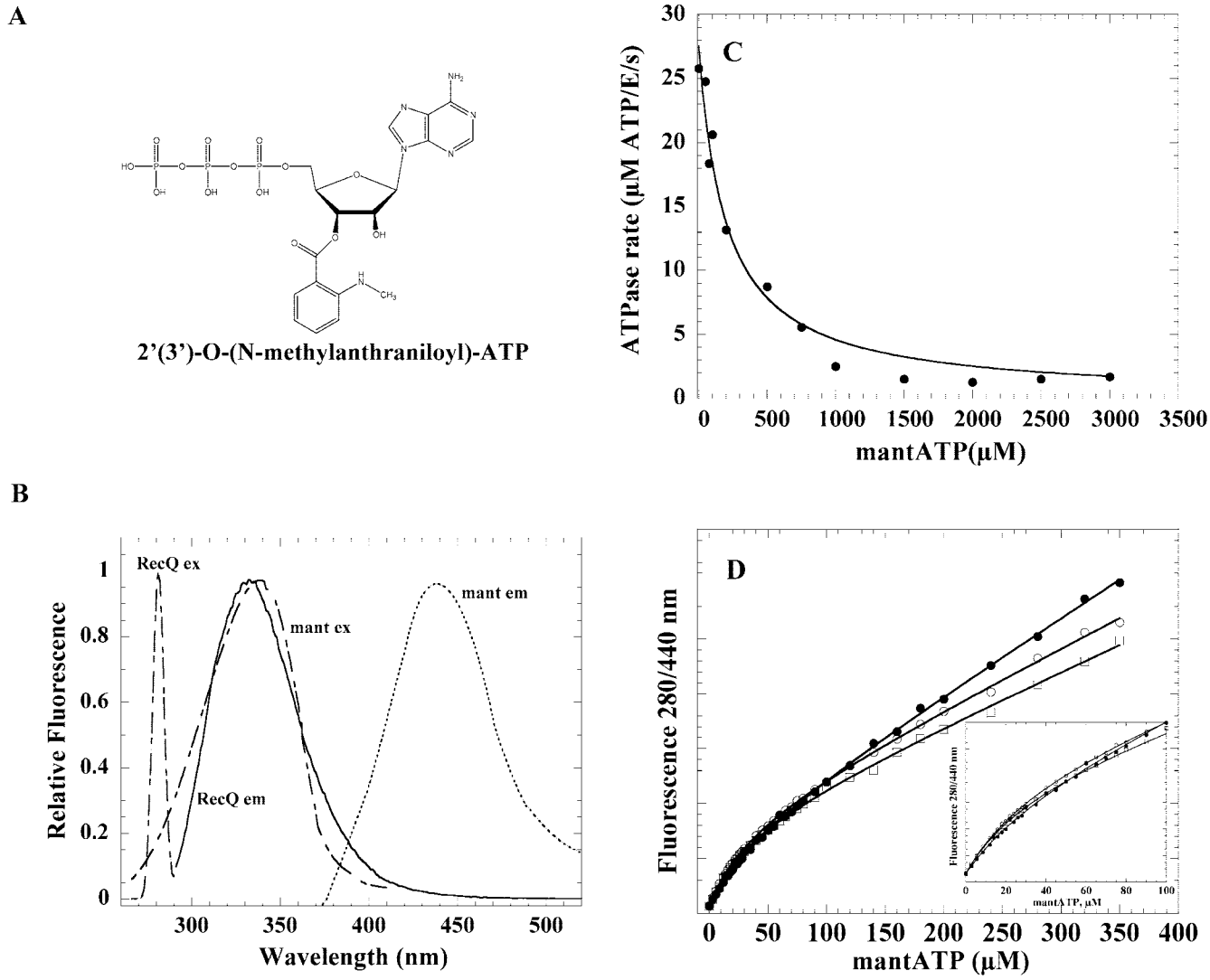


FIG. 3. **Binding equilibrium of mantATP to wild-type and mutant RecQ helicases.** *A*, structure of mantATP. *B*, the overlap of the RecQ emission spectrum (λ_{ex} = 280 nm) and the mantATP excitation spectrum (λ_{em} = 440 nm). The excitation wavelength for the mantATP emission spectrum is λ_{ex} = 345 nm. *C*, determination of the competitive inhibition constant (K_i) of mantATP. The ATPase reaction was initiated by adding 2 mM ATP to a mixture of 10 nM RecQ protein, 6 μM ssDNA (nucleotide, 60-mer), and an increasing concentration of mantATP. The initial ATPase rate was measured, plotted *versus* inhibition concentration, and fitted to Equation 7 using K_m = 250 μM (determined from Fig. 4*A*). *D*, changes in fluorescence intensity when 0.5 μM wild-type (*squares*), double mutant (*closed circles*), and zinc-demetalated (*open circles*) helicases were titrated with mantATP. The *inset* shows the titration curves obtained with low concentrations of mantATP. *Solid lines* represent the best fit of the data to Equations 5 and 6.

Structural Characterization of the Mutant Protein—It is well established that metal ions have important effects on secondary structure formation. We were wondering whether the purified double mutant RecQ helicase (C397N/C400N) has a normal structure. We first performed CD studies to check the effect of mutation on the secondary structure of the protein. The replacement of both cysteines with asparagines leads to a subtle modification in the secondary structure of RecQ helicase as judged from the CD spectra (Fig. 2*A*), suggesting that the double mutation induced slightly a local conformational change. In contrast, no significant CD spectra change was observed with the zinc-demetalated RecQ helicase. We next performed the limited proteolysis experiments on wild-type, mutant, and zinc-demetalated helicases under the same experimental conditions. Fig. 2*B* shows that both mutant and zinc-demetalated proteins display similar proteolysis-resistant patterns as the wild-type RecQ helicase, suggesting that the three proteins assume similar structures. These results have been further confirmed by size-exclusion chromatographic studies. Because both ultracentrifugation analyses and three-dimensional structure studies have shown that RecQ helicase takes a globular shape (17, 20), the apparent molecular masses of wild-type, double mutant, and zinc-demetalated helicases were used to estimate their Stokes radii, thus determining their overall spherical shapes. As shown in Table I, these helicases have similar Stokes radii. Taken together, these results indicate that these enzymes (wild-type, double mutant, and zinc-demetalated helicases) possess similar three-dimensional structures.

Zinc Finger Motif Is Not Required for ATP Binding—We next studied the binding of ATP to the double mutant using a fluorescent nucleotide analogue (Fig. 3*A*, *mantATP*). The spectral properties of the mant fluorophore are ideally suited for monitoring nucleotide binding by FRET from the intrinsic tryptophan fluorescence of RecQ to the mant fluorophore bound at the ATP-binding site. The comparison between the fluorescence excitation and emission spectra of RecQ and those of mantATP showed that the emission spectrum of RecQ overlaps with the excitation spectrum of mantATP (Fig. 3*B*), indicating the possibility of FRET.

Although the three-dimensional structure of RecQ helicase has shown that the enzyme has only one ATP-binding site, we want to first determine whether mantATP binds to the same ATP-binding site of RecQ. For this purpose, the competitive inhibition constant, K_i, for mantATP was determined by measuring the ATPase rate of the helicase as a function of mantATP concentration (Fig. 3*C*). The data were fit to a competitive inhibition equation with a K_i of 85 μM, indicating that mantATP binds competitively to the ATP-binding site. The apparent K_d values for the wild-type, double mutant, and zinc-demetalated helicases were measured using standard fluorimetric titration methods. From the titration curves as shown in Fig. 3*D*, the apparent K_d values determined are 43.6 μM for the wild-type helicase, 56.8 μM for the double mutant helicase, and 47.6 μM for the zinc-demetalated helicase, revealing that neither the zinc finger nor zinc ion is essential to ATP binding. This study sheds light not only on ATP binding but also on folding of the mutant protein. The fact that the double mutant protein binds ATP normally indicates that the overall three-dimensional structure of the double mutant was not altered.

It is also interesting to note that the apparent mantATP binding constant determined in this study (K_d = 43.6 μM) is lower than that of ATP determined from bulk ATPase (250 μM, Fig. 4*A*) and helicase assays (200 μM) (28). However, the constant is close to the value determined from a single molecule assay (50 μM).[2] The discrepancy among these values may be due to different experimental approaches and different experimental conditions. Regardless, the FRET method used in this study for determining the ATP binding constant is a more direct approach compared with that through the measurement of enzymatic activities.

The Zinc Finger Motif Is Important for DNA-dependent ATPase and Helicase Activities—Previous studies have shown that the ATPase activity of wild-type RecQ is greatly stimulated by ssDNA and, to a less extent, by dsDNA (10). To test the role of the zinc finger motif in ATP hydrolysis, the ATPase activities of the double mutant RecQ and zinc-demetalated helicases were compared with the wild-type enzyme in the presence of ssDNA (60-mer). The mutant protein was severely compromised in DNA-dependent ATPase activities, exhibiting no detectable ATPase activities when compared with wild-type and zinc-demetalated helicases (Fig. 4*A* and Table I). Similar results were obtained with 3-kb linear plasmid DNA (results not shown).

The above observations demonstrate that the integrity of the zinc finger motif of RecQ is important for ATPase activity. We reasoned that the helicase activity should also be affected when the zinc binding motif is altered. As expected, although the wild-type enzyme unwinds the duplex DNA substrate completely within 5 min, no helicase activity was detectable under the same conditions for the double mutant protein (Fig. 4*B* and Table I). These results demonstrate that the zinc finger motif is needed for both ATPase and helicase activities.

The Zinc Finger Motif Is Required for Stable DNA Binding—To further understand the molecular basis of the observed decrease in ATPase and helicase activities for the double mutant protein, the effect of the mutation on DNA binding was first investigated using the gel mobility shift assay under the best experimental condition that we determined previously (25). Whereas a shift of 3 kb of DNA was observed as wild-type and zinc-demetalated helicase concentrations increases (Fig. 5*A*, *lanes 2–5*), no protein-DNA complexes were observed under the same condition for mutant enzyme (Fig. 5*A*, *lanes 6* and *7*),

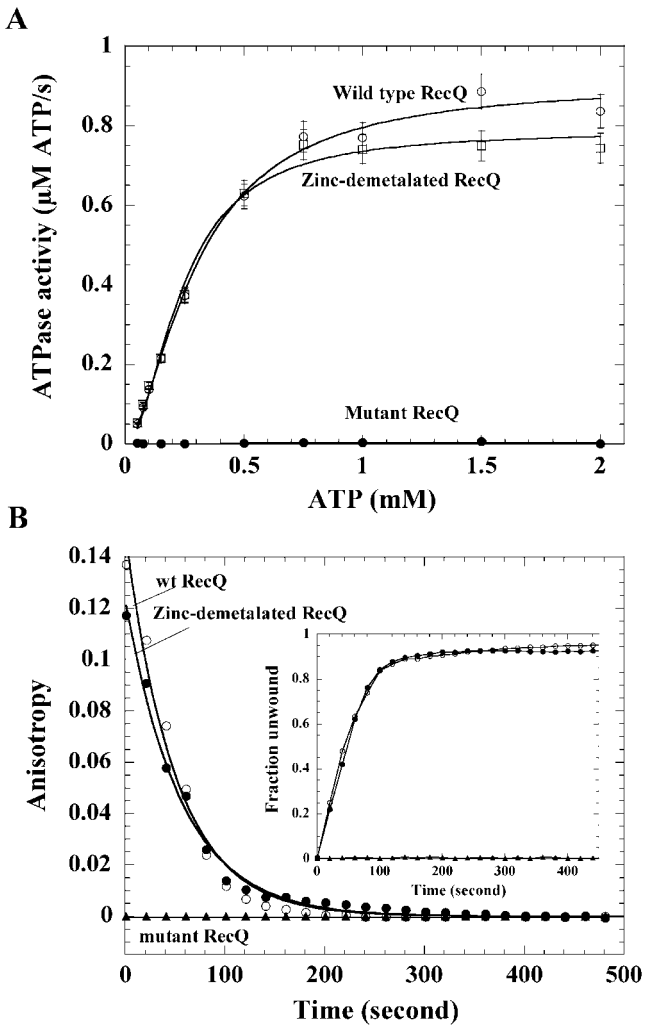


FIG. 4. *A*, ATPase activity of RecQ helicase as a function of ATP concentration. Experiments were performed at 37 °C with 10 nM protein for each helicase preparation and 6 μM ssDNA (nucleotide, 60-mer oligonucleotide). *B,* kinetics of RecQ helicase-mediated DNA unwinding. 50 nM RecQ helicases (wild-type (*wt*), mutant, and zinc-demetalated protein) were incubated with 2 nM DNA substrate (36 bases in duplex length). DNA unwinding was initiated by the addition of 1 mM ATP at 25 °C. The time courses from the wild-type (*open circles*), zinc-demetalated (*closed circles*), and double mutant (*triangle*) RecQ helicases were fit to the exponential equation: $A = A_0\exp(-k_{obs}t)$. The fraction of unwound DNA substrate is shown in the *inset*.

suggesting that the DNA binding ability of mutant protein is completely compromised. However, it is still possible that the mutant protein displays weak DNA binding activities that cannot be detected by the electrophoretic mobility shift assay method due to the physical separation of free DNA from the protein. Therefore, we measured the DNA binding activities of both wild-type and mutant protein under equilibrium conditions using fluorescence anisotropy assays as performed previously (24). Fig. 5*B* shows that both wild-type and zinc-demetalated helicases display a high affinity for the 5′-fluorescein-labeled ssDNA. The apparent K_d values of both wild-type and zinc-demetalated helicases determined from this experiment are very close to each other (Table I). In contrast, no DNA binding activity is detectable for the mutant helicase, even at a high protein concentration (3 μM). Similar results were obtained with dsDNA (Fig. 5*B*, *inset*, and Table I). Together, these results demonstrate that the zinc finger motif plays an essential role in DNA binding.

Role of the Zinc Atom—The zinc ion may play roles both in

[2] M.-N. Dessinges, T. Lionnet, X. G. Xi, D. Bensimon, and V. Croquette, unpublished observation.

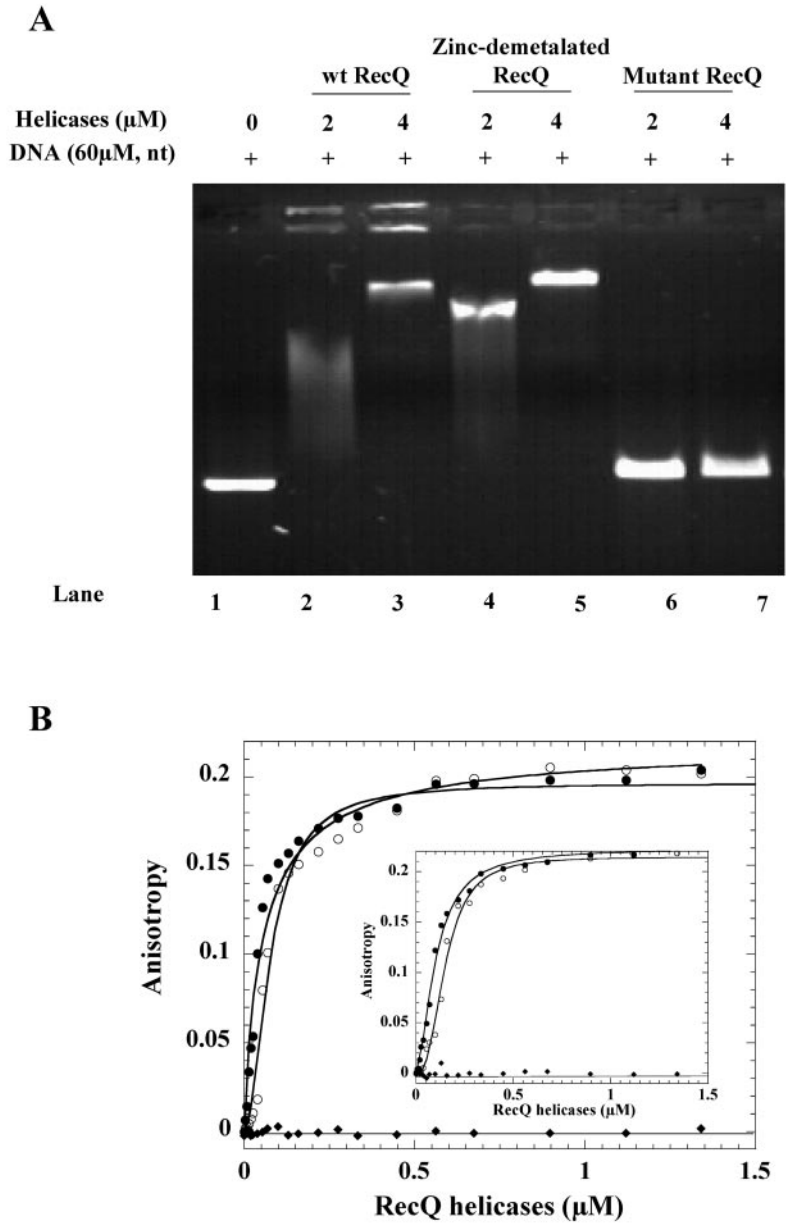


FIG. 5. *A*, DNA binding affinity of wild-type (*wt*), double mutant, and zinc-demetalated helicases detected by a gel mobility shift assay. The different helicases were bound to DNA at the indicated concentrations for 30 min at 25 °C in 20 mM Tris acetate at pH 7.5. All of the complexes were analyzed by electrophoresis through a native 0.8% agarose gel as described under "Materials and Methods." The concentration of DNA was 30 μM (nucleotide, *nt*). *B*, binding equilibrium of different RecQ helicases to DNA. Binding of wild-type (*closed circles*), mutant (*rhombuses*), and zinc-demetalated (*open circles*) helicases to ssDNA (36-mer) were determined by measuring the changes in fluorescence anisotropy as described under "Materials and Methods." Varying amounts of the proteins were added to a binding assay buffer containing 1 nM DNA. Fluorescence anisotropy data were fit to a hyperbola using KaleidaGraph software. Given in the *inset* are the titration curves of the different RecQ helicases with 36-mer dsDNA. The association constant values obtained from the best fit are summarized in Table I.

structure and in enzymatic catalysis of RecQ. To better define the role of zinc atom, we performed three kinds of experiments. We first compared the properties of both wild-type and zinc-demetalated RecQ helicases. For this purpose, the zinc-demetalated RecQ helicase was obtained by EDTA extraction protocol (17) and analyzed in parallel with wild-type and mutant enzymes. As can be seen from Figs. 2–5 and Table I, the zinc-demetalated RecQ helicase displays very similar properties with wild-type helicase in terms of the full tertiary structure of holoenzyme, DNA binding, ATPase, and helicase activities. We next investigated the effect of the zinc atom on ATPase activity of both wild-type and zinc-demetalated RecQ helicases with increasing zinc concentrations. The results revealed that the zinc atom does not significantly influence the activities of both wild-type and zinc-demetalated RecQ helicases (Fig. 6*A*). Finally, the stabilities of the wild-type and zinc-demetalated helicases were assessed by measuring ATPase activity at different temperatures ranging from 25 to 54 °C. Fig. 6*B* shows that both enzymes display similar thermostability except at 54 °C where the zinc-demetalated enzyme displays a modest decrease in K_{cat} and an increase in K_m. The similar thermostabilities of both enzymes indicate that zinc ion is not essential to protein stability. Taken together, these observations indicate that the zinc atom is not absolutely required for enzymatic catalysis, DNA binding, or stabilization of the protein conformation.

DISCUSSION

The three-dimensional structure of the *E. coli* RecQ helicase (17) reveals the existence of a zinc binding motif. In this study, the importance of this motif to RecQ helicase function was highlighted by the loss-of-function mutations at highly conserved residues within the zinc finger motif by site-directed mutagenesis. Consistent with the notion that zinc fingers are structural modules of a major ubiquitous class of DNA binding motifs (29), our data illustrated that a mutation at the zinc finger, which drastically reduces zinc binding, abrogates DNA binding to the helicase and leads to decreased ATPase and helicases activities. Furthermore, this motif is also crucial for the integrity of the whole protein.

The Zinc Finger Motif Is Essential to DNA Binding—The most striking observation in this report is that alterations of

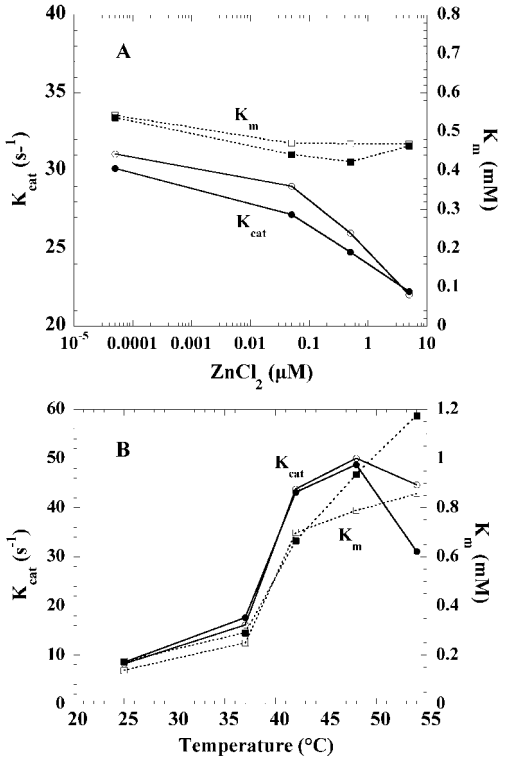


FIG. 6. **The effects of zinc concentration (*A*) and temperature (*B*) on K_{cat} and K_m of wild-type and zinc-demetalated RecQ helicases.** The experiments were performed under the same conditions as in Fig. 4*A* in the presence of different concentrations of $ZnCl_2$ or at different temperatures as indicated. The values of K_{cat} (*open circles*, wild type; *closed circles*, zinc-demetalated) and K_m (*open squares*, wild type; *closed squares*, zinc-demetalated) were determined by fitting each ATP saturation curve to the Michaelis-Menten equation.

the zinc finger motif by site-directed mutagenesis lead to a complete loss of RecQ-DNA binding ability. In view of the fact that RecQ helicase is a DNA-stimulated ATPase and an ATP-dependent helicase, the impairment of DNA binding should be the primary cause of the observed defects in ATPase and helicase activities. These observations indicate that the zinc finger motif is essential to DNA binding. Alternatively, the replacement of amino acids in the zinc finger motif leads to an overall conformational change of the helicase, which resulted in the observed defects in helicase functions. Consistent with this possibility, all of the single-point mutations were rapidly degraded, making functional analysis impossible. However, a careful characterization of the double mutant helicase indicated that the protein undergoes a subtle conformational change rather than a radical modification of the overall protein architecture. First, both wild-type and mutant enzymes displayed very similar limited proteolysis pattern. Second, the size-exclusion chromatography analysis indicated that both wild-type and mutant helicases have similar Stokes radii, suggesting that these proteins fold in overall similar fashions. Third, the fact that the mutant protein possesses almost the same affinity for mantATP binding as wild-type helicase suggests that the tertiary structure of the mutant protein is not dramatically altered. These results clearly demonstrate that a local subtle conformational change in the zinc finger motif abrogates DNA binding capability, and as a consequence, the ATPase and helicase activities were abolished. This interpretation gains support from CD experiments where the small reduction in α-helix may be attributed to the altered structure of the zinc finger motif because of the replacement of two cysteine amino acids with asparagines. The zinc finger motif thus has a dominant effect in DNA binding. We hypothesize that some conserved positively charged residues (Arg^{382} and Lys^{408}) and solvent-exposed hydrophobic residues (Phe^{374} and Phe^{389}) harbored by this zinc motif could be directly involved in the recognition and binding of DNA.

For other helicases without zinc finger motif within the molecules, such as PrcA, Rep, UvrD, and HCV (hepatitis C virus) helicases, their DNA binding surfaces are composed primarily of motifs Ia, IV, and V. Several lines of experimental evidence suggest that the same situation may be held in the RecQ family of helicases (13). It is also interesting to note that most RecQ family of helicases harbor a HRDC domain, which possesses DNA binding activities (16). Because the double mutant that we studied appears to keep its overall three-dimensional structure and because the helicase domain and HRDC domain could still bind DNA, the observation that DNA binding activity is completely compromised is not expected. How can we reconcile the observation that alterations of the zinc finger motif lead to the RecQ helicase losing its DNA binding activity almost completely? One of the answers to this question lies in the fact that, for RecQ helicase, the zinc finger motif plays an essential role in DNA binding, whereas both the helicase and HRDC domains may function as auxiliary DNA-binding domains. Without zinc finger motif, both helicase and HRDC domains display very low affinities for DNA binding. Indeed, in work to be published elsewhere,[3] it has been shown that the isolated helicase domain and HRDC domain fragment proteins display very low affinity for ssDNA and dsDNA. It is likely that when DNA is bound to zinc finger motif, the helicase domain plays an essential role in unwinding activity, whereas HRDC domain may direct DNA binding specificity. Thus, it is tempting to speculate that the coordination among the zinc finger motif, the helicase domain, and HRDC will determine the DNA substrate specificity, the kinetics, and the processivity of the helicase. Here again, these analyses further enforce the notion that the zinc finger motif plays an essential role in DNA binding.

The Zinc Finger Motif Plays an Important Role in the Tertiary Structural Stability—The results from this study and the three-dimensional structural analysis reveal that the zinc finger motif is stabilized by four cysteine residues. It is important to note that altering one of the four conserved cysteine residues leads to highly unstable proteins. These results showed that the zinc finger motif is unexpectedly involved in the integrity of the whole enzyme. It is possible that, in addition to its DNA binding function, the zinc finger motif also functions in the folding cascade, linking the domains to each other to stabilize the protein tertiary structure. In addition to the four highly conserved cysteine residues, the zinc finger motif could be further stabilized through very important interactions among α-helices 16, 17, and 18. It appears that the hydrogen bonds between Phe^{374} and Arg^{381} and between Arg^{381} and Asp^{401} play an important role in stabilizing the zinc finger motif and the whole protein structure. Consistent with this postulation, the mutation of Arg^{381} or Asp^{401} leads to the enzyme becoming very sensible to protease degradation. Similar results were observed with the Bloom syndrome protein (30). The replacement of Asp^{1064} (which is located at a position equivalent to that of Asp^{401} of RecQ helicase) with Ala substantially reduced DNA binding and helicase activities, whereas the protein displayed essentially the same activities as the wild-type enzyme when Asp^{1064} was replaced by Asn. This phenomenon is probably the result of the properties of the side chain of the replacing residues. Asn probably could still possibly establish hydrogen bond with Arg^{1037}, thus stabilizing the protein structure, whereas

[3] J. L. Liu and X. G. Xi, unpublished observation.

Ala cannot. Thus, both the cysteines, C1 and C4, and the hydrogen bonds between Phe^{374} and Arg^{381} and between Arg^{381} and Asp^{401} contribute to the stabilization of the zinc finger motif and increase the stability of the tertiary structure of the protein.

The observation that the wild-type and zinc-demetalated forms of RecQ are very similar to each other by virtue of their enzymatic activities suggests that the zinc atom is not directly implicated in DNA binding, ATPase, and helicase activities. It appears that the zinc ion is not essential for stabilizing the overall conformation of the protein, because both the wild-type and zinc-demetalated helicases display very similar thermostabilities between 25 and 54 °C. Nevertheless, the zinc ion may have a quite relevant role in determining the full development of secondary structure of the zinc finger motif during the protein folding. Once the zinc finger motif has folded correctly in the presence of zinc ion, its structure remains stable even in the absence of zinc ion; thus, DNA can bind to the residues involved in base-specific contacts. There are several examples in which metal ions play an important role in the formation of secondary structural elements in metalloproteins, whereas demetalated proteins still have well defined tertiary structures (31).

Biological Relevance of the Cysteine Cluster to the RecQ Helicase Family—Amino acid sequence alignment of several RecQ helicases revealed that the cysteine residues involved in the formation of zinc finger in *E. coli* helicases are conserved among the RecQ family of helicases (Fig. 1). Available evidence indicates that mutations of the cysteine residues in the zinc finger motif in most RecQ family of helicases can have dramatic effects on the enzymatic activities. Two disease-causing Bloom missense mutations map to Cys^{1036} and Cys^{1055}, respectively (32, 33), among four conserved cysteine residues in the zinc finger motif. *In vitro* analysis shows that these mutations abolish BLM ATPase and helicase activities (34). *In vitro* studies of the RecQ core of the Bloom syndrome protein have shown that when three of the four cysteines in the zinc finger motif were mutated, respectively, the resulting mutants were very unstable. Furthermore, even mutations of residues near the conserved cysteine residues such as R1038A and D1064A prevented the modified enzymes from binding DNA and caused them to lose ATPase and helicase activities (29). It has also been shown that this region is essential to *in vitro* function of the yeast RecQ homologue Sgs1 (35, 36).

Sequence comparisons among the DNA helicases suggest that the zinc finger motif appears to be unique to the RecQ helicase family. Both the sequence analysis and three-dimensional structural studies showed that other DNA helicases, such as T7 gene 4 helicase (37), Rep (38), PcrA (39), and hepatitis C virus NS3 helicases (40), do not use the zinc finger motif for DNA binding. Whether the use of zinc finger motif for DNA binding is a unique feature of RecQ family of helicases is currently being investigated in our laboratory.

In summary, the present study reveals that the zinc finger motif in the RecQ helicase plays important roles both in DNA binding and folding of the whole enzyme. Thus, evolution has selected a complex mechanism to link all of the functions of the enzyme to this small subdomain. Although much remains to be discovered, RecQ enzymes without perfect folding of the zinc finger motif would indeed be unable to ensure the helicase functions.

Acknowledgements—We thank an anonymous reviewer who meticulously reviewed the paper and provided us with extremely constructive comments. We thank Dr. Camille Kotton for careful reading of the paper, Marie Claude Vernières for the synthesis of mantATP, Drs. Loussiné Zargarian and Serge Fermandjian for generosity and expert assistance in the CD spectroscopy measurements, Drs. Robert Pansu and X. D. Zhang for assistance in the fluorescence measurements, S. Lu for the role in the early stage of this work, and Drs. Eric Deprez and Rongbing Guo for insightful discussion. We are very grateful to Prof. C. Auclair for continuous support.

REFERENCES

1. Lohman, T. M., and Bjornson, K. P. (1996) *Annu. Rev. Biochem.* **65,** 169–214
2. Soultanas, P., and Wigley, D. B. (2000) *Curr. Opin. Struct. Biol.* **10,** 124–128
3. Singleton, M. R., and Wigley, D. B. (2002) *J. Bacteriol.* **184,** 1819–1826
4. Singleton, M. R., Wentzell, L. M., Liu, Y., West, S. C., and Wigley, D. B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13492–13497
5. Betterton, M. D., and Jülicher, F. (2003) *Phys. Rev. Lett.* **91,** 258103
6. Hall, M. C., and Matson, S. W. (1999) *Mol. Microbiol.* **34,** 867–877
7. Chakraverty, R. K., and Hickson, I. D. (1999) *BioEssays* **21,** 286–294
8. Furuichi, Y. (2001) *Ann. N. Y. Acad. Sci.* **928,** 121–131
9. Cantor, S., Drapkin, R., Zhang, F., Lin, Y., Han, J., Pamidi, S., and Livingston, D. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 2357–2362
10. Umezu, K., Nakayama, K., and Nakayama, H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5363–5367
11. Hanada, K., Iwasaki, M., Ihashi, S., and Ikeda, H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5989–5994
12. Harmon, F. G., and Kowalczykowski, S. C. (1998) *Genes Dev.* **12,** 1134–1144
13. Bennett, R. J., and Keck, J. L. (2004) *Crit. Rev. Biochem. Mol. Biol.* **39,** 79–97
14. Caruthers, J. M., and McKay, D. B. (2002) *Curr. Opin. Struct. Biol.* **12,** 123–133
15. Morozov, V., Mushegian, A. R., Koonin, E. V., and Bork, P. (1997) *Trends Biochem. Sci.* **22,** 417–418
16. Liu, Z., Macias, M. J., Bottomley, M. J., Stier, G., Linge, J. P., Nilges, M., Bork, P., and Sattler, M. (1999) *Struct. Fold. Des.* **7,** 1557–1566
17. Bernstein, D. A., Zittel, M. C., and Keck, J. L. (2003) *EMBO J.* **22,** 4910–4921
18. Berg, J. M., and Shi, Y. (1996) *Science* **271,** 1081–1085
19. Hiratsuka, T. (1982) *Biochim. Biophys. Acta* **719,** 509–517
20. Xu, H. Q., Deprez, E., Zhang, A. H., Tauc, P., Ladjimi, M. M., Brochon, J. C., Auclair, C., and Xi, X. G. (2003) *J. Biol. Chem.* **278,** 34925–34933
21. Iwaasa, M., Umeda, S., Ohsato, T., Takamatsu, C., Fukuoh, A., Iwasaki, H., Shinagawa, H., Hamasaki, N., and Kang, D. (2002) *J. Neurochem.* **82,** 30–37
22. Hunt, J. B., Neece, S. H., and Ginsburg, A. (1985) *Anal. Biochem.* **146,** 150–157
23. Levin, M. K., Gurjar, M. M., and Patel, S. S. (2003) *J. Biol. Chem.* **278,** 23311–23316
24. Dou, S. X., Wang, P. Y., Xu, H. Q., and Xi, X. G. (2004) *J. Biol. Chem.* **279,** 6354–6363
25. Dessinges, M. N., Lionnet, T., Xi, X. G., Bensimon, D., and Croquette, V. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6439–6444
26. Xu, H. Q., Zhang, A. H., Auclair, C., and Xi, X. G. (2003) *Nucleic Acids Res.* **31,** e70
27. Simpson, R. J., Cram, E. D., Czolij, R., Matthews, J. M., Crossley, M., and Mackay, J. P. (2003) *J. Biol. Chem.* **278,** 28011–28018
28. Harmon, F. G., and Kowalczykowski, S. C. (2001) *J. Biol. Chem.* **276,** 232–243
29. Coleman, J. E. (1992) *Annu. Rev. Biochem.* **61,** 897–946
30. Janscak, P., Garcia, P. L., Hamburger, F., Makuta, Y., Shiraishi, K., Imai, Y., Ikeda, H., and Bickle, T. A. (2003) *J. Mol. Biol.* **330,** 29–42
31. Banci, L., Bertini, I., Cramaro, F., Del Conte, R., and Viezzoli, M. S. (2003) *Biochemistry* **42,** 9543–9553
32. Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M., and German, J. (1995) *Cell* **83,** 655–666
33. Foucault, F., Vaury, C., Barakat, A., Thibout, D., Planchon, P., Jaulin, C., Praz, F., and Amor-Gueret, M. (1997) *Hum. Mol. Genet.* **6,** 1427–1434
34. Bahr, A., De Graeve, F., Kedinger, C., and Chatton, B. (1998) *Oncogene* **17,** 2565–2571
35. Mullen, J. R., Kaliraman, V., and Brill, S. J. (2000) *Genetics* **154,** 1101–1114
36. Onoda, F., Seki, M., Miyajima, A., and Enomoto, T. (2000) *Mutat. Res.* **459,** 203–209
37. Singleton, M. R., Sawaya, M. R., Ellenberger, T., and Wigley, D. B. (2000) *Cell* **101,** 589–600
38. Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) *Cell* **90,** 635–647
39. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) *Cell* **97,** 75–84
40. Yao, N., Hesson, T., Cable, M., Hong, Z., Kwong, A. D., Le, H. V., and Weber, P. C. (1997) *Nat. Struct. Biol.* **4,** 463–467

2. Régulation de l'hélicase RECQ5 humaine par le motif à doigt de zinc

2.1. Présentation

Afin d'étudier le rôle du motif à doigt de zinc des hélicases RecQ, nous avons choisi l'hélicase RECQ5 humaine comme modèle. Cette hélicase existe sous trois isoformes, RECQ5α, RECQ5β et RECQ5γ (Article 2, Fig. 1), qui résultent de l'épissage alternatif lors de la transcription du gène *RECQ5* (Shimamoto et al., 2000 ; Sekelsky *et al.,* 1999). Les trois protéines sont identiques dans leur région N-terminale correspondant au domaine hélicase. RECQ5β contient en plus un domaine RecQ-Ct (RQC) putatif suivi d'une longue région C-terminale qui semble être non homologue aux domaines C-terminaux des autres helicases RecQ, tandis que RECQ5γ a une extension C-terminale de 25 acides aminés qui n'est pas présente dans RECQ5β. En outre, le domaine HRDC de RecQ d'*E. coli* n'est pas présent dans les protéines RECQ5 (Bennett et Keck, 2004 ; Bachrati et Hickson, 2003). Seul RECQ5β a été caractérisé sur le plan biochimique (Garcia *et al.,* 2004). Janscak et collègues ont montré que RECQ5β est une ATPase ADN-dépendante et une ADN hélicase de polarité 3'-5' (Garcia *et al.,* 2004). Ils ont aussi montré que RECQ5β possède une activité favorisant l'hybridation de l'ADN, activité qui réside dans la moitié C-terminale de la protéine (Garcia *et al.,* 2004). Les activités enzymatiques du domaine hélicase isolé des hélicases de la famille RecQ n'ont pas été caractérisées. Ainsi, les protéines RECQ5 offrent un modèle pour étudier la régulation des activités enzymatiques du domaine hélicase de la famille RecQ par son domaine RQC, en particulier son doigt de zinc.

Dans cette étude, nous avons quantitativement caractérisé les propriétés enzymatiques multiples des trois isoformes RECQ5 humaines, à savoir, la fixation de l'ATP, l'activité ATPase, la fixation de l'ADN, l'activité hélicase et l'activité à favoriser l'hybridation de l'ADN. Nous avons montré que les trois protéines RECQ5 lient l'ATP avec une affinité semblable, mais l'ADN avec des affinités nettement différentes. Les activités ATPase et hélicase de ces enzymes sont correlées à leur affinité pour l'ADN. RECQ5α et RECQ5γ, qui fixent l'ADN faiblement, sont déficientes ou possèdent une activité faible ATPase et hélicase, tandis que RECQ5β, qui fixe l'ADN fortement, est une ATPase et une hélicase active. Remarquablement, nous avons montré que RECQ5α possède une activité forte à favoriser l'hybridation de l'ADN. C'est la première démonstration qu'un domaine hélicase de la famille RecQ possède une activité intrinsèque à favoriser l'hybridation de l'ADN. Nos études

sur des protéines tronquées montrent que RECQ5β possède deux domaines, le noyau hélicase N-terminal et le fragment C-terminal, capables de catalyser l'hybridation de l'ADN. D'ailleurs, nous avons prouvé que le doigt de zinc augmente la fixation à l'ADN, les activités ATPase et hélicase, mais supprime l'activité à favoriser l'hybridation de l'ADN du domaine hélicase de la protéine RECQ5. La caractérisation additionnelle suggère que l'interaction protéine-ADN faible pourrait être importante pour catalyser l'hybridation de l'ADN. Pris ensemble, nos résultats sur les protéines RECQ5 humaines démontrent que le doigt de zinc joue un rôle important dans l'activité ATPase et la fixation à l'ADN et par conséquent dans l'activité hélicase.

L'ensemble de ces résultats est présenté dans l'article № 2.

2.2. Article № 2:

Modulation of human RECQ5 helicase function by a zinc finger-containing motif

Xing-Dong Zhang, Shuo-Xing Dou, Jin-Shan Hu, Jie lin Liu, Peng Xie, Peng-Ye Wang, and Xu Guang Xi

Modulation of human RECQ5 helicase function by a zinc finger-containing motif

Xing-Dong Zhang[2], Shuo-Xing Dou[2], Jin-Shan Hu[3], Jie Lin Liu[1], Peng Xie[2], Peng-Ye Wang[2], and Xu Guang Xi[1],

[1] Laboratoire de Biotechnologies et Pharmacologie Génétique Appliquée CNRS UMR 8113, Ecole Normale Supérieure (ENS) Cachan, 61 avenue du Président Wilson, 94235 Cachan cedex, France

[2] Laboratory of Soft Matter Physics, Beijing National Laboratory for Condensed Matter Physics, Institute of Physics, Chinese Academy of Sciences, Beijing 100080, China

[3] Department of Chemistry and Biochemistry, and the Center for Biomolecular Structure and Organization, University of Maryland, College Park, MD 20742, USA

Running title: Modulation of RECQ 5 function by a Zinc Finger

Correspondence should be addressed to X.G.X (xi@lbpa.ens-cachan.fr)

[1] The abbreviations used are: BS, Bloom syndrome protein; BLM, Bloom syndrome protein; BSA, bovine serum albumin; CD, circular dichroism; dsDNA, double-stranded DNA; EMSA, electrophoretic mobility shift assay; FRET, fluorescence resonance energy transfer; HRDC, helicase-and-ribonuclease D-C-terminal domain; mantATP, 2′(3′)-*O*-(*N*-methlanthraniloyl) adenosine-5′-triphosphate; PAR, 4-(2-pyridylazo)resorcinol; RPA, replication protein A; RQC, RecQ carboxy-terminal conserved; ssDNA, single-stranded DNA; WH, winged helix-turn-helix; WS, Werner syndrome, WRN, Werner syndrome protein.

SUMMARY

RecQ helicases function as caretakers to preserve genomic integrity. Mutations in three (*BLM*, *WRN* and *RECQ4*) of the five human RecQ genes have been linked to Bloom, Werner, and Rothmund-Thomson syndromes, characterized by genome instability and cancer predisposition. Interestingly, RECQ5 exists in three isoforms, RECQ5α, RECQ5β and RECQ5γ, which share an identical N-terminal helicase core, but differ in the C-terminal domains. This unique property is not shared by any other human RecQ helicase and offers an attractive model to study the function of a RecQ helicase core in isolation and in an association with other motifs on the same polypeptide. Here we show that three proteins bind ATP with a similar affinity, but DNA with significantly different affinities. RECQ5α and RECQ5γ are either deficient or low in ATPase and helicase activities, while RECQ5β is an active ATPase and helicase. Remarkably, RECQ5α possesses a strong strand-annealing activity, and this is the first demonstration that the RecQ helicase core has strand-annealing activity. Further characterization shows that RECQ5β harbours a Zn finger that regulates the multiple enzymatic activities of the RECQ5 helicase core. The application of this novel model to three human disorders caused by the defective RecQ helicases is discussed.

INTRODUCTION

Helicases are the enzymes that use the chemical energy derived from the binding and hydrolysis of ATP to catalyse the unwinding of nucleic acid duplexes. Helicases play essential roles in many nucleic acid transaction processes, including DNA replication, recombination, repair, and RNA transcription, rearrangement, and splicing [1,2]. In order to accomplish these complex biological roles, helicases often combine with other functional domains or interact with other proteins. However, helicases are usually studied in isolation by biochemical and structural methods [3], the mechanism how a helicase is regulated is poorly understood.

The RecQ helicase family enzymes are required for the maintenance of genome integrity [4-7]. Prokaryotes and unicellular eukaryotes have a single RecQ homolog, while multicellular organisms usually have multiple. In humans, five family members, RECQ1 [8,9], BLM [10], WRN [11], RECQ4 [12] and RECQ5 [13], have been identified. Defects in BLM, WRN and RECQ4 are associated with human hereditary disorders, Bloom syndrome (BS), Werner syndrome (WS) and Rothmund-Thomson syndrome (RTS), respectively. All these diseases are characterized by genome instability and cancer predisposition. Interestingly, no genetic diseases have yet been linked to RECQ1 and RECQ5. These results suggest that human RecQ proteins are not redundant in function and each plays a unique role in preventing genomic instability [14]. However, the exact roles of these helicases *in vivo* and how their enzymatic activities are regulated remain elusive.

The RecQ helicases share a conserved DExH helicase core containing seven conserved motifs involved in ATP-Mg^{2+} and DNA binding. In some cases, the RecQ helicases also contain a RQC domain or both RQC and HRDC domains. The conserved HRDC domain functions as an auxiliary DNA-binding domain [15]. The RQC domain appears to be unique to the RecQ helicase family. The crystallographic studies show that the RQC domain of *E. coli* RecQ is composed of a Zn finger and a winged helix-turn-helix (WH) domain [16]. Recently, we have shown that the *E. coli* RecQ and human BLM proteins indeed contain Zn-binding domains which regulate helicase activities of the RecQ and BLM helicases[17,18]. Biochemical mapping experiments lead Bohr and co-workers to propose that WRN has a WH-like domain,

which binds to both structure-specific DNA and a number of DNA processing proteins [19,20]. This prediction has been confirmed by our NMR studies of this multifunctional DNA- and protein-binding domain [21]. Whether a Zn-binding domain or a WH domain or a RQC domain exists in any other RecQ helicase remains to be demonstrated.

To investigate whether the Zn finger motif is involved in the regulation of the RecQ helicases, we have chosen human RECQ5 as a model system. The striking feature is that the human RECQ5 exists in three isoforms, namely, RECQ5α, RECQ5β and RECQ5γ (**Fig. 1**), which result from alternative splicing of the *RECQ5* transcript [13,22]. The three proteins are identical in the N-terminal regions with 410 residues and all contain the conserved seven DExH helicase motifs. In addition to the helicase core, RECQ5β contains a putative RQC domain, which followed by a long C-terminal region that appears to be nonhomologous to the other RecQ helicases, while RECQ5γ has a C-terminal extension of 25 amino acids that is not present in RECQ5β. At present, only RECQ5β has been characterized biochemically [23]. Janscak and co-workers have shown that RECQ5β functions as a DNA-dependent ATPase and an ATP-dependent 3′→5′ DNA helicase [23]. They have also shown that RECQ5β has a novel ssDNA annealing activity, which resides in the C-terminal half of the protein [23]. Interestingly, in *Drosophila Melanogaster*, dmRECQ5 also exists in three isoforms, dmRECQ5a, dmRECQ5b and dmRECQ5c (**Fig. 1**) [22]. Similar to human RECQ5β, dmRECQ5a is the largest isoform with 1057 residues, while dmRECQ5b and dmRECQ5c are smaller isoforms, which differ by only 2 C-terminal residues and stop right after the putative Zn finger, instead of the helicase core (**Fig. 1**). Both dmRECQ5a dmRECQ5b have been characterized biochemically and dmRECQ5b has been shown to possess strand-exchange activity in addition to other enzymatic activities demonstrated for human RECQ5β [24-26]. In addition, the HRDC domain is not present in RECQ5 proteins [27,28]. The enzymatic activities of an isolated helicase core in the RecQ helicase family have not yet been carefully characterized. Thus, RECQ5 proteins offer an attractive model to study the regulation of the enzymatic activities of a RecQ helicase core by a RQC domain, in particular a Zn finger.

We hypothesized that, if the RECQ5 helicase were not regulated, similar enzymatic activities would be observable for all three RECQ5 isoforms. In this study, we have extensively and quantitatively characterized the multiple enzymatic properties, namely, ATP binding, ATPase, DNA binding, helicase, strand annealing and strand exchange, of the three human RECQ5 isoforms. We have found that three RECQ5 proteins bind ATP with a similar affinity, but DNA with dramatically different affinities. The ATPase and helicase activities of these enzymes correlate with their DNA binding affinities. RECQ5α and RECQ5γ, which bind DNA poorly, are either deficient or low in ATPase and helicase activities; while RECQ5β, which binds DNA strongly, is an active ATPase and helicase. Remarkably, RECQ5α possesses a strong ssDNA annealing activity. This is the first demonstration that a RecQ helicase core has an intrinsically high annealing activity. Further studies show that RECQ5β possesses two motifs, the N-terminal helicase core and the C-terminal fragment, which can mediate strand annealing. Moreover, we have shown that the Zn finger enhances the DNA-binding, ATPase and helicase activities, but suppresses the annealing activity of the RECQ5 helicase core. Additional characterization suggests that the weak protein-DNA interaction is important for mediating strand annealing. Taken together, our results on the human RECQ5 proteins support the proposal that the Zn finger plays an important role in regulating the enzymatic activities of its associated RecQ helicase core. The application of this model to three human RecQ helicases associated with human disorders has been discussed.

RESULTS

Of three RECQ5 proteins, only RECQ5β harbours a Zn finger

Three human RECQ5 proteins, RECQ5α, RECQ5γ and RECQ5β, were overproduced in the *E. coli* as histidine-tagged proteins, and purified to more than 95% homogeneity as judged by SDS-PAGE (results not show). RECQ5α, RECQ5γ and RECQ5β, are largely α-helical proteins. This prediction based on the amino acid sequences has been supported by the NMR (data not shown) and circular dichroism (CD) studies. The CD studies show that the proteins contain 43.4% (RECQ5α), 38.5% (RECQ5β) and 44.2% (RECQ5γ) α-helical structures, respectively (**Supplementary Fig. 1A** online). As the near-UV CD spectrum is sensitive to the tertiary structure of a protein [29], the near-UV CD spectra for both RECQ 5α and RECQ 5γ isoforms were recorded at two different temperatures (25 and 75°C). The results show that both RECQ5α and RECQ5γ proteins have well folded structures (**Supplementary Fig. 1B** online).

The sequence alignments of the RECQ5 isoforms with other RecQ family proteins show that RECQ5β could contain a Zn finger ligated by the conserved Cys^{411}, Cys^{427}, Cys^{431} and Cys^{434} residues (**Fig. 1B**). This prediction was confirmed by the sensitive PAR assay (**Fig. 1C**), which is widely used to exclusively determine zinc quantity [30]. RECQ5β was shown to contain 0.98 ± 0.18 equivalent Zn^{2+} ion (**Fig. 1D** and **Table 1**). The similar results were obtained for $RECQ5\beta^{1-475}$ and $RECQ5\beta^{1-662}$, the C-terminal deletion variants (**Table 1**). The former mutant consists of the helicase core and the Zn finger [23], while the latter includes the additional 188 residues, which would contains the C-terminal half of the putative RQC domain. The replacement of Cys^{431} with a Ser reduced the protein-ligated Zn^{2+} to 0.35 ± 0.12 equivalents (**Table 1**). However, under the identical conditions, the PAR assay shows that RECQ5α and RECQ5γ do not contain any Zn^{2+} ion, with measured Zn^{2+} values of 0.032 ± 0.01 and 0.015 ± 0.02 (**Table 1**), respectively.

Three RECQ5 proteins bind ATP with a similar affinity, but hydrolyze ATP with different rates

The ATP-binding abilities of RECQ5α, RECQ5β, and RECQ5γ were first evaluated by the UV cross-linking method [18], using the ^{32}P-labelled nucleotides. The densitometer analysis showed that the same amount of ATP was cross-linked to each of the three proteins (data not shown), suggesting that all three proteins bind to ATP with a similar affinity. To quantify their ATP-binding affinities, the mantATP, a fluorescent analogue of ATP, was used. The mantATP binds competitively to the ATP binding site for each protein with an apparent K_i value of 56.0 ± 1.2 μM for RECQ5β. The fluorescence excitation and emission spectra of the three RECQ5 helicases and mantATP were then measured. The fact that the emission spectrum of each protein overlaps with the excitation spectrum of mantATP makes it possible to determine the mantATP-binding constant for each protein (**Fig. 2A**). The measured apparent dissociation constants (K_d) (**Fig. 2B**) were 48.4 ± 2.5, 51.0 ± 4.9, and 41.6 ± 2.0 μM for RECQ5α, RECQ5β and RECQ5γ, respectively (**Table 1**). These results confirm that the three isoforms not only fold structurally, but also bind ATP with a similar affinity.

The ATPase activities of three RECQ5 proteins in the presence of the denatured dsDNA (2.9 kb) were then determined by measuring the release of inorganic P_i from ATP (**Fig. 2C**). The k_{cat}/K_M ratio can be estimated from the plot of the initial ATP hydrolysis rate versus ATP concentration, where k_{cat} is the maximum DNA-activated ATP hydrolysis rate, and K_M is the ATP concentration required for a half-maximum activation. As expected, RECQ5β exhibits high ATPase activity [23]. The measured k_{cat} of 15.6 ± 1.4 sec^{-1} for ATP hydrolysis is comparable to those observed for human BLM [31] and dmRECQ5b[24]. Surprisingly, under identical conditions, RECQ5γ displayed significantly lower, but detectable ATPase activity (**Fig. 2C** and **Table 1**), while RECQ5α had no measurable activity (**Fig. 2C** and **Table 1**), even in presence of a variety of other different DNA substrates (data not shown). The results were unexpected since RECQ5α and RECQ5γ possess all necessary RecQ helicase motifs.

To evaluate the role of the C-terminal part of the protein in the ATPase reaction, the C-terminal truncated RECQ5β variants (RECQ5β$^{1-475}$ and RECQ5β$^{1-662}$) were assayed for their ATPase activities (**Fig. 2C**). We found that these two C-terminal truncated mutants had essentially identical ATPase activity with respect to the full-length RECQ5β protein, suggesting that the Zn finger plays an important role

in the regulating the ATPase activity of the RECQ5 helicase core (**Table 1**). To further evaluate the role of the Zn finger in the ATPase reaction of RECQ5, the ATPase activity of the RECQ5β^{C431S} mutant, by replacing one of four conserved Cys residues of RECQ5β with Ser, was measured (**Fig. 2C**). We found that the mutation significantly decreased the ATPase activity of RECQ5β (**Table 1**). Taken together, our data suggest that the Zn finger regulates the ATPase activity of the RECQ5 helicase core.

The Zn finger stimulates DNA binding activity of the RECQ5 helicase core

Electrophoretic mobility shift assay (EMSA) was first used to study the DNA binding properties of the RECQ5 proteins. We found that RECQ5β binds to both ssDNA and dsDNA, with a preference for ssDNA over dsDNA (**Supplementary Fig. 2A,B** online). However, under the identical conditions, no significant binding for either ss- or dsDNA could be detected for either RECQ5α or RECQ5γ, even at 10-fold higher protein concentration (**Supplementary Fig. 2A,B** online).

The DNA-binding affinities of the RECQ5 proteins were then measured quantitatively using the fluorescence-labelled oligonucleotides. This method allows the apparent dissociation constant for a protein-DNA complex to be measured under an equilibrium condition, and it is therefore intrinsically more sensitive than the EMSA method. The results show that RECQ5β binds to both ss- and dsDNA quickly to form stable protein-DNA complexes, and the fluorescence anisotropies for the resulting complexes increase as the protein concentration increases (**Fig. 3**). However, under similar experimental conditions, both RECQ5α and RECQ5γ display about 6- and 4-fold lower binding affinities for ss- and dsDNA compared to RECQ5β, respectively (**Fig. 3** and **Table 2**).

To evaluate the role of the Zn finger in the DNA binding of the RECQ5 helicase core, the RECQ5β$^{1-475}$ and RECQ5β$^{1-662}$ truncation mutants were assayed for their DNA-binding activities. We found that these two C-terminal truncated mutants had essentially identical DNA-binding affinities for both ss- and dsDNA with respect to the WT RECQ5β protein, suggesting that the Zn finger plays an important role in the regulating the DNA-binding activity of the RECQ5 helicase core (**Table 2**). To

further evaluate the role of the Zn finger in the DNA-binding reaction of RECQ5, the site-specific mutant RECQ5β^{C431S} was used. We found that the mutation decreased the DNA-binding affinities by 2.0 and 3.5 folds for ss- and dsDNA, respectively, compared to WT RECQ5β (**Table 2**).

It has been shown that a triphosphate nucleotide such as ATP can enhance the DNA binding affinity of some helicases [32]. Therefore the DNA binding activities of the RECQ5 proteins were measured in the presence of an unhydrolyzable analogue ATP, AMPPNP. The results show that while the ssDNA binding affinity of RECQ5β can be enhanced in about two folds in the presence of 2 mM AMPPNP, no noticeable effect could be observed for either RECQ5α or RECQ5β, under the identical conditions (**Table 2**). To compare DNA binding specificity for the RECQ5 proteins, three additional substrates, namely, 3′-overhang, 5′-overhang, and forked dsDNA, were used (**Table 2**). The results show that RECQ5β binds as well to these three substrates as to ss- or ds-DNA as described previously, with about two-folds higher affinity for the forked dsDNA (**Table 2**). In contrast, both RECQ5α and RECQ5γ display lower binding affinities to these DNA substrates, comparable to those determined for ssDNA and dsDNA (**Table 2**). Taken together, these results indicate that, under the identical conditions, both RECQ5α and RECQ5γ bind to different DNA substrates with significantly lower affinities compared to RECQ5β, and the Zn finger enhances the DNA-binding affinity of the RECQ5 helicase core.

The Zn finger enhances DNA helicase activity of RECQ5

To characterize the effect of the Zn^{2+} finger motif on the helicase activity of RECQ5, we compared the helicase activities of the three isoforms using a radiometric helicase assay (**Fig. 4A**). The results show that RECQ5β efficiently unwinds a partial DNA duplex in a protein concentration-dependent manner, and RECQ5γ displays only a low unwinding activity. However, under the identical conditions, RECQ5α has no detectable helicase activity (**Fig. 4A**), even at the enzyme concentration as high as 2 μM (data not shown).

To quantitatively measure the helicase activities of three RECQ5 isoforms, the DNA unwinding kinetics mediated by the three isoforms were measured by the fluorimetric helicase assay, which allows the helicase-mediated DNA unwinding

kinetics to be measured continuously in real time in the stopped-flow experiments [33]. Consistent with the results obtained from the radiometric assay, RECQ5β unwinds a partial DNA duplex in a time- and concentration-dependent manner, while RECQ5α and RECQ5γ isoforms display either a low or nondetectable helicase activity (**Fig. 4B**). Under the single turnover conditions, the unwinding kinetics of a 16 bp duplex mediated by RECQ5β was biphasic. The fitting using a sum of two exponential terms gave two rate constants of 4.54 and 0.84 min^{-1}, for the fast and slow phases, respectively. The corresponding unwinding rates were 2.1 and 0.22 bp/s, respectively. The same method was applied to the RECQ5γ and RECQ5α proteins. A monophasic DNA unwinding kinetics with a rate of 0.05 min^{-1} was determined for RECQ5γ, and the unwinding rate was too low to be measured accurately for RECQ5α, under the identical conditions (**Fig. 4B** and **Table 1**).

To evaluate the role of the Zn finger in the helicase activity of the RECQ5 helicase core, the RECQ5β^{1-475} and RECQ5β^{1-662} mutants were used. We found that these two mutants had essentially identical unwinding activities for the same substrate with respect to the WT RECQ5β protein, with the monophasic DNA unwinding rates of 5.42 and 5.32 min^{-1} for RECQ5β^{1-475} and RECQ5β^{1-662}, respectively. These results suggest that the Zn finger modulates the DNA-helicase activity of the RECQ5 helicase core. This conclusion was further supported by studying the unwinding reaction catalysed by the RECQ5β^{C431S} mutant. We found that the mutation decreased the helicase activity, compared to that of the WT RECQ5β, with a measured unwinding rate of 1.38 min^{-1}.

The helicase activity of RECQ5β has been shown to be enhanced by RPA [23], a ssDNA-binding protein, the effects of RPA on helicase activities of the three RECQ5 proteins were characterized. As shown previously [23], RPA greatly stimulated RECQ5β-mediated DNA unwinding reaction in a concentration-dependent manner. However, RPA had no effect on the RECQ5α- or RECQ5γ-mediated DNA unwinding activity under the identical conditions (data not shown), indicating that the C-terminal extension of RECQ5β is responsible for the interaction with RPA. To map the RPA binding site, RECQ5β^{1-475} was used, and results showed that RPA failed to stimulate the helicase activity of RECQ5β^{1-475}, suggesting that the C-terminal region of RECQ5β is involved in the functional interaction with RPA. This is consistent with

the recent observation that both BLM and WRN interact with RPA through their N or C-terminal regions [34].

RECQ5 helicase core alone can catalyze strand annealing, but not strand exchange

To measure the RECQ5-mediated ssDNA annealing activities of all three isoforms, the two complementary ssDNA oligonucleotides were used. The experiments were first carried out in the absence of ATP using the EMSA method. Remarkably, RECQ5α promotes annealing more efficiently than RECQ5β (**Fig. 5A**). These results are surprising since the RECQ5β-mediated strand-annealing activity has been previously shown to reside in the C-terminal region of RECQ5β [23]. Interestingly, no significant strand-annealing activity could be detected for RECQ5γ (**Fig. 5A**), suggesting that that the 25 amino acid extension to the helicase core of RECQ5γ strongly suppresses the annealing activity of helicase core.

The effect of ATP on the annealing activities of three RECQ5 proteins was examined. The experiments were performed in the absence or in the presence of 2 mM ATP, ADP, or ATPγS, a slow-hydrolyzable ATP analogue. We found that ADP had little effect on the RECQ5α-mediated annealing. In contrast, ATP and ATPγS dramatically inhibited the efficient annealing activity of RECQ5α (**Fig. 5B**), implying that the observed inhibitory effect is due to ATP-bound protein conformation, as RECQ5α is a poor ATPase. For RECQ5β, ATP and ADP had no or little effect on the RECQ5β-mediated annealing. However, ATPγS significantly suppressed the annealing activity of RECQ5β, consistent with the previous observation [23] (**Fig. 5B**). The effect of ATP on the RECQ5γ-mediated annealing could not be determined as the intrinsic annealing activity of RECQ5γ was too low to observe (**Fig. 5B**). The fact that RECQ5 mediated annealing activity is regulated by the nucelotides suggests that helicase core indeed possesses intrinsic annealing activity.

To quantitatively compare the annealing kinetics mediated by the RECQ5 isoforms, a fluorimetric assay was used (**Fig. 5C**). The assay is essentially identical to the helicase assay as described previously [33], and the only difference is that the annealing assay explores the decrease, instead of the increase, in the fluorescence

emission due to energy transfer. The method allows the annealing to be measured in real time to extract the kinetic parameters. The measured annealing reaction rates were 0.95, 0.14 and 0.082 min^{-1} for RECQ5β, RECQ5α and RECQ5γ, respectively. Thus, RECQ5β promoted strand annealing about seven- and eleven- fold faster than RECQ5 α and RECQ5γ, respectively (**Fig. 5D)**. However, when evaluated by the annealing percentage (**Fig. 5D and Table 1**), RECQ5α is actually more efficient than RECQ5β. Interestingly, the fluorimetric assay shows that RECQ5γ displays a low, but measurable annealing activity (**Fig. 5D)**, which could not be observed by the EMSA method (**Fig. 5A**).

We next exploited whether RECQ5 isoforms could catalyse a strand exchange by combining dsDNA unwinding and ssDNA annealing activities. As most RecQ family helicases can efficiently unwind forked duplex, but not 5'-overhanged dsDNA [4]. Thus, a forked duplex was used as an unwinding substrate, and a third complementary ssDNA was used as an acceptor to form a stable exchange product, 5'-overhanged dsDNA, which is a poor substrate for the unwinding reaction (**Fig. 6A**). We found that RECQ5β could mediate complete conversion of a forked duplex into a partial duplex within 20 min (**Fig. 6B,C**). However, neither RECQ5α nor RECQ5γ could catalyse the same conversion (data not shown). This is probably because the helicase activity of RECQ5α is too poor to compensate its high annealing activity, while the weak helicase and annealing activities of RECQ5γ make it impossible to produce strand-exchange products. We then tested whether the high annealing activity of RECQ5α could overcome the weak helicase activity of RECQ5γ in the strand exchange. The results show that no exchange products could be detected under the experimental conditions when combining RECQ5α and RECQ5γ (data not shown), suggesting that both helicase and annealing activities are required for the strand exchange.

RECQ5β comprises two motifs that mediate annealing throught weak DNA binding

The fact that RECQ5α has a higher annealing activity than RECQ5β raises an intriguing possibility that the Zn finger motif could suppress the helicase core-mediated strong annealing activity. To test this hypothesis, the $RECQ5\beta^{1-475}$

variant, the RECQ5β C-terminal deletion fragment containing the RECQ5 helicase core and the Zn finger, was used. Indeed, consistent with the previous observation [23], RECQ5β$^{1-475}$ displayed low annealing activity, but still considerably higher than that in the absence of a RECQ5 protein (**Table 1**). However, when RECQ5β$^{1-662}$ deletion mutant was used, a significantly higher annealing activity was recovered (**Fig. 7A** and **Table 1**). In fact, the annealing activity of RECQ5β$^{1-662}$ is still lower than that mediated by RECQ5α. These results suggest that the C-terminal region of RECQ5β, most likely in the fragment RECQ5β$^{454-662}$, harbours another domain, which can catalyse ssDNA annealing, as previously proposed [23].

To define the C-terminal region of RECQ5β responsible for the annealing activity, three N-terminal deletion variants, RECQ5β$^{379-991}$, RECQ5β$^{454-991}$, and RECQ5β$^{454-662}$, were tested for their ability to catalyse the annealing using both radiometric and fluorimetric assays (**Fig. 7A,B**). The RECQ5β$^{379-991}$ variant, which stops right after the helicase core, consists the entire C-terminal half of RECQ5β including the Zn finger. RECQ5β$^{454-991}$ represents the C-terminal half of RECQ5β lacking the Zn finger, and RECQ5β$^{454-662}$ is produced by further deletion of the C-terminal region of the RECQ5β$^{454-991}$ fragment. The results measured by both methods (**Fig. 7A,B** and **Table 1**) show that the N-terminal helicase core and the C-terminal fragment can both mediate ssDNA annealing with efficiency higher or comparable to that catalysed by WT RECQ5β. Remarkably, the Zn finger strongly suppresses the annealing activity of the helicase core, but slightly enhances the annealing activity of the C-terminal motif (**Table 1**).

To understand the mechanism of the RECQ5 fragment mediated ssDNA annealing, we studied the relationship between the DNA binding affinity and annealing activity of the various RECQ5β-derived fragments. The DNA-binding affinities for both ds- and ssDNA were first qualitatively evaluated by EMSA method (**Supplementary Fig. 2C,D**), and then quantitatively measured by fluorescence assay under the equilibrium conditions (**Fig. 7C,D)**. We found that RECQ5β-derived fragments displayed significantly lower DNA binding affinities than RECQ5β (**Table 2**), suggesting that the weak protein-DNA interaction could catalyze ssDNA annealing.

DISCUSSION

In this study, we have characterized the enzymatic activities of three human RECQ5 isoforms. RECQ5 offers an attractive model to study the function of a RECQ helicase in isolation and in an association with other motifs on the same peptide. This unique property, which is not shared by any other human RecQ helicase, allows us to address some interesting questions that could not be readily answered with other RecQ helicases, namely, the intrinsic enzymatic activities of the RecQ helicase core and their regulations by the Zn finger-containing motif.

Human RECQ5 helicase core has a high intrinsic ssDNA annealing activity

In the RecQ helicase family, the ssDNA annealing activity was first discovered in RECQ5β [23]. The similar activity has been observed in BLM, WRN, RECQ1, RECQ4, and *Drosophila* RECQ5b (dmRECQ5b) [26,35-37]. In addition, WRN, BLM and dmRECQ5b have been shown to catalyze strand exchange by combining the DNA unwinding and annealing activities [26]. Surprisingly, it has been proposed that the C-terminal regions of these proteins are responsible for the strand annealing [23,26,36] and strand exchange [26]. The mechanism of the C-terminus-mediated strand annealing in the RecQ helicase family remains elusive. Structurally, this region of the RecQ family helicases shares little sequence homology. On the other hand, the helicase domain of the RecQ helicases, which possesses the DNA-binding motifs, more likely functions as an enzyme to catalyse the ssDNA annealing reaction. Indeed, we have discovered that RECQ5 helicase core has an intrinsically high annealing activity. We have further shown that the helicase core-mediated annealing can be strongly inhibited by ATP, ATPγS or the Zn finger of RECQ5β, but not ADP.

RECQ5α offers a nature-made RecQ helicase core to study its enzymatic activities, in particular ssDNA annealing. The discovery that the RECQ5 helicase core has intrinsically high annealing activity is not surprising. Several lines of evidence strongly support our conclusion. First, the nucleotide modulates the annealing activity of RECQ5β [23], BLM [23,26,36], WRN [26], and dmRECQ5b [26], implying that the helicase cores of these RecQ proteins are involved in the strand annealing. The fact that ATP binding to the helicase domain of RECQ5β suppresses strand

annealing based upon the site-directed mutagenesis [23] also supports this conclusion. Second, both previous studies [23] and the results reported in this study (**Fig. 7A,B** and **Table 1**) indicate that the full length RECQ5β catalyses ssDNA strand annealing more efficiently than the isolated C-terminal fragment, which was proposed to be the sole motif to catalyze strand annealing [23]. This observation suggests that the helicase core contributes to the observed annealing activity of the WT RECQ5β. Third, dmRECQ5b, which consists of the helicase core and zinc finger, has been shown to possess, although weak, strand-annealing activity [26].

Interestingly, the strong strand-annealing activity of the RECQ5 helicase core can be suppressed by the Zn finger, since RECQ5β$^{1-475}$ has a significantly lower activity than RECQ5α, but it is still considerably (23.5%) higher than the reaction carried out in the absence of any RECQ5 protein (**Table 1**). We have also noticed similar results were reported by Garcia et al. [23], although the annealing activity of RECQ5β$^{1-475}$ is only slightly higher than the nonenzymatic reaction under their conditions. Furthermore, dmRECQ5b, similar to human RECQ5β$^{1-475}$ (**Fig. 1A**), has weak strand-annealing activity [26]. Human RECQ4, which lacks the putative Zn finger and helicase activity, has been shown to possess the strand-annealing activity [37].

More interestingly, the loss of the strand-annealing activity of RECQ5α by the Zn finger extension in the RECQ5β$^{1-475}$ fragment can be significantly recovered by adding another 186 residues in the RECQ5β$^{1-662}$ fragment (**Table 1**), suggesting that the C-terminal region of the protein harbours another domain that could mediate strand annealing. Indeed, we have shown that RECQ5β$^{454-662}$ possesses a strand-annealing activity, although its activity is still considerably lower than that of the entire C-terminal domain (RECQ5β$^{454-991}$ or RECQ5β$^{379-991}$) (**Table 1**). These results suggest that RECQ5β has two motifs, the N-terminal helicase core and the C-terminal fragment, which catalyse the strand annealing. Comparison of the annealing activity of different RECQ5 fragments further suggest that these two motifs cooperatively catalyse the strand annealing, since the activity of RECQ5β is only slightly higher than that of the C-terminal fragment, but considerably lower than that of the RECQ5 helicase core (RECQ5α) (**Table 1**). Other motifs such as the Zn finger regulate the strand-annealing activity of the full-length enzyme (RECQ5β).

Further characterization indicates that the RECQ5 fragments-mediated strand-annealing activity correlates with their DNA binding affinity and the weak DNA binding is enough to catalyse annealing (**Fig. 7C,D**). Therefore, RECQ5 proteins or fragments likely function as a 'molecular crowding agent' [38] that increases the effective concentrations of the ssDNA substrates in the annealing reaction through the mechanism of the intramolecular catalysis [39]. This model based upon our studies on RECQ5 proteins appears to be similar to that proposed for BLM [36].

We have also observed that RECQ5β can mediate strand exchange. However, either RECQ5α or RECQ5γ has no detectable activity under identical conditions. This is probably because the helicase unwinding activity of either protein is too low to catalyse the strand exchange even though RECQ5α can strongly mediate strand annealing. Thus, the helicase core is essential for catalysing strand exchange, although the C-terminal domain plays an important role in the regulating the DNA-binding, helicase, strand-annealing, and strand-exchange reactions. It is quite logical that annealing and exchange functions would be associated with RECQ5 and other RecQ hleicases because these enzymes are postulated to act in recombination or replication restart pathways that involve swapping of DNA strands [4].

The Zn finger-containing motif modulates the enzymatic activities of RECQ5

Comprehensive and quantitative comparison of the multiple enzymatic activities of the RECQ5α, RECQ5β and RECQ5γ proteins leads us to propose that the Zn finger motif plays an important role in regulating the enzymatic activities of the RECQ5 helicase. The ATP binding to the helicase core appears to be determined by the helicase core motifs, the Walker A and B motifs, which are present in all three RECQ5 isoforms. Thus, RECQ5α, RECQ5β and RECQ5γ bind ATP with a similar affinity. Like other helicases, the conserved helicase motifs, namely Ia, IV and V, of the RecQ helicases comprise a DNA binding surface [40]. Consistent with this view, RECQ5α, RECQ5β and RECQ5γ bind DNA (**Table 2**). Kinetic studies show that RECQ5α and RECQ5γ bind DNA weakly, while RECQ5β binds DNA rapidly and forms a stable protein-DNA complex, suggesting that the C-terminal domains provide additional DNA binding affinity. The RQC and HRDC domains in the RecQ

members have been shown to be involved in the DNA binding [28]. As the HRDC domain is not present in RECQ5β [28], the RQC domain would be the primary site for interacting with DNA. Interestingly, RECQ5β appears to lack the WH domain [23], or at least the putative WH domain in RECQ5β is not readily identifiable solely based upon sequence alignment [21]. Consistent with this notion, the C-terminal deletion mutants RECQ5β$^{1-475}$ and RECQ5β$^{1-662}$ displayed similar DNA binding affinity as the full length of RECQ5β. These studies support the proposal that the Zn finger in the putative RQC domain is the key element that enhances DNA binding in RECQ5β.

As the nucleotide-dependent ATPases, the ATPase and helicase activities of the RECQ5 proteins correlate with their DNA-binding affinities. Both activities require the C-terminal motifs of RECQ5β, as RECQ5α and RECQ5γ have no detectable or weak ATPase and helicase activities, while RECQ5β is a robust ATPase and helicase. Specifically, the Zn finger in the RQC domain is the key regulatory element that enhances ATPase and helicase activities of the RECQ5 helicase core, but suppresses the intrinsic annealing activity of the helicase core by enhancing the DNA binding. Moreover, the Zn finger also plays an important role in the RECQ5β-catalyzed strand-exchange activity by coordinating the unwinding and annealing activities (**Fig. 8A**). The site-directed mutagenesis provides additional evidence that the Zn finger regulates the enzymatic activities of the RECQ5 helicase core. Our proposal is further supported by the existence of the evident correlation between the presence of the Zn finger in the RecQ helicases and the observed enzymatic activities (**Table 3**).

The Zn finger-containing motifs may regulate the RecQ helicase functions

The motivation to study RECQ5 helicase is with the expectation that it will help to understand the regulation of the RecQ helicases by the Zn finger-containing motifs. The model (**Fig. 8A**) proposed for the human RECQ5 can be extended to the RecQ family helicases. We therefore propose that the multiple enzymatic activities of the RecQ helicases play important roles in the DNA metabolism, and the enzymatic activities of the helicase cores are regulated by the helicase-associated functional motifs, in particular the Zn finger-containing RQC domains. This model appears to be consistent with all known biochemical and structural data, and can be used to explain

not only common biochemical and genetic properties shared by all RecQ helicases, but also distinct cellular defects associated with each RecQ helicase. In the following sections, we will limit the application of this model to other four human RecQ helicases (**Fig. 8** and **Table 3**).

BLM have been characterized extensively. The DNA-stimulated ATPase and helicase activities have been studied[28]. Recently, it has been shown that BLM also possess ssDNA annealing and strand-exchange activities[26,36]. Many of mutations in *BLM* are nonsense or frame-shift mutations that would express truncated proteins. However, seven missense mutations associated with BS have been identified[10,41-43]. Five of the seven point mutations map to the helicase core region of BLM: Q672R, I841T, C878R, G891E, and C901Y[10,41-43]. Three of these mutations have been shown to inactivate the murine BLM protein *in vitro* [44], implying that the missense mutations cause disease as a result of enzymatic failure. Other mutations could also impair BLM helicase enzyme function [16]. The two remaining BS-linked point mutations, C1036F and C1055S [10,42], are involved in the zinc finger formation[16,18]. The mutated proteins completely lost their enzymatic activities[18]. These results clearly support that the Zn^{2+} finger of BLM regulates the enzymatic activities of the BLM helicase core (**Fig. 8B**). The ssDNA annealing activity of BLM has been mapped to a 60-residue C-terminal fragment (amino acids 1291-1350)[36]. It would be interesting to show that the isolated fragment indeed has the annealing activity as proposed [36]. We predict that the BLM helicase core possesses a strong ssDNA annealing activity, but weak ATPase, helicase, and strand-exchange activities, which are subject to regulation by the Zn finger (**Fig. 8B**).

WRN is another extensively characterized human RecQ helicase. Although the conserved Cys residue at the position 2 is replaced by Ser, the presence of a Zn finger in WRN has been confirmed biochemically (Xi et al., unpublished). Interestingly, a multifunctional DNA- and protein-binding domain (DPBD) has been mapped out [19,20]. Our recent NMR structural studies show that the DPBD contains a WH-like domain [21]. The fragment containing the helicase core and RQC domain has a similar helicase activity compared to the full-length WRN [34], but the activity decreases when K1016 is replaced with Ala [19]. Moreover, when the DPBD is deleted from the fragment, the helicase activity of the fragment decreases dramatically[19],

suggesting that the DPBD rather than the Zn finger of WRN functions as a regulatory motif to regulate the enzymatic activities of WRN (**Fig. 8C**) [21].

Human RECQ1 helicase has recently being characterized. DNA helicase and ssDNA annealing activities have been observed for the protein [35]. RECQ1 does not contain a HRDC domain, but appears to possess a RQC domain [28]. We predict that the putative Zn finger (**Fig. 8D**) would regulate the enzymatic activities of the helicase core of RECQ1.

The clinical features associated with RTS have been recently recognized to be caused by the defects in *RECQ4*. Outside of the helicase domain, RECQ4 shows no obvious identity with the other RecQ family members and lack both RQC and HRDC domains (**Fig. 8E**) [28]. It has been shown that RECQ4, either in the isolated form [37] or in the complex with UBR1 and UBR2 [45], fails to unwind DNA, but catalyses ssDNA annealing. These apparent surprising results can be understood using the model derived from our RECQ5 studies. In this case, the Zn finger or DPBD is not available in RECQ4 to activate the helicase activity and to suppress the ssDNA annealing activity of the RECQ4 helicase core. Interestingly, the ATPase activity of RECQ4 is observable in the absence of the Zn finger [37].

Concluding remarks

In this study, we have used human RECQ5 proteins as a model system to characterize the functional modulation of a RecQ helicase core by a zinc finger-containing motif, by taking advantage of three isoforms of human RECQ5 helicase produced by alternative splicing. We have shown for the first time that the RECQ5 helicase core has an intrinsically high ssDNA annealing activity, and RECQ5β has two separate motifs that mediate ssDNA annealing. Based upon comprehensive and quantitative characterization of the multiple enzymatic properties of the RECQ5 proteins, we conclude that the Zn finger-containing motif of RECQ5β functions as a regulatory motif that regulates the DNA binding, ATPase, unwinding, strand-annealing and strand-exchange activities of the RECQ5 helicase core. This model appears to be applicable to other four human RecQ helicases to explain the similarity and difference of the molecular functions of these RecQ helicases.

METHODS

Materials

ATP, AMPPNP, ATPγS, EDTA, 4-(2-Pyridylazo) resorcinol disodium salt (PAR) and PMSF were obtained from Sigma. [γ-^{32}P]ATP and Western blot reagents were purchased from Amersham Pharmacia Biotech. Chelex 100 resin was obtained from Bio-Rad. The N-methylanthraniloyl derivative of adenine nucleotides (mantATP) was synthesized according to Hiratsuka [46], and purified on DEAE-celllose using a gradient of triethylammonium bicarbonate. Polyclonal antibody against six histidine residues was from Cell Signalling. Ni-NTA resin was from Qiagen.

Plasmid construction

RECQ5γ cDNA was kindly provided by Dr. Janscak, and was used as a PCR template to reconstruct RECQ5α. RECQ5β cDNA was obtained by PCR using RECQ5γ cDNA and the PP1045 cDNA (kindly provided by Dr. Gu, China) as templates. The resulting cDNA fragments were digested with *Nde*I and *Xho*I and ligated into pET15b (Novagen). Site-directed mutageneses were used to construct the desired mutations and mutations were confirmed by DNA sequencing.

Protein expression, purification and Western blot

The plasmids encoding human RECQ5 isoforms and variants were transformed into the *E. coli* strain BL21 (Novagen). The cells were grown to the mid-exponential phase (A_{600} of 0.5-0.6) at 37 °C and the protein expression was induced by 0.25 mM of isopropyl-D-thiogalactoside at 15 °C for 18 h. The cells were lysed in a buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, PMSF 0.1μM and 10% ethylene glycerol). The cell lysis was cleared by centrifugation and the supernatant was applied to a 20 ml Ni^{2+}-column equipped with AKATA FPLC system. The bound proteins were eluted with 300 ml of the linear gradient of imidazole (0.02-0.4 M). Fractions containing the proteins were identified by SDS-PAGE and were further purified using size exclusion chromatography (Superdex 200, Amersham). The protein concentrations were determined by the Bio-Rad dye method using bovine serum albumin (BSA) as a standard.

Human RPA protein containing all three subunits (RPA70, RPA32, and RPA14) was expressed in and purified from the *E. coli* according to Henricksen et al. [47]. The protein concentration is expressed as a trimeric complex.

Western blot analysis was performed using His_6-Tag polyclonal antibody purified from rabbit. A peroxidase labelled goat anti-rabbit IgG antibody (Amersham) was used as the secondary antibody. The signals were detected with an ECL Western Blotting Kit (Amersham).

DNA substrate preparation

The PAGE purified DNA substrates (**Supplementary Table 1**) were purchased from Proligo. The DNA duplex substrates were prepared as described previously [32]. Briefly, 250 μM DNA substrates were denatured in the 1× TE buffer containing 1 M NaCl or KCl by heating at 95 °C for 10 min. The denatured DNA was then annealed at 37 °C for 48 h. The annealed products were separated on 8% native PAGE containing 10 mM KCl running at 4 °C for 12 h with constant current of 20 mA.

CD spectroscopy

The CD measurements were performed with a Jobin-Yvon Marker IV high sensitivity dichrography at 25 °C. Some of the experiments were also confirmed with a PiStar-180 spectrometer. The samples were extensively dialyzed against the dialysis buffer (50 mM Tris-HCl, pH 7.4, 30 mM NaCl) and were analyzed in the quartz cells with path-lengths of 1 mm for far-UV CD measurements. Near-UV wavelength scans were recorded from 250 to 350 nm. All of the CD spectra were corrected by subtracting the background obtained with the buffer alone. An average of three to six wavelength scans is presented. The ellipticity results were expressed as mean residue ellipticity, in degrees·cm^2·$dmol^{-1}$.

$$MRE = \theta_{obs}(m \text{ deg}) / 10 \times n \times Cp \times l$$

where θ_{obs} is the CD in millidegree; n is the number of amino acid residues; l is the pathlength of the cell in centimeters and Cp is the molar fraction. The relative content in α-helix was deduced according to Zhong and Johnson [48]: % α-helix = $\Delta\varepsilon_{222\ nm} \times (-10)$, where $\Delta\varepsilon_{222\ nm}$ is the circular dichroism per residue at 222 nm and is related to $[\theta]_{222}$ by $\Delta\varepsilon_{222\ nm} = [\theta]_{222}/3300$. A protein concentration of 0.18 mg/ml and 1.8 mg/ml were used for far-UV and near-UV CD measurements, respectively.

Quantification of the RECQ5 protein-bound Zn^{2+} ion

The protein-ligated Zn^{2+} was measured using PAR, a reporter dye that absorbs light at 490 nm when bound to Zn^{2+}. To precisely quantify the Zn^{2+} content, all buffers were treated with Chelex®-100 resin. The proteins were dialysed against the EDTA-free Chelex®-treated buffer, passed over a 10 cm column of Chelex®-100 and re-concentrated. To facilitate the Zn^{2+} release, the protein (about 1 nmole in 40 µl) was first denatured with Chelex®-treated 7 M guanidine-HCl, and was then transferred to a 1 ml cuvette. PAR was added to the cuvette for a final concentration of 100 µM and the volume was adjusted to 1 ml with the PAR buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl). The absorbance was recorded from 350 to 600 nm on a UVIKON spectrophotometer 941 (Kontron) at 25 °C. The Zn^{2+} quantity was determined using the absorbance coefficient for the $Zn(PAR)_2$ complex ($\varepsilon_{500} = 6.6\times10^4\ M^{-1}cm^{-1}$). As a control, 20 nmoles of the pure $ZnCl_2$ was quantified under the identical conditions.

ATP binding assay

a) UV cross-linking assay

The UV cross-linking assay was carried out as previously describe [18]. 1 µg of a protein was added to a 10 µl of the buffer containing 20 mM HEPES-KOH, pH 7.5, and 5 mM $Mg(OAc)_2$. 2.5 µCi (2 pmol) of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and 150 pmol of cold ATP were then added to the solution. After incubated on ice for 10 min, the reaction mixture was then irradiated using a UV cross-linker (254 nm) (Stratagene) situated at a distance of 4 cm for 5 min. After boiled in the sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 20%

β-mercaptoethanol, 20% glycerol, 4 mM EDTA, and 0.01% bromophenol blue) for 5 min, the sample was then separated by SDS-12.5% PAGE. The gels were stained with Coomassie brilliant blue, dried, and processed for autoradiography.

b) Fluorescence measurements

A fluorescent nucleotide analogue (mantATP) was used to determine the apparent dissociation constant of ATP binding to the RECQ5 proteins. The fluorescence spectra were measured using PiStar-180 spectrometer (Applied Photophysics) or Fluoro Max-2 spectrofluorimeter (Jobin Yvon, Spex Instruments S.A., Inc) at 25 °C. In a 10×10×40 quartz cuvette, 0.5 μM protein in 1 ml reaction buffer was excited at 280 nm and fluorescence emission was monitored at 350 nm. The binding of mantATP to RECQ5 was measured by exciting RECQ5 at 280 nm and measuring the fluorescence of mantATP at 440 nm due to FRET. Both sample dilution and inner filter effects were taken into account and the observed fluorescence intensity was corrected.

The apparent K_d values were determined by fitting the fluorescence intensity values to Equation 1:

$$F = F_s + f_d x + f_c \frac{(x + 0.5 + K_d) - \sqrt{(x + 0.5 + K_d)^2 - 2x}}{2} \qquad \text{(Eq. 1)}$$

where, Fs is the starting fluorescence of the reaction mixture, f_d is the fluorescence coefficient of free mantATP, f_c is the fluorescence coefficient of complex formed, x is the total concentration of mantATP, and K_d is the apparent constant dissociation.

ATPase assay

The ATPase activity of the RECQ5 proteins and fragments was detected by measuring the release of the inorganic phosphate produced from ATP hydrolysis [18]. The reaction was carried out in an ATPase reaction buffer (50 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 0.5 mM DTT) at 37 °C in a volume of 100 μl. The reactions were initiated by the addition of an enzyme into a reaction mixture containing 1.5 μM heat-denatured Hind III-cut pGEM-7Zf linear DNA and the indicated ATP concentration. The reaction was stopped by pipetting an 80 μl aliquot from the

reaction mixture every 30 s into a HCl solution of ammonium molybdate. The liberated $^{32}P_i$ is extracted with a solution of 2-butanol/benzene/acetone/ammonium molybdate (750:750:15:1) saturated with water. An aliquot of 400 µl was removed from the organic phase and the radioactivity was quantified using a liquid scintillation counter.

DNA binding assay

a) Electrophoretic mobility shift assay (EMSA)

The binding reactions (20 µl) were conducted in a binding buffer (40 mM Tris-HCl, pH 7.5, 3 mM $Mg(OAc)_2$, 20 mM NaCl, 8% glycerol and 20 µg/ml BSA). The protein and DNA concentrations were indicated in the figure legends. Reactions were incubated for 30 min at 25 °C. After adding 4 µl of the native loading dye (0.25% bromphenol blue in 30% glycerol), the reactions were loaded on 5% native PAGE (37.5:1). Electrophoresis was carried out at a constant voltage of 14V/cm at 4 °C in the 1× TBE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 3 h. The gel was dried and processed for autoradiography.

b) Fluoresence polarization assay

The binding of a protein to DNA was analyzed by fluorescence polarization using a Beacon fluorescence polarization spectrophotometer (PanVera) as described previously [32]. A 36-mer ssDNA labelled with 5'-fluorescein was used. A 1:1 mixture of the 5'-fluorescein labelled 36-mer and its complementary 36-mer was first heated to 95 °C, and then slowly cooled to form a 36-bp dsDNA. Varying amounts of the protein were added to a 150 µl aliquot of the binding buffer (25 mM Tris-HCl, pH 8.0, 30 mM NaCl, 3 mM $Mg(OAc)_2$, and 0.1 mM DTT) containing 2 nM of the fluorescein-labelled ssDNA or dsDNA. Each sample was allowed to equilibrate in the solution for 5 min before the fluorescence polarization was measured. A second reading was taken after 10 min to ensure that the mixture was equilibrated. A less than 5% change between a 30-min and a 5-min measurement indicated that equilibrium was reached. The equilibrium dissociation constant was determined by plotting polarization as a function of the protein concentration and fitting the data to the

Michaelis-Menten or Hill equation using the program KaleidaGraph (Synergy Software).

DNA helicase activity assay

a) Radiometric assay

DNA unwinding reaction was carried out at 37 °C in a reaction mixture containing 25 mM HEPES-NaOH, pH 7.5, 25 mM NaOAc, 7.5 mM $Mg(OAc)_2$, 2 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, and indicated ^{32}P-labelled partial DNA duplex substrate (10 fmol, 3000 c.p.m/fmol). Where required, RPA was added at the indicated concentration. The reaction was initiated by addition of indicated concentration of the RECQ5 protein or fragment at 37 °C for 20 min. The reaction was terminated by the addition of 5 μl of 5× loading buffer (50 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol). The reaction products were resolved on a 12% (w/v) PAGE run in a TBE buffer (90 mM Tris, 90 mM boric acid, pH 8.3, and 1 mM EDTA) at 100 V for 2h at 4 °C.

b) Fluorimetric assay

Stopped-flow DNA unwinding assay was performed according to Zhang et al [33]. Briefly, the experiment was carried out using a Bio-logic SFM-400 mixer with a 1.5 mm × 1.5 mm cell (Bio-Logic, FC-15) and a Bio-Logic MOS450/AF-CD optical system equipped with a 150-W mercury-xenon lamp. The assay was performed in a two-syringe mode, where the protein and DNA substrates were preincubated in the syringe #1 for 5 min, while ATP in syringe #4. Each syringe contained the unwinding reaction buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM DTT). The unwinding reaction was initiated by rapid mixing. The sequences of the two hexachlorofluorescein- and fluorescein-labeled strands are listed in the **Supplementary Table 1**. For converting the output data from volts to percent unwinding, we performed another four-syringe mode experiment, where the helicase in syringe #1, hexachlorofluorescein-labeled ssDNA in syringe #2, and fluorescein-labeled ssDNA in the syringe #3 were incubated in unwinding reaction buffer, and the solution in syringe #4 (ATP). The fluorescent signal of the mixed solution from the four syringes was corresponding to 100% unwinding. The standard reaction was carried out at 25 °C unless noted otherwise.

DNA strand-annealing assay

a) Radiometric assay

The DNA annealing activity of the RECQ5 isoforms was assayed using complementary synthetic oligonucleotides (0.5 nM) with one strand 5′-labelled using [γ-^{32}P]ATP and T4 DNA kinase. In a standard strand-annealing assay, the labelled oligonucleotide was added to a reaction buffer (20 µl) containing 20 mM Tris-acetate, pH 7.9, 50 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, 50 µg/ml BSA and the indicated protein concentration. If indicated, 2 mM of ATP, ATPγS, AMPPNP or ADP was also added. The reaction was initiated by adding the unlabelled oligonucleotide, immediately followed by incubation at 37 °C for 15 min. The resulting DNA products were analysed as described for the helicase reactions.

b) Fluorimetric assay

The stopped-flow DNA strand-annealing assay was carried out under the similar conditions used for the helicase assay. The reaction was performed in a three-syringe mode, where the helicase was in syringe #1 while fluorescein- and hexachlorofluorescein-labeled complementary ssDNA in syringe #2 and syringe #3, respectively, in the reaction buffer comprising 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM DTT. The sequences of the two 45-mer ssDNA substrates are indicated in the **Supplementary Table 1**. The reaction was initiated by rapidly mixing the three syringes. For converting the output data from volts to percent annealing, we performed another experiment in a two-syringe mode, where the helicase was in the syringe #1, and the annealed dsDNA in the syringe #4. The fluorescent signal of the mixed solution from the two syringes corresponded to 100% annealing. The reaction was carried out 25 °C.

Strand-exchange assay

The strand-exchange assays were performed essentially as described by Machwe et al.[26] with some modifications. Briefly, the labelled forked substrate (UA/UB) and ssDNA (1 nM) were incubated in a 20 µl helicase reaction buffer for 15 min with an indicated concentration of a protein, as indicated in the figure legends. After addition of 1 mM ATP, the DNA products were subsequently monitored over time.

ACKNOWLEDGEMENTS

We thank Drs. Robert Pansu and Luossiné Zargarian for assistance in the fluorescence and CD measurements, Dr. Pascal Rigolet for help in the sequence alignments, Rong bin Guo for suggestion about radiometric assay, and Yan He for carrying some preliminary studies. X. G. X. is a research staff of the Centre National de la Recherche Scientifique (CNRS). This research was supported by the grants from la Ligue Contre Le cancer and Institut National du Cancer to X. G. X.; the National Natural Science Foundation of China (Grants 60025516 and 10334100) and the Innovation Project of the Chinese Academy of Sciences to P.-Y. W.; and the grants from the American Cancer Society (RSG-03-238-01) and the American Heart Association (RSG-03-238-01) to J.-S. H.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

References

1. Lohman,T.M. & Bjornson,K.P. Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169-214 (1996).

2. Soultanas,P. & Wigley,D.B. DNA helicases: 'inching forward'. *Curr. Opin. Struct. Biol.* **10**, 124-128 (2000).

3. von Hippel,P.H. & Delagoutte,E. A general model for nucleic acid helicases and their "coupling" within macromolecular machines. *Cell* **104**, 177-190 (2001).

4. Hickson,I.D. RecQ helicases: caretakers of the genome. *Nat. Rev. Cancer* **3**, 169-178 (2003).

5. Nakayama,H., Nakayama,K., Nakayama,R., Irino,N., Nakayama,Y., & Hanawalt,P.C. Isolation and genetic characterization of a thymineless death-resistant mutant of Escherichia coli K12: identification of a new mutation (recQ1) that blocks the RecF recombination pathway. *Mol. Gen. Genet.* **195**, 474-480 (1984).

6. Umezu,K., Nakayama,K., & Nakayama,H. Escherichia coli RecQ protein is a DNA helicase. *Proc. Natl. Acad. Sci. U. S. A* **87**, 5363-5367 (1990).

7. Hanada,K., Ukita,T., Kohno,Y., Saito,K., Kato,J., & Ikeda,H. RecQ DNA helicase is a suppressor of illegitimate recombination in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A* **94**, 3860-3865 (1997).

8. Puranam,K.L. & Blackshear,P.J. Cloning and characterization of RECQL, a potential human homologue of the Escherichia coli DNA helicase RecQ. *J. Biol. Chem.* **269**, 29838-29845 (1994).

9. Seki,M., Miyazawa,H., Tada,S., Yanagisawa,J., Yamaoka,T., Hoshino,S., Ozawa,K., Eki,T., Nogami,M., Okumura,K., & . Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12. *Nucleic Acids Res.* **22**, 4566-4573 (1994).

10. Ellis,N.A., Groden,J., Ye,T.Z., Straughen,J., Lennon,D.J., Ciocci,S., Proytcheva,M., & German,J. The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* **83**, 655-666 (1995).

11. Yu,C.E., Oshima,J., Fu,Y.H., Wijsman,E.M., Hisama,F., Alisch,R., Matthews,S., Nakura,J., Miki,T., Ouais,S., Martin,G.M., Mulligan,J., & Schellenberg,G.D. Positional cloning of the Werner's syndrome gene. *Science* **272**, 258-262 (1996).

12. Kitao,S., Ohsugi,I., Ichikawa,K., Goto,M., Furuichi,Y., & Shimamoto,A. Cloning of two new human helicase genes of the RecQ family: biological significance of multiple species in higher eukaryotes. *Genomics* **54**, 443-452 (1998).

13. Shimamoto,A., Nishikawa,K., Kitao,S., & Furuichi,Y. Human RecQ5beta, a large isomer of RecQ5 DNA helicase, localizes in the nucleoplasm and interacts with topoisomerases 3alpha and 3beta. *Nucleic Acids Res.* **28**, 1647-1655 (2000).

14. Karow,J.K., Wu,L., & Hickson,I.D. RecQ family helicases: roles in cancer and aging. *Curr. Opin. Genet. Dev.* **10**, 32-38 (2000).

15. Morozov,V., Mushegian,A.R., Koonin,E.V., & Bork,P. A putative nucleic acid-binding domain in Bloom's and Werner's syndrome helicases. *Trends Biochem. Sci.* **22**, 417-418 (1997).

16. Bernstein,D.A., Zittel,M.C., & Keck,J.L. High-resolution structure of the E.coli RecQ helicase catalytic core. *EMBO J.* **22**, 4910-4921 (2003).

17. Liu,J.L., Rigolet,P., Dou,S.X., Wang,P.Y., & Xi,X.G. The zinc finger motif of Escherichia coli RecQ is implicated in both DNA binding and protein folding. *J. Biol. Chem.* **279**, 42794-42802 (2004).

18. Guo,R.B., Rigolet,P., Zargarian,L., Fermandjian,S., & Xi,X.G. Structural and functional characterizations reveal the importance of a zinc binding domain in Bloom's syndrome helicase. *Nucleic Acids Res.* **33**, 3109-3124 (2005).

19. Lee,J.W., Kusumoto,R., Doherty,K.M., Lin,G.X., Zeng,W., Cheng,W.H., von Kobbe,C., Brosh,R.M., Jr., Hu,J.S., & Bohr,V.A. Modulation of Werner syndrome protein function by a single mutation in the conserved RQC domain. *J. Biol. Chem.* (2005).

20. Lee,J.W., Harrigan,J., Opresko,P.L., & Bohr,V.A. Pathways and functions of the Werner syndrome protein. *Mech. Ageing Dev.* **126**, 79-86 (2005).

21. Hu,J., Zeng,W., Feng,H., Lin, G., & Xi,X.G. Solution structure of a multifunctional DNA-and protein-binding motif of human Werner syndrome protein. Proc. Natl. Acad. Sci. U. S. A. (2005, in press).

22. Sekelsky,J.J., Brodsky,M.H., Rubin,G.M., & Hawley,R.S. Drosophila and human RecQ5 exist in different isoforms generated by alternative splicing. *Nucleic Acids Res.* **27**, 3762-3769 (1999).

23. Garcia,P.L., Liu,Y., Jiricny,J., West,S.C., & Janscak,P. Human RECQ5beta, a protein with DNA helicase and strand-annealing activities in a single polypeptide. *EMBO J.* **23**, 2882-2891 (2004).

24. Ozsoy,A.Z., Sekelsky,J.J., & Matson,S.W. Biochemical characterization of the small isoform of Drosophila melanogaster RECQ5 helicase. *Nucleic Acids Res.* **29**, 2986-2993 (2001).

25. Kawasaki,K., Maruyama,S., Nakayama,M., Matsumoto,K., & Shibata,T. Drosophila melanogaster RECQ5/QE DNA helicase: stimulation by GTP binding. *Nucleic Acids Res.* **30**, 3682-3691 (2002).

26. Machwe,A., Xiao,L., Groden,J., Matson,S.W., & Orren,D.K. RecQ family members combine strand pairing and unwinding activities to catalyze strand exchange. *J. Biol. Chem.* **280**, 23397-23407 (2005).

27. Bennett,R.J. & Keck,J.L. Structure and function of RecQ DNA helicases. *Crit Rev. Biochem. Mol. Biol.* **39**, 79-97 (2004).

28. Bachrati,C.Z. & Hickson,I.D. RecQ helicases: suppressors of tumorigenesis and premature aging. *Biochem. J.* **374**, 577-606 (2003).

29. Venyaminov,S.Y. & Vassilenko,K.S. Determination of protein tertiary structure class from circular dichroism spectra. *Anal. Biochem.* **222**, 176-184 (1994).

30. Hunt,J.B., Neece,S.H., & Ginsburg,A. The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from Escherichia coli aspartate transcarbamoylase. *Anal. Biochem.* **146**, 150-157 (1985).

31. Janscak,P., Garcia,P.L., Hamburger,F., Makuta,Y., Shiraishi,K., Imai,Y., Ikeda,H., & Bickle,T.A. Characterization and mutational analysis of the RecQ core of the bloom syndrome protein. *J. Mol. Biol.* **330**, 29-42 (2003).

32. Dou,S.X., Wang,P.Y., Xu,H.Q., & Xi,X.G. The DNA binding properties of the Escherichia coli RecQ helicase. *J. Biol. Chem.* **279**, 6354-6363 (2004).

33. Zhang,X.D., Dou,S.X., Xie,P., Wang,P.Y., & Xi,X.G. RecQ helicase-catalyzed DNA unwinding detected by fluorescence resonance energy transfer. *Acta Biochim. Biophys. Sin. (Shanghai)* **37**, 593-600 (2005).

34. Doherty,K.M., Sommers,J.A., Gray,M.D., Lee,J.W., von Kobbe,C., Thoma,N.H., Kureekattil,R.P., Kenny,M.K., & Brosh,R.M., Jr. Physical and functional mapping of the replication protein a interaction domain of the werner and bloom syndrome helicases. *J. Biol. Chem.* **280**, 29494-29505 (2005).

35. Sharma,S., Sommers,J.A., Choudhary,S., Faulkner,J.K., Cui,S., Andreoli,L., Muzzolini,L., Vindigni,A., & Brosh,R.M., Jr. Biochemical analysis of the DNA unwinding and strand annealing activities catalyzed by human RECQ1. *J. Biol. Chem.* **280**, 28072-28084 (2005).

36. Cheok,C.F., Wu,L., Garcia,P.L., Janscak,P., & Hickson,I.D. The Bloom's syndrome helicase promotes the annealing of complementary single-stranded DNA. *Nucleic Acids Res.* **33**, 3932-3941 (2005).

37. Macris,M.A., Krejci,L., Bussen,W., Shimamoto,A., & Sung,P. Biochemical characterization of the RECQ4 protein, mutated in Rothmund-Thomson syndrome. *DNA Repair (Amst)* (2005).

38. Minton,A.P. Influence of excluded volume upon macromolecular structure and associations in 'crowded' media. *Curr. Opin. Biotechnol.* **8**, 65-69 (1997).

39. Jencks,W.P. Economics of enzyme catalysis. *Cold Spring Harb. Symp. Quant. Biol.* **52**, 65-73 (1987).

40. Caruthers,J.M. & McKay,D.B. Helicase structure and mechanism. *Curr. Opin. Struct. Biol.* **12**, 123-133 (2002).

41. Foucault,F., Vaury,C., Barakat,A., Thibout,D., Planchon,P., Jaulin,C., Praz,F., & Amor-Gueret,M. Characterization of a new BLM mutation associated with a topoisomerase II alpha defect in a patient with Bloom's syndrome. *Hum. Mol. Genet.* **6**, 1427-1434 (1997).

42. Barakat,A., Ababou,M., Onclercq,R., Dutertre,S., Chadli,E., Hda,N., Benslimane,A., & Amor-Gueret,M. Identification of a novel BLM missense mutation (2706T>C) in a Moroccan patient with Bloom's syndrome. *Hum. Mutat.* **15**, 584-585 (2000).

43. Rong,S.B., Valiaho,J., & Vihinen,M. Structural basis of Bloom syndrome (BS) causing mutations in the BLM helicase domain. *Mol. Med.* **6**, 155-164 (2000).

44. Bahr,A., De Graeve,F., Kedinger,C., & Chatton,B. Point mutations causing Bloom's syndrome abolish ATPase and DNA helicase activities of the BLM protein. *Oncogene* **17**, 2565-2571 (1998).

45. Yin,J., Kwon,Y.T., Varshavsky,A., & Wang,W. RECQL4, mutated in the Rothmund-Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. *Hum. Mol. Genet.* **13**, 2421-2430 (2004).

46. Hiratsuka,T. Biological activities and spectroscopic properties of chromophoric and fluorescent analogs of adenine nucleoside and nucleotides, 2',3'-O-(2,4,6-trinitrocyclohexadienylidene) adenosine derivatives. *Biochim. Biophys. Acta* **719**, 509-517 (1982).

47. Henricksen,L.A., Umbricht,C.B., & Wold,M.S. Recombinant replication protein A: expression, complex formation, and functional characterization. *J. Biol. Chem.* **269**, 11121-11132 (1994).

48. Zhong,L. & Johnson,W.C., Jr. Environment affects amino acid preference for secondary structure. *Proc. Natl. Acad. Sci. U. S. A* **89**, 4462-4465 (1992).

FIGURE LEGENDS

Figure 1 Identification of the Zn finger motif of the RECQ5 helicases. (**A**) Schematic representation of human RECQ5 (RECQ5α, RECQ5β and RECQ5γ) and *Drosophile melanogaster* RECQ5 (DmRECQ5a, DmRECQ5b and DmRECQ5c) isoforms. The conserved helicase domain, zinc finger and the C-terminal domain are shown as blue, red, and open boxes. (**B**) Amino acid sequence alignments of the putative zinc finger among the RecQ family helicases. The multiple alignments were performed with the program ClustalW and refined manually. The conserved Cys and other residues are shadowed in red and blue, respectively. (**C**) Typical absorbance spectra of PAR in the absence and in the presence of $ZnCl_2$. (**D**) Protein-bound Zn^{2+} concentrations are plotted against the RECQ5β protein concentration. The absorption spectra were scanned from 300 to 600 nm at 25 °C. As a control, 20 nmol $ZnCl_2$ complexed with PAR was scanned under the identical conditions.

Figure 2 ATP-binding affinity and ATPase activity of the RECQ5 proteins by fluorescence assays. (**A**) The overlap of the RECQ5 protein emission spectrum after excited at 280 nm and the mantATP excitation spectrum at 345 nm (emission at 445 nm). For clarity, only excitation-emission spectra of RECQ5β are shown. (**B**) Changes in fluorescence intensity when 0.5 μM enzymes were titrated with increasing concentration of mantATP. Solid lines represent the best fit of the data to Equation 1 and the apparent K_d values are summarized in **Table 1**. (**C**) ATPase activities of the RECQ5 proteins and mutants. Reactions were carried out as described in the Methods. 250 nM of the RECQ5 protein and 1.5 μM of heated dsDNA were used for each assay. The initial rate of ATP hydrolysis by the RECQ5 proteins was shown as function of the ATP concentration. The lines in the figure correspond to the best fits by Michaelis-Menton equation: $V = V_{max}[ATP]/(K_M + [ATP])$, where V is the initial reaction rate, [ATP] is the concentration of ATP, and K_M is the Michaelis-Menton constant. Each value represents the mean of at least three independent measurements.

Figure 3 The determination of DNA binding affinity of the RECQ5 proteins under the equilibrium conditions. The anisotropy based binding isotherms were

obtained by varying protein concentration in the presence of 1 nM 3'-fluorescent labelled (**A**) ssDNA (BA) and (**B**) dsDNA (BA/BB) (**Supplementary Table 1**), and then were fitted to Hill equation. The apparent K_d values are summarized in **Table 2**.

Figure 4 The helicase unwinding assays of the RECQ5 proteins. (**A**) Radiometric assay of DNA unwinding using 0.5 nM [$5'^{-32}$P]-labelled 23-base DNA duplex (UA/UB) (**Supplementary Table 1**). The reaction was carried out at 30 °C for 30 min under condition as described in the Methods. The reaction products were analyzed by 12% nondenatured PAGE. Radiolabelled species were visualized quantitatively using a Storm 860 Phosphoimager (Amersham). (**B**) The stopped-flow DNA unwinding assay. As described in the Methods, 1 nM 56:16-mer DNA substrate (UFA/UFB) (**Supplementary Table 1**) was pre-incubated with 20 nM helicase for 5 min at 25 °C. Unwinding reaction was initiated by mixing with 1 mM ATP. The fluorescence emission of fluorescein at 520 nm (excited at 492 nm) was monitored. Fraction of DNA unwound was obtained by normalization of the fluorescence signal.

Figure 5 The RECQ5-mediated ssDNA strand annealing. (**A**) Effect of the protein concentration of the RECQ5 proteins on the annealing of the [5'-^{32}P]-labelled SPA (0.5 nM) and SPB ssDNA substrates (**Supplementary Table 1**) to generate a duplex DNA. The reactions were incubated with the indicated protein concentrations for 30 min at 25 °C. The reaction products were separated by 12% nondenaturing PAGE and quantified by PhosphoImager. (**B**) Effect of nucleotides on the RECQ5-mediated ssDNA annealing. The reactions were carried out using 50 nM of the RECQ protein under the same condition as indicated in **Fig. 5A** in the absence or presence of the indicated nucleotide (2 mM). (**C**) Fluorimetric assay of the ssDNA annealing. The annealing was measured by monitoring the decreasing of the fluorescent signal of fluorescein at 520 nm as function of time. At beginning, the fluorescent signal of fluorescein is high because the two labelled ssDNA substrates are separated. After annealing, the fluorescein and hexachlorofluorescein of the resulted dsDNA are in close proximity, the fluorescent signal of fluorescein decreases due to FRET. The curve is obtained by mixing 0.5 nM fluorescein-labeled SPFA DNA, 0.5 nM hexachlorofluorescein-labeled complementary SPFB DNA (**Supplementary Table 1**), and 30 nM RECQ5β. (**D**) Time courses of ssDNA annealing reactions with the RECQ5 proteins obtained with the above fluorimetric

assays. The 30 nM of each protein was used. Fraction of DNA annealing (**Table 1**) was obtained by normalization of the fluorescence signal.

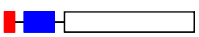
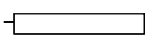
Figure 6 The RECQ5-mediated strand exchange. (**A**) Shown is a schematic diagram of the strand-exchange reaction between a labelled forked dsDNA and a partial complementary 23-nt ssDNA. The resulted products are an unlabelled ssDNA and a 5'-tail dsDNA, which is a poor unwinding substrate for the RECQ5 helicase. (**B**) Strand-exchange reaction was performed as described in the Methods. 1 nM [5'-^{32}P]-labelled forked dsDNA(UA/UB) and 1 nM ssDNA (SPC) (**Supplementary Table 1**) were incubated in 20 μl helicase reaction buffer for 15 min. After addition of 1 mM ATP, the resulted DNA products were analyzed at the time indicated in the figure. (**C**) The observed percentage of the original forked dsDNA, and unwinding product (ssDNA), and the exchange product (partial dsDNA) in the RECQ5β-mediated strand exchange are plotted as function of the reaction time. The products were quantified from three independent experiments using ImagerQuant software.

Figure 7 The DNA-binding affinity and ssDNA annealing activity of the RECQ5β-derived fragments. (**A**) The strand-annealing reactions of the RECQ5β-derived fragments were assayed with 2, 10, 50 nM RECQ5 proteins using radiometric assay. (**B**) Strand-annealing activities of the RECQ5β-derived fragments were quantified by the fluorimetric assay. The reactions were performed under the identical conditions as in **Figure 7A** using 50 nM of the proteins. (**C**) and (**D**) DNA-binding isotherms (**Table 2**) of the RECQ5β-derived fragments using 1 nM of 3'- fluorescent of 36nt ssDNA (BA) (**C**) and dsDNA (BA/BB) (**D**) (**Supplementary Table 1**).

Figure 8 Models for the regulation of human RecQ helicases by RQC motif. Only three functional domains, helicase core (HC), the Zn-binding domain (ZBD), and DNA- and protein-binding domain (DPBD) of the RecQ helicases are shown. The active site of HC is shown. (**A**) **RECQ5**. RECQ5α or the RECQ5 helicase core has no or low DNA-binding, ATPase, unwinding and strand-exchange activities, but high strand-annealing activity. The RECQ5 fragment such as RECQ5β$^{1-475}$ containing

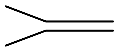
HC and ZBD has high DNA-binding, ATPase, unwinding and strand-exchange activities, comparable to those of RECQ5β. The putative DPBD domain on the RECQ5β$^{1-662}$ fragment has no observable effect on the activities, except the strand-annealing activity, of RECQ5β$^{1-475}$ fragment. (**B**) **BLM**. The ZBD of BLM enhances the helicase activity of the BLM helicase [18] and may regulate the DNA-binding, ATPase, strand-annealing and strand-exchange activities of the BLM helicase core. (**C**) **WRN**. The WRN fragment containing only HC and ZBD has very low helicase activity [19], while the fragment containing additional DPBD has essentially identical helicase activity compared to the full-length enzyme [34]. The DPBD likely functions as main regulatory motif to regulate the DNA-binding, ATPase, strand-annealing and strand-exchange activities of the WRN helicase core [21]. (**D**) **RECQ1**. The ZBD may regulate DNA-binding, ATPase, strand-annealing and strand-exchange activities of the RECQ1 helicase core. (**E**) **RECQ4**. RECQ4 has no ZBD or DPBD to stimulate the helicase activity and to suppress the ssDNA annealing activity of the RECQ4 helicase core.

Table 1. Summary of the measured parameters of human RECQ5 isoforms and mutants

RECQ5 Protein	Structural Feature	[Zn^{2+}]/ [Protein][a]	ATP binding K_d (μM)	ATPase k_{cat} (s^{-1})	Helicase Unwinding (%)	Strand Annealing (%)[b]
RECQ5β		0.98 ± 0.18	51.0 ± 4.9	15.6 ± 1.4	84.1 ± 3.5	72.3 ± 5.6
RECQ5β$^{1-662}$		1.02 ± 0.15	45.2 ± 3.5	16.3 ± 1.3	92.5 ± 5.6	65.3 ± 10.5
RECQ5β$^{1-475}$		0.96 ± 0.11	46.5 ± 6.3	14.8 ± 1.1	90.3 ± 4.9	35.5 ± 6.5
RECQ5β^{C431S}		0.35 ± 0.12	52.2 ± 3.6	4.8 ± 0.6	25.6 ± 3.6	58.6 ± 3.5
RECQ5γ		0.01 ± 0.02	41.6 ± 2.0	0.15 ± 0.1	27.2 ± 2.3	40.1 ± 6.6
RECQ5α		0.03 ± 0.02	48.4 ± 2.5	ND[c]	ND	95.2 ± 8.6
RECQ5β$^{379-991}$						65.2 ± 9.5
RECQ5β$^{454-991}$						61.5 ± 9.3
RECQ5β$^{454-662}$						48.2 ± 7.2
No enzyme						12.3 ± 8.2

The assays were performed as described in the Methods. [a] Number of bound Zn per molecule of the protein. [b] The values are averages of the data obtained with radiometric and fluorescence asays. [c] ND represents the value that is too low to be determined correctly.

Table 2. Equilibrium dissociation constants of DNA substrates (K_d, nM)

RECQ5 Protein	DNA substrates					
	ssDNA 5′———	ssDNA/ AMP-PNP 5′———	dsDNA 5′═══	3′ overhange Duplex ═══5′	5′ overhange Duplex ———5′	Long forked Duplex  5′
RecQ5α	265 ± 12	285 ± 32	299 ± 26	289 ± 18	298 ± 15	299 ± 26
RecQ5γ	276 ± 6	293 ± 8	285 ± 6	201 ± 12	266 ± 36	220 ± 16
RecQ5β	45 ± 2	24 ± 5	82 ± 3	62 ± 3	52 ± 3	35 ± 6
RecQ5β$^{1-475}$	55 ± 5		68 ± 5			
RecQ5β$^{1-662}$	50 ± 7		89 ± 3			
RecQ5β^{C431S}	90 ± 10		289 ± 27			
RecQ5β$^{379-991}$	102 ± 3		360 ± 28			
RecQ5β$^{454-991}$	152 ± 2		650 ± 56			
RecQ5β$^{454-662}$	558 ± 41		684 ± 28			

All experiments were performed under equilibrium condition as indicated in the Methods.
1 nM 3'-fluorescent labelled DNA substrates were used. The K_d is expressed in nM.

Table 3. The correlation between the observed enzymatic activities of the RecQ helicases and the presence of the zinc fingers

RECQ helicase	Structural Feature	ATPase	Unwinding	Strand annealing	Reference
RECQ1		+	+	+	35
BLM		+	+	+	26,36
WRN		+	+	+	26
DmRECQ5a		+	+	NA	25
DmRECQ5b		+	+	+	35
DmRECQ5c		NA	NA	NA	
RECQ5β		+	+	+	23, this work
RECQ5γ		-	-	-/+	this work
RECQ5α		-	-	+	this work
RECQ4		+	-	+	39,40

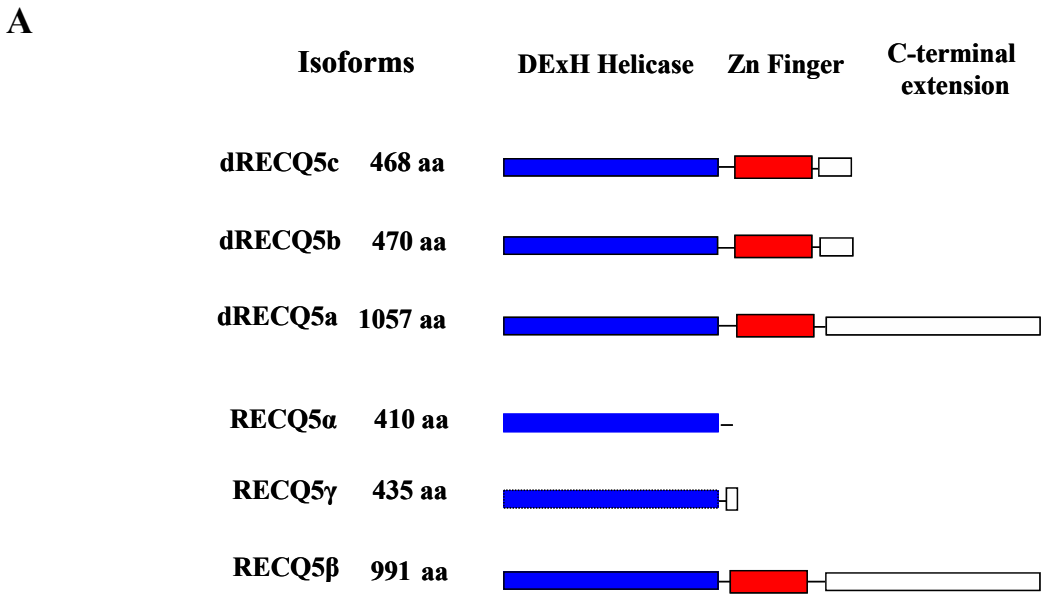
A summary of available information about ATPase, helicase and strand annealing activities of the RecQ helicases and their structural features. The observed enzymatic activity is identified by a (+) sign, the deficiency in the enzymatic activity is identified as (-) sign, and a weak activity is shown as -/+. The presence of the Zn finger appears to be required for the ATPase and helicase activities, but not the strand annealing activity of the helicases.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 Far-UV (**a**) and near-UV (**b**) circular dichroism (CD) spectra of human RECQ5 proteins. Experiments were performed under conditions as indicated in "Methods".

Figure S2 RECQ5 isoforms bind to DNA substrates as detected by gel electrophoresis mobility shift assays under equilibrium condition. Increasing concentration of the isoforms proteins were preincubated with 1 nM [5'-^{32}P]-labelled 36-mer ssDNA (BA oligo for A and D) and 1 nM 36-bp dsDNA (BA/BB for B and C) (**Supplementary Table 1**) for 15 min on ice in binding buffer. The protein-DNA complexes were then resolved on 12 % nondenatured PAGE. Radiolabelled bands were detected by phosphoimager analysis.

Fig. 1



B

```
hRECQ5   390 ASDKATIMAFDALVTFCEE-LGCRHAAIAKYFGDA---LPACAKG------CDHCQNPTAVRRRLEALE
DmRECQ5  387 LLTERAIKQFEKITEFCER-TTCRHKLFSDFFGDP---TPDCSGQ------CDVCKRPKKAEKALEMFH
BLM     1013 HTRETHFNNLYSMVHYCENITECRRIQLLAYFGENGFNPDFCKKHP-DVS-CDNCCKTKDYKT--RDVT
RECQ1    433 ---NVGQQKLYEMVSYCQNISKCRRVLMAQHFDEV-WNSEACNKM------CDNCCKDSAFER--KNIT
RqhI     866 ETKERQRQMLRQVIQFCENKTDCRRKQVLAYFGEN-FDKVHCRKG------CDICCEEATYIK--QDMT
WRN      886 KFRLYKLKMMAKMEKYLHS-SRCRRQIILSHFEDK-QVQKASLGIMGTEKCCDNCRSRLDHCYSMDDSE
SgsI    1024 ENKEKHLNKLQQVMAYCDNVTDCRRKLVLSYFNED-FDSKLCHKN------CDNCRNSANVIN--EE--
RecQ     357 QLQDIERHKLNAMGAFAEA-QTCRRLVLLNYFGEG--RQEPCGN------CDICLDPPKQYDGSTDAQ
```

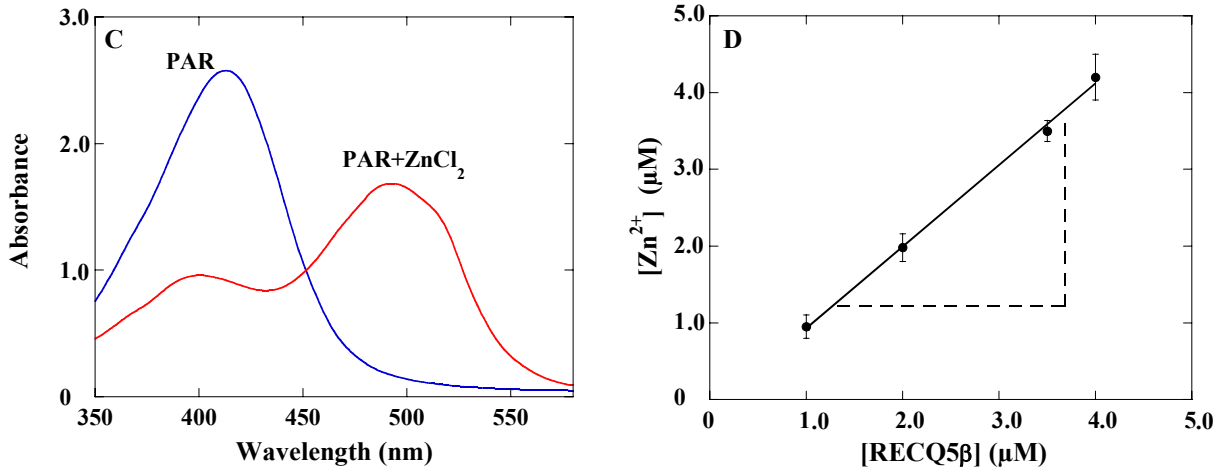


Fig. 2

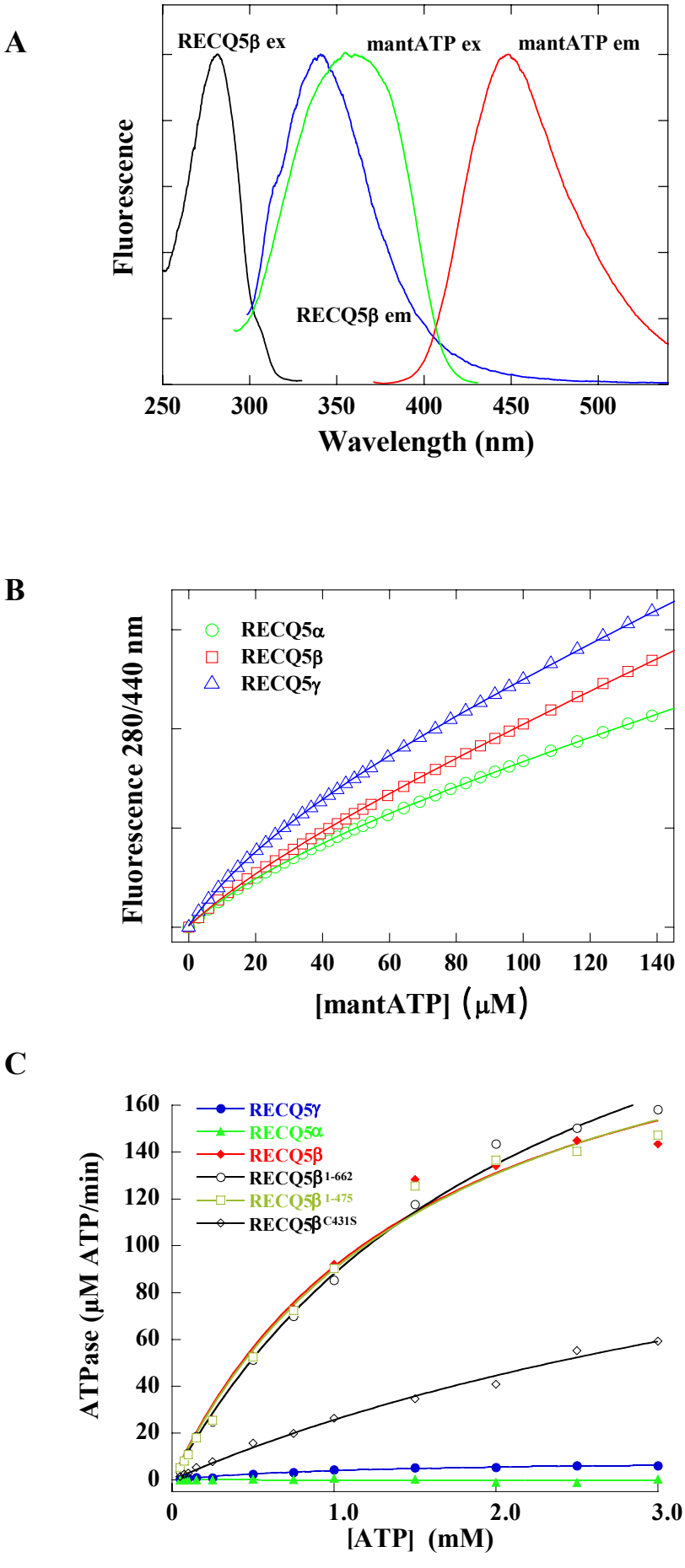
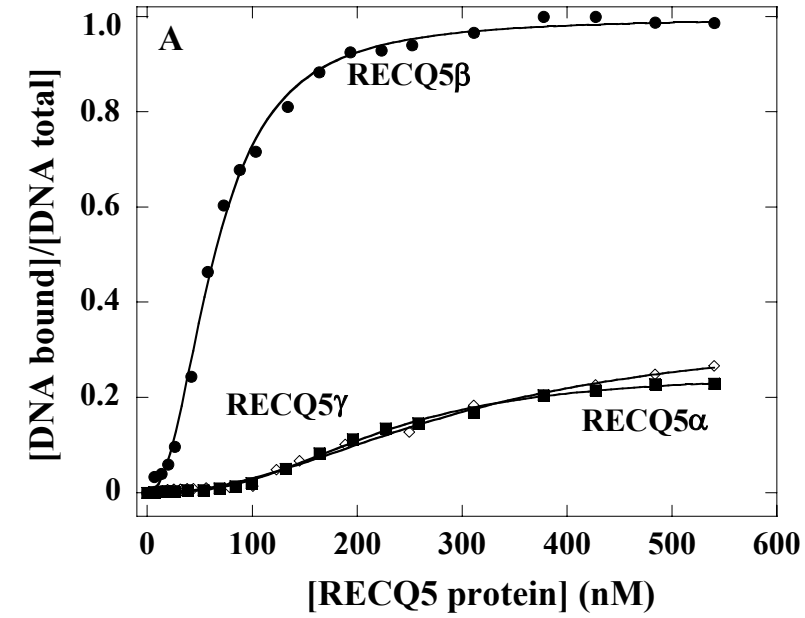

A
RECQ5β ex
mantATP ex
mantATP em
RECQ5β em
Fluorescence
250
300
350
400
450
500
Wavelength (nm)
B
RECQ5α
RECQ5β
RECQ5γ
Fluorescence 280/440 nm
0
20
40
60
80
100
120
140
[mantATP] (μM)
C
RECQ5γ
RECQ5α
RECQ5β
RECQ5β 1-662
RECQ5β 1-475
RECQ5β C431S
ATPase (μM ATP/min)
160
140
120
100
80
60
40
20
0
0
1.0
2.0
3.0
[ATP] (mM)

Fig. 3


A
RECQ5β
RECQ5γ
RECQ5α
[DNA bound]/[DNA total]
[RECQ5 protein] (nM)
0
0.2
0.4
0.6
0.8
1.0
0
100
200
300
400
500
600

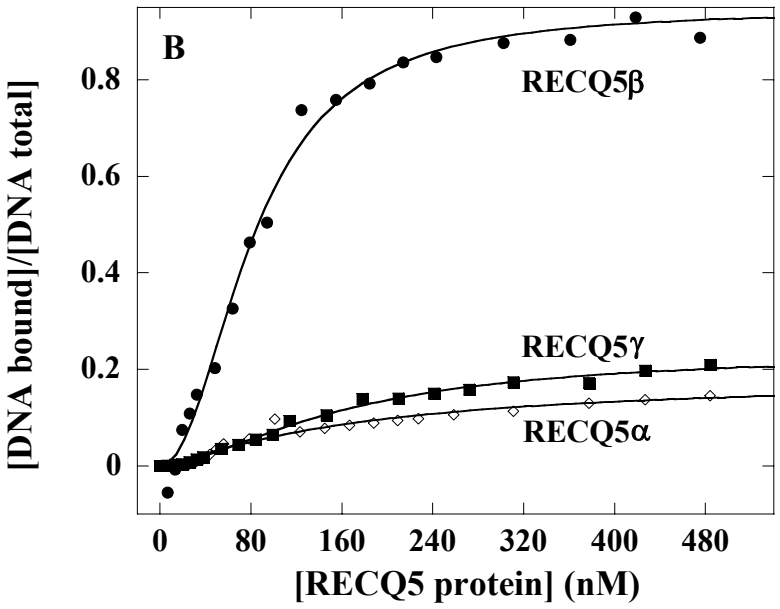

B
RECQ5β
RECQ5γ
RECQ5α
[DNA bound]/[DNA total]
[RECQ5 protein] (nM)
0
0.2
0.4
0.6
0.8
0
80
160
240
320
400
480

Fig. 4

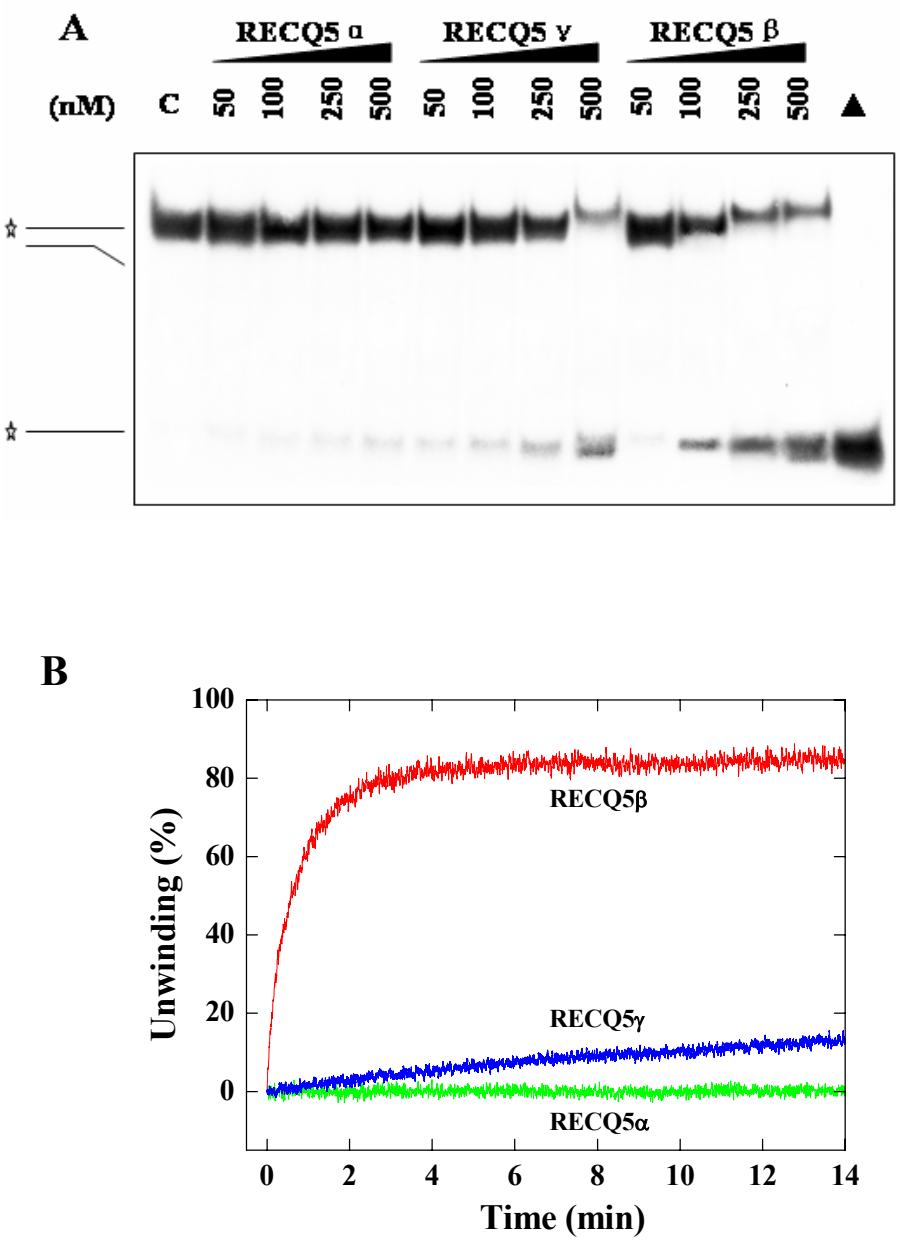
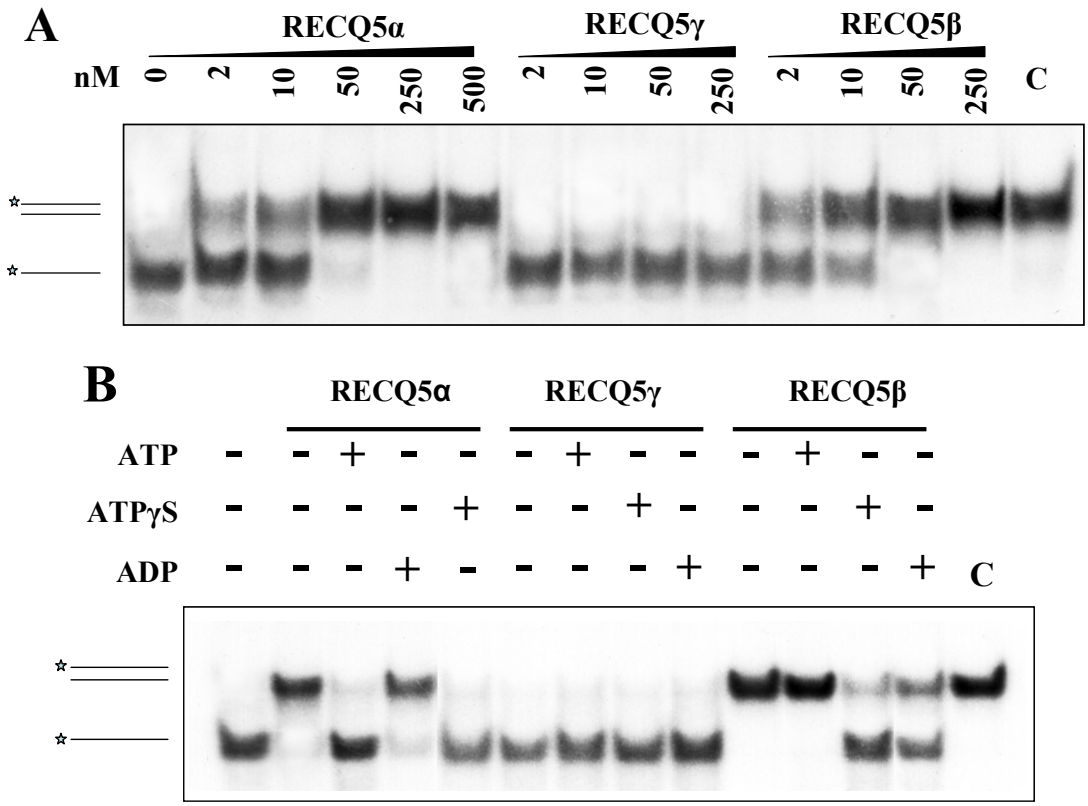

A
RECQ5 α
RECQ5 γ
RECQ5 β
(nM)
C
50
100
250
500
50
100
250
500
50
100
250
500
▲
B
Unwinding (%)
100
80
60
40
20
0
RECQ5β
RECQ5γ
RECQ5α
0
2
4
6
8
10
12
14
Time (min)

Fig. 5


A
RECQ5α
RECQ5γ
RECQ5β
nM
0
2
10
50
250
500
2
10
50
250
2
10
50
250
C
B
RECQ5α
RECQ5γ
RECQ5β
ATP
ATPγS
ADP
C

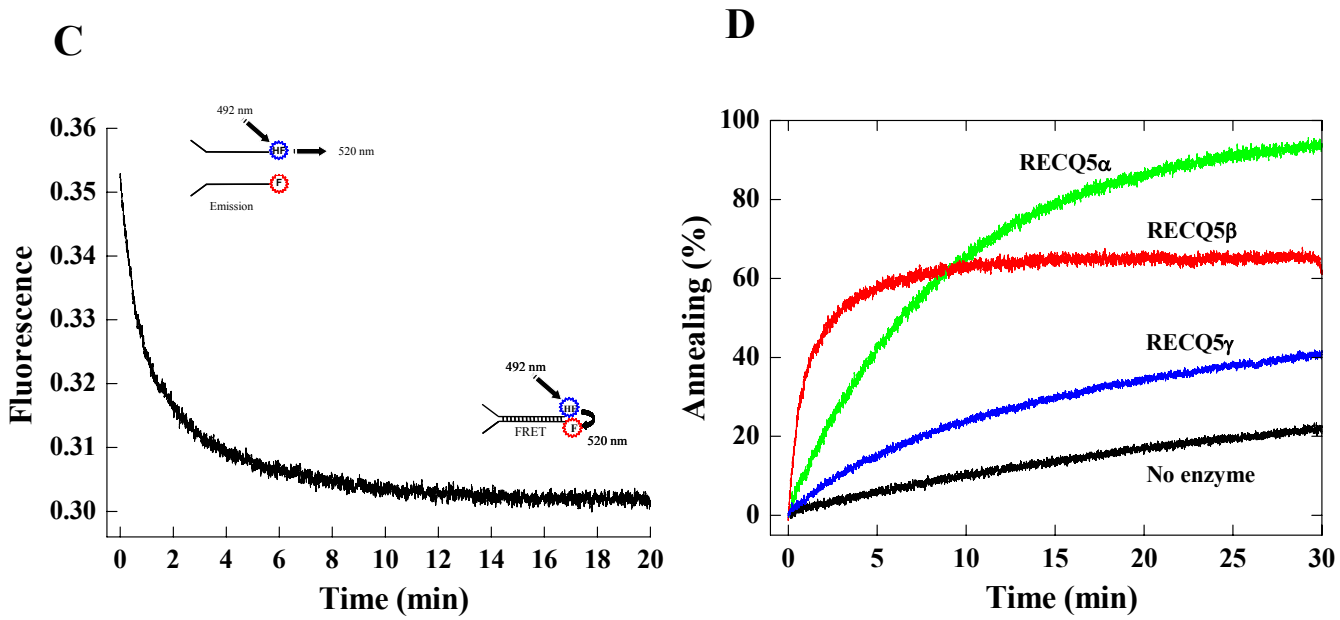

C
492 nm
520 nm
Emission
FRET
Fluorescence
Time (min)
D
RECQ5α
RECQ5β
RECQ5γ
No enzyme
Annealing (%)
Time (min)

Fig. 6

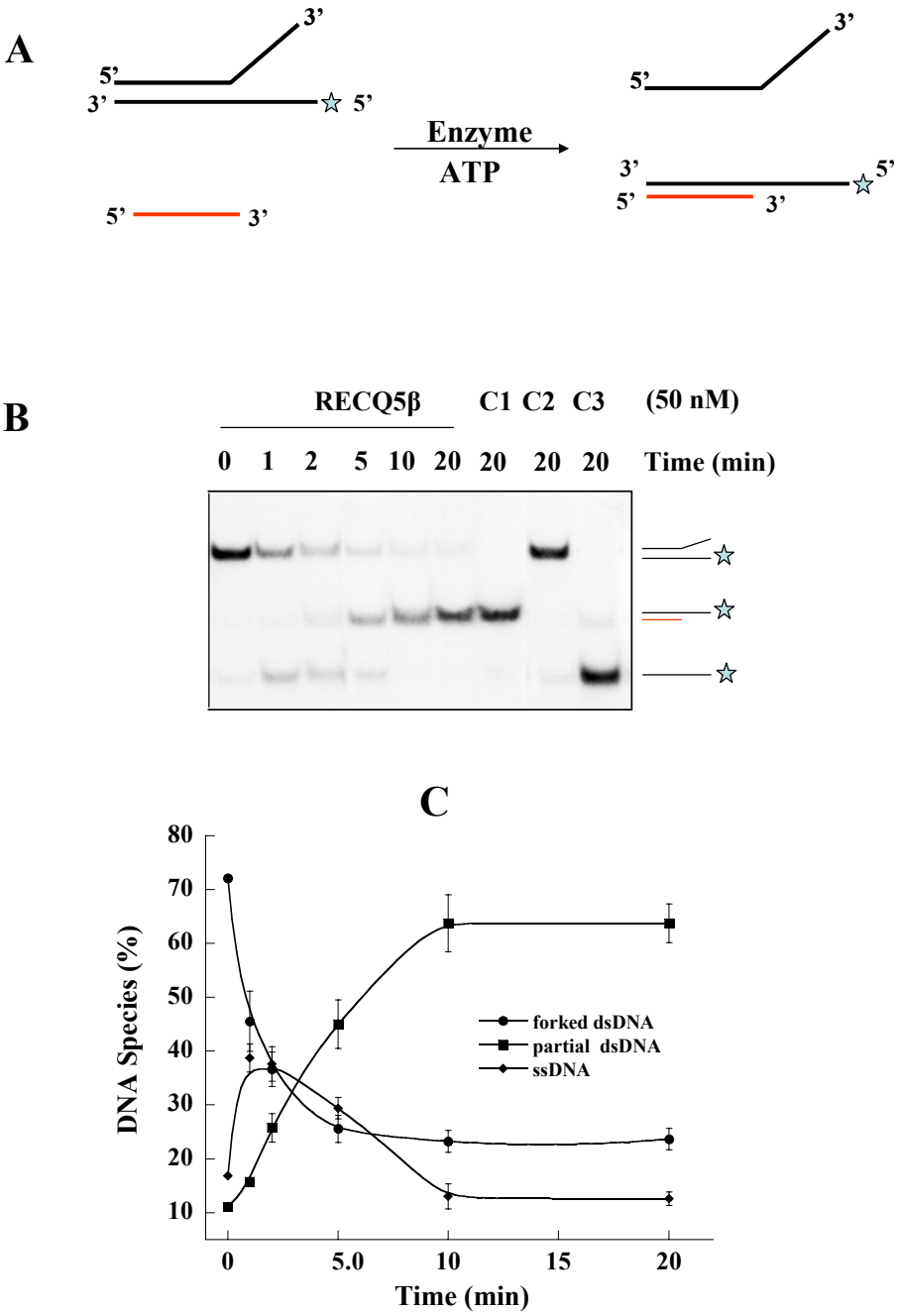

A
3'
5'
3'
5'
Enzyme
ATP
5'
3'
5'
3'
5'
3'
B
RECQ5β
C1 C2 C3
(50 nM)
0 1 2 5 10 20 20 20 20
Time (min)
C
DNA Species (%)
80
70
60
50
40
30
20
10
0
5.0
10
15
20
Time (min)
forked dsDNA
partial dsDNA
ssDNA

Fig. 7

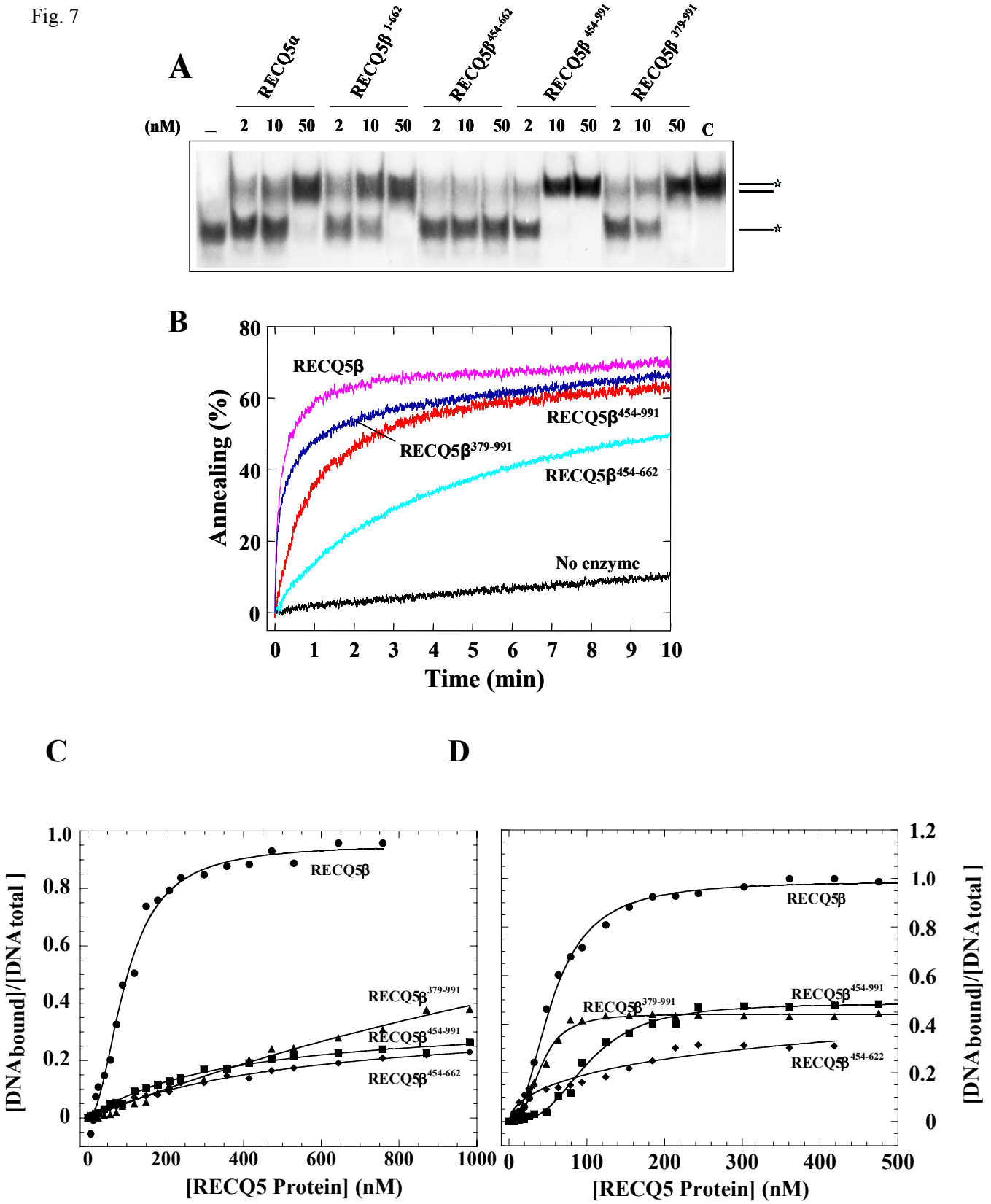

A
RECQ5α
RECQ5β 1-662
RECQ5β454-662
RECQ5β 454-991
RECQ5β 379-991
(nM) – 2 10 50 2 10 50 2 10 50 2 10 50 2 10 50 C
B
Annealing (%)
RECQ5β
RECQ5β454-991
RECQ5β379-991
RECQ5β454-662
No enzyme
Time (min)
C
D
[DNAbound]/[DNAtotal]
RECQ5β
RECQ5β379-991
RECQ5β454-991
RECQ5β454-662
[RECQ5 Protein] (nM)
RECQ5β454-622

Fig. 8

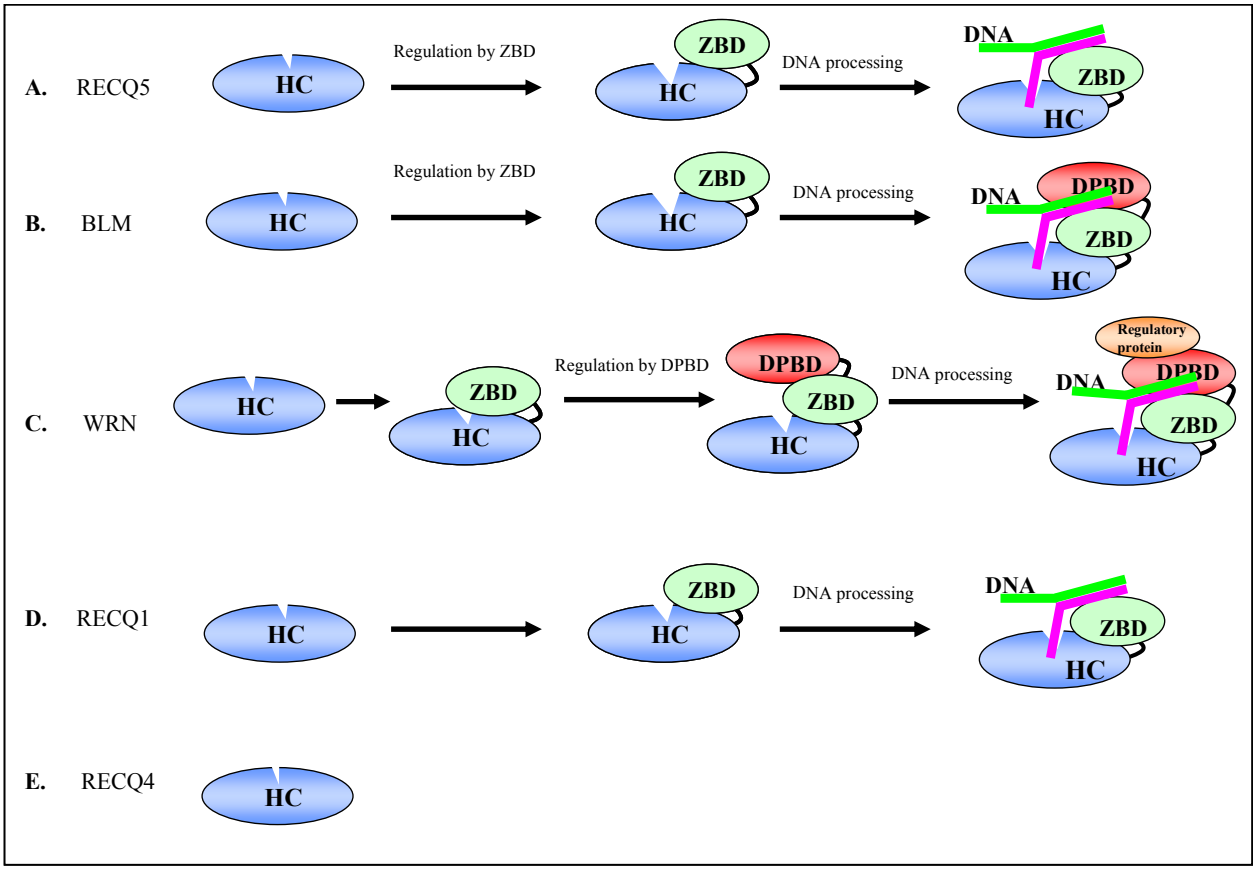

A. RECQ5
HC
Regulation by ZBD
ZBD
HC
DNA processing
DNA
ZBD
HC
B. BLM
HC
Regulation by ZBD
ZBD
HC
DNA processing
DNA
DPBD
ZBD
HC
C. WRN
HC
ZBD
HC
Regulation by DPBD
DPBD
ZBD
HC
DNA processing
Regulatory protein
DNA
DPBD
ZBD
HC
D. RECQ1
HC
ZBD
HC
DNA processing
DNA
ZBD
HC
E. RECQ4
HC

Fig. S1

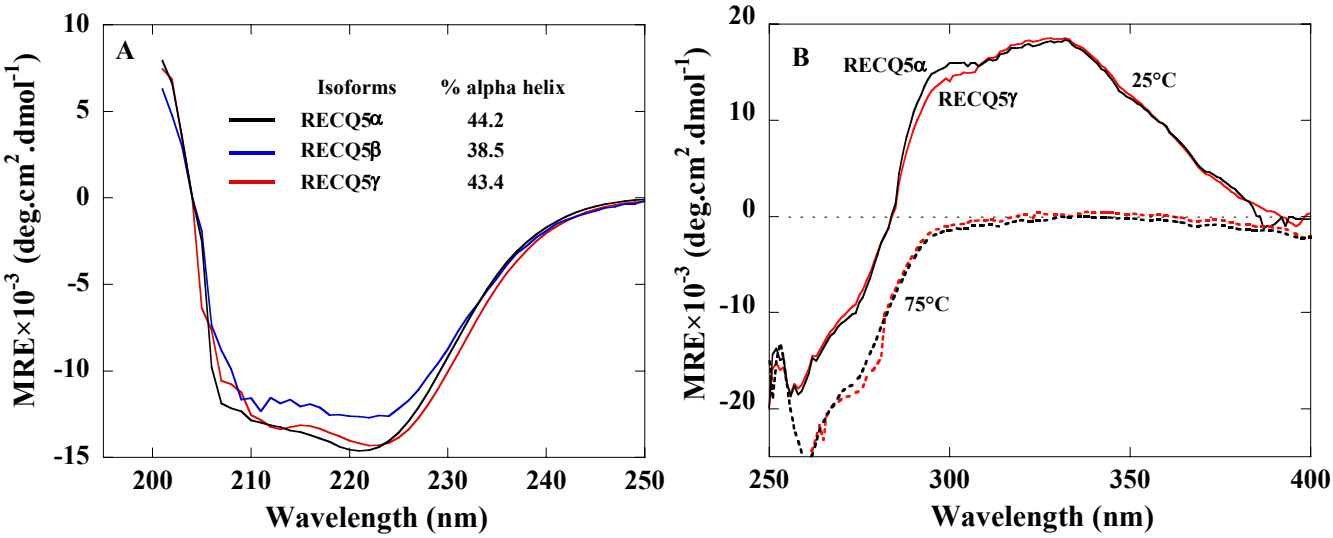
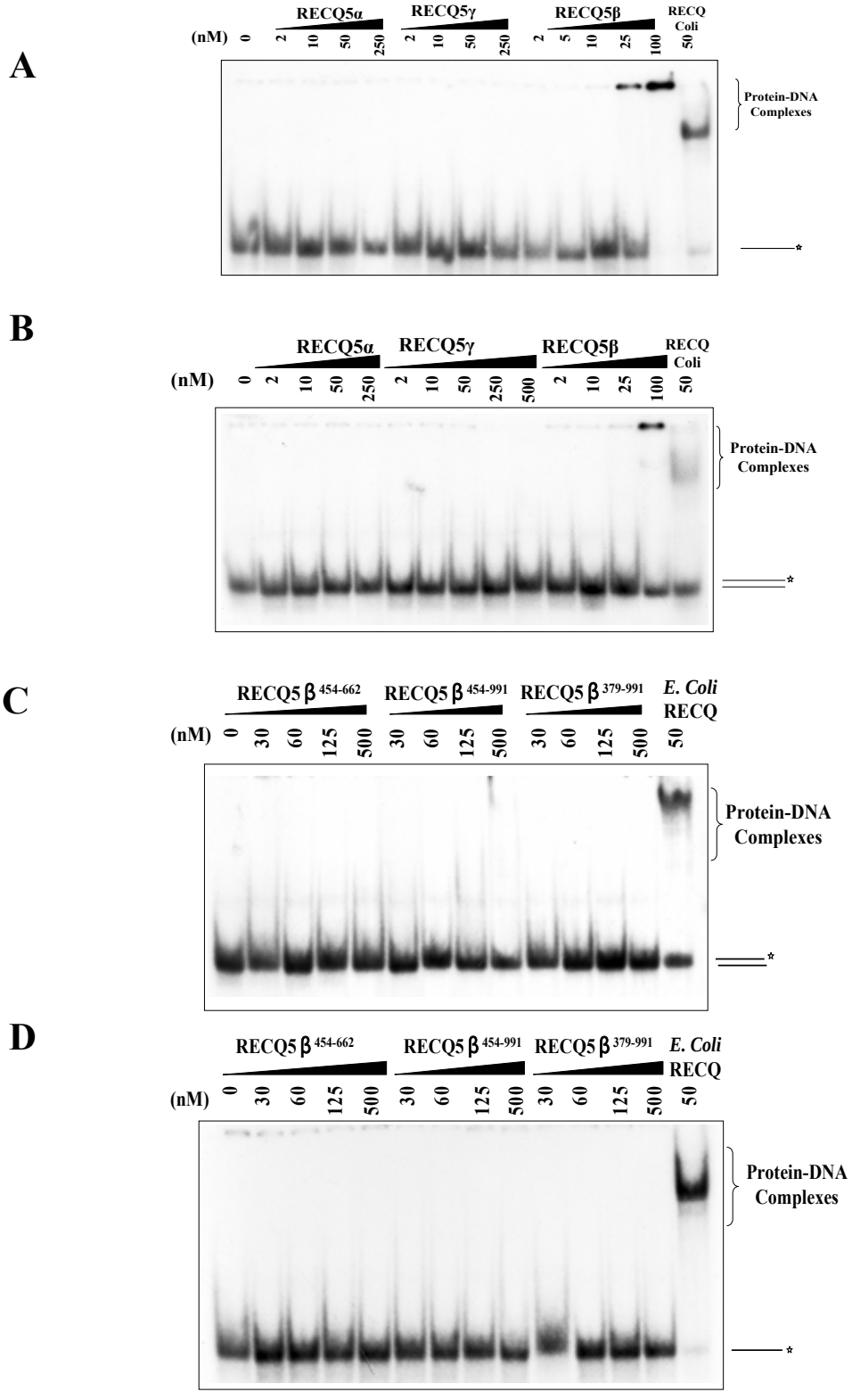

A
Isoforms
% alpha helix
RECQ5α
44.2
RECQ5β
38.5
RECQ5γ
43.4
MRE×10-3 (deg.cm2.dmol-1)
Wavelength (nm)
B
RECQ5α
RECQ5γ
25°C
75°C
MRE×10-3 (deg.cm2.dmol-1)
Wavelength (nm)

Fig. S2


A
RECQ5α
RECQ5γ
RECQ5β
RECQ Coli
(nM) 0 2 10 50 250 2 10 50 250 2 5 10 25 100 50
Protein-DNA Complexes
*
B
RECQ5α
RECQ5γ
RECQ5β
RECQ Coli
(nM) 0 2 10 50 250 2 10 50 250 500 2 10 25 100 50
Protein-DNA Complexes
*
C
RECQ5β 454-662
RECQ5β 454-991
RECQ5β 379-991
E. Coli RECQ
(nM) 0 30 60 125 500 30 60 125 500 30 60 125 500 50
Protein-DNA Complexes
*
D
RECQ5β 454-662
RECQ5β 454-991
RECQ5β 379-991
E. Coli RECQ
(nM) 0 30 60 125 500 30 60 125 500 30 60 125 500 50
Protein-DNA Complexes
*

Supplementary Table 1. DNA used in this study

Oligo name	Length (nt)	Sequence (5'-3')	Comment
BA	36	AATCCGTCGAGCAGAGTTAGGGTTAGGGTTAGGGTT	BA and BB were used for both radiometric and fluorescence binding assays, except that BA was labelled with 3'-fluorescein instead of ^{32}P labelled at 5' end for fluorescence assay
BB	36	AACCCTAACCCTAACCCGAAGTCTGCTCGACGGATT	
UA	44	GCACTGGCCGTCGTTTTACTTGG TGACTGGGAAAACCCTGGCG	UA was ^{32}P-labelled at 5'-end and hybridized with UB for radiometric unwinding and Strand-exchange assay. UFA and UFB were used for fluorescence unwinding assay.
UB	44	TTTTTTTTTTTTTTTTTTTTTCCAAGTAAAACGACGGCCAGTGC	
UFA	56	H[a]-AATCCGTCGAGCAGAG (dT_{40})	
UFB	16	CTCTGCTCGACGGATT-F[a]	
SPA	50	TCAAAGTCACGACCTAGACACTGCGAGCTCGAATTCACTGGAGTGACCTC	SPA was labelled using [γ-^{32}P]ATP and SPFA was labelled at 3'-end with fluorescein.
SPB	50	GAGGTCACTCCAGTGAATTCGAGCTCGCAGTGTCTAGGTCGTGACTTTGA	
SPFA	45	AGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-F	
SPFB	45	H-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTCTGAGGGATCT	
SPC	23	GCACTGGCCGTCGTTTTACTTGG	SPC was used for strand-exchange assay.

[a] F and H represent fluorescein and hexachlorofluoresein group respectively.

3. Etude de l'hélicase de la famille RecQ chez *Bacillus subtilis* I. La protéine SubL

3.1. Présentation

Selon des données génomiques, *Bacillus subtilis* possède deux hélicases de la famille RecQ. L'hélicase SubL est la forme la plus longue et contient les domaines helicase, RecQ-Ct et HRDC communs des ADN hélicases de la famille RecQ (Fig.1A de l'article 3). Cette hélicase putative n'a pas été caractérisée. L'objective de ce travail a été de caractérriser cette hélicase chez *Bacillus subtilis*.

Nous avons déterminé la capacité de la protéine SubL purifiée à fixer l'ion zinc en employant la technique de PAR (4-(2-Pyridylazo)resorcinol) et nous avons montré que 1 mole d'hélicase SubL fixe effectivement 1 mole d'ion zinc. Nous avons montré que SubL est une ATPase ADN dépendante et que cette activité nécessite l'ion Mg^{2+}, l'enzyme est inactive en présence de Mn^{2+}, Ca^{2+}, Zn^{2+}. Cette activité ATPase est optimale entre pH 7.0 et 8.8. L'activité ATPase de SubL (k_{cat}: 6.87/s, Kd: 699µM) est plus faible que celle de la protéine RecQ d'*E. coli* (k_{cat}: 35.2/s, Kd: 250µM) (Liu *et al.,* 2004).

Nous avons ensuite montré que que SubL est une ADN hélicase. La protéine SubL peut dérouler efficacement un ADN duplex avec une extrémité 3' sortante mais pas 5' sortante. Donc, la protéine SubL est une hélicase de polarité 3'-5'. L'hélicase SubL ne peut pas dérouler un ADN duplex avec des extrémités franches, mais peut dérouler une structure de type fourche et un ADN duplex avec un flap 5'. L'hélicase SubL peut faiblement dérouler un substrat d'ADN à 4 branches de type jonction de Holliday. Cette protéine est également capable de favoriser l'hybridation de l'ADN. La fixation à l'ADN par la protéine SubL a été étudiée en utilisant l'analyse de la mobilité électrophorétique. La fixation à un ADN de type fourche aboutit à la formation d'un complexe protéine-ADN plus stable.

En résumé, la protéine SubL est une ADN hélicase et une ATPase ADN dépendante.

L'ensemble de ces résultats est présenté dans l'article № 3.

3.2. Article № 3:

Studies on Helicase of RecQ family from *Bacillus subtillis*: I. SubL protein.

Jie Lin Liu and Xu Guang Xi

Studies on Helicase of RecQ family from *Bacillus subtillis*: I. SubL protein.[#]

Jie Lin Liu[+], and Xu Guang Xi*

Laboratoire de Biotechnologies et Pharmacologie Génétique Appliquée CNRS UMR 8113, Ecole Normale Supérieure de Cachan, 61 Avenue du Président Wilson, 94235 Cachan cedex, France

Running title: Biochemical property of Sub-L helicase from *Bacillus subtillis*

* To whom correspondence should be addressed. Tel.: 33-01-47-40-68-92; Fax: 33-01-47-40-76-71; E-mail: xi@lbpa.ens-cachan.fr.

This work was supported by the Centre National de la Recherche Scientifique.

[+] Supported by the China Scholarship Council.

[1] The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; PAR, 4-(2-Pyridylazo)resorcinol; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 5'-adenyl-β,γ-imidodiphosphate; TFA, time-resolved fluorescence anisotropy; FPLC, fast protein liquid chromatography; NTP, nucleotide triphosphate; DNP, nucleotide diphosphate.

Acknowledgement: We are very grateful to Drs. P. Fosse, F. Chaminade and Y.Y. Zhang for help with analytical EMSA and autoradiography experiments, Drs E. Deprez, P. Tauc and J.-C. Brochon for help with TFA experiments, Dr. H. Leh for help with FPLC, and A. Vignes for preparation of the figures. We thank Professor C. Auclair for his continuous support.

ABSTRACT

Proteins belonging to the highly conserved RecQ helicase family are essential for the maintenance of genomic integrity. Here, the biochemical properties of the SubL protein of the RecQ family from *Bacillus subtilis* have been described. To analyze its biochemical properties, the *SubL* gene product has been overexpressed in *Escherichia coli* and purified to near homogeneity. The recombinant SubL protein possesses an ATPase activity that is strongly stimulated by either single- or double-stranded DNA. Moreover, SubL exhibits ATP- and Mg^{2+}-dependent DNA helicase activity that displays 3'-5' polarity. The efficiency of unwinding was found to correlate inversely with the length of the duplex region of DNA. In addition, the recombinant SubL was found to bind more tightly to a forked DNA substrate than to single-stranded DNA and to possess an intrinsic DNA strand-annealing activity. SubL is not capable of unwinding duplex DNA from a blunt-ended terminus. However, the enzyme efficiently unwind the same blunt-ended duplex containing a synthetic X-structure (a model for the Holliday junction recombination intermediate) in which each 'arm' of the 4-way junction is blunt-ended. SubL can recognize elements of the fork structure to initiate unwinding and unwind two important intermediates of replication/repair, a 5'-ssDNA flap substrate and a synthetic replication fork. For the 5'-flap structure, the 5'-flap oligonucleotide could be specifically displaced by SubL, suggesting its role in Okazaki fragment processing. The ability of SubL to target DNA replication/repair intermediates may be relevant to its role in ensuring the genome stability maintenance.

INTRODUCTON

DNA helicases are the motor proteins responsible for unwinding the double stranded DNA and play an important part in DNA metabolism processes such as DNA replication, repair, and recombination (1, 2). An important group of DNA helicases is the RecQ family (3); members of which exist in different organisms, including bacteria (4), yeast (5-7), fungi (8), fly (9), frog (10), and humans (11-15). In microorganisms such as *E. coli, Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe*, only one member of the family per species is present, whereas higher eukaryotes contain diversity of RecQ helicase. For example, five members of the RecQ family have been discovered so far in human cells, i.e. RECQ1,WRN, BLM, RECQ4, and RECQ5 (3). In all organisms, defective function of RecQ helicase is associated with genomic instability, which is generally manifested as an increase in the frequency of inappropriate recombination events. Mutations in genes encoding the human RecQ helicases BLM, WRN and RECQ4 give rise to the hereditary disorders as Bloom's syndrome, Werner's syndrome and Rothmund−Thomson syndrome, respectively (16). These diseases are associated with cancer predisposition and variable aspects of premature aging. Although the precise DNA metabolism mediated by RecQ helicases remain elusive, the enzymes have been implicated in the processing of aberrant DNA structures arising during DNA replication and repair (16). Very interestingly, two helicases rather than one, of the RecQ family are present in *Bacillus subtillis*. SubL helicase is a longer protein form of these two helicases. And it contains helicase, RecQ-Ct, and HRDC domains (Fig.1A) of the RecQ helicase family, whereas little information is available so far on its catalytic characteristics. The previous studies indicated that the zinc finger motif of RecQ-Ct is very important for the protein structure and the enzyme activity of the RecQ helicases (17, 18). In the present study, Subl protein has been purified and the enzymatic activity and the DNA substrate specificity were investigated. The results indicated that SubL protein is the DNA-dependent ATPase, helicase, and possess DNA annealing activity.

EXPERIMENTAL PROCEDURES

Reagents—Chemicals were reagent grade and all solutions were prepared using ELGA pure water. [γ-^{32}P] ATP was obtained from Amersham Biosciences. EDTA, 2-mercaptoethanol, ATP, and AMPPNP were purchased from Sigma. T4 polynucleotide kinase was from New England Biolabs. TE: 10mM Tris-HCl (pH8.0), 1mM EDTA (pH8.0).

Nucleic Acid Substrates—Oligonucleotides were purchased from Genset and further purified by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides used for helicase activity measurement in this study are listed in Table I. The DNA or RNA used for a cofactor of ATPase activity of the helicase are: 60nt ssDNA, 7kb PN6 DNA linear, 5.5kb GC70-plasmid DNA, 5.5kb GC70-plasmid DNA denatured, 678nt RNA. For each substrate (see figures below) a single oligonucleotide was 5'-end-labbelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified with Sephadex-G25-TE column. The labeled oligonucleotides were annealed to their unlabelled complementary strands at a 1:1-2 molar ratio by incubation at 95℃ for 5 min followed by slow cooling to room temperature. For purification of the substrates, the annealed oligonucleotides were purified with Sephadex-G50, G75, and G100 TE columns respectively, according to their length.

Protein Expression—A full coding region of SubL helicase DNA was prepared by PCR using *Bacillus subtilis* chromosome DNA as template. A 1.8-kb PCR product was cloned into pGEM®-T easy vector, and the sequence of this insert was shown to be identical to the published Sub-L helicase gene sequence. The DNA corresponding to the coding sequence of the SubL helicase gene was amplified again by PCR-mediated amplification, using a set of oligonucleotides corresponding to the 5'- and 3'-ends of the gene. These primers had additional bases at each end for creation of restriction sites: NdeI at the 5'-end (5'-CAT ATG TTA CAT AGA GCC CAA TCC CTT CTG GCT CAT-3') and XhoI at the 3'-end of the gene (5'-ATA CAG GCT TAT GCA AGG ATG ACA GAC TAA CTC GAG-3') for expression of the SubL helicase bearing a hexahistidine tag at the NH_2-terminal end. The amplified product was

digested with NdeI and XhoI enzymes, and the appropriate size fragment (1776 bp) was gel-purified and subcloned, respectively, into NdeI/XhoI sites of the vector PET-15b. The PET-15b vector encodes a hexahistidine tag at the amino terminus that allows purification of the expressed protein on a nickel-chelating column. A thrombin cleavage site was adjacent to the histidine tag. The constructed plasmid was transformed in *E. coli* strain BL21 (DE3)/Rosetta. The bacteria were grown at 37 °C in Luria-Bertani (LB) broth supplemented with 50μg/ml ampicillin and 34 μg/ml chloramphenicol. The expression of the protein was induced by adding isopropylthio-D-galactoside to 0.2 mM at low log phase (A600 = 0.6). The culture was then incubated for 12-14 h at 15-18 °C. Cells were harvested by centrifugation at 3,000 × g for 15 min at 4 °C.

Protein Purification—His-tagged SubL helicase was overexpressed in *E. coli* BL21 (DE3)/Rosetta and purified under native conditions. Briefly, harvested cells were suspended in 30 ml of suspension buffer (20mM Tris-HCl, pH 7.9, 5mM imidazole, 500mM NaCl) and were lysed using a French pressure cell. The lysate was then sonicated to shear DNA into small fragments. Followed by centrifugation at 70,000 x g for 30 min at 4 °C, the supernatant was applied to the column charged with histidine binding resin (Novagen). The column was washed with 20mM Tris-HCl (pH 7.9) buffer containing 500mM NaCl, 5 mM imidazole. The proteins bound to the column were eluted stepwise using 20mM Tris-HCl (pH 7.9) buffer containing 50, 65, 80, 200, or 500mM imidazole. Sub-L helicase-containing fractions, identified by both DNA-dependent ATP hydrolysis and helicase activity assays, were pooled and further purified by FPLC size exclusion chromatography (Superdex 200; Amersham Biosciences). The pooled active fractions were dialyzed against storage buffer (20mM Tris-HCl, pH 7.9, 500mM NaCl, 2mM dithiothreitol, 10% glycerol), and stored at –80 °C. The protein was pure as judged by Coomassie staining and electrospray mass spectrometry. Protein concentration was determined spectrophotometrically using a Bio-Rad Protein Assay.

Quantification of Zinc Ion Bound to Sub-L Helicase—The zinc content of SubL helicase was measured by the PAR (4-(2-Pyridylazo)resorcinol) assay as described

by Hunt *et al.* (19). PAR has a low absorbance at 500 nm in the absence of zinc ion. However, in the presence of zinc ion, the absorbance at 500 nm increases dramatically due to the formation of the $PAR_2 \cdot Zn^{2+}$ complex. To more precisely quantify the zinc content of SubL helicase, all of the buffers were treated with Chelex 100 resin. The enzymes were dialyzed against the EDTA-free Chelex-treated buffer passed over a 10-cm column of Chelex-100 and reconcentrated. To facilitate zinc release, the enzymes (1 nmol in a volume of 20 μl) were first denatured with Chelex-treated 7 M guanidine HCl and then transferred to a 1-ml cuvette and the volume was adjusted to 0.9 ml with buffer A (20 mM Tris-HCl at pH 8.0, 150 mM NaCl). PAR was added into the cuvette for a final concentration of 100 μM. The absorbance at 500 nm was measured. The quantity of zinc ion was determined from a standard curve of $ZnCl_2$ samples in a range of concentrations using the sample preparation procedure as described above with the SubL helicase omitted. The zinc ion concentration was also determined using the absorbance coefficient for the $(PAR)_2 \cdot Zn^{2+}$ complex ($\varepsilon_{500nm} = 6.6 \times 10^4\ M^{-1}\ cm^{-1}$).

pH Measurements—pH measurements were performed with a Knick 654 pH meter and an Ingold microelectrode. For calibration three standard buffers were used, and the precision was about ± 0.01 pH unit at 25 °C. To obtain a pH range from 5 to 10, 30mM sodium citrate, MES, and Tris acetate were employed for pH intervals 5.0–6.0, 5.5–7.2, and 6.8–10, respectively.

ATPase Assay — The ATPase activity was determined by measuring the radioactive ^{32}Pi liberated during hydrolysis (20). The measurement was carried out at 37 °C for 10 min in a 40 μl reaction mixture （50mM Tris-HCl pH8.0, 35mM NaCl, 3mM $MgCl_2$, 0.1mg/ml BSA, 1 mM DTT, 2mM ATP） containing the indicated concentration of DNA or RNA at the indicated concentration of SubL helicase. The reactions were initiated by the addition of Sub-L helicase into 40 μl of reaction mixture and stopped by pipetting 35 μl of aliquots from the reaction mixture every 30 seconds into a hydrochloric solution of ammonium molybdate. The liberated radioactive ^{32}Pi was extracted with a solution of 2-butanol-cyclohexane-acetone-ammonium molybdate (250:250:50:0.1) saturated

with water. An aliquot of the organic phase was counted.

DNA helicase assay---The unwound γ-^{32}P-labeled DNA fragment from a partial duplex DNA molecule was first determined in 20 μl reaction mixture containing 50 mM Tris–HCl, pH 8.0, 1 mM DTT, 35mM NaCl, 3 mM $MgCl_2$, 2 mM ATP, 100 μg/ml BSA and ^{32}P-labeled helicase substrate (~0.45 nM). The recombinant SubL helicase of indicated concentration was then added to the mixture and incubated at 37°C for 30min. The reaction was terminated by the addition of 2% SDS, 150 mM EDTA, 30% glycerol and 0.1% bromphenol blue. The products of the reaction were fractionated by electrophoresis on a 12 or 6% non-denaturing polyacrylamide gel. The gel was dried and exposed by autoradiography.

DNA strand annealing and exchange assays---The DNA strand-annealing activity of Sub-L was measured by using complementary synthetic oligonucleotides (each at a concentration of 0.5 nM), of which one was labeled at the 5'-end using [γ-^{32}P]ATP and T4 polynucleotide kinase. Annealing reactions (typically 20 μl) were carried out at 37°C in buffer (50 mM Tris–HCl, pH 8.0, 35 mM NaCl, 3 mM $MgCl_2$, 100 μg/ml BSA and 1 mM DTT) containing the indicated amounts of Sub-L. Where required, ATP (2 mM), ATP-γ-S (2 mM) and ADP (2 mM) were added. Reactions were usually initiated by adding the unlabeled DNA strand and were incubated for 20 min. For the exchange assays, labeled fork substrate Tstem25/Flap26 (1nM) was incubated with or without SubL (at the concentrations indicated in figure legends) and oligonucleotide U25 (1nM) for 0-20min at 37℃ in 20 μl reaction buffer plus ATP (1mM). Reactions were analyzed essentially as the helicase reactions (see above).

EMSA assay---The recombinant SubL of indicated concentration was incubated with 5'-[γ -^{32}P]- labeled ssDNA or dsDNA (~0.45nM) in a 20 μl reaction mixture containing 50 mM Tris–HCl, pH 8.0, 35 mM NaCl, 3 mM $MgCl_2$, 100 μg/ml BSA and 1 mM DTT. Incubation was for 30 min at room temperature. The resulting mixture was then resolved by 6% non-denaturing PAGE and visualized by autoradiography. Gels were run at 200 V and 25°C in 0.5×TBE buffer.

DNA Binding Assay under Equilibrium Condition—The binding of SubL helicase to DNA was also analyzed by fluorescence anisotropy using a Beacon 2000

fluorescence polarization spectrophotometer (PanVera) as described previously (21). An appropriate quantity of fluorescein-labeled ssDNA or dsDNA was added to a standard titration buffer (150 μl of total volume) in a temperature-controlled cuvette at 25 °C. The anisotropy of the fluorescein-labeled DNA was measured successively until it stabilized. An appropriate quantity of SubL helicase then was added. The anisotropy then was measured continuously until it reached a stable plateau. To determine the concentration of the helicase-DNA complex, fluorescence signals observed in these SubL helicase titrations were subtracted by those observed in the absence of enzyme. The increase in sample volume during the titration was taken into account in the analysis of the data. The reported values represent the averages of two to three measurements.

RESULTS

Purification of a Recombinant Sub-L Protein---- To study the enzyme functions, the SubL helicase was overexpressed in and purified from *E. coli*. Gel electrophoresis of the protein in denaturing conditions gave a single band corresponding to a molecular mass of about 68kDa (Fig. 1B), consistent with the value determined from the amino acid sequence (66,783Da). Immunoblotting with an anti-His monoclonal antibody confirmed that the overexpressed protein was the desired product (data not shown). Large-scale preparations of the recombinant protein were then carried out, and the protein was purified to near homogeneity (Fig. 1B).

We have determined the ability of the purified SubL protein to bind zinc by using the PAR assay. 1 mol of the SubL helicase binds 1.11±0.17 mol of Zn^{2+}. This result is consistent with the prediction from the primary amino acid sequence of SubL helicase, indicating that the enzyme contains a zinc-binding motif.

DNA-dependent ATPase Activity---- To determine the ATPase activity, the purified recombinant SubL protein was tested for its ability to hydrolyze ATP in the presence and absence of DNA and RNA. As shown in Fig. 2, a DNA-dependent ATPase activity was readily detected. Hydrolysis of ATP was almost equally efficient in the presence of several different DNA cofactors, including either single- or double-stranded DNA but not in the presence of RNA or in the absence of DNA (Fig. 2A). Therefore, SubL is an ATP dependent DNA helicase but not a RNA helicase. Whereas the previous study on the *E. coli* RecQ ATPase was shown that single-stranded DNA was a much more effective cofactor than double-stranded DNA (22).

Previous studies indicated that the ATPase activity of the helicase is Mg^{2+} dependent. Fig. 2B shows that the ATP hydrolysis by SubL is dependent on divalent cations such as Mg^{2+}, but not Mn^{2+}, Ca^{2+}, Zn^{2+}.

The pH dependence of ATPase activity of SubL protein has been next investigated. The SubL helicase displays an optimum ATPase activity between pH 7.0 and 8.8 (Fig. 2C).

The ssDNA-stimulated ATPase activity of the SubL helicase has been studied as a function of ATP concentration. As come from Fig. 2D, the k_{cat} value is 6.87 /s, the Kd value is 699μM. This result indicates that the ATPase activity of SubL is less than that of RecQ protein from *E. coli* (k_{cat}: 35.2/s, Kd: 250μM) (17).

DNA Unwinding Activities Assay versus Sub-L Helicase Concentration----To determine the optimal amount of the SubL protein required unwinding dsDNA, its concentration was varied from 2.5nM to 80nM. As shown in Fig. 3A, 2.5 to 80nM of the SubL protein can unwind forked DNA with the increasing efficiency by using the energy derived from the ATP hydrolysis. This indicated that SubL is ATP dependent DNA helicase.

DNA Unwinding is ATP dependent---- It is well established that RecQ helicase requires ATP hydrolysis to promote DNA unwinding. AMPPNP and ATP-γ-S, the non-hydrolysable analogs of ATP, inhibited the RecQ-mediated DNA unwinding (22). Fig. 3B shows that the SubL enzyme displays no detectable helicase activity in the presence of 2mM AMPPNP. Except ATP and dATP, UTP, GTP, CTP, dGTP, dCTP and dTTP cannot be used by the SubL helicase as the energy source to catalyze DNA unwinding.

Polarity of the DNA Helicase----To determine the polarity of the helicase activity, the DNA substrates shown in Fig. 4 were used and the recombinant SubL protein was found to efficiently unwind the 3'-tailed but not 5'-tailed duplex DNA. Generally, the helicase of RecQ family unwinds duplex DNA with a 3' to 5' directionality. This result is consistent with that the SubL protein is a unidirectional helicase with 3' to 5' polarity.

The RecQ helicase of *E. coli* expresses a blunt ended duplex DNA unwinding in its higher concentrations. Whereas, the SubL helicase can't unwind the duplex DNA with double blunt ends (data not shown).

The Dependence of the efficiency of DNA unwinding on the length of double Strands--- To investigate the dependence of the efficiency of DNA unwinding on the length of the duplex region of partial duplex DNA, several DNA substrates were prepared by using ^{32}P-labeled fragments of 20 and 70 nucleotides annealing to

PhiX174 virion DNA. A time course of the SubL-mediated strand unwinding reaction is shown in Fig. 5A and 5B. With the short partial duplex (20bp) substrate, the labeled fragment was unwound within 5 min, and the amount of fragment unwound in reaction was increased with the time. However, with the long partial duplex (70bp) substrate, the labeled fragment was not unwound by the SubL helicase within 40 min. Therefore, it was found that its length markedly affected the efficiency of strand unwinding.

SubL Helicase Activity on Partial Duplex DNA ---- Our following studies were focused on determining the importance of the length of the noncomplementary 5'-ssDNA tail of a forked dsDNA substrate for the efficiency of unwinding by SubL (Fig. 6A). Because SubL has been found to unwind dsDNA with a 3'-ssDNA tail, the length of the 5'-ssDNA tail may also be an important factor in the SubL helicase reaction. As expected, SubL can unwind the forked DNA duplexes with increasing 5'-tail length.

In the other hand, a series of linear substrates with 3'-ssDNA tails of 1, 5, 10, and 25 nucleotides were made to study the effect of 3'-tail length on the unwinding activity of the SubL helicase (Fig. 6B). The studies done with this series of substrates clearly showed that all the substrates with 3'-tails, even if it is 1nt long could be efficiently unwound by the Sub-L helicase (Fig. 6B).

Following this observation, a set of substrates of the partial duplex DNA with different structures and with various lengths of the double-strand regions were prepared to obtain novel information on the substrate specificity of the Sub-L helicase. Substrates with a 20bp oligonucleotide annealed to PhiX174 virion ssDNA were unwound by the SubL helicase, regardless of the presence or absence of mismatched hanging tails at the 5'-end or the 3'-end (Fig. 6C). However, if the duplex region was increased to 70bp, the substrate could not be unwound (Fig. 5B).

SubL Unwinds a 5'-Flap Substrate Flanked by Adjacent Duplex DNA. ---- To assess whether the SubL helicase is able to unwind a 5'-flap substrate, we tested a DNA substrate related to the forked duplex with 26- and 25-nucleotide 5'- and 3'-ssDNA tails, respectively, which also contains an upstream 25-mer hybridized to the

upstream ssDNA region that resides below the flap (Fig.7A, substrate A). A nick separating the adjacent oligonucleotides characterizes substrate A. The results of unwinding by using substrate A is shown in Fig. 7A. 0.2μM SubL can efficiently unwind the 5'-flap substrate, releasing the duplex DNA with a labeled 5'-tail. This is consistent with the 3' to 5' polarity of the SubL helicase.

SubL Unwinds a Synthetic Replication Fork DNA Substrate----We also tested a forked duplex in which both the 5'- and 3'-tails were double-stranded (Fig.7A, substrate C). This substrate mimics a synthetic DNA replication fork with double-stranded leading and lagging strands. The duplex forked DNA substrate (substrate C) was unwound by the SubL at the concentration tested (0.2μM), however, only releasing the duplex DNA with a labeled 5'-tail. This is also consistent with the 3' to 5' polarity of the SubL helicase. Hence SubL is able to unwind the synthetic replication fork.

SubL helicase can disrupt Kappa joint molecule and Holliday Junction---- The properties of Kappa joint molecule suggested that it could be composed of a linear dsDNA molecule that was invaded by homologous ssDNA; the resultant joint molecule would resemble the letter K and, hence, is referred to as a Kappa intermediate. To test the unwinding of Kappa intermediate catalyzed by the SubL helicase, we prepared a substrate (Fig. 7A, substrate B) which mimics Kappa joint molecule. The result shows that the Sub-L helicase can unwind it (Fig. 7A, 7B). Meanwhile, the SubL helicase could unwind the four-way junction DNA substrate that mimics a Holliday junction (Fig. 7B).

Sub-L helicase possesses DNA strand-annealing activity but can't promote DNA exchange----We incubated two complementary oligonucleotides (those used to make the blunt end double-strands DNA substrate) with increasing amounts of SubL in the absence of ATP, and subsequently analyzed the reaction products by 12% nondenaturing polyacrylamide gel electrophoresis (PAGE). We found that SubL promoted an efficient annealing reaction (Fig. 7C). Using 1 nM ssDNA, we found that annealing was dependent on protein concentration. But the ATP can inhibit the DNA strand-annealing promoted by SubL protein (data not shown). Therefore the

DNA strand-exchange catalyzed by SubL hasn't been observed (data not shown).

DNA Binding by SubL protein---- To understand the mechanism of SubL mediated ssDNA annealing, we studied the relationship between the DNA binding affinity and annealing activity of SubL. The DNA binding activity of the purified recombinant protein was investigated using the electrophoretic mobility shift assay (EMSA) (Fig. 8A,8B). The SubL protein was found to bind single-stranded DNA (ssDNA), since the presence of increasing amounts of the protein progressively reduced the gel electrophoretic mobility of the DNA (Fig. 8A).

To test the possibility that binding to the single-stranded DNA might be facilitated by secondary structures within it, the SubL binding to a forked DNA substrate was examined. The forked DNA was prepared by the annealing of two partially complementary oligonucleotides, 44 nucleotides in length. It was found that incubation of ^{32}P-labeled forked DNA with the SubL protein resulted in the formation of a more stable protein-DNA complex (Fig. 8B), as detected by polyacrylamide gel electrophoresis. These results are consistent with the preference of the forked DNA bound by SubL helicase analyzed with fluorescence polarization (Fig. 8C). The weak SubL protein-ssDNA interaction could catalyze ssDNA annealing.

DISCUSSION

We have shown that a purified recombinant SubL protein possesses helicase and ATPase activity. As the zinc finger motif of RecQ-Ct is necessary for the enzyme activity of the RecQ helicases (17, 18), the function of the SubL protein is likely related with its zinc finger motif. The DNA unwinding by SubL is dependent on the presence of ATP or dATP which is hydrolyzed to provide the energy for the unwinding. Unlike the *E. coli* RecQ helicase, which is stimulated by single-stranded DNA but not by double-stranded DNA, the hydrolysis of ATP by the recombinant Sub-L protein is strongly stimulated by both forms of DNA and is dependant on Mg^{++}. Sub-L helicase displays an optimum ATPase activity between pH 7.0 and 8.8.

The polarity of unwinding by SubL helicase is 3' to 5' with respect to the overhanging single-stranded DNA to which the Sub-L is binding. Consistent with this directionality, it was shown that SubL unwinds a duplex DNA substrate with a 3'-ssDNA tail but not a substrate with a 5'-ssDNA tail. These findings might lead one to believe that Sub-L requires a free 3'-ssDNA tail to initiate unwinding of duplex DNA substrates. However, the ability of SubL to unwind 5'-flap and synthetic replication fork substrates indicates that SubL does not require a preexisting 3'-ssDNA tail in the DNA substrate for either loading or initiating of unwinding. The characteristics of the unwinding activity by SubL are similar to those by *E. coli* RecQ protein (22), which shares homology with SubL within the helicase domain. Of the other members of the RecQ family of DNA helicases, the human RecQL protein has previously been purified and found to have DNA unwinding activity *in vitro* (11,23). Recently, the BLM and WRN proteins (human homologues of RecQ) were also overexpressed and shown to possess a DNA helicase function (24-26). It thus seems that all of the members of the RecQ family likely possess a common helicase activity.

It has been shown here that the unwinding of blunt-ended double-stranded DNA substrates by SubL is undetectable under the assay conditions employed and hence SubL helicase can't unwind the duplex DNA with double blunt ends. In contrast, *E.coli* RecQ protein, like several other helicases such as RecBCD, can initiate duplex

DNA unwinding from a blunt-ended terminus (27; reviewed in 2, 28). The addition of a single-stranded tail (a 3'-tail for 3'→5' helicase or a 5'-tail for a 5'→3' helicase) to a duplex DNA could be enough to produce a good substrate for many helicases, including those incapable of unwinding from blunt ends. In their studies Bennett *et al.* (29) concluded that Sgs1p, the *Saccharomyces cerevisiae* homologue of SubL, had a requirement for a 3'-tail of at least 3 nt and used the junction of both single-stranded and double-stranded DNA as a key site in the substrate recognition process. In our studies, as expected, a 5'-tailed duplex DNA is not a substrate for SubL helicase but 3'- tailed duplex is.

The efficiency of strand unwinding by the recombinant SubL protein decreases sharply with increasing length of the strand to be unwound. The unwinding assay used here, however, detects only complete unwinding, and partially unwound fragments would not be scored. It is therefore plausible that the longer DNA strands reannealing behind the translocating SubL protein could reduce the observed efficiency of unwinding. Alternatively, the recombinant SubL helicase might exhibit a low processivity and might fall off the DNA before completing the unwinding of the annealed fragment.

SubL helicase studies with a synthetic replication fork substrate demonstrate that unwinding specifically occurs in the direction of the fork and that the double-stranded 3'-arms of the fork are not unwound. The unidirectional movement of SubL in a 3' → 5' direction and disruption of the complementary Watson-Crick base pair of the duplex region ahead of the fork structure would result in the release of the complementary DNA species, whether it is a single strand or a partially duplex molecule that retains a double-stranded region within the arm of the original DNA substrate.

The ability of SubL helicase to unwind 5'-flap and synthetic replication fork substrates efficiently suggests that SubL has the capacity to load onto duplex DNA by virtue of a junction recognition property. Because the synthetic replication fork substrate was detectably unwound by SubL. Thus, it may initiate unwinding of DNA duplex substrates by a mechanism that does not require an interaction between the

protein and the ssDNA tails of the forked duplex substrate.

A 3'-ssDNA tail in DNA structure is not only requirement for unwinding of B form duplex DNA by SubL. The protein SubL may also act as a helicase on a synthetic X structure (a model for the Holliday junction recombination intermediate). The former structure forms a stacked X structure in the presence of Mg^{2+} (present during the helicase assay) and therefore lacks ssDNA, even at its core (30). Thus SubL certainly does not require a free 3'-ssDNA tail or any preexisting ssDNA tracts in the substrate to catalyze efficient unwinding.

The action of the SubL helicase on the 5'-flap substrate is interesting from a genetic viewpoint because this structure is a key DNA intermediate during lagging strand synthesis of DNA replication. The average size of an Okazaki fragment (100-150 nucleotides) (31) may be influenced by the action of SubL helicase on the flap structure. In this study, we have demonstrated that SubL is able to displace the 5'-ssDNA flap. The ability of SubL helicase to unwind the duplex ahead of a fork structure that contains fully double-stranded leading and lagging strands may be important for the maintenance of fork progression when replication undergoes pausing. Precisely how SubL helicase acts *in vivo* to maintain genome stability remains to be defined, but it is likely to involve its helicase function on a structural intermediate of an important DNA metabolic pathway.

Using an assay based on the electrophoretic mobility of a DNA bound to protein, the recombinant SubL protein was shown to have a higher affinity for a forked DNA substrate than single-stranded DNA. This may have implications for the mechanism of DNA unwinding by SubL, since it has been suggested that preferential binding of the enzyme to such a junction might be important for helicase action (2). Meanwhile, the weak SubL protein-ssDNA interaction could catalyze ssDNA annealing.

Most importantly, the present study revealed that SubL protein exhibits an efficient DNA strand-annealing activity. At the present time, the biological significance of these findings cannot be readily assessed because of the lack of information on the phenotypic consequences of *SubL* deficiency in *Bacillus subtilis* cells. The biochemical activities of SubL suggest that the helicase may be implicated in a DNA

reparation that demands the cooperation between DNA unwinding and DNA strand-annealing. Cooperation of the DNA helicase and reannealing activities can facilitate regression of replication fork while the annealing activity promotes base pairing of the newly synthesized strands during the fork regression (32). Consistent with this, the SubL protein can unwind preferentially the 5'-flap DNA and synthetic replication fork. Further analysis of the roles of SubL *in vivo* will be important to understand the functions of the RecQ helicase in maintaining genomic integrity.

REFERENCES

1. Matson, S. W., and Kaiser-Rogers, K. A. (1990) DNA helicases. Annu. Rev. Biochem. 59, 289-329

2. Lohman, T. M., and Bjornson, K. P. (1996) Mechanisms of helicase-catalyzed DNA unwinding. Annu Rev Biochem. Annu. Rev. Biochem. 65, 169-214

3. Karow, J. K., Wu, L., and Hickson, I. D. (2000) RecQ family helicases: roles in cancer and aging. Curr. Opin. Genet. Curr. Opin. Genet. & Dev. 10, 32-38

4. Irino, N., Nakayama, K., and Nakayama, H. (1986) The recQ gene of *Escherichia coli* K12: primary structure and evidence for SOS regulation. Mol. Gen. Genet. 205, 298-304

5. Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L., and Rothstein, R. (1994) The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. Mol. Cell. Biol. 14, 8391-8398

6. Watt, P. M., Louis, E. J., Borts, R. H., and Hickson, I. D. (1995) Sgs1: a eukaryotic homolog of E. coli RecQ that interacts with topoisomerase II in vivo and is required for faithful chromosome segregation. Cell 81, 253-260

7. Stewart, E., Chapman, C. R., Al-Khodairy, F., Carr, A. M., and Enoch, T. (1997) rqh1+, a fission yeast gene related to the Bloom's and Werner's syndrome genes, is required for reversible S phase arrest. EMBO J. 16, 2682-2692

8. Cogoni, C., and Macino, G. (1999) Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. Science 286, 2342-2344

9. Kusano, K., Berres, M. E., and Engels, W. R. (1999) Evolution of the RECQ family of helicases: A drosophila homolog, Dmblm, is similar to the human bloom syndrome gene. Genetics 151, 1027-1039

10. Fry, M., and Loeb, L. A. (1998) The three faces of the WS helicase. Nat. Genet. 19, 308-309

11. Puranam, K. L., and Blackshear, P. J. (1994) Cloning and characterization of RECQL, a potential human homologue of the *Escherichia coli* DNA helicase RecQ. J. Biol. Chem. 269, 29838-29845

12. Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M., and German, J. (1995) The Bloom's syndrome gene product is homologous to RecQ helicases. Cell 83, 655-666

13. Yu, C. E., Oshima, J., Fu, Y. H., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G. M., Mulligan, J., and Schellenberg, G. D. (1996) Positional cloning of the Werner's syndrome gene. Science 272, 258-262

14. Kitao, S., Shimamoto, A., Goto, M., Miller, R. W., Smithson, W. A., Lindor, N. M., and Furuichi, Y. (1999) Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. Nat. Genet. 22, 82-84

15. Ozsoy, A. Z., Sekelsky, J. J., and Matson, S. W. (2001) Biochemical characterization of the small isoform of *Drosophila melanogaster* RECQ5 helicase. Nucleic Acids Res. 29, 2986-2993

16. Bachrati CZ, Hickson ID (2003) RecQ helicases: suppressors of tumorigenesis and premature aging. *Biochem J* **374**: 577–606

17. Liu, J. L., Rigolet, P., Dou, S. X., Wang, P. Y., and Xi, X. G. (2004) The zinc finger motif of *Escherichia coli* RecQ is implicated in both DNA binding and protein folding. J. Biol. Chem. 279. 42794-42802.

18. Guo, R. B., Rigolet, P., Zargarian, L., Fermandjian, S., and Xi, X. G. (2005) Structural and functional characterizations reveal the importance of a zinc binding domain in Bloom's syndrome helicase. *Nucleic Acids Res.* 33. 3109-3124

19. Hunt, J. B., Neece, S. H., and Ginsburg, A. (1985) The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from Escherichia coli aspartate transcarbamoylase. *Anal. Biochem.* **146,** 150–157

20. Dessinges, M. N., Lionnet, T., Xi, X. G., Bensimon, D., and Croquette, V. (2004) Single-molecule assay reveals strand switching and enhanced processivity of UvrD. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6439–6444

21. Dou, S. X., Wang, P. Y., Xu, H. Q., and Xi, X. G. (2004) The DNA binding properties of the Escherichia coli RecQ helicase. *J. Biol. Chem.***279,**6354–6363

22. Umezu, K., Nakayama, K., and Nakayama, H. (1990) *Escherichia coli* RecQ

protein is a DNA helicase. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5363-5367

23. Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., and Yamaoka, T. (1994) Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12. *Nucleic Acids Res.* **22**, 4566-4573

24. Suzuki, N., Shimamoto, A., Imamura, O., Kuromitsu, J., Kitao, S., Goto, M., and Furuichi, Y. (1997) DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. *Nucleic Acids Res.* **25**, 2973-2978

25. Gray, M. D., Shen, J-C., Kamath-Loeb, A. S., Blank, A., Sopher, B. L., Martin, G. M., Oshima, J., and Loeb, L. A. (1997) The Werner syndrome protein is a DNA helicase. *Nat. Genet.* **17**, 100-103

26. Karow, J. K., Chakraverty, R. K., and Hickson, I. D. (1997) The Bloom's syndrome gene product is a 3'-5' DNA helicase. *J. Biol. Chem.* **272**, 30611-30614

27. Harmon,F.G. and Kowalczykowski,S.C. (1998) RecQ helicase, in concert with RecA and SSB proteins, initiates and disrupts DNA recombination. *Genes Dev.*, **12**, 1134–1144.

28. West,S.C. (1996) DNA helicases: new breeds of translocating motors and molecular pumps. *Cell*, **86**, 177–180.

29. Bennett,R.J., Keck,J.L. and Wang,J.C. (1999) Binding specificity determines polarity of DNA unwinding by the Sgs1 protein of *S. cerevisiae*. *J. Mol. Biol.*, **289**, 235–248.

30. Lilley,D.M.J. (1999) Structures and interactions of helical junctions in nucleic acids. In Neidle,S. (ed.), *Oxford Handbook of Nucleic Acid Structure*. Oxford University Press, New York, NY, pp. 471–498.

31. Bae, S. H., Bae, K. H., Kim, J. A., and Seo, Y. S. (2001) RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes. Nature 412,456-461.

32. Garcia PL, Liu YL, Jiricny J, West SC and Janscak P (2004) Human RECQ5β, a protein with DNA helicase and strand-annealing activities in a single polypeptide. *The EMBO Journal* **23,** 2882–2891

TABLE I

Oligonucleotide sequences used for helicase measurement in this study

Name	length (nt)	sequence
174-1	20	5’- TCAGCACCAGCACGCTCCCA-3’
174-2	35	5’- TCAGCACCAGCACGCTCCCAttttttttttttT-3’
174-4	35	5’-aaaaaaaaaaaaaaTCAGCACCAGCACGCTCCCA-3’
Bubble-F	70	5’-TGC-AGT-AGC-GCC-AAT-ATG-AGA-AGA-GCC ATA-CCG-CTG-ATT-CTG-CGT-TTG-CTG-ATG- AAC-TAA-GTC-AAC-CTC-A-3’
Tstem01	20	5’-GCA-CTG-GCC GTC GTT TTA CG-3’
Tstem05	24	5’-GCA-CTG-GCC GTC GTT TTA CGG TCG-3’
Tstem10	29	5’-GCA-CTG-GCC GTC GTT TTA CGG TCG TGA CT-3’
Tstem25	44	5’-GCA-CTG-GCC GTC GTT TTA CGG TCG TGA CTG GGA AAA CCC TGG CG-3’
FLAP00	19	5’-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP01	20	5’-A-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP05	24	5’-TC-CAA-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP10	29	5’-T-TTT-TTC-CAA-GTA-AAA-CGA-CGG- CCA-GTG-C-3’
FLAP26	45	5’-TTTTTTTTTTTTTTTTTT-TTT-TTC-CAA-GTA -AAA-CGA-CGG-CCA-GTG-C
U25	25	5’-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC-C-3’
U25comp		44
5’-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC-CAA-CCC-CTT-TTT-TTT-TTC-AA-3’		
FLAP26Comp	26	5’-TTG-GAA-AAAAAAAAAAAAAAAAAAA-3’
Sub03	19	5’-GAC-GAA-CCT-TTG-CCC-ACG-T-3’
02Bis	40	5’-ACG-TGG-GCA-AAG-GTT-CGT-CAA-TGG- ACT-GAC-AGC-TGC-ATG-G-3’
Nick0	21	5’-CCA-TGC-AGC-TGT-CAG-TCC-ATT-3’
Nick2	19	5’-CCA-TGC-AGC-TGT-CAG-TCC-A-3’
Nick4	16	5’-CCA-TGC-AGC-TGT-CAG-T-3’
H1	41	5’-GCC-GTG-ATC-ACC-AAT-GCA-GAT-TGA-CGA- ACC-TTT-GCC-CAC-GT-3’
H2	41	5’-GAC-GTG-GGC-AAA-GGT-TCG-TCA-ATG-GAC- TGA-CAG-CTG-CAT-GG-3’
H6	41	5’-GCC-ATG-CAG-CTG-TCA-GTC-CAT-TGT-CAT- GCT-AGG-CCT-ACT-GC-3’
H7	41	5’-GGC-AGT-AGG-CCT-AGC-ATG-ACA-ATC-TGC- ATT-GGT-GAT-CAC-GG-3’
E21A	21	5’-GTG-TGG-AAA-ATC-TCT-AGC-ATC-3’
E21B	21	5’-ACT-GCT-AGA-GAT-TTT-CCA-CAC-3’
PhiX174		
SubA20	50	5’-TCA-AAG-TCA-CGA-CCT-AGA-CAC-TGC-GAG-CTC -GAA-TTC-ACT-GGA-GTG-ACC-TC
SubB20	50	5’-GAG-GTC-ACT-CCA-GTG-AAT-TCG-AGC-TCG-CAG -TGT-CTA-GGT-CGT-GAC-TTT-GA

Figure legends

Figure 1 (A). Amino acid sequence alignment of *E. coli* RecQ , *Bacillus subtilis* SubL. The multiple alignments were performed with the program ClustalW and refined manually. The numbers at the beginning and end of each sequence correspond, respectively, to positions of the first and the last amino acid residues. Highly conserved amino acid residues are *shadowed* in *gray*. The protein accession numbers used by the NCBI are as follows: RecQ, P15043 [GenBanK] (*E. coli*); SubL, F69901 [GenBanK] (B. *Subtilis*). **(B).Purification of SubL protein.** A Coomassie Blue-stained 10% SDS-polyacrylamide gel of a crude cell extract and the purified SubL protein by FPLC size exclusion chromatography (lane2 and lane3).The molecular mass standards (lane M) are shown on both sides. Lane2: 0.5μg protein, lane3: 1μg protein.

Figure 2 (A) Effect of different DNA or RNA on ATPase activity of SubL helicase. DNA-dependent ATP hydrolysis activity of SubL helicase as a function of enzyme concentration.▽: no DNA, ■: 60nt ssDNA 24μM(nt), ●: 7kb PN6 DNA 28μM(bp),♦ 5.5kb GC70 plasmid DNA 27.5μM(bp), ○: 5.5kb GC70 plasmid DNA denatured 27.5μM(bp), Δ: 678nt RNA 24μM(nt). ATP 2mM. **(B) Requirement for ATP hydrolysis catalyzed by SubL protein.** Experiments were performed at 37 °C with 0.1μM protein and 24 μM ssDNA (nucleotide, 60-mer oligonucleotide) for each helicase preparation , 3mM Mg^{2+}, 3mM Mn^{2+},3mM Ca^{2+}, 3mM Zn^{2+}, 15mM EDTA, 2mM AMPPNP and 2 mM ATP respectively. **(C) Effect of different pH on ATPase activity of SubL helicase.** Experiments were performed at 37 °C with 0.1μM protein for each helicase preparation , 24 μM ssDNA (nucleotide, 60-mer oligonucleotide) and 2 mM ATP. **(D) ATPase activity of the SubL helicase as a function of ATP concentration.** Experiments were performed at 37 °C with 0.1μM protein for each helicase preparation and 24 μM ssDNA (nucleotide, 60-mer oligonucleotide).

Figure 3 (A) DNA unwinding in the presence of varying concentrations of the SubL helicase. DNA unwinding assays were performed as described in "Experimental Procedures" using DNA substrate (0.45nM, Tstem25*/Flap26); each reaction contained 2.5-80nM helicases respectively and 2mM ATP, * denote γ-^{32}P

labeled. lane △ ,boiled substrate. **(B) Effects of nucleotides on SubL helicase-catalyzed DNA unwinding activity**. DNA unwinding assays were performed as described in "Experimental Procedures" using DNA substrate (0.45nM, Tstem25*/Flap26); each reaction contained 0.1μM helicases and one of the listed nucleotides at a final concentration of 2mM. * denote γ-^{32}P labeled. Lane C, negative control, lane △,boiled substrate.

Figure 4 (A) Polarity of DNA unwinding by the recombinant SubL protein. Reaction mixtures (20 μl each) containing ~0.45nM DNA substrate were incubated with 0.1μM of the recombinant SubL protein and incubated at 37 °C for 30 min. The products of the reaction were analyzed by electrophoresis in a 12% polyacrylamide gel and autoradiography. The substrates are shown in the figure (Tstem25*/Flap00, Tstem25*/U25). * denote γ-^{32}P labeled. Lane C, negative control, lane -, no DNA unwinding, lane +, DNA unwinding, lane △,boiled substrate.

Figure 5 Time course of the strand displacement reaction mediated by the recombinant SubL protein. (A). A reaction mixture (20 μl) containing 0.45 nM substrate (ΦX174 virion DNA annealed with a ^{32}P-labeled 20-nucleotide fragment, PhiX174-1*/PhiX174) in the helicase assay buffer was incubated at 37 °C with the addition of the SubL protein (0.2μM). **(B).** A reaction mixture (20 μl) containing 0.45nM substrate (ΦX174 virion DNA annealed with a ^{32}P-labeled 70-nucleotide fragment, Bubble-F*/PhiX174) in the helicase assay buffer was incubated at 37 °C with the addition of the SubL protein (0.2μM). Following the addition of the recombinant SubL protein, 20-μl reaction mixtures were mixed with excess EDTA to terminate the reactions at the times indicated. The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. lane △,boiled substrate.

Figure 6 (A) 5'-Tail length dependence studies. The unwinding were determined with four DNA substrates having an 3'-ssDNA tail of 26nt and an 5'-ssDNA tails of 1, 5, 10 and 26nt (Tstem25*/Flap00, Tstem25*/Flap01, Tstem25*/Flap05, Tstem25*/Flap10, Tstem25*/Flap26), respectively. The concentration of the SubL helicase used in all studies was 0.1μM. *Asterisks* denote the ^{32}P-labeled end. **(B)**

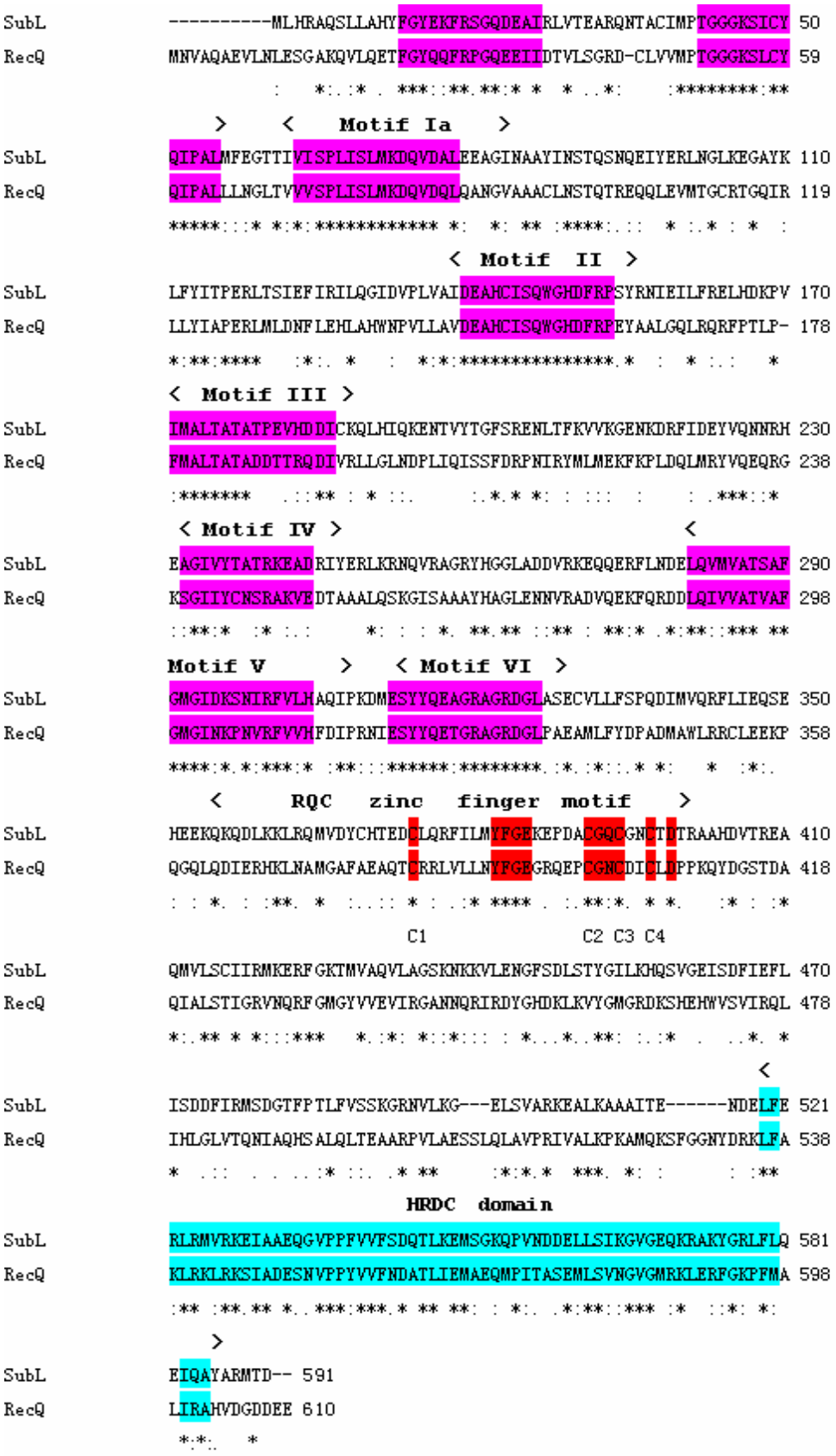
3'-Tail length dependence studies. The unwinding were determined with four DNA substrates having an ssDNA tails of 1, 5, 10 and 25nt (Flap00*/Tstem01, Flap00*/Tstem05, Flap00*/Tstem10, Flap00*/Tstem25). The concentration of the SubL helicase used in all studies was 0.2μM. *Asterisks* denote the ^{32}P-labeled end. **(C) Helicase activity with various substrates.** Each panel shows the structure of the substrate used and an autoradiogram of the gel. A reaction mixture (20 μl) containing 0.45nM indicated substrate (PhiX174-1*/PhiX174, PhiX174-4*/PhiX174, PhiX174-2*/PhiX174) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the SubL protein (0.2μM). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. Lane C, negative control, lane +, DNA unwinding, lane △, boiled substrate.

Figure 7 (A) SubL unwinds a 5'-ssDNA flap substrate, Kappa joint molecule and forked DNA substrates with 5'- and 3'-tails dsDNA. A reaction mixture (20 μl) containing 0.45nM indicated substrate (Tstem25*/Flap26/U25, Tstem25*/Flap26/U25Comp, Tstem25*/Flap26/U25/Flap26Comp) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the SubL protein (0.2μM). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. Lane C, negative control, lane +, DNA unwinding, lane △, boiled substrate. **(B) SubL unwinds the four-way junction DNA substrate.** A reaction mixture (20 μl) containing 0.45nM indicated substrate (H1*/H2, H1*/H2/H7, H1*/H2/H7/H6) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the SubL protein (0.2μM). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. Lane C, negative control, lane +, DNA unwinding, lane △, boiled substrate. **(C) SubL helicase promotes DNA strand annealing.** Formation of 50-bp blunt end duplex in the presence of varying concentrations of SubL. The SubA20 and SubB20 complementary oligonucleotides (1 nM), of which SubA20 was radiolabeled at its 5'-end, were incubated with the indicated concentrations of SubL for 20 min at 37°C. The reaction products were separated by 12% nondenaturing PAGE and visualized by autoradiography. lane +, DNA pairing positive control.

Figure 8 (A) ssDNA binding by the recombinant SubL protein. Electrophoretic mobility shift measurements were carried out to determine the binding of the SubL with single-stranded DNA (ssDNA, Tstem25*). Reaction mixtures (20 µl each) contained DNA (0.45nM) and Sub-L (0, 5, 10, 20, 40, 80 and 160nM) in binding buffer as described under "Experimental Procedures." Reactions were incubated for 30 min at room temperature before loading onto a 6% nondenaturing polyacrylamide gel to separate the protein-DNA complexes electrophoretically. DNA was visualized by autoradiography. * denote γ-^{32}P labeled. **(B) Preference for binding of forked DNA by the SubL protein.** 5'-^{32}P-end-labeled forked DNA substrates (Flap26/ Tstem25*) were incubated with the Sub-L protein in binding buffer as described under "Experimental Procedures." Reaction mixtures (20 µl) contained DNA (0.45nM) and Sub-L (0, 5, 10, 20, 40 and 80nM). Protein-DNA complexes were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel and autoradiography. * denote γ-^{32}P labeled. **(C) Preference for binding of forked DNA by the SubL protein analyzed by TFA method.**

Figure 1

A


SubL ----------MLHRAQSLLAHYFGYEKFRSGQDEAIRLVTEARQNTACIMPTGGGKSICY 50
RecQ MNVAQAEVLNLESGAKQVLQETFGYQQFRPGQEEIIDTVLSGRD-CLVVMPTGGGKSLCY 59
Motif Ia
SubL QIPALMFEGTTIVISPLISLMKDQVDALEEAGINAAYINSTQSNQEIYERLNGLKEGAYK 110
RecQ QIPALLLNGLTVVVSPLISLMKDQVDQLQANGVAAACLNSTQTREQQLEVMTGCRTGQIR 119
Motif II
SubL LFYITPERLTSIEFIRILQGIDVPLVAIDEAHCISQWGHDFRPSYRNIEILFRELHDKPV 170
RecQ LLYIAPERLMLDNFLEHLAHWNPVLLAVDEAHCISQWGHDFRPEYAALGQLRQRFPTLP- 178
Motif III
SubL IMALTATATPEVHDDICKQLHIQKENTVYTGFSRENLTFKVVKGENKDRFIDEYVQNNRH 230
RecQ FMALTATADDTTRQDIVRLLGLNDPLIQISSFDRPNIRYMLMEKFKPLDQLMRYVQEQRG 238
Motif IV
SubL EAGIVYTATRKEADRIYERLKRNQVRAGRYHGGLADDVRKEQQERFLNDELQVMVATSAF 290
RecQ KSGIIYCNSRAKVEDTAAALQSKGISAAAYHAGLENNVRADVQEKFQRDDLQIVVATVAF 298
Motif V
Motif VI
SubL GMGIDKSNIRFVLHAQIPKDMESYYQEAGRAGRDGLASECVLLFSPQDIMVQRFLIEQSE 350
RecQ GMGINKPNVRFVVHFDIPRNIESYYQETGRAGRDGLPAEAMLFYDPADMAWLRRCLEEKP 358
RQC zinc finger motif
SubL HEEKQKQDLKKLRQMVDYCHTEDCLQRFILMYFGEKEPDACGQCGNCTDTRAAHDVTREA 410
RecQ QGQLQDIERHKLNAMGAFAEAQTCRRLVLLNYFGEGRQEPCGNCDICLDPPKQYDGSTDA 418
C1 C2 C3 C4
SubL QMVLSCIIRMKERFGKTMVAQVLAGSKNKKVLENGFSDLSTYGILKHQSVGEISDFIEFL 470
RecQ QIALSTIGRVNQRFGMGYVVEVIRGANNQRIRDYGHDKLKVYGMGRDKSHEHWVSVIRQL 478
SubL ISDDFIRMSDGTFPTLFVSSKGRNVLKG---ELSVARKEALKAAAITE------NDELFE 521
RecQ IHLGLVTQNIAQHSALQLTEAARPVLAESSLQLAVPRIVALKPKAMQKSFGGNYDRKLFA 538
HRDC domain
SubL RLRMVRKEIAAEQGVPPFVVFSDQTLKEMSGKQPVNDDELLSIKGVGEQKRAKYGRLFLQ 581
RecQ KLRKLRKSIADESNVPPYVVFNDATLIEMAEQMPITASEMLSVNGVGMRKLERFGKPFMA 598
SubL EIQAYARMTD-- 591
RecQ LIRAHVDGDDEE 610

B

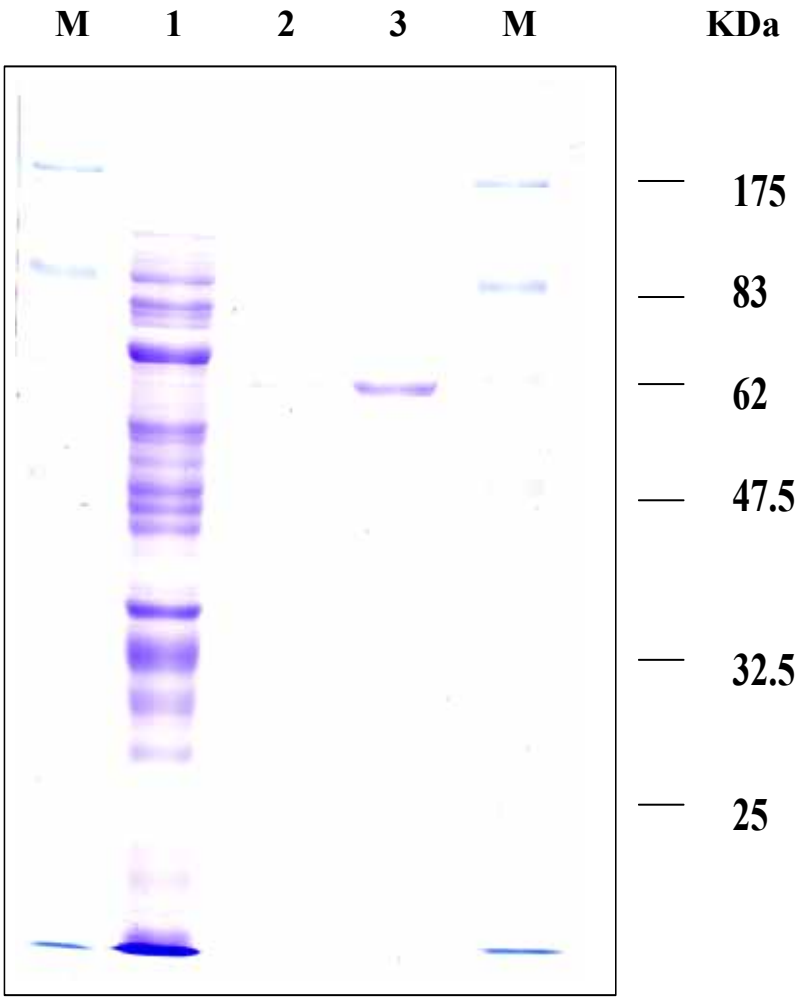
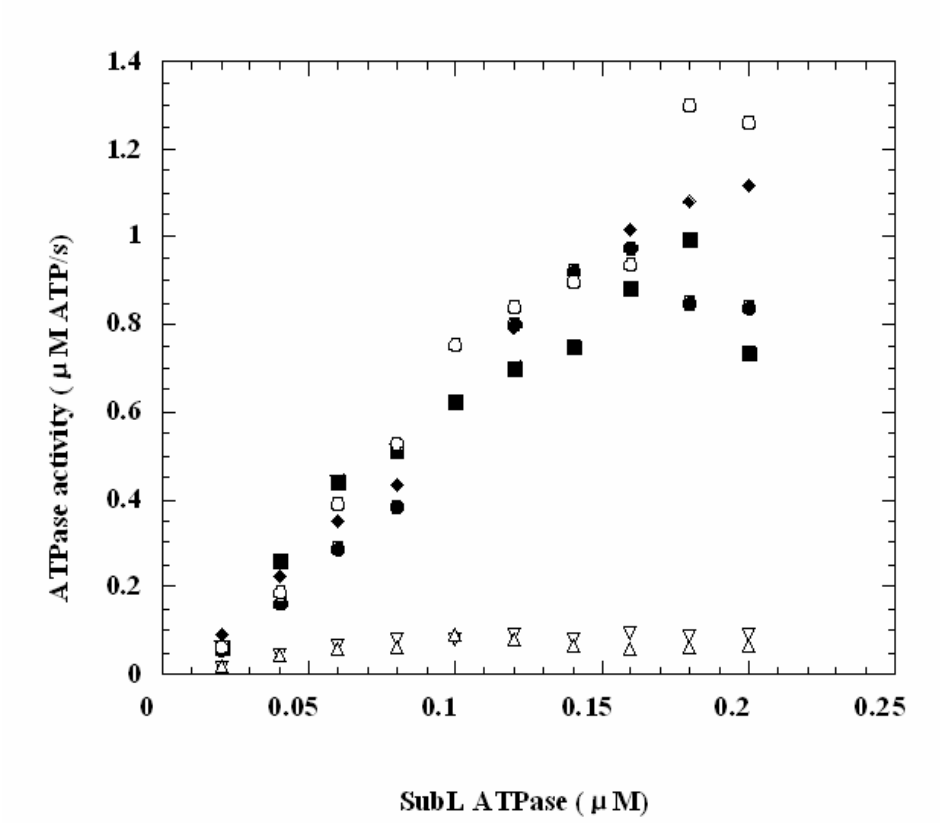
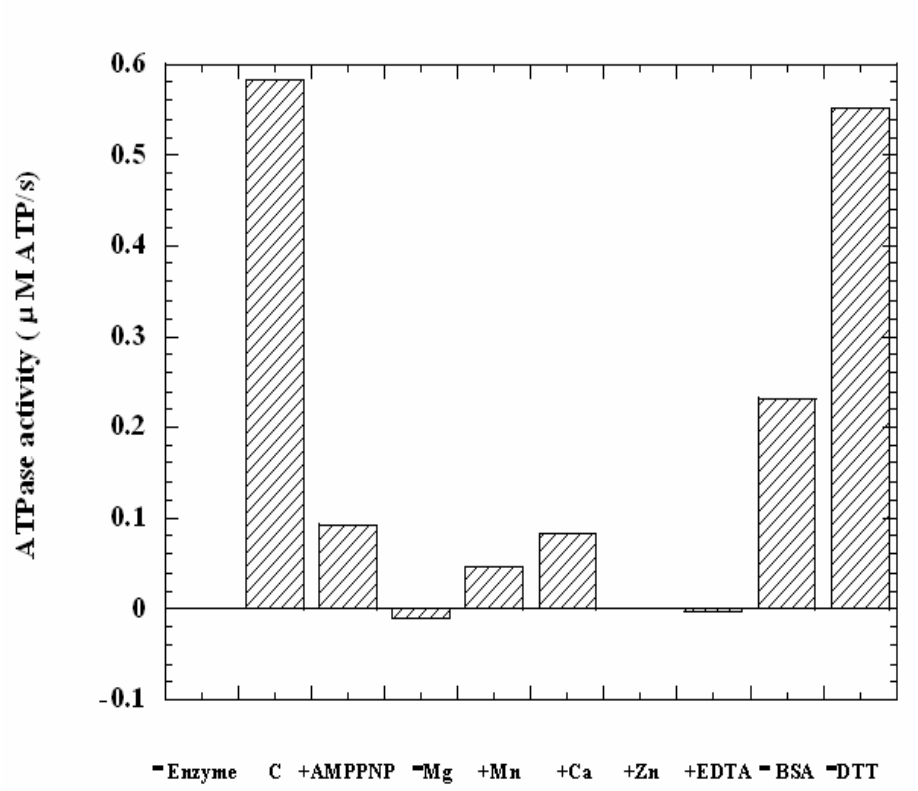


Figure 2

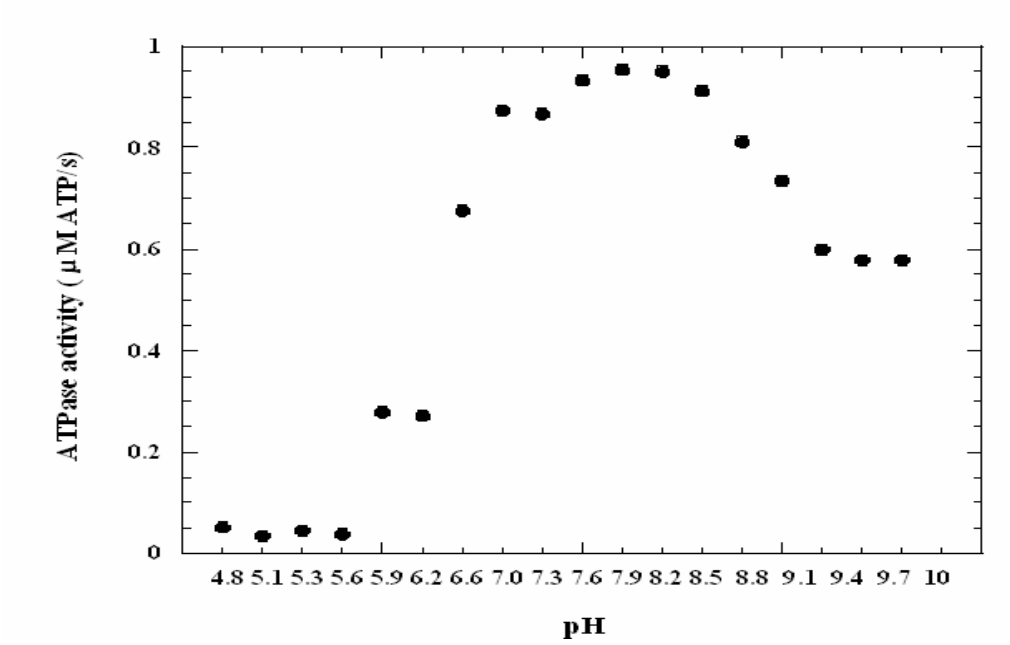
A


ATPase activity (μM ATP/s)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.15
0.2
0.25
SubL ATPase (μM)

B


ATPase activity (μM ATP/s)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Enzyme
C
+AMPPNP
Mg
+Mn
+Ca
+Zn
+EDTA
BSA
DTT

C


ATPase activity (μM ATP/s)
1
0.8
0.6
0.4
0.2
0
4.8 5.1 5.3 5.6 5.9 6.2 6.6 7.0 7.3 7.6 7.9 8.2 8.5 8.8 9.1 9.4 9.7 10
pH

D

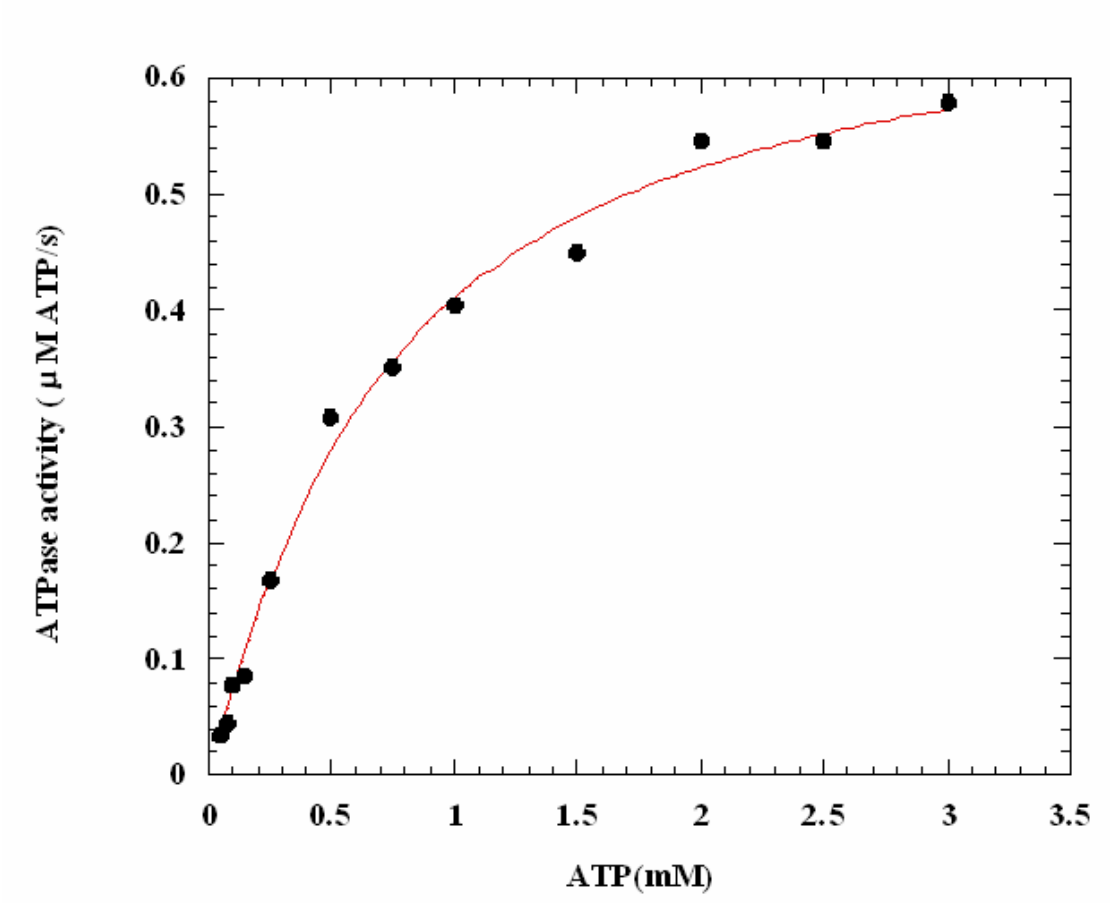
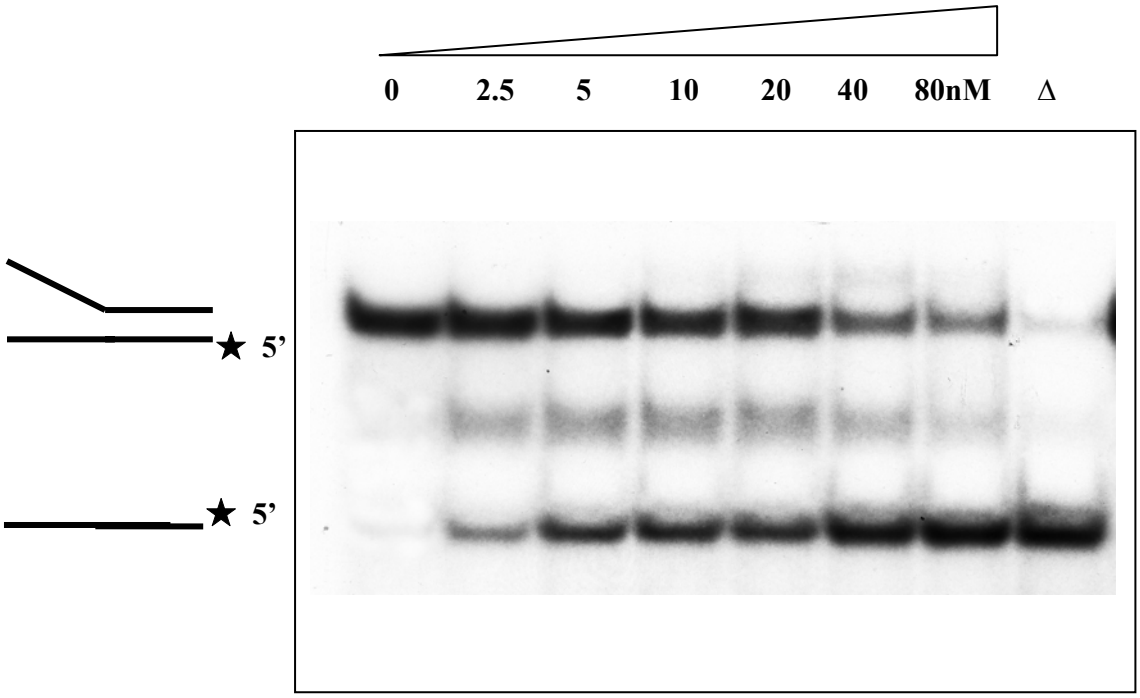

ATPase activity (μM ATP/s)
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.5 1 1.5 2 2.5 3 3.5
ATP(mM)

Figure 3

A


0
2.5
5
10
20
40
80nM
Δ
5'
5'

B

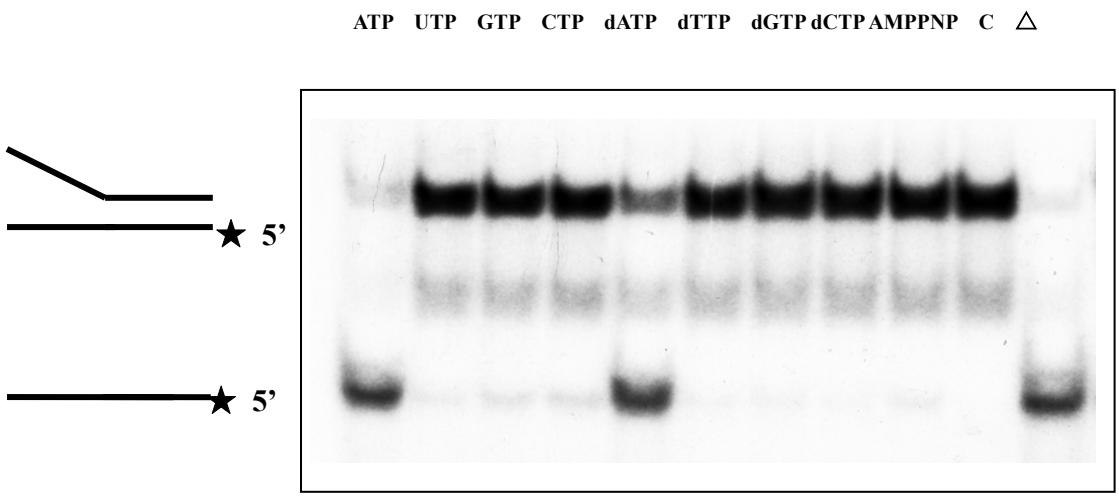

ATP
UTP
GTP
CTP
dATP
dTTP
dGTP
dCTP
AMPPNP
C
△
5'
5'

Figure 4

A

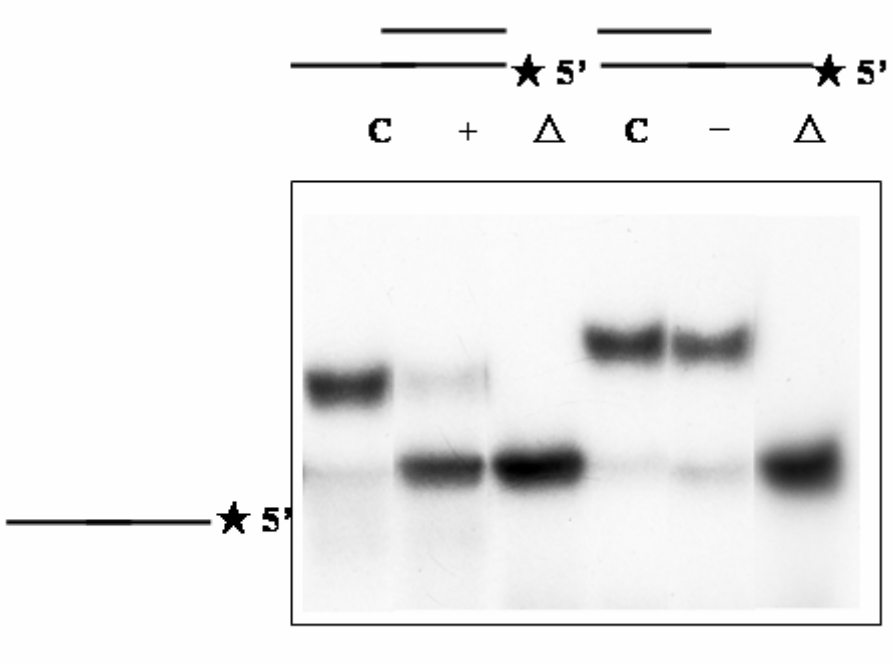
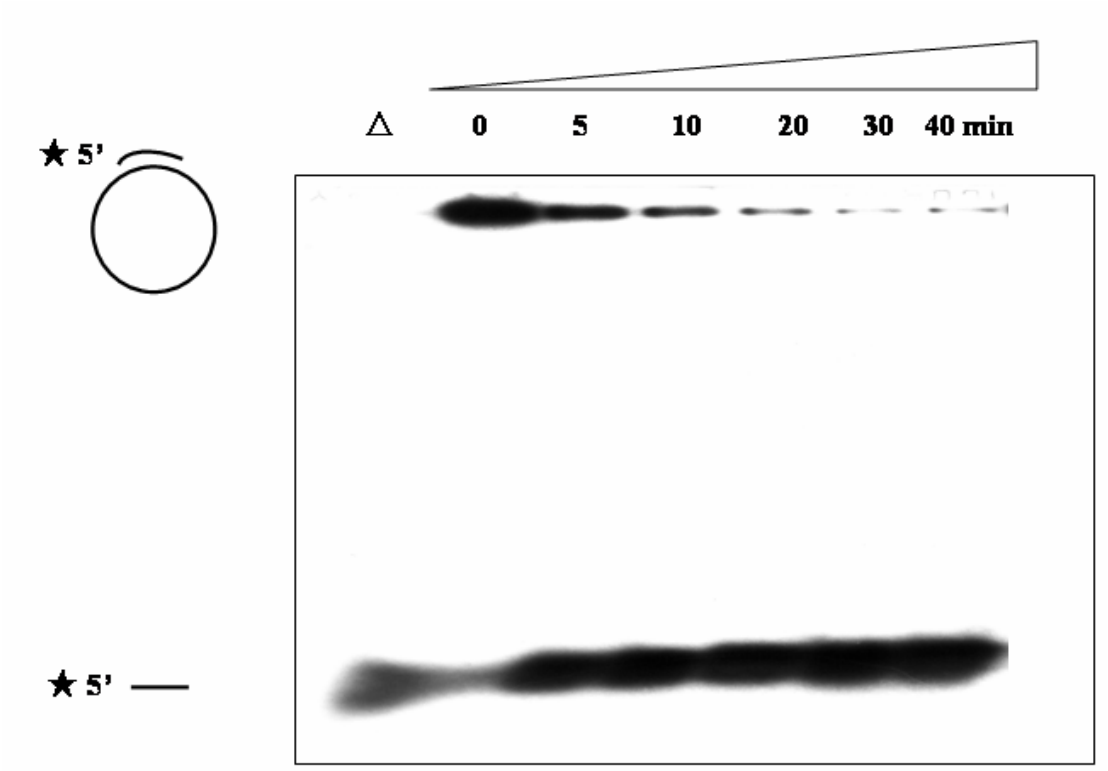


Figure 5

A



B

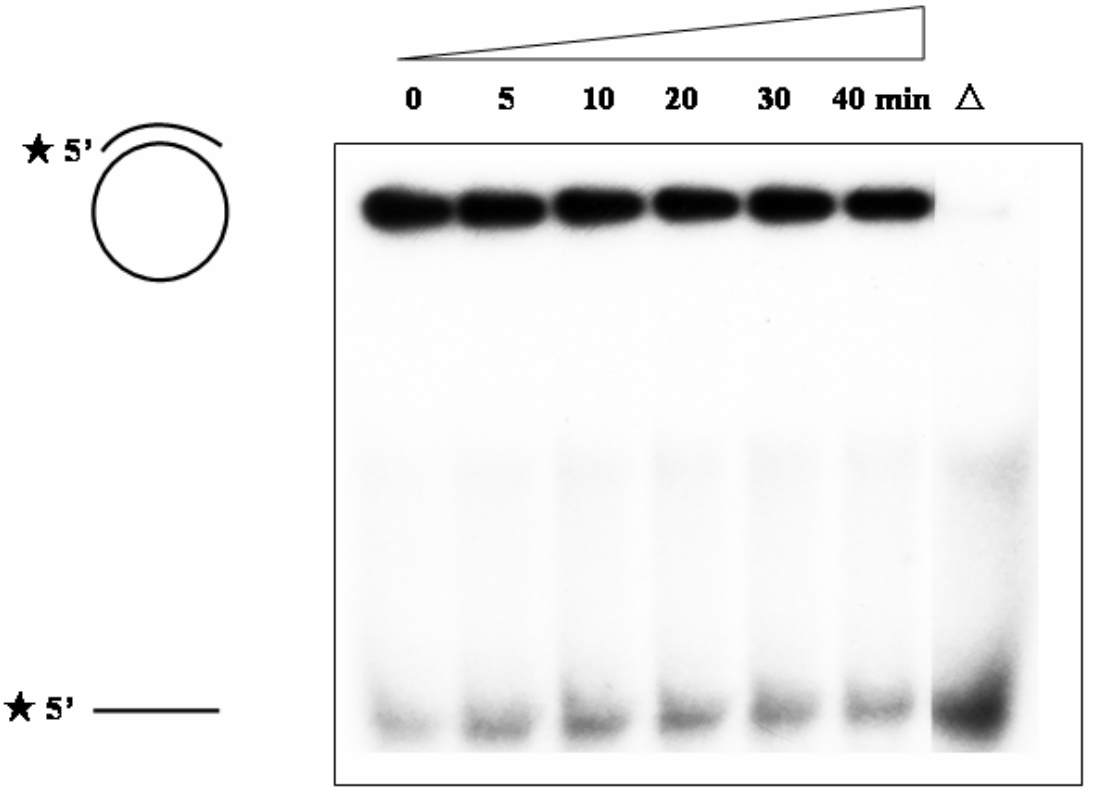
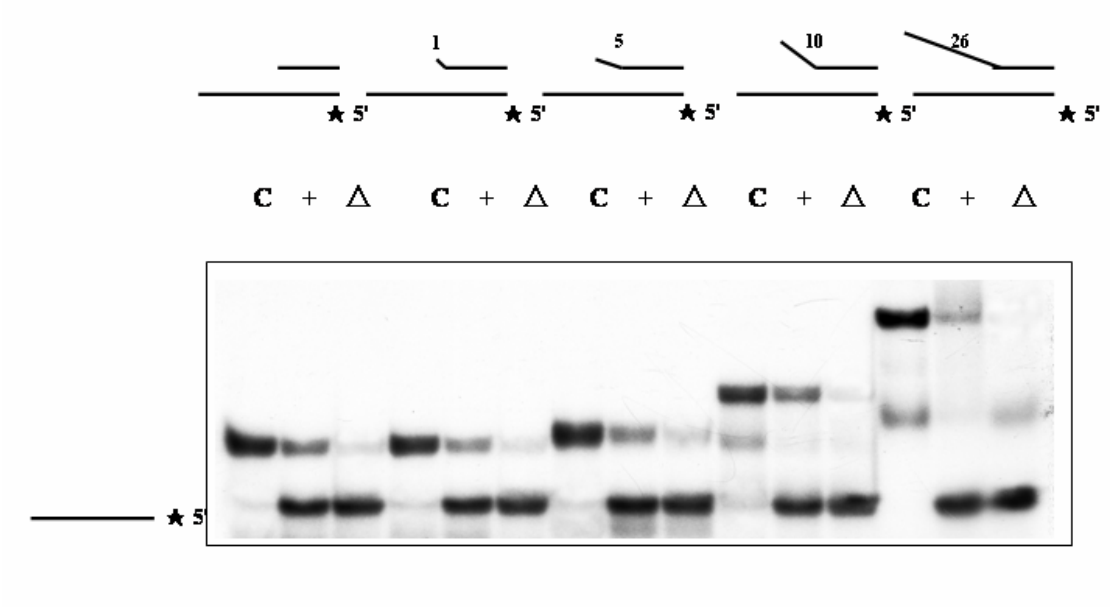
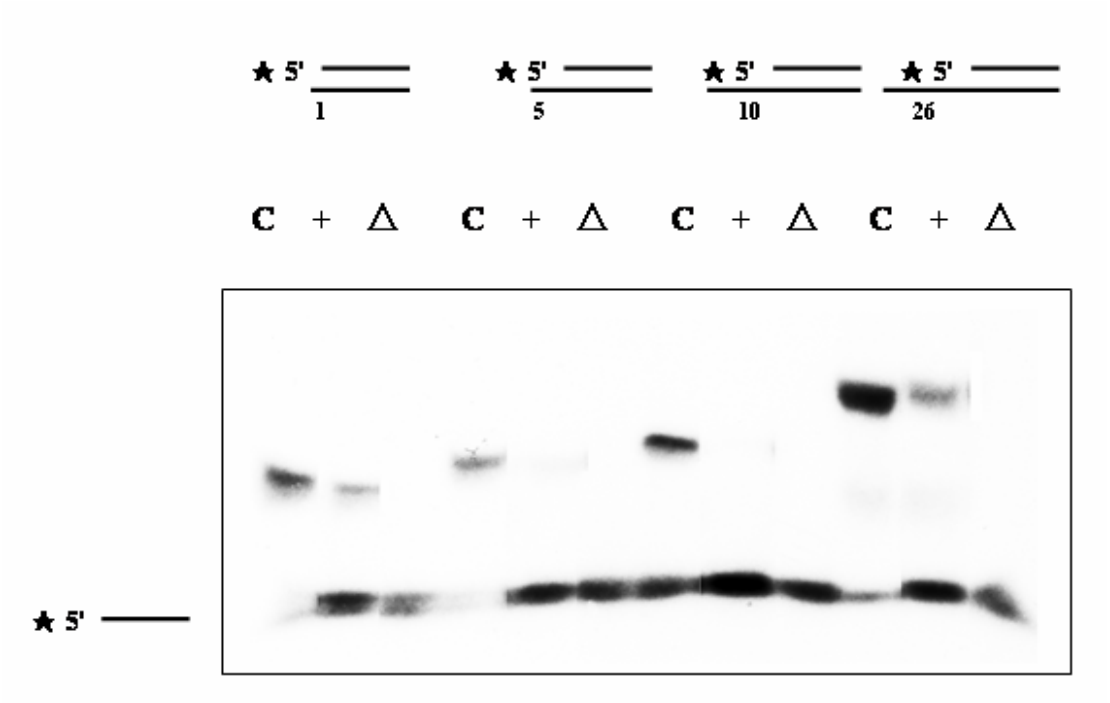


Figure 6

A



B



C

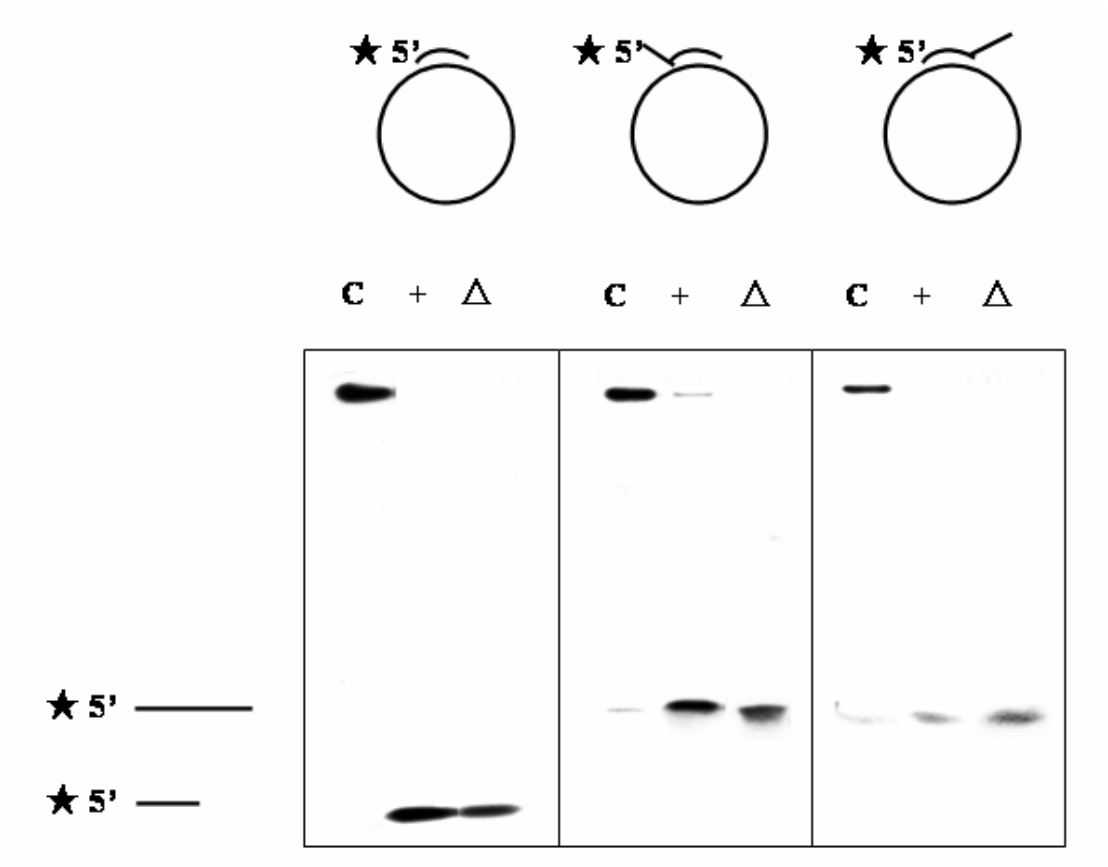
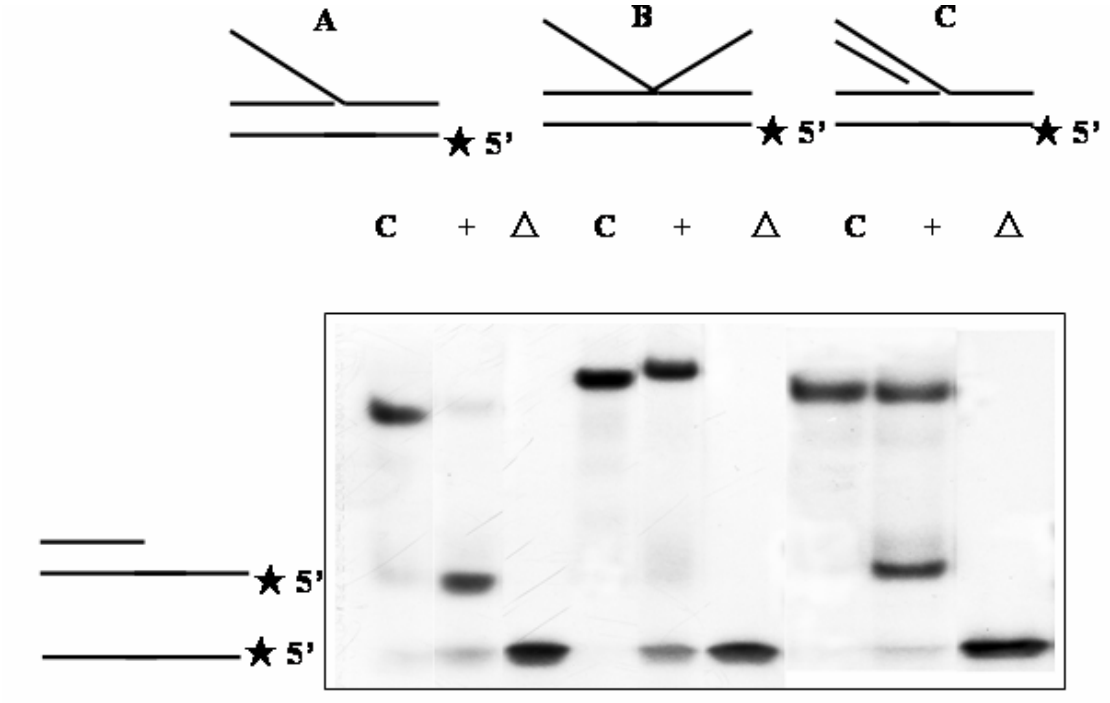
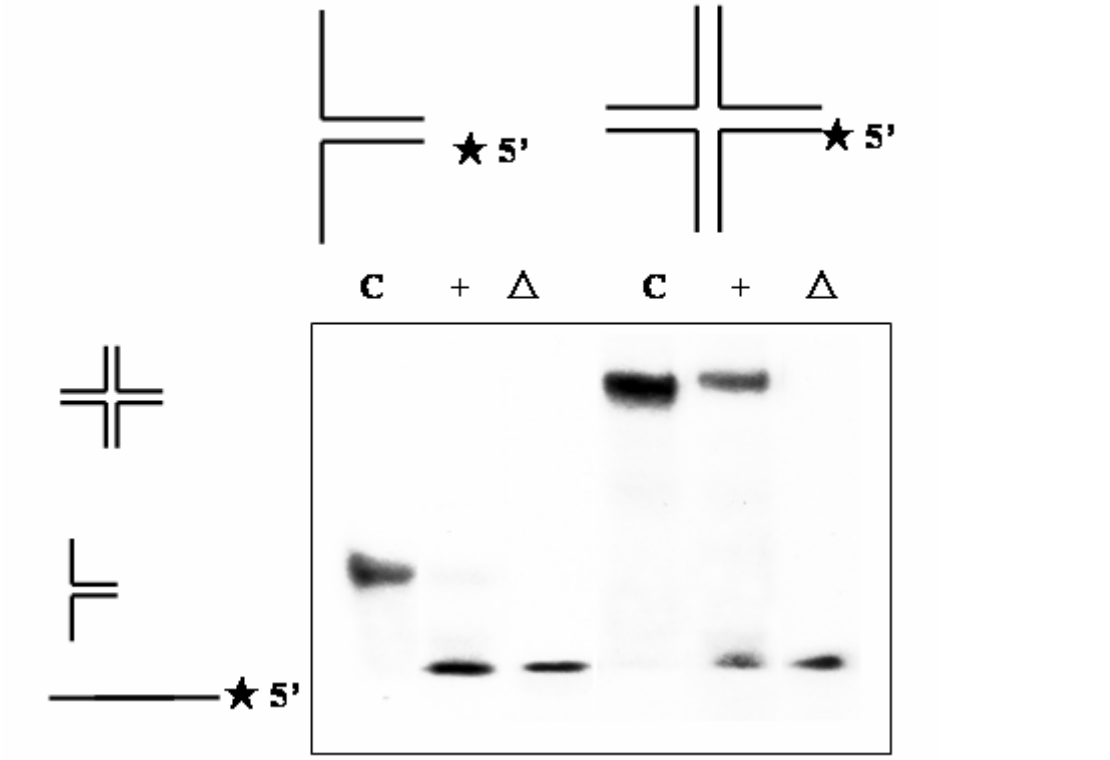


Figure 7

A



B



C

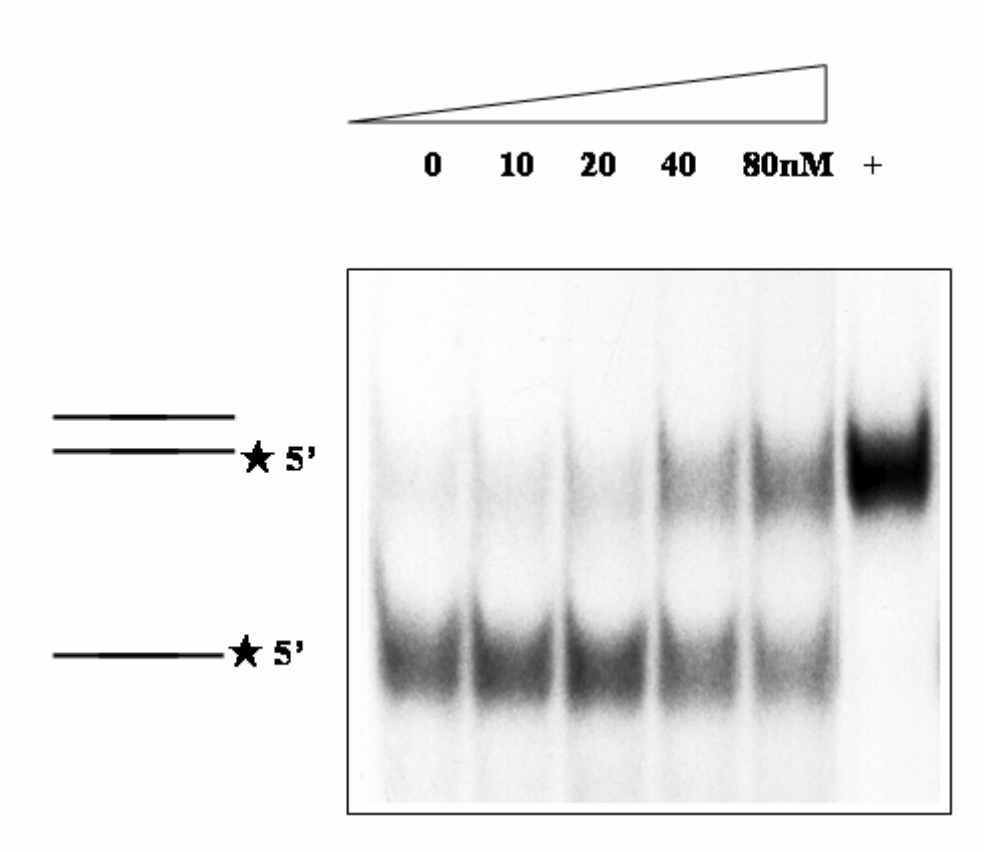
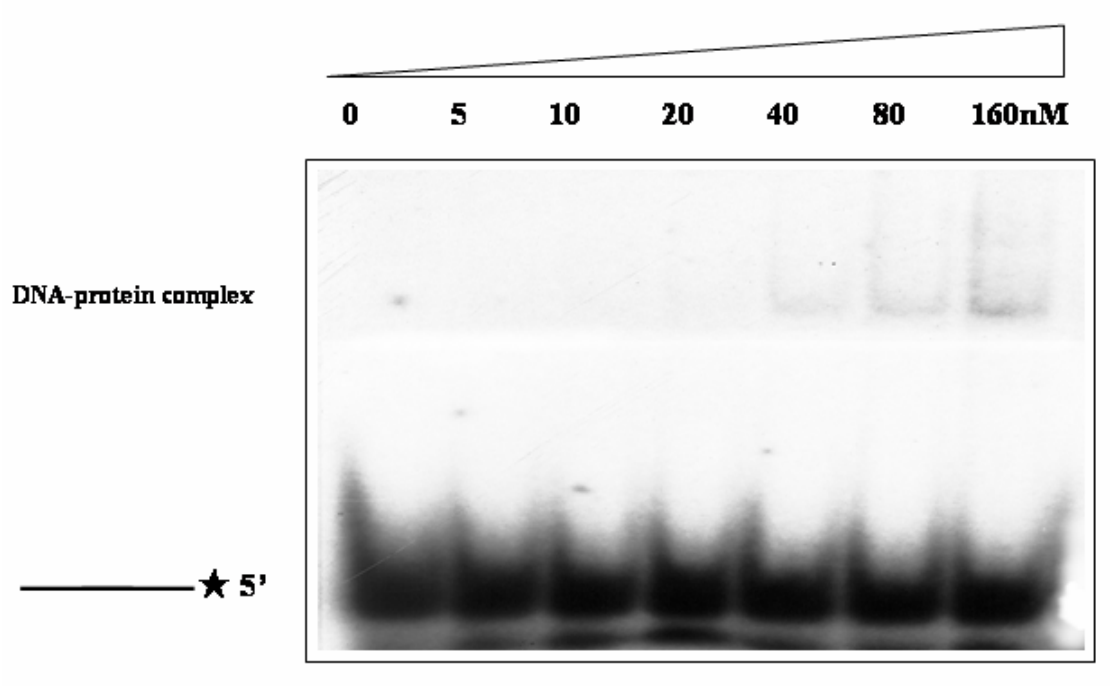
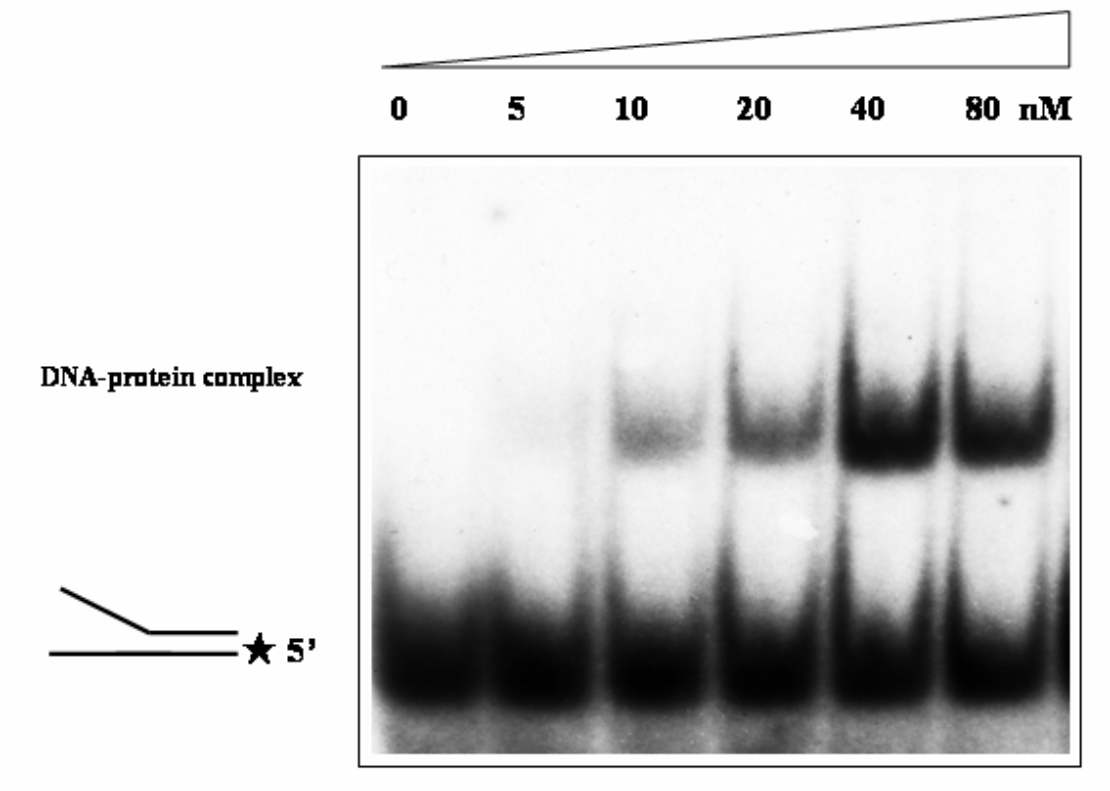


Figure 8

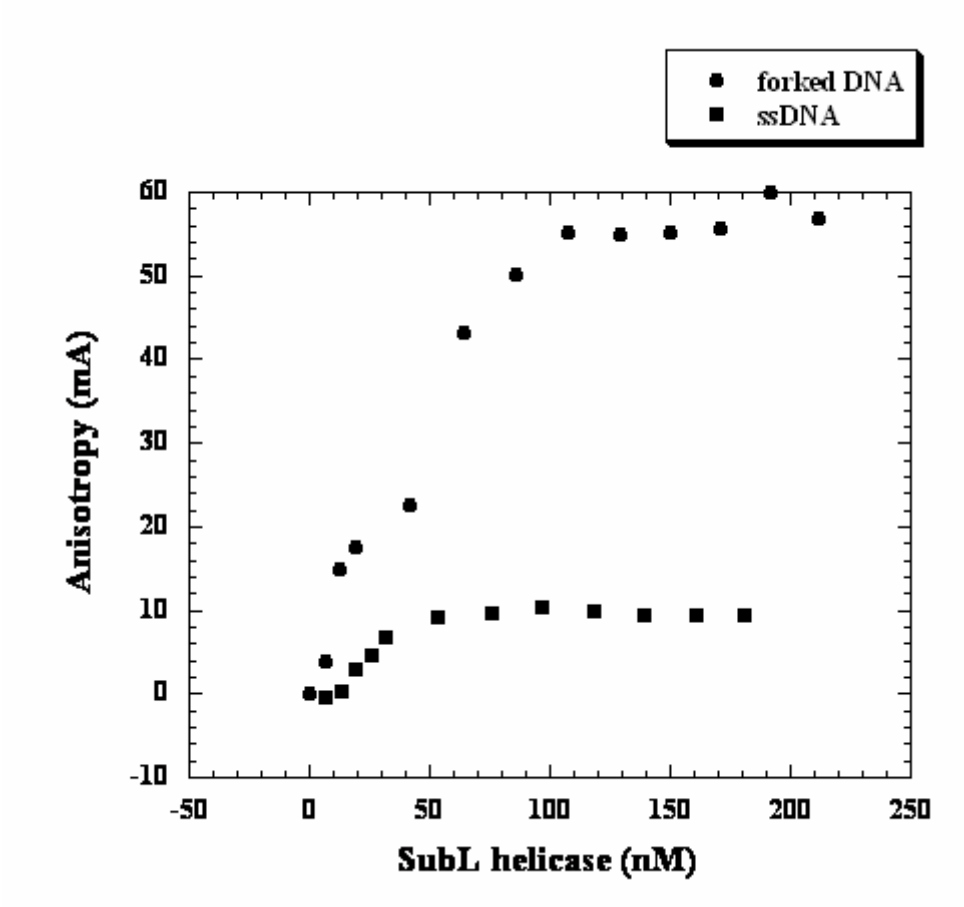
A


0
5
10
20
40
80
160nM
DNA-protein complex
5'

B


0
5
10
20
40
80 nM
DNA-protein complex
5'

C


forked DNA
ssDNA
Anisotropy (mA)
SubL helicase (nM)
60
50
40
30
20
10
0
-10
-50
0
50
100
150
200
250

4. Etude de l'hélicase de la famille RecQ chez *Bacillus subtilis* II. La protéine SubS

4.1. Présentation

Selon des données génomiques, *Bacilus subtilis* possède deux hélicases de la famille RecQ. L'hélicase SubS est la forme la plus courte. Selon sa séquence d'acides aminés (Fig. 1A de l'article 4), la protéine SubS de *Bacilus subtilis* possède un domaine helicase et un domaine RecQ-Ct mais pas de domaine HRDC. Cette hélicase putative n'a pas été caractérisée. Pour étudier la fonction de SubS, la protéine a été exprimée chez *E. coli,* purifiée et caractérisée par les méthodes biochimiques.

Nous avons détecté des ions zinc fixés par la protéine SubS en utilisant la technique de PAR (4-(2-Pyridylazo)resorcinol) et nous avons montré que 1 mole d'hélicase SubS fixe effectivement 1 mole d'ion zinc. Ce résultat indique que l'enzyme contient un motif fixant un ion zinc. Nous avons aussi étudié l'activité ATPase catalysée par SubS en présence ou absence d'ADN et d'ARN. Les résultats montrent que SubS est une ATPase ADN dépendante mais pas une ATPase ARN dépendante. Nos données démontrent que l'hydrolyse de l'ATP catalysée par SubS est dépendante du Mg^{2+}, mais pas du Mn^{2+}, Ca^{2+}, Zn^{2+}. L'hélicase SubS montre une activité ATPase optimale entre pH 7.0 et 8.8. L'activité ATPase de SubS (kcat : 0.4/s) est plus faible que celle de la protéine RecQ d'*E. coli* (kcat : 35.2/s) (Liu *et al.*, 2004).

Ensuite, nous avons étudié l'activité hélicase de SubS en utilisant l'électrophorèse et l'autoradiographie. Les résultats indiquent que la protéine SubS est une ADN hélicase. Nous avons aussi montré que la protéine SubS est une hélicase de polarité 3'-5'. L'hélicase SubS n'est pas capable de dérouler un ADN duplex avec des extrémités franches et un ADN duplex avec une cassure. Nous avons démontré que l'hélicase SubS ne peut pas dérouler un ADN duplex avec un flap 5', une structure de type fourche de réplication, une structure Kappa ou une structure à 4 branches mimant une jonction de Holliday.

Enfin, nous avons montré que SubS ne peut pas favoriser l'hybridation de l'ADN. Elle fixe plus fortement l'ADN simple brin que l'ADN double brin contenant une fourche.

En résumé, la protéine SubS est aussi une ADN hélicase et une ATPase ADN dépendante, mais ses activités hélicase et ATPase sont faibles.

L'ensemble de ces résultats est présenté dans l'article № 4.

4.2. Article № 4:

Studies on Helicase of RecQ family from *Bacillus subtillis*: II. SubS protein.

Jie Lin Liu and Xu Guang Xi

Studies on Helicase of RecQ family from *Bacillus subtilis*: II. SubS protein.[#]

Jie Lin Liu+, and Xu Guang Xi*

Laboratoire de Biotechnologies et Pharmacologie Génétique Appliquée CNRS UMR 8113, Ecole Normale Supérieure de Cachan, 61 Avenue du Président Wilson, 94235 Cachan cedex, France

Running title: Biochemical property of Sub-L helicase from *Bacillus subtillis*

* To whom correspondence should be addressed. Tel.: 33-01-47-40-68-92; Fax: 33-01-47-40-76-71; E-mail: xi@lbpa.ens-cachan.fr.

This work was supported by the Centre National de la Recherche Scientifique.

[+] Supported by the China Scholarship Council.

[1] The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; PAR, 4-(2-Pyridylazo)resorcinol; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 5'-adenyl- β,γ-imidodiphosphate; TFA, time-resolved fluorescence anisotropy; FPLC, fast protein liquid chromatography; NTP, nucleotide triphosphate; DNP, nucleotide diphosphate.

Acknowledgement: We are very grateful to Drs. P. Fosse, F. Chaminade and Y.Y. Zhang for help with analytical EMSA and autoradiography experiments, Drs E. Deprez, P. Tauc and J.-C. Brochon for help with TFA experiments, Dr. H. Leh for help with FPLC, and A. Vignes for preparation of the figures. We thank Professor C. Auclair for his continuous support.

ABSTRACT

The Bacteria *Bacillus subtilis* SubS protein is a member of the RecQ family of DNA helicases that include the *Escherichia coli* RecQ protein and the products of human Bloom's syndrome and Werner's syndrome genes. To study the enzymatic characteristics of the protein, a recombinant SubS protein was overexpressed in *E. coli* and purified to near homogeneity. The purified protein exhibits an ATPase activity in the presence of single- or double-stranded DNA, but single-stranded DNA is more effective cofactor. In the presence of ATP, unwinding of duplex DNA by the recombinant SubS was readily observed. Similar to the *E. coli* RecQ helicase, unwinding of the DNA strand occurs in the 3' to 5' direction with respect to the single-stranded DNA to which the SubS binds. In addition, the recombinant SubS were found to bind more tightly to a single-stranded DNA substrate than to a forked DNA. Because there is a zinc finger motif of RecQ-Ct domain but not HRDC domain in the SubS amino acids sequence, the efficiencies of unwinding and ATPase activity of SubS are about 15 times less than that of SubL which contains both a zinc finger motif and a HRDC domain. Moreover, an intrinsic DNA strand-annealing activity has not been detected in SubS. This study demonstrated that duplex DNA cannot be unwinded by SubS from either an internal nick or a blunt-ended terminus. It is also resemble to the two important intermediates of replication/repair, a 5'-ssDNA flap substrate and a synthetic replication fork as well as a synthetic X-structure (a model for the Holliday junction recombination intermediate).

INTRODUCTION

Helicases are ubiquitous enzymes that use the free energy of nucleotide triphosphate hydrolysis to unwind double-stranded DNA into the constituent single strands (1-3). They play essential roles in nearly all DNA metabolic processes, including DNA replication, recombination and repair.

DNA helicases of the RecQ family, named after the 3'−5' DNA helicase RecQ of *Escherichia coli*, unwind a wide variety of potentially recombinogenic DNA structures, including four-way junctions, D-loops and G-quadruplex DNA. RecQ helicases are highly conserved from bacteria to humans. Generally, prokaryotes and unicellular eukaryotes such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* possess only a single RecQ homolog, while multicellular organisms have severals (4-6).

Unlike *E. coli* containing only one RecQ protein with DNA helicase activity (7), *Bacillus subtilis* could possess more than one such putative helicase (8). This is similar with the fact that human cells contain five RecQ helicases (RecQ1, BLM, WRN, RecQ4 and RecQ5) (5, 9). Although the *B. subtilis* "*recQ*" gene has not yet been tested, the gene seems to be nonessential for cell viability (9, 10).

In all organisms, defective RecQ helicase function is associated with genomic instability, which is generally manifested as an increase in the frequency of inappropriate recombination events. Mutations in genes encoding the human RecQ helicases BLM, WRN and RECQ4 give rise to the hereditary disorders Bloom's syndrome, Werner's syndrome and Rothmund−Thomson syndrome, respectively (4). These diseases are associated with cancer predisposition and premature aging. Although the precise DNA metabolism mediated by RecQ helicases remain elusive, the enzymes have been implicated in DNA replication, recombination and reparation (4).

RecQ helicases possess the helicase and RecQ-Ct (RecQ C-terminal) domains, which form the catalytic core of the enzyme (4, 11). The former is homologous to the superfamily 2 helicases, while the latter is unique to the RecQ family. Recent structural studies on the *E. coli* RecQ helicase have shown that the RecQ-Ct region forms two subdomains (12). The proximal part of this region folds into a platform of

four helices containing a Zn^{2+}-binding site and the distal part forms a specialized helix−turn−helix motif, called the winged-helix (WH) domain (12).

According to its amino acids sequence (Fig. 1A), there is both a helicase domain and a RecQ-Ct domain but not a HRDC domain in the SubS protein (RecQ homologue) from *B. subtilis*. The previous studies indicated that the zinc finger motif of RecQ-Ct is very important for the protein structure and the enzyme function of the RecQ helicases (13, 14). In this paper, we showed the DNA-dependent ATPase, helicase, and DNA annealing activities of the purified protein.

EXPERIMENTAL PROCEDURES

Reagents—Chemicals were reagent grade and all solutions were prepared using ELGA pure water. [γ-^{32}P] ATP was purchased from Amersham Biosciences. EDTA, 2-mercaptoethanol, ATP, and AMPPNP were obtained from Sigma. T4 polynucleotide kinase was from New England Biolabs. TE: 10mM Tris-HCl (pH8.0), 1mM EDTA (pH8.0).

Nucleic Acid Substrates—Oligonucleotides were purchased from Genset and further purified by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides used for helicase activity measurement in this study are listed in Table I. The DNA or RNA used for a cofactor of ATPase activity of Inf helicase are: 60nt ssDNA, 7kb PN6 DNA linear, 5.5kb GC70-plasmid DNA, 5.5kb GC70-plasmid DNA denatured, 678nt RNA. For each substrate (see figures below) a single oligonucleotide was 5'-end-labbelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified with Sephadex-G25-TE column. The labeled oligonucleotides were annealed to their unlabelled complementary strands at a 1:1-2 molar ratio by incubation at 95℃ for 5 min followed by slow cooling to room temperature. For purification of the substrates, the annealed oligonucleotides were purified with Sephadex-G50, G75, and G100 TE columns respectively, according to their length.

Protein Expression—A full coding region of Sub-S helicase DNA was prepared by PCR using *Bacillus subtilis* chromosome DNA. A 1.5-kb PCR product was cloned into pGEM®-T easy vector, and the sequence of this insert was shown to be identical to the published Sub-S helicase gene sequence. The DNA corresponding to the coding sequence of the SubS helicase gene was amplified again by PCR-mediated amplification, using a set of oligonucleotides corresponding to the 5'- and 3'-ends of the gene. These primers had additional bases at each end for creation of restriction sites: NdeI at the 5'-end (5'-CAT ATG ACT AAA TTA CAG CAA ACG TTA TAT CAG TTT-3') and XhoI at the 3'-end of the gene (5'-AGC TTG CAG ACT GTA GGT GAG CTG AAC TGA CTC GAG-3') for expression of the Sub-S helicase bearing a hexahistidine tag at the NH_2-terminal end. The amplified product was digested with NdeI and XhoI enzymes, and the appropriate size fragment (1488bp) was gel-purified and subcloned, respectively, into NdeI/XhoI sites of the vector PET-15b. The PET-15b vector encodes a hexahistidine tag at the amino terminus that allows purification of the expressed protein on a nickel-chelating column. A thrombin

cleavage site was adjacent to the histidine tag. The constructed plasmid was transformed in E. coli strain BL21(DE3)/TrxB. The bacteria were grown at 37 °C in terrific broth supplemented with 50μg/ml ampicillin and 15μg/ml kanamycin. The expression of the protein was induced by adding isopropylthio-D-galactoside to 0.2 mM at low log phase (A600 = 0.6). The culture was then incubated for 12-14 h at 15-18 °C. Cells were harvested by centrifugation at 3,000 × g for 15 min at 4 °C.

Protein Purification—His-tagged SubS helicase was overexpressed in E. coli BL21 (DE3)/TrxB and purified under native conditions. Briefly, harvested cells were suspended in 30 ml of suspension buffer (20mM Tris-HCl, pH 7.9, 5mM imidazole, 500mM NaCl) and were lysed using a French pressure cell. The lysate was then sonicated in order to shear DNA into small fragments. The lysate was cleared by centrifugation at 70,000 × g for 30 min at 4 °C. The supernatant was applied to the column charged with histidine binding resin (Novagen). The column was washed with 20mM Tris-HCl (pH 7.9) buffer containing 500mM NaCl, 5mM imidazole. The proteins bound to the column were eluted stepwise using 20mM Tris-HCl (pH 7.9) buffer containing 40, 50, 80, 200, or 500mM imidazole. SubS helicase-containing fractions, identified by both DNA-dependent ATP hydrolysis and helicase activity assays, were pooled. SubS helicase was further purified by FPLC size exclusion chromatography (Superdex 200; Amersham Biosciences). The active fractions were pooled, dialyzed against storage buffer (20mM Tris-HCl, pH 7.9, 500mM NaCl, 2mM dithiothreitol, 10% glycerol), and stored at –80 °C. The protein was pure as judged by Coomassie staining and electrospray mass spectrometry. Protein concentration was determined spectrophotometrically using a Bio-Rad Protein Assay.

Quantification of Zinc Ion Bound to SubS Helicase—The zinc content of SubS helicase was measured by the PAR (4-(2-Pyridylazo)resorcinol) assay as described by Hunt *et al.* (15). PAR has a low absorbance at 500 nm in the absence of zinc ion. However, in the presence of zinc ion, the absorbance at 500 nm increases dramatically due to the formation of the $PAR_2 \cdot Zn^{2+}$ complex. To more precisely quantify the zinc content of SubS helicase, all of the buffers were treated with Chelex 100 resin. The enzymes were dialyzed against the EDTA-free Chelex-treated buffer passed over a 10-cm column of Chelex-100 and reconcentrated. To facilitate zinc release, the enzymes (1 nmol in a volume of 20 μl) were first denatured with Chelex-treated 7 M guanidine HCl and then transferred to a 1-ml cuvette and the volume was adjusted to 0.9 ml with buffer A (20 mM Tris-HCl at pH 8.0, 150 mM NaCl). PAR was added

into the cuvette for a final concentration of 100 μM. The absorbance at 500 nm was measured. The quantity of zinc ion was determined from a standard curve of $ZnCl_2$ samples in a range of concentrations using the sample preparation procedure as described above with the SubS helicase omitted. The zinc ion concentration was also determined using the absorbance coefficient for the $(PAR)_2 \cdot Zn^{2+}$ complex (ε_{500nm} = 6.6 x 10^4 M^{-1} cm^{-1}).

pH Measurements—pH measurements were performed with a Knick 654 pH meter and an Ingold microelectrode. For calibration three standard buffers were used, and the precision was about ± 0.01 pH unit at 25 °C. To obtain a pH range from 5 to 10, 30mM sodium citrate, MES, and Tris-acetate were employed for pH intervals 5.0–6.0, 5.5–7.2, and 6.8–10, respectively.

ATPase Assay — The ATPase activity was determined in an assay by measuring the radioactive ^{32}Pi liberated during hydrolysis (16). The measurement was carried out at 37 °C for 10 min in a 40 μl reaction mixture (50mM Tris-HCl pH8.0, 35mM NaCl, 3mM $MgCl_2$, 0.1mg/ml BSA, 1mM DTT, 0.2mM ATP) containing the indicated concentration of DNA or RNA at the indicated concentration of SubS helicase. The reactions were initiated by the addition of SubS helicase into 40 μl of reaction mixture and stopped by pipetting 35 μl of aliquots from the reaction mixture every 30 s into a hydrochloric solution of ammonium molybdate. The liberated radioactive ^{32}Pi was extracted with a solution of 2-butanol-cyclohexane-acetone-ammonium molybdate (250:250:50:0.1) saturated with water. An aliquot of the organic phase was counted.

DNA helicase assay---The helicase assay measures the unwinding of a γ-^{32}P-labeled DNA fragment from a partial duplex DNA molecule. The 20 μl reaction mixture contained 30mM Tris–acetic, pH 7.9, 1mM DTT, 20mM NaCl, 3mM $MgCl_2$, 2mM ATP, 100 μg/ml BSA and ^{32}P-labeled helicase substrate (~0.45nM). The recombinant SubS helicase of indicated concentration was added to the mixture and incubated at 37°C for 30min. The reaction was terminated by the addition of 2% SDS, 150mM EDTA, 30% glycerol and 0.1% bromphenol blue. The products of the reaction were fractionated by electrophoresis on a 12 or 6% non-denaturing polyacrylamide gel. The gel was dried and exposed by autoradiography.

DNA strand annealing and exchange assays---The DNA strand-annealing activity of SubS was measured using complementary synthetic oligonucleotides (each at a concentration of 0.5 nM) of which one was labeled at the 5'-end using [γ-^{32}P]ATP and

T4 polynucleotide kinase. Annealing reactions (typically 20 µl) were carried out at 37°C in buffer (50 mM Tris–HCl, pH 8.0, 35 mM NaCl, 3 mM $MgCl_2$, 100 µg/ml BSA and 1 mM DTT) and contained the indicated amounts of SubS. Where required, ATP (2 mM), ATPγS (2 mM) and ADP (2 mM) were added. Reactions were usually initiated by adding the unlabeled DNA strand and were incubated for 20 min. For the exchange assays, labeled fork substrate Tstem25/Flap26 (1nM) was incubated with or without SubS (at the concentrations indicated in figure legends) and oligonucleotide U25 (1nM) for 0-20min at 37℃ in 20 µl reaction buffer plus ATP (1mM). Reactions were analyzed essentially as the helicase reactions (see above).

EMSA assay---The recombinant SubS of indicated concentration was incubated with 5'-[γ-^{32}P]- labeled ssDNA or dsDNA (~0.45nM) in a 20 µl reaction mixture containing 30mM Tris–HCl, pH 7.9, 20mM NaCl, 3mM $MgCl_2$, 100 µg/ml BSA and 1mM DTT. Incubation was for 30 min at room temperature. The resulting mixture was then resolved by 6% non-denaturing PAGE and visualized by autoradiography. Gels were run at 200 V and 25°C in 0.5×TBE buffer.

RESULTS

Purification of a Recombinant SubS Protein---- To study the enzyme functions, the SubS helicase was overexpressed in and purified from *E. coli*. Gel electrophoresis of the protein in denaturing conditions gave a single band corresponding to a molecular mass of about 56kDa (Fig. 1B), consistent with the value determined from the amino acid sequence (56048Da). Immunoblotting with an anti-His monoclonal antibody confirmed that the overexpressed protein was the desired product (data not shown). Large-scale preparations of the recombinant protein were then carried out, and the protein was purified to near homogeneity (Fig. 1B).

We have detected zinc ions bound in the purified SubS protein by using the PAR assay. 1 mol of the SubS helicase binds 0.90±0.14 mol of zinc. This result is consistent with the prediction from the primary amino acid sequence of SubS helicase, indicating that the enzyme contains a zinc-binding motif.

DNA-dependent ATPase Activity-- Generally the DNA helicase possesses an ATPase activity. To determine it, the purified recombinant SubS protein was tested for its ability to hydrolyze ATP in the presence and absence of DNA and RNA. As shown in Fig. 2A, a DNA-dependent ATPase activity was readily detected and hydrolysis of ATP was differentially efficient in the presence of several different DNA cofactors, including either single- or double-stranded DNA (Fig. 2A). Thus, SubS is a DNA dependent ATPase but not RNA dependent. In consistent with the *E. coli* RecQ ATPase, single-stranded DNA was a much more effective cofactor than double-stranded DNA for the SubS ATPase (17).

Previous studies indicated that the ATPase activity of the helicase is Mg^{2+} dependent. Fig. 2B shows that the ATP hydrolysis by SubS was dependent on DTT, BSA and divalent cations such as Mg^{2+}, but not Mn^{2+}, Ca^{2+}, Zn^{2+}. It is also shown that the ATPase activity of SubS protein is pH dependent. The SubS helicase displays an optimum ATPase activity between pH 7.0 and 8.8 (Fig. 2C).

The ssDNA-stimulated ATPase activity of the SubS helicase has been studied as a function of ATP concentration. As come from Fig. 2D, the k_{cat} value is 0.4 /s, the Kd value is 164μM. This result indicates that the ATPase activity of SubS is less than that of SubL protein from *E. coli* (k_{cat}: 6.87/s) (unpublished data).

DNA Unwinding Activities Assay versus SubS Helicase Concentration----To determine the optimal amount of the SubS helicase required unwinding dsDNA, its

concentration was varied from 25nM to 800nM. As shown in Fig. 3A, 25 to 800nM of the SubS protein can unwind forked DNA with the increasing efficiency by using the energy derived from the ATP hydrolysis. This indicated that SubS is ATP dependent DNA helicase.

DNA Unwinding is ATP dependent---- It is well established that RecQ helicase requires ATP hydrolysis to promote DNA unwinding. AMPPNP and ATP-γ-S, the non-hydrolysable analogs of ATP, inhibited the RecQ-mediated DNA unwinding (17). Fig.3B shows that the Sub-S enzyme displays no detectable helicase activity in the presence of 2mM AMPPNP. In addition, UTP, GTP, CTP, dATP, dGTP, dCTP and dTTP cannot be used by the SubS hélicase as the energy source to catalyze DNA unwinding.

Polarity of the DNA Helicase-- To determine the polarity of the helicase activity, the DNA substrates shown in Fig. 4A were used. The recombinant SubS protein was found to efficiently unwind the 3'-tailed but not 5'-tailed duplex DNA. Generally, the helicase of RecQ family unwinds duplex DNA with a 3' to 5' directionality. This result is consistent with the fact that the SubS protein is a unidirectional helicase with 3' to 5' polarity.

The RecQ helicase of *E. coli* can unwind a blunt ended duplex DNA in its higher concentrations. Whereas the result shown in Fig. 4B indicates that the SubS helicase can't unwind the duplex DNA with double blunt ends and the nicked duplex DNA.

The Dependence of the Efficiency of Strand Unwinding on the Length of the Strand-- To investigate the dependence of the efficiency of DNA unwinding on the length of the double strands of partial duplex DNA, several DNA substrates were prepared using ^{32}P-labeled fragments of 20 and 70 nucleotides annealing to PhiX174 virion DNA. A time course of the SubS-mediated strand unwinding reaction is shown in Fig. 5A and 5B. With the short partial duplex (20bp) substrate and the long partial duplex (70bp) substrate, the labeled fragments were not unwound by the SubS helicase within 40 min. This suggested that its efficiency of strand unwinding is very weak.

SubS Helicase Activity on Partial Duplex DNA -- A series of linear substrates with 3'-single-strand tails of 1, 5, 10, and 25 nucleotides were made to study the effect of tail length on the unwinding activity of the SubS helicase (Fig. 6A). It showed that all the substrates with tails more than 1nt could be efficiently unwound by the SubS (Fig. 6A).

Following this observation, a set of substrates with different structures and with

various lengths of the double-strand regions were prepared to obtain novel information on the substrate specificity of the SubS helicase. Substrates with a 20-bp oligonucleotide annealed to PhiX174 virion ssDNA were unwound by the SubS, regardless of the presence or absence of mismatched hanging tails at the 5'-end or the 3'-end (Fig. 6B). However, if the duplex region was increased to 70bp, the substrate could not be unwound (Fig. 5B).

SubS Can't Unwind a 5'-Flap Substrate Flanked by Adjacent Duplex DNA. –To assess whether the SubS is able to unwind a 5'-flap substrate, we tested a DNA substrate related to the forked duplex with 26- and 25-nucleotide 5'- and 3'-ssDNA tails, respectively, which also contains an upstream 25-mer hybridized to the upstream ssDNA region that resides below the flap (Fig.7A, substrate A). A nick separating the adjacent oligonucleotides characterizes substrate A. The results from a typical SubS helicase assay using substrate A is shown in Fig. 7A. 0.4μM SubS can't efficiently unwind the 5'-flap substrate.

Sub-S Can't Unwind a Synthetic Replication Fork DNA Substrate---We also tested a forked duplex in which both the 5'- and 3'-tails were double-stranded (Fig.7A, substrate C). This substrate mimics a synthetic DNA replication fork with double-stranded leading and lagging strands. The duplex forked DNA substrate (substrate C) was not unwound by the SubS at the concentration tested (0.4μM, Fig. 7A).

Sub-S helicase can't disrupt Kappa joint molecule and Holliday Junction--- The properties of Kappa joint molecule suggested that it could be composed of a linear dsDNA molecule that was invaded by homologous ssDNA; the resultant joint molecule would resemble the letter K and, hence, is referred to as a Kappa intermediate. To test the unwinding of Kappa intermediate catalyzed by the SubS helicase, we prepared a substrate (Fig. 7A, substrate B) which mimics Kappa joint molecule. The result shows that the SubS helicase can't unwind it (Fig. 7A, 7B). Meanwhile, the SubS helicase could not unwind the four-way junction DNA substrate that mimics a Holliday junction (Fig. 7B).

SubS hélicase doesn't possess DNA strand-annealing activity and can't promote DNA exchange---We incubated two complementary oligonucleotides (those used to make

the blunt end double-strands DNA substrate) with increasing amounts of SubS in the absence of ATP, and subsequently analyzed the reaction products by 12% nondenaturing polyacrylamide gel electrophoresis (PAGE). We found that SubS didn't promote an efficient annealing reaction (data not show). Therefore, the DNA strand-exchange catalyzed by SubS was not observed (data not shown).

DNA Binding by SubS protein-- The DNA binding activity of the purified recombinant protein was investigated using the electrophoretic mobility shift assay (Fig. 8A, 8B). The SubS protein was found to bind single-stranded DNA (ssDNA), since the presence of increasing amounts of the protein progressively reduced the gel electrophoretic mobility of the DNA (Fig. 8A).

To test the possibility that binding to the single-stranded DNA might be facilitated by secondary structures within it, the SubS binding to a forked DNA substrate was examined. The forked DNA was prepared by the annealing of two partially complementary oligonucleotides, 44 nucleotides in length. It was found that incubation of ^{32}P-labeled forked DNA with the SubS protein resulted in the formation of a less stable protein-DNA complex (Fig. 8B), as detected by polyacrylamide gel electrophoresis.

DISCUSSION

We have purified to near homogeneity the product of the gene *SubS* in *Bacillus subtilis* and demonstrated that the SubS protein is both a DNA-dependent ATPase and an ATP-dependent DNA helicase that unwinds DNA in a 3' to 5' direction. As the zinc finger motif of RecQ-Ct is necessary for the enzyme activity of the RecQ helicases (13, 14).The function of the SubS protein is consistent with that it possess a zinc finger motif. Hydrolysis of the ATP appears to be obligatory, as AMPPNP can not substitute for ATP and inhibits the helicase activity in the presence of excess ATP. Like the *E. coli* RecQ helicase, which is stimulated by ssDNA but not double-stranded DNA, the hydrolysis of ATP by the recombinant SubS protein is strongly stimulated by ssDNA and is dependant on Mg^{++}. Moreover, the hydrolysis of ATP by the recombinant SubS protein is dependant on the presence of BSA and DTT. Generally BSA and DTT are helpful for a stability of protein in the solution. Therefore the dependence of ATPase activity on BSA and DTT may be that the SubS is not stable in the solution without BSA and DTT. Moreover, deficiency of HDRC domain in SubS sequence may be the major reason of its instability. SubS helicase displays an optimum ATPase activity between pH 7.0 and 8.8.

The polarity of unwinding by SubS helicase is 3' to 5' with respect to the overhanging single-stranded DNA to which the protein binds. Consistent with this directionality, it was shown that SubS unwinds a duplex DNA substrate with a 3'-ssDNA tail but not a substrate with a 5'-ssDNA tail. These findings might lead one to believe that SubS requires a free 3'-ssDNA tail to initiate unwinding of duplex DNA substrates. The characteristics of the unwinding activity are similar to those of the *E. coli* RecQ protein (17), which shares homology with SubS within the helicase domain. Of the other members of the RecQ family of DNA helicases, the human RecQL protein has been purified and shown to have DNA unwinding activity *in vitro* (18, 19). Recently, human WRN and BLM proteins were also overexpressed and shown to possess a DNA helicase activity (20-22). Therefore, all of the members of the RecQ family will likely possess a common helicase function.

We have shown here that the unwinding of blunt-ended double-stranded DNA by SubS is below the level of detection under the assay conditions employed. SubS helicase can't unwind the duplex DNA with double blunt ends. In contrast, *E. coli* RecQ protein, like several other helicases such as RecBCD, can initiate duplex DNA unwinding from a blunt-ended terminus (23, reviewed in 1, 24). The addition of a short single-stranded tail (a 3'-tail for 3'→5' helicases or a 5'-tail for a 5'→3' helicases) to a duplex molecule is enough to create a good substrate for many helicases, including those incapable of unwinding from blunt ends. In their studies Bennett *et al.* (25) showed that Sgs1, the *Saccharomyces cerevisiae* homologue of SubS, had a requirement for a 3'-tail of at least 3 nt and used the junction of single-stranded and double-stranded DNA as a key site in the substrate recognition process. In our studies, as expected, a 5'-tailed duplex DNA is not a substrate for SubS helicase but 3'- tailed duplex is even if the 3'-tail is only 5nt long. However, SubS helicase didn't show appreciable unwinding of the nicked duplex DNA.

The efficiency of strand unwinding by the recombinant SubS protein is very weak. The unwinding assay used here, however, detects only complete unwinding, and partially unwound fragments would not be scored. It is therefore plausible that the DNA strands reannealing behind the translocating protein could reduce the observed efficiency of unwinding. Alternatively, the recombinant SubS helicase might show a low processivity in its reaction and might fall off the DNA before completing the unwinding of the annealed fragment.

In this study, we have demonstrated that SubS is not able to unwind the 5'-ssDNA flap substrate, a synthetic replication fork substrate, Kappa structure DNA substrate and a synthetic X structure (a model for the Holliday junction recombination intermediate). But it can make the 5'-ssDNA flap substrate and Kappa structure DNA substrate to produce a particular DNA structure which leads to some incompact DNA bands on the gel. Because there is not HRDC domain in the SubS amino acids sequence, the efficiency of unwinding and ATPase activity of SubS are about 15 times less than that of Sub-L which contains a zinc finger motif in its RecQ-Ct domain and HRDC domain (unpublished data). The previous study has indicated that the HRDC domain is required for stable DNA binding (11) which is necessary for DNA unwinding activity of helicase. Recently there is a report that the HRDC domain of

BLM is necessary for the DNA unwinding of double Holliday junctions (26). Therefore, the deficiency of HRDC domain in SubS may be a major cause of none unwinding the DNA substrate mentioned above.

Using an assay based on the electrophoretic mobility of a DNA bound by protein, the recombinant SubS protein was shown to have a higher affinity for a single-stranded DNA than a forked DNA substrate. This is consistent with that the hydrolysis of ATP catalyzed by the recombinant SubS protein is more strongly stimulated by ssDNA than by dsDNA.

Most importantly, our analyses revealed that SubS protein can't exhibit an efficient DNA strand-annealing activity. This is because the Zinc finger of SubS could strongly suppress the annealing activity of the helicase core by enhancing ssDNA binding (Xi et al, unpublished). The biochemical activities of SubS suggest that the protein may be implicated in a DNA metabolism pathway. Further analysis of the roles *in vivo* of SubS will explicate the functions of the RecQ helicases in maintenance of genomic integrity and stability.

REFERENCES

1. Lohman TM, Bjornson KP (1996) Mechanisms of helicase-catalyzed DNA unwinding. *Annu Rev Biochem* **65**: 169–214
2. Hall MC, Matson SW (1999) Helicase motifs: the engine that powers DNA unwinding. *Mol Microbiol* **34**: 867–877
3. von Hippel PH, Delagoutte E (2001) A general model for nucleic acid helicases and their 'coupling' within macromolecular machines. *Cell* **104**: 177–190
4. Bachrati CZ, Hickson ID (2003) RecQ helicases: suppressors of tumorigenesis and premature aging. *Biochem J* **374**: 577–606
5. Hickson ID (2003) RecQ helicases: caretakers of the genome. *Nat Rev Cancer* **3**: 169–178
6. Khakhar RR, Cobb JA, Bjergbaek L, Hickson ID, Gasser SM (2003) RecQ helicases: multiple roles in genome maintenance. *Trends Cell Biol* **13**: 493–501
7. Umezu K, Kolodner R D. (1994) Protein interactions in genetic recombination in *Escherichia coli*. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. J Biol Chem. **269**:30005–30013.
8. Kunst F, Ogasawara N, Moszer I, Albertini A M, Alloni G, Azevedo V, Bestero M G, et al. The complete genome sequence of the Gram-positive model organism *Bacillus subtilis*. Nature. 1997;**390**:249–256.
9. Ellis N A, German J. (1996) Molecular genetics of Bloom's syndrome. Hum Mol Genet. **5**:1457–1463.
10. Kowalczykowski S C, Dixon D A, Eggleston A K, Lauder S D, Rehrauer W M. (1994) Biochemistry of homologous recombination in *Escherichia coli*. Microbiol Rev. **58**:401–465.
11. Bernstein DA, Keck JL (2003) Domain mapping of *Escherichia coli* RecQ defines the roles of conserved N- and C-terminal regions in the RecQ family. *Nucleic Acids Res* **31**: 2778–2785
12. Bernstein DA, Zittel MC, Keck JL (2003) High-resolution structure of the *E.coli* RecQ helicase catalytic core. *EMBO J* **22**: 4910–4921
13. Liu, J.L., Rigolet, P., Dou, S.X., Wang, P.Y., Xi, X.G. (2004) The zinc finger motif of *Escherichia coli* RecQ is implicated in both DNA binding and protein folding *J. Biol. Chem.*, **279**, 42794–42802

14. Rong-bin Guo, Pascal Rigolet, Loussiné Zargarian, Serge Fermandjian and Xu Guang Xi (2005) Structural and functional characterizations reveal the importance of a zinc binding domain in Bloom's syndrome helicase. Nucleic Acids Res. 33(10):3109-24.

15. Hunt, J. B., Neece, S. H., and Ginsburg, A. (1985) The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from Escherichia coli aspartate transcarbamoylase. *Anal. Biochem.* **146,** 150–157

16. Dessinges, M. N., Lionnet, T., Xi, X. G., Bensimon, D., and Croquette, V. (2004) Single-molecule assay reveals strand switching and enhanced processivity of UvrD. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6439–6444

17. Umezu, K., Nakayama, K., and Nakayama, H. (1990) *Escherichia coli* RecQ protein is a DNA helicase. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5363-5367

18. Puranam, K. L., and Blackshear, P. J. (1994) Cloning and characterization of RECQL, a potential human homologue of the Escherichia coli DNA helicase RecQ. J. Biol. Chem. 269, 29838-29845

19. Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., and Yamaoka, T. (1994) Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12. *Nucleic Acids Res.* **22**, 4566-4573

20. Suzuki, N., Shimamoto, A., Imamura, O., Kuromitsu, J., Kitao, S., Goto, M., and Furuichi, Y. (1997) DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. *Nucleic Acids Res.* **25**, 2973-2978

21. Gray, M. D., Shen, J-C., Kamath-Loeb, A. S., Blank, A., Sopher, B. L., Martin, G. M., Oshima, J., and Loeb, L. A. (1997) The Werner syndrome protein is a DNA helicase. *Nat. Genet.* **17**, 100-103

22. Karow, J. K., Chakraverty, R. K., and Hickson, I. D. (1997) The Bloom's syndrome gene product is a 3'-5' DNA helicase. *J. Biol. Chem.* **272**, 30611-30614

23. Harmon,F.G. and Kowalczykowski,S.C. (1998) RecQ helicase, in concert with RecA and SSB proteins, initiates and disrupts DNA recombination. *Genes Dev.*, **12**, 1134–1144.
24. West,S.C. (1996) DNA helicases: new breeds of translocating motors and molecular pumps. *Cell*, **86**, 177–180.
25. Bennett,R.J., Keck,J.L. and Wang,J.C. (1999) Binding specificity determines polarity of DNA unwinding by the Sgs1 protein of *S. cerevisiae*. *J. Mol. Biol.*, **289**, 235–248.
26. Leonard Wu, Kok Lung Chan, Christine Ralf, Douglas A Bernstein, Patrick L Garcia, Vilhelm A Bohr, Alessandro Vindigni, Pavel Janscak, James L Keck and Ian D Hickson (2005) The HRDC domain of BLM is required for the dissolution of double Holliday junctions. *The EMBO Journal* **24,** 2679–2687.

TABLE I

Oligonucleotide sequences used for helicase measurement in this study

Name	length (nt)	sequence
174-1	20	5’- TCAGCACCAGCACGCTCCCA-3’
174-2	35	5’- TCAGCACCAGCACGCTCCCAttttttttttttT-3’
174-4	35	5’-aaaaaaaaaaaaaaTCAGCACCAGCACGCTCCCA-3’
Bubble-F	70	5’-TGC-AGT-AGC-GCC-AAT-ATG-AGA-AGA-GCC ATA-CCG-CTG-ATT-CTG-CGT-TTG-CTG-ATG- AAC-TAA-GTC-AAC-CTC-A-3’
Tstem01	20	5’-GCA-CTG-GCC GTC GTT TTA CG-3’
Tstem05	24	5’-GCA-CTG-GCC GTC GTT TTA CGG TCG-3’
Tstem10	29	5’-GCA-CTG-GCC GTC GTT TTA CGG TCG TGA CT-3’
Tstem25	44	5’-GCA-CTG-GCC GTC GTT TTA CGG TCG TGA CTG GGA AAA CCC TGG CG-3’
FLAP00	19	5’-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP01	20	5’-A-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP05	24	5’-TC-CAA-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP10	29	5’-T-TTT-TTC-CAA-GTA-AAA-CGA-CGG-CCA-GTG-C-3’
FLAP26	45	5’-TTTTTTTTTTTTTTTTTT-TTT-TTC-CAA-GTA -AAA-CGA-CGG-CCA-GTG-C
U25	25	5’-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC-C-3’
U25comp	44	5’-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC- CAA-CCC-CTT-TTT-TTT-TTC-AA-3’
FLAP26Comp	26	5’-TTG-GAA-AAAAAAAAAAAAAAAAAAAA-3’
Sub03	19	5’-GAC-GAA-CCT-TTG-CCC-ACG-T-3’
02Bis	40	5’-ACG-TGG-GCA-AAG-GTT-CGT-CAA-TGG- ACT-GAC-AGC-TGC-ATG-G-3’
Nick0	21	5’-CCA-TGC-AGC-TGT-CAG-TCC-ATT-3’
Nick2	19	5’-CCA-TGC-AGC-TGT-CAG-TCC-A-3’
Nick4	16	5’-CCA-TGC-AGC-TGT-CAG-T-3’
H1	41	5’-GCC-GTG-ATC-ACC-AAT-GCA-GAT-TGA-CGA-ACC-TTT-GCC-CAC-GT-3’
H2	41	5’-GAC-GTG-GGC-AAA-GGT-TCG-TCA-ATG-GAC-TGA-CAG-CTG-CAT-GG-3’
H6	41	5’-GCC-ATG-CAG-CTG-TCA-GTC-CAT-TGT-CAT-GCT-AGG-CCT-ACT-GC-3’
H7	41	5’-GGC-AGT-AGG-CCT-AGC-ATG-ACA-ATC-TGC-ATT-GGT-GAT-CAC-GG-3’
E21A	21	5’-GTG-TGG-AAA-ATC-TCT-AGC-ATC-3’
E21B	21	5’-ACT-GCT-AGA-GAT-TTT-CCA-CAC-3’
PhiX174		
SubA20	50	5’-TCA-AAG-TCA-CGA-CCT-AGA-CAC-TGC-GAG-CTC -GAA-TTC-ACT-GGA-GTG-ACC-TC
SubB20	50	5’-GAG-GTC-ACT-CCA-GTG-AAT-TCG-AGC-TCG-CAG -TGT-CTA-GGT-CGT-GAC-TTT-GA

Figure legends

Figure 1 (A). Amino acid sequence alignment of *E. coli* RecQ , *Bacillus subtilis* SubS. The multiple alignments were performed with the program ClustruW and refined manually. The numbers at the beginning and end of each sequence correspond, respectively, to positions of the first and the last amino acid residues. Highly conserved amino acid residues are *shadowed* in *gray*. In *boldface* are the four conserved cysteine residues and the fully conserved arginine, aspartic acid, and aromatic residues involved in three very important hydrogen bonds. The protein accession numbers used by the NCBI are as follows: RecQ, P15043 [GenBank] (*E. coli*); SubS, P50729 [GenBanK] (*B. subtilis*); SubL, F69901 [GenBanK] (*B. subtilis*) **(B). Purification of SubS protein.** A Coomassie Blue-stained 10% SDS-polyacrylamide gel of a crude cell extract and the purified SubS protein by FPLC size exclusion chromatography (lane2 and lane3).The molecular mass standards (lane M) are shown on the left side. Lane2: 0.25µg protein, lane3: 0.5µg protein, Lane4: 0.75µg protein, lane5: 1µg protein.

Figure 2 (A).Effect of different DNA or RNA on ATPase activity of SubS helicase.

DNA-dependent ATP hydrolysis activity of SubS helicase as a function of enzyme concentration. ●: no DNA, ■: 60nt ssDNA 24µM(nt), ♦: 7kb PN6 DNA 28µM(bp), ▽: 5.5kb GC70 plasmid DNA 27.5µM(bp), ▲: 5.5kb GC70 plasmid DNA denatured 27.5µM(bp), Δ: 678nt RNA 24µM(nt). 0.2mM ATP. **(B). Requirement for ATP hydrolysis catalyzed by SubS protein.** Experiments were performed at 37 °C with 0.15µM protein and 24 µM ssDNA (nucleotide, 60-mer oligonucleotide) for each helicase preparation , 3mM Mg^{2+}, 3mM Mn^{2+},3mM Ca^{2+}, 3mM Zn^{2+}, 15mM EDTA, 2mM AMPPNP and 2 mM ATP respectively. **(C). Effect of different pH on ATPase activity of SubS hélicase.** Experiments were performed at 37 °C with 0.15µM protein for each helicase preparation and 24 µM ssDNA (nucleotide, 60-mer oligonucleotide) and 0.2mM ATP. **(D). ATPase activity of the SubS helicase as a function of ATP concentration.** Experiments were performed at 37 °C with 0.15µM protein for each helicase preparation and 24 µM ssDNA (nucleotide, 60-mer oligonucleotide).

Figure 3 (A). DNA unwinding in the presence of varying concentrations of the SubS helicase. DNA unwinding assays were performed as described in "Experimental Procedures" using DNA substrate (0.45nM, Tstem25*/Flap26); each reaction contained 25-800nM helicases respectively and 2mM ATP, * denote γ-^{32}P labeled. **(B). Effects of nucleotides on Sub-S helicase-catalyzed DNA unwinding activity**. DNA unwinding assays were performed as described in "Experimental Procedures" using DNA substrate (0.45nM, Tstem25*/Flap26); each reaction contained 0.8μM helicases and one of the listed nucleotides at a final concentration of 2mM. * denote γ-^{32}P labeled. Lane C, negative control, lane △, boiled substrate.

Figure 4 (A). Polarity of DNA unwinding by the recombinant Sub-S protein. Reaction mixtures (20 μl each) containing ~0.45nM DNA substrate were incubated with 0.4μM of the recombinant SubS protein and incubated at 37 °C for 30 min. The products of the reaction were analyzed by electrophoresis in a 12% polyacrylamide gel and autoradography. The substrates are shown in the figure (Tstem25*/U25/Flap00, Tstem25*/Flap00, Tstem25*/U25). * denote γ-^{32}P labeled. **(B). Sub-S can't unwinds the nicked duplex DNA and blunt ends duplex DNA.** A reaction mixture (20 μl) containing 0.45nM indicated substrate (E21A*/E21B, 02Bis*/Sub03/Nick0, 02Bis*/Sub03/Nick2, 02Bis*/Sub03/Nick4) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the SubS protein (0.8μM). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. Lane C, negative control, lane -, no DNA unwinding, lane +, DNA unwinding, lane △, boiled substrate.

Figure 5 (A). Time course of the strand displacement reaction mediated by the recombinant Sub-S protein. A reaction mixture (20 μl) containing 0.45 nM substrate (PhiX174 virion DNA annealed with a ^{32}P-labeled 20-nucleotide fragment, PhiX174-1*/PhiX174) in the helicase assay buffer was incubated at 37 °C with the addition of the SubS protein (0.8μM). **(B). Time course of the strand displacement reaction mediated by the recombinant Sub-S protein.** A reaction mixture (20 μl) containing 0.45nM substrate (PhiX174 virion DNA annealed with a ^{32}P-labeled 70-nucleotide fragment, Bubble-F*/PhiX174) in the helicase assay buffer was

incubated at 37 °C with the addition of the SubS protein (0.8μM). Following the addition of the recombinant Sub-S protein, 20-μl reaction mixtures were mixed with excess EDTA to terminate the reactions at the times indicated. The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. Lane C, negative control.

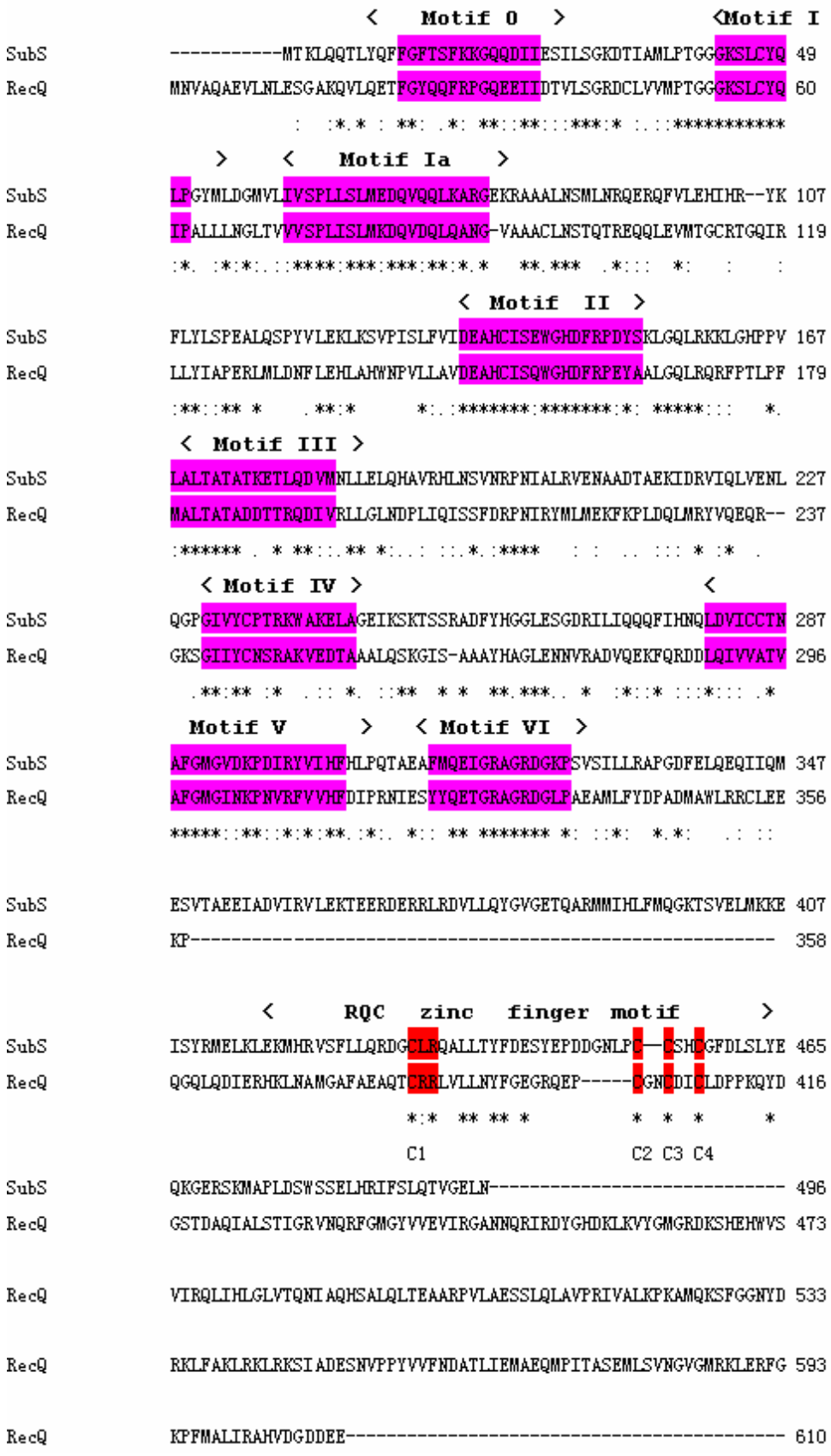
Figure 6 (A). 3'-Tail length dependence studies. The unwinding were determined with four DNA substrates having an ssDNA tails of 1, 5, 10 and 25nt (Flap00*/Tstem01, Flap00*/Tstem05, Flap00*/Tstem10, Flap00*/Tstem25). The concentration of the SubS helicase used in all studies was 0.8μM. *Asterisks* denote the ^{32}P-labeled end. **(B). Helicase activity with various substrates.** Each panel shows the structure of the substrate used and an autoradiogram of the gel. A reaction mixture (20 μl) containing 0.45nM indicated substrate (PhiX174-1*/PhiX174, PhiX174-4*/PhiX174, PhiX174-2*/PhiX174) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the SubS protein (0.8M). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. Lane C, negative control, lane -, no DNA unwinding, lane +, DNA unwinding, lane △, boiled substrate.

Figure 7 (A). Sub-S can't unwind a 5'-ssDNA flap substrate, Kappa joint molecule and forked DNA substrates with dsDNA 5'- and/or 3'-tails. A reaction mixture (20 μl) containing 0.45nM indicated substrate (Tstem25*/Flap26/U25, Tstem25*/Flap26/U25Comp, Tstem25*/Flap26/U25/Flap26Comp) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the Sub-S protein (0.4μM). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. **(B) Sub-S can't unwind the four-way junction DNA substrate.** A reaction mixture (20 μl) containing 0.45nM indicated substrate (H1*/H2, H1*/H2/H7, H1*/H2/H7/H6) in the helicase assay buffer was incubated for 30min at 37 °C with the addition of the SubS protein (0.8μM). The products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis and by autoradiography. *Asterisks* denote the ^{32}P-labeled end. Lane C, negative control, lane -, no DNA unwinding, lane +, DNA

unwinding, lane △, boiled substrate.

Figure 8 (A). Single strand DNA binding by the recombinant SubS protein. Electrophoretic mobility shift measurements were carried out to determine the binding of the SubS with single-stranded DNA (ssDNA, Tstem25*). Reaction mixtures (20 µl each) contained DNA (0.45nM) and SubS (0, 25, 50, 100, 200, 400 and 800nM) in binding buffer as described under "Experimental Procedures." Reactions were incubated for 30 min at room temperature before loading onto a 6% nondenaturing polyacrylamide gel to separate the protein-DNA complexes electrophoretically. DNA was visualized by autoradiography. * denote γ-^{32}P labeled. **(B). Binding of forked DNA by the SubS protein.** 5'-^{32}P-end-labeled forked DNA substrates (Flap26/ Tstem25*) were incubated with the Sub-S protein in binding buffer as described under "Experimental Procedures." Reaction mixtures (20 µl) contained DNA (0.45nM) and SubS (0, 25, 50, 100, 20, 400 and 800nM). Protein-DNA complexes were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel and autoradiography. * denote γ-^{32}P labeled.

Figure 1
A


Motif 0
Motif I
Motif Ia
Motif II
Motif III
Motif IV
Motif V
Motif VI
RQC zinc finger motif
C1
C2 C3 C4
SubS
RecQ

B

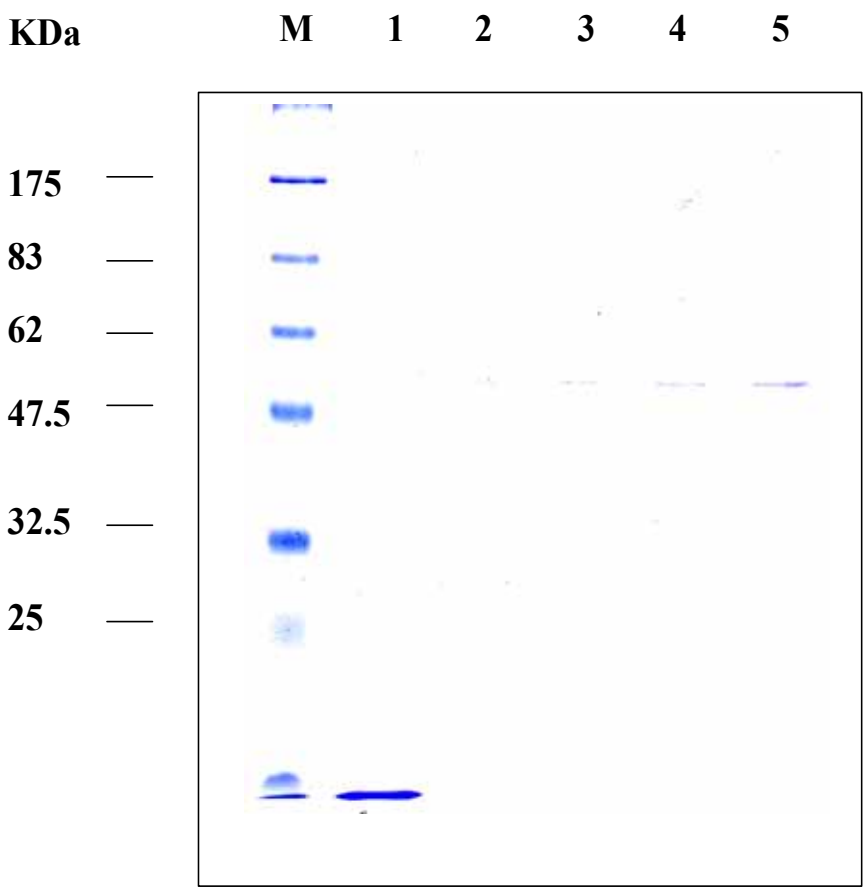
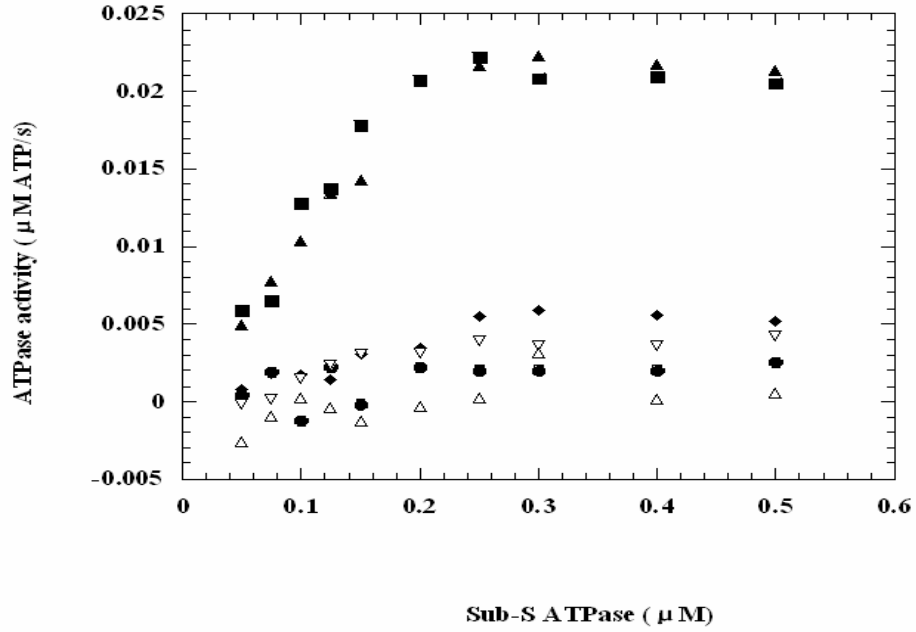
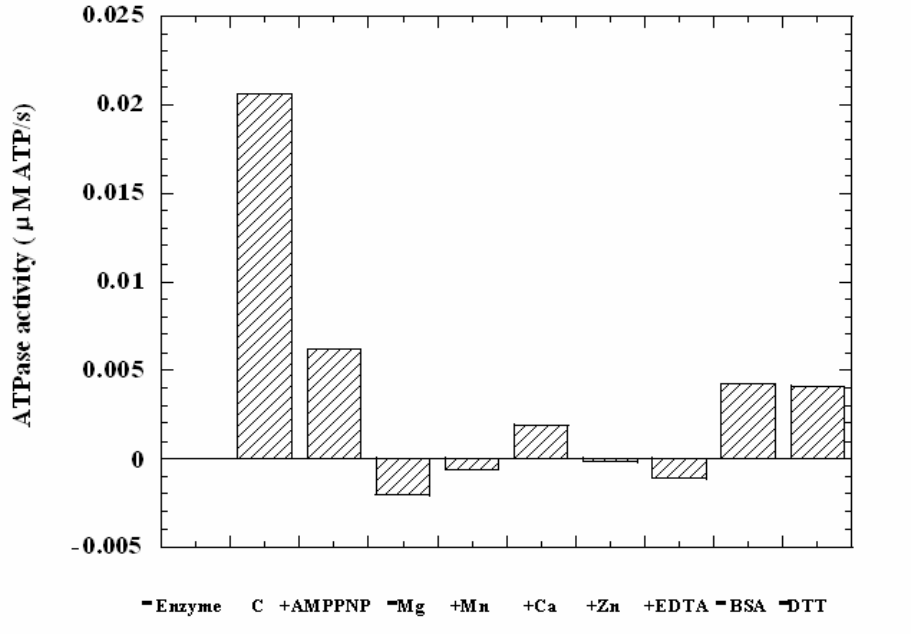


Figure 2

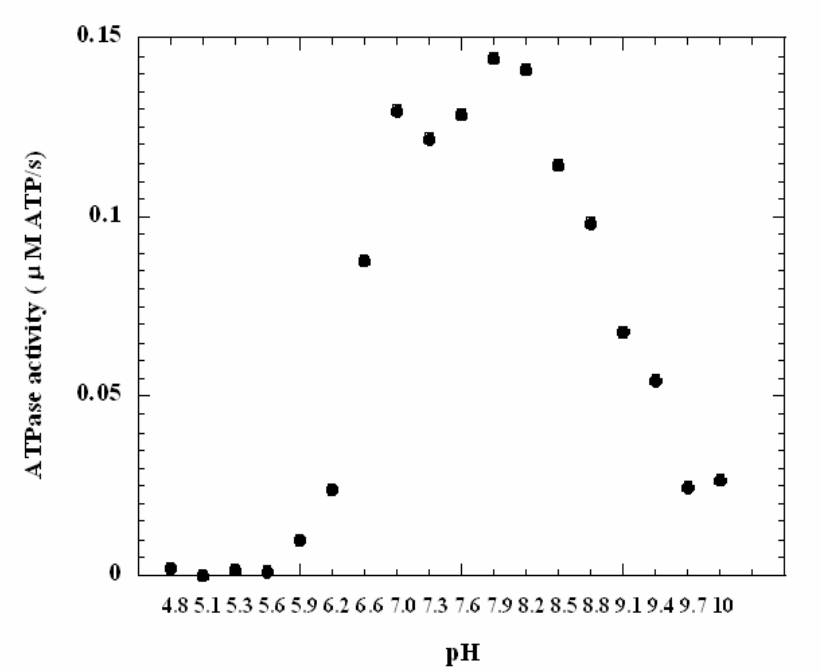
A


0.025
0.02
0.015
0.01
0.005
0
-0.005
ATPase activity (μM ATP/s)
0
0.1
0.2
0.3
0.4
0.5
0.6
Sub-S ATPase (μM)

B


0.025
0.02
0.015
0.01
0.005
0
-0.005
ATPase activity (μM ATP/s)
Enzyme
C
+AMPPNP
Mg
Mn
Ca
Zn
EDTA
BSA
DTT

C


0.15
0.1
0.05
0
ATPase activity (μ M ATP/s)
4.8 5.1 5.3 5.6 5.9 6.2 6.6 7.0 7.3 7.6 7.9 8.2 8.5 8.8 9.1 9.4 9.7 10
pH

D

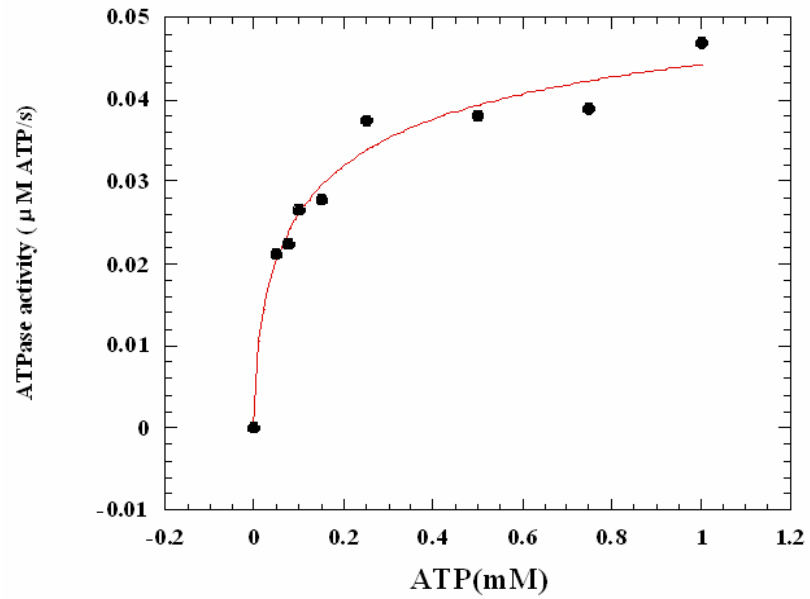
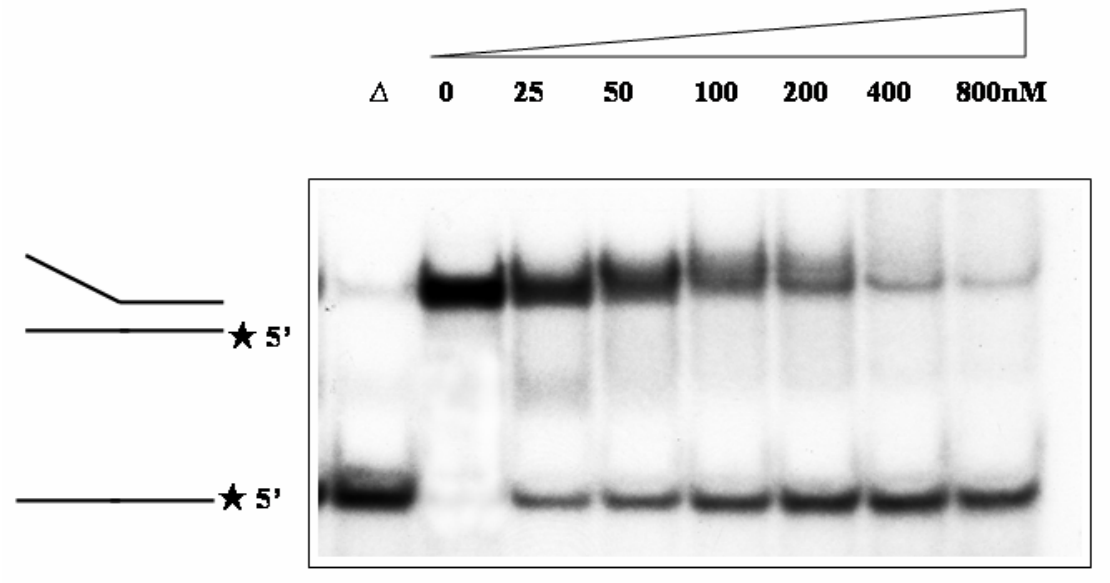

0.05
0.04
0.03
0.02
0.01
0
-0.01
ATPase activity (μ M ATP/s)
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
ATP(mM)

Figure 3

A


Δ
0
25
50
100
200
400
800nM
5'
5'

B

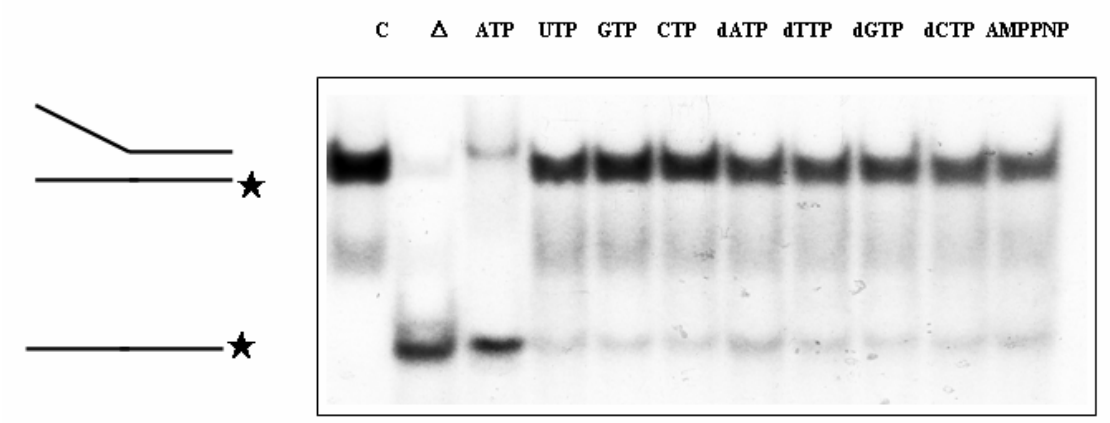
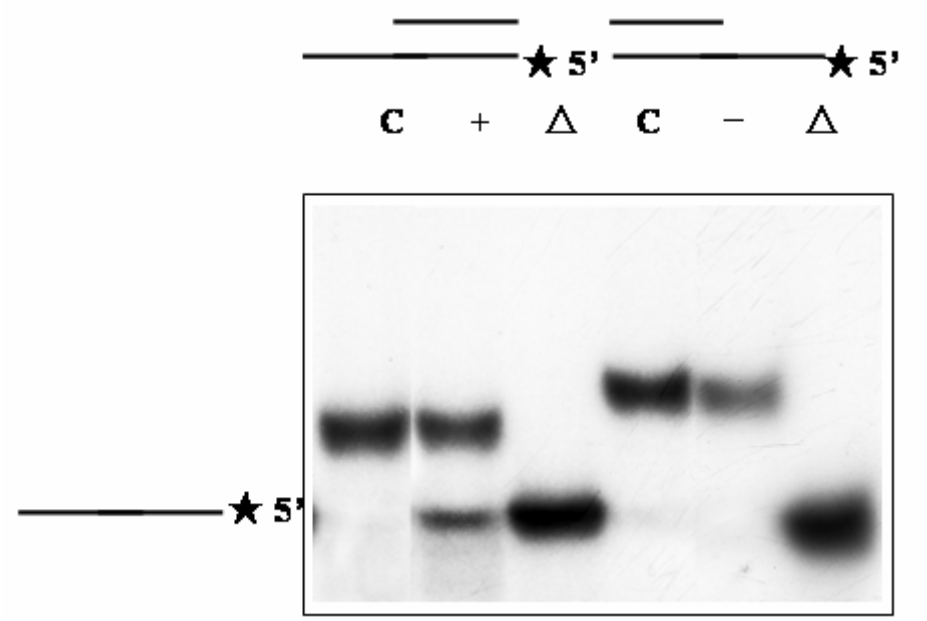

C
Δ
ATP
UTP
GTP
CTP
dATP
dTTP
dGTP
dCTP
AMPPNP

Figure 4

A



B

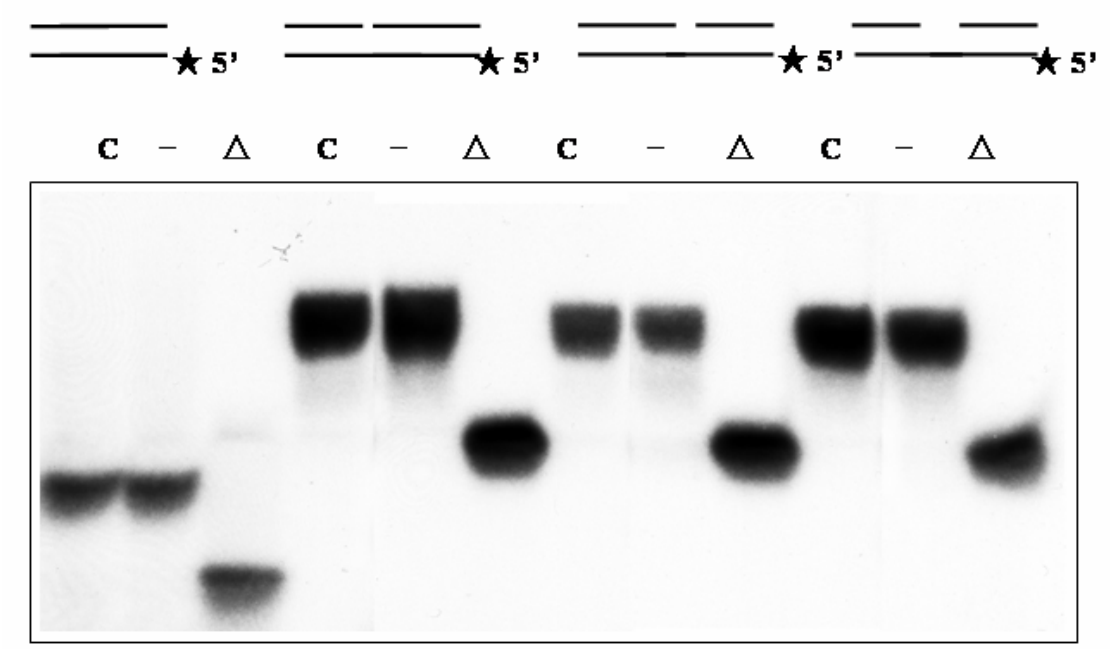
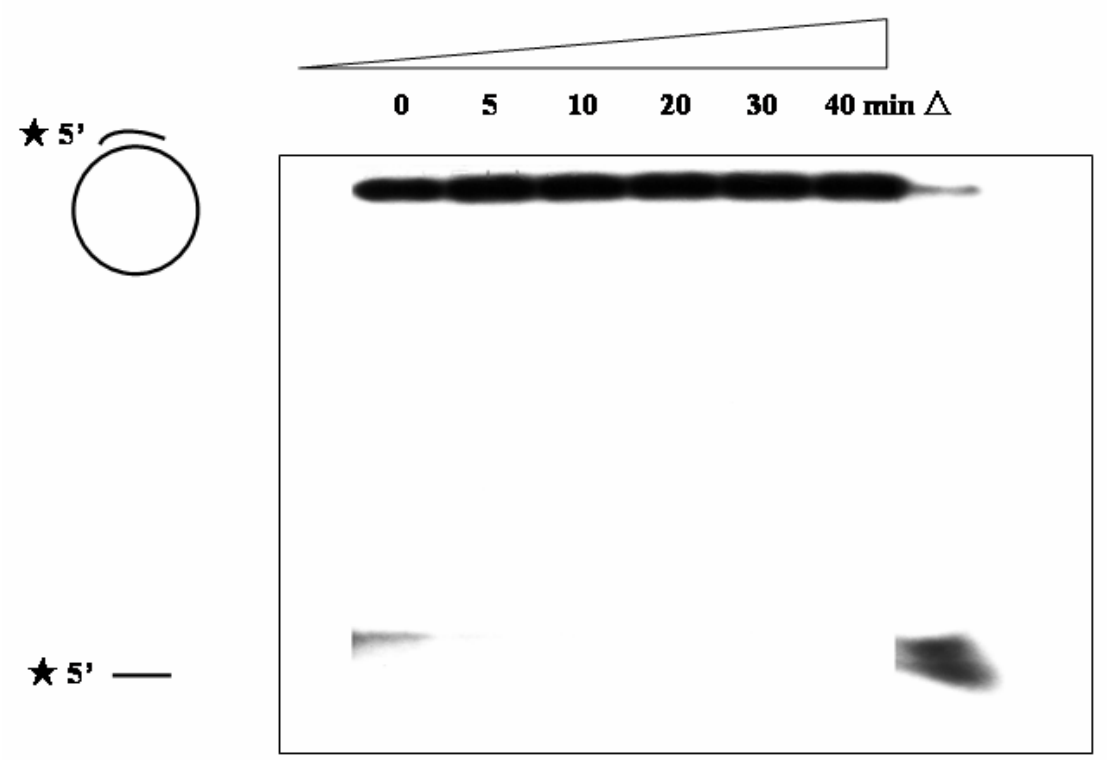


Figure 5

A



B

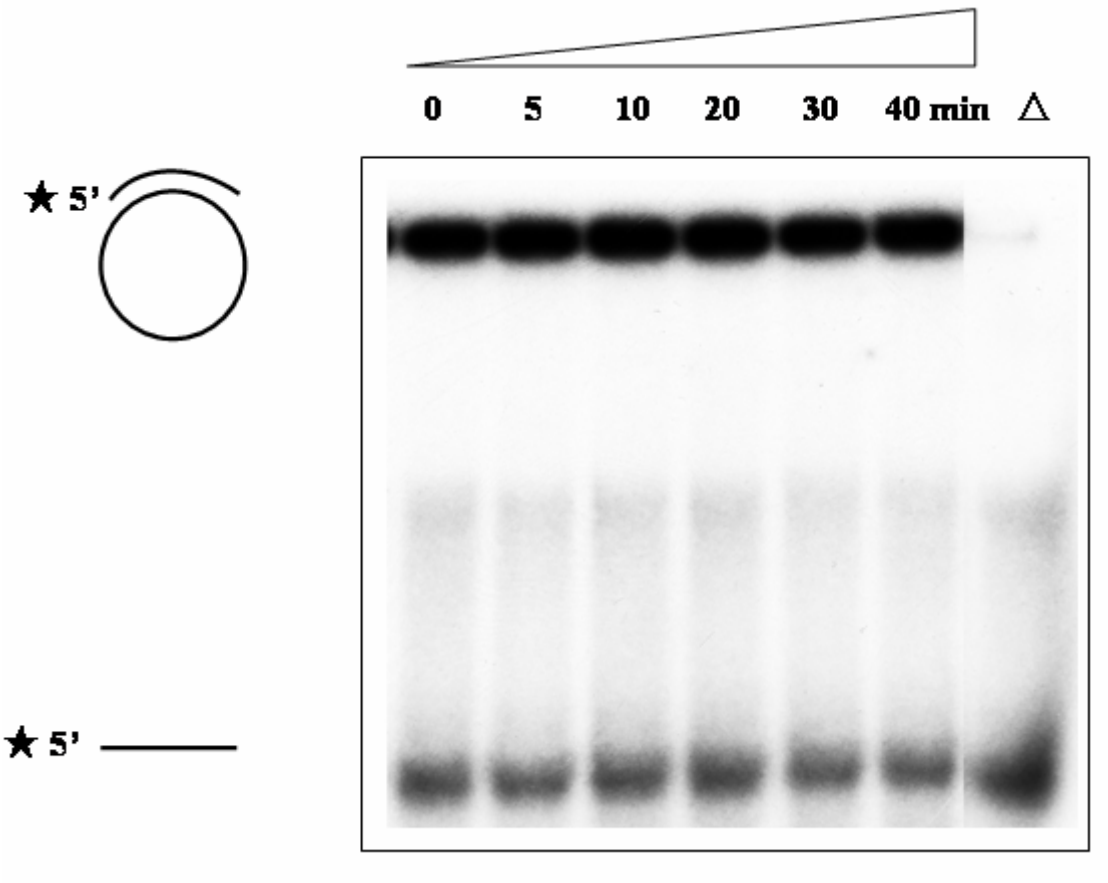
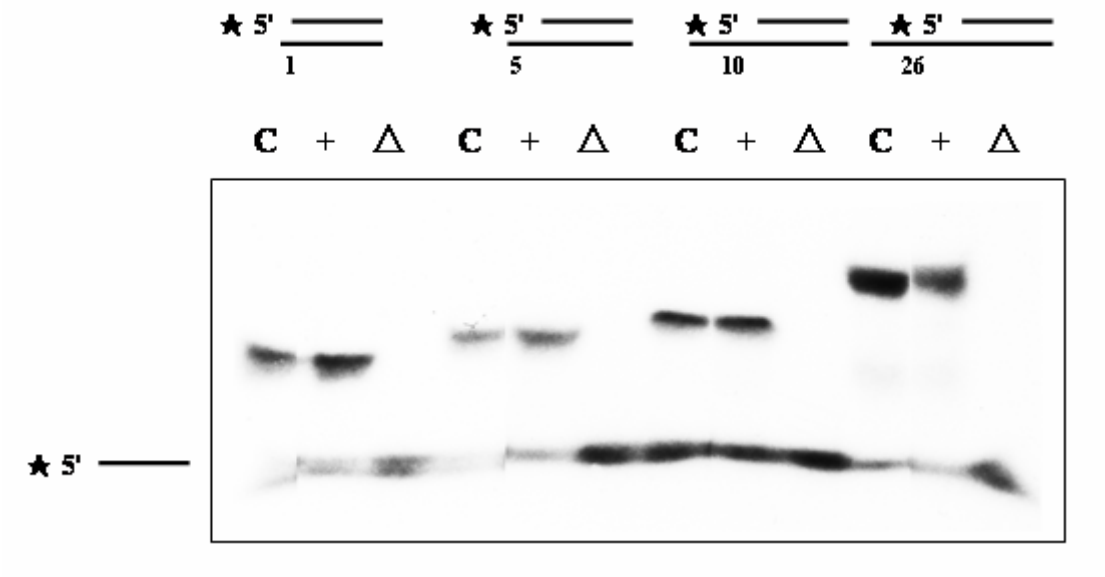


Figure 6

A



B

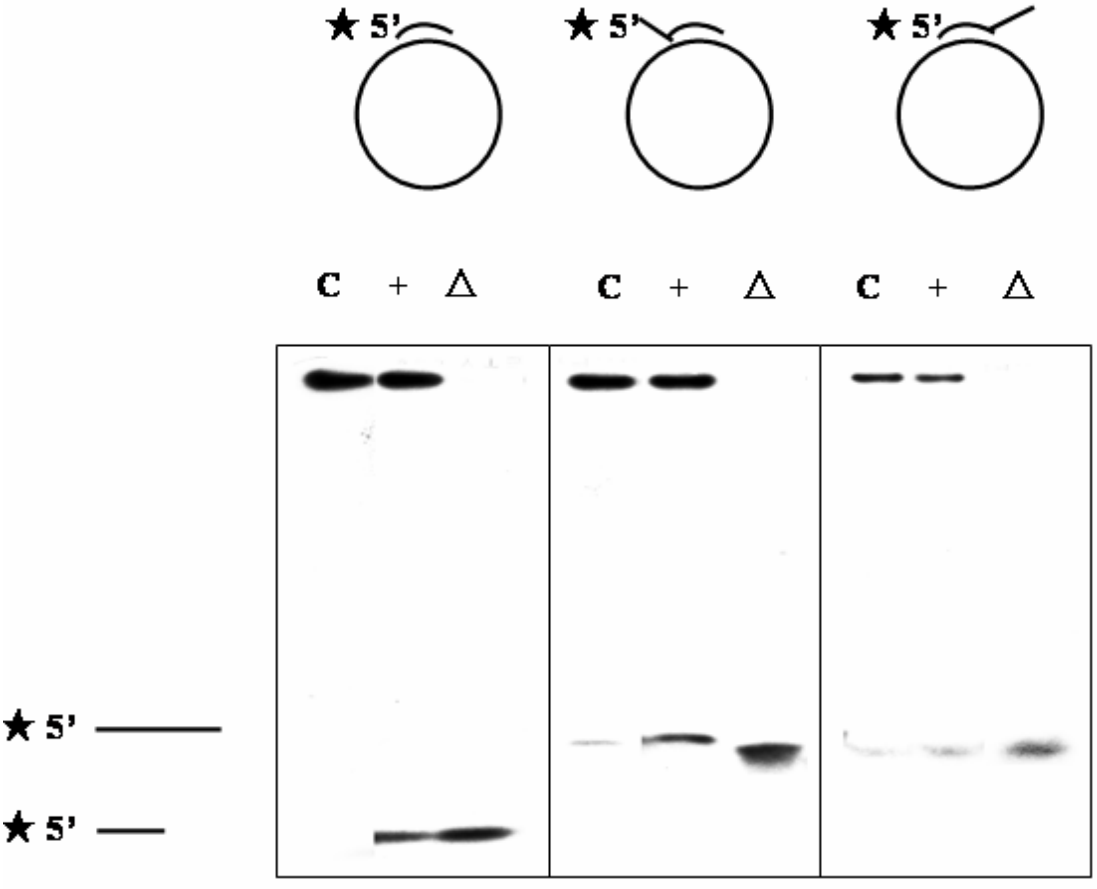
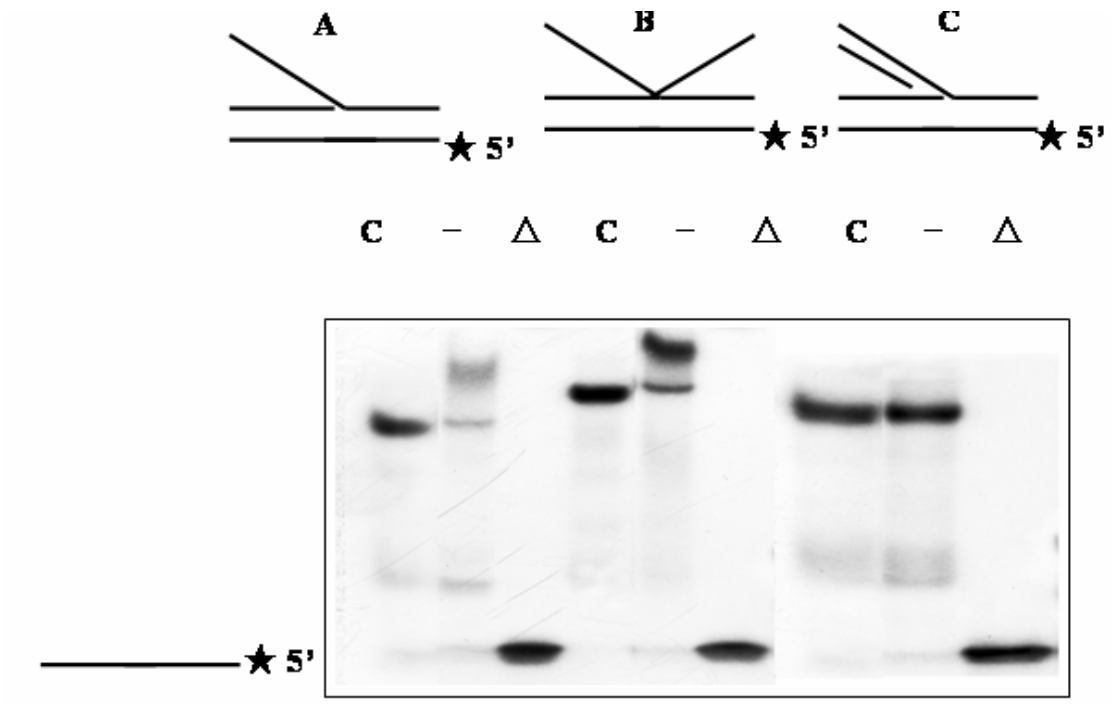


Figure 7

A



B

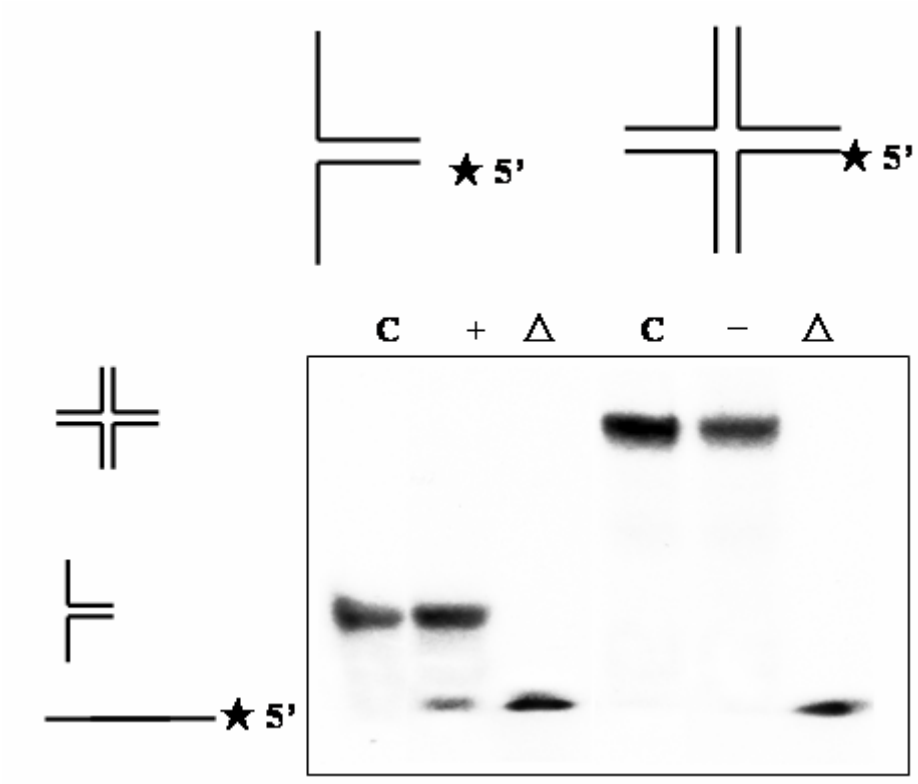
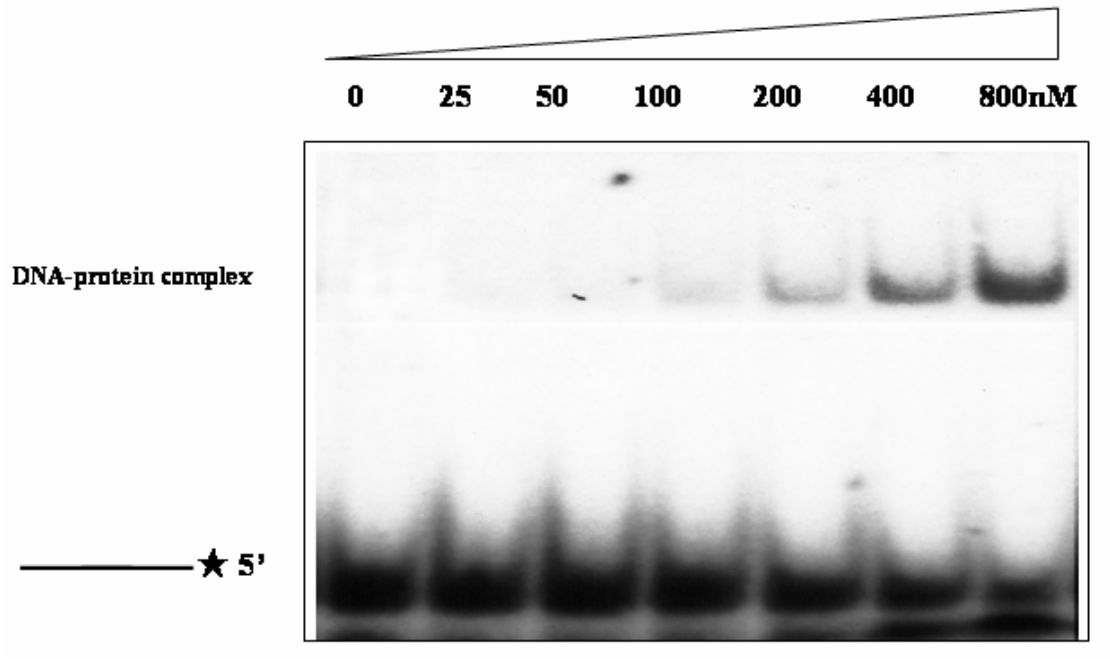
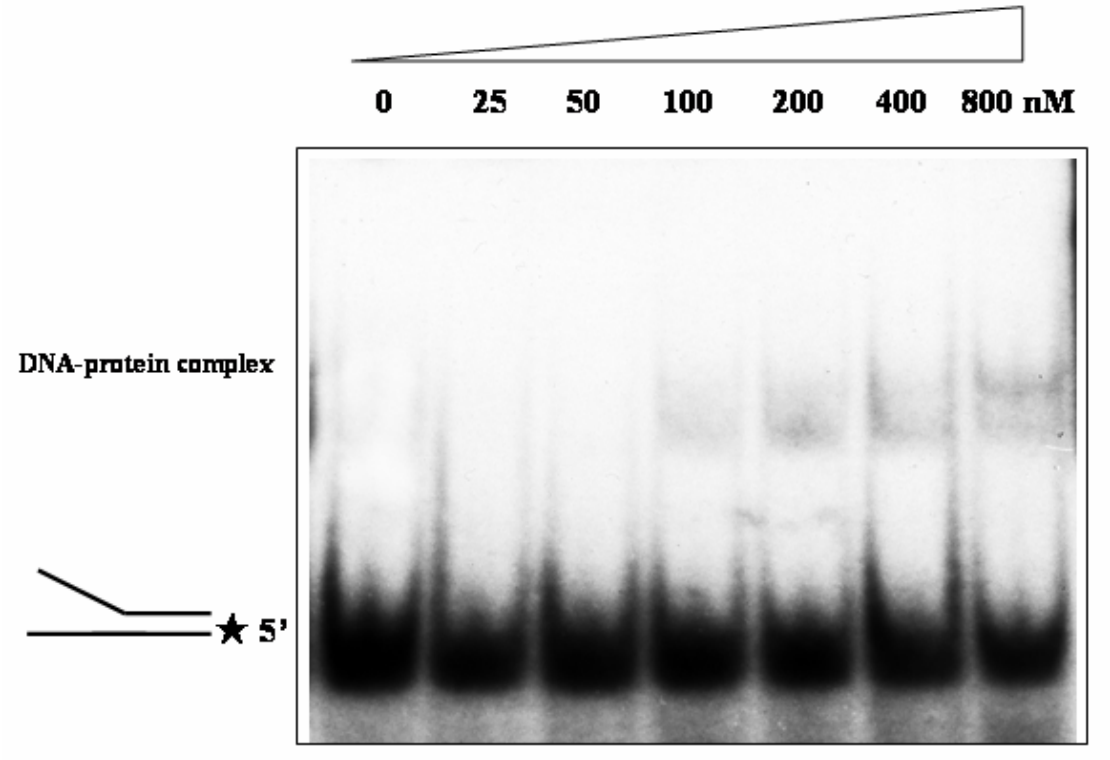


Figure 8

A


0
25
50
100
200
400
800nM
DNA-protein complex
★ 5'

B


0
25
50
100
200
400
800 nM
DNA-protein complex
★ 5'

Chapitre 3 : Discussions

Les hélicases sont des enzymes ubiquitaires qui déroulent l'ADN duplex en utilisant l'énergie dérivée de l'hydrolyse de l'ATP (Soultanas et Wigley, 2001). La famille RecQ des ADN hélicases est nécessaire à la maintenance de l'intégrité des génomes (Chakraverty et Hickson, 1999; Karow *et al.*, 2000b; van Brabant *et al.*, 2000a; Mohaghegh et Hickson, 2001a). Généralement, les membres de la famille RecQ contiennent les domaines hélicase, RecQ-Ct et HRDC dont la structure et la fonction sont importantes pour l'activité enzymatique. Les caractéristiques biochimiques du domaine hélicase ont été élucidées, mais celles du domaine RecQ-Ct restent à déterminer. Dans ce travail, nous avons étudié la structure primaire et la fonction du motif à doigt de zinc du domaine RecQ-Ct de l'hélicase RecQ d'*E. coli* (Article 1) ainsi que les isoformes RECQ5 humaines (Article 2), les activités hélicase et ATPase, la spécificité de substrat des hélicases homologues à RecQ chez *Bacillus subtilis* (Article 3 et Article 4).

1. Importance du motif doigt de zinc dans la fixation de l'ADN et les activités hélicase et ATPase de l'hélicase RecQ d'*E. coli*

La structure tridimensionnelle de l'hélicase RecQ d'*E. coli* (Bernstein *et al.*, 2003a) indique l'existence d'un motif fixant le zinc. Dans cette étude, l'importance de ce motif pour la fonction de l'hélicase RecQ a été montrée par des mutations de résidus fortement conservés dans le motif à doigt de zinc par mutagenèse dirigée. En accord avec la notion que les doigts de zinc sont les modules structuraux d'une classe majeure de motifs fixant l'ADN (Coleman, J. E. 1992), nos données ont illustré qu'une mutation dans le doigt de zinc, qui réduit fortement la fixation du zinc, abroge la liaison de l'hélicase à l'ADN et diminue les activités ATPase et hélicase. En outre, ce motif est également crucial pour l'intégrité de la protéine entière.

1.1. Le motif à doigt de zinc est essentiel pour fixation à l'ADN

La plus frappante observation est que les changements du motif à doigt de zinc par mutagenèse dirigée mènent à une perte complète des capacités de RecQ à fixer l'ADN. L'hélicase RecQ étant une ATPase ADN-dépendante et une hélicase ATP-dépendante, l'altération de l'affinité pour l'ADN devrait être la cause primaire des défauts observés dans des activités ATPase et hélicase. Ces observations indiquent que le motif à doigt de zinc est essentiel à la fixation de l'ADN. Alternativement, le remplacement des acides aminés dans le motif à doigt de zinc

mène à un changement local de conformation de la protéine, qui produit les défauts observés dans les fonctions d'hélicase. Tous les mutants ponctuels obtenus ont donné des protéines instables, rendant l'analyse fonctionnelle impossible. Cependant, un double mutant stable a pu être obtenu et nous avons montré que la protéine possède une modification subtile et locale de conformation plutôt qu'une modification radicale de l'architecture globale de la protéine. D'abord, les enzymes sauvages et mutantes possèdent un pattern de protéolyse limitée très semblable. En second lieu, nous avons montré par chromatographie d'exclusion que les hélicases sauvages et mutantes ont des rayons de Stokes semblables, suggérant que ces protéines ont des modes de repliement semblables. Troisièmement, le fait que la protéine mutante possède presque la même affinité pour l'ATP que la protéine sauvage suggère que la structure tertiaire de la protéine mutante n'est pas totalement modifiée. Ces résultats démontrent clairement qu'un changement local subtil de conformation du motif à doigt de zinc abroge les capacités de fixation à l'ADN, et en conséquence, les activités ATPase et hélicase. Cette interprétation reçoit l'appui des expériences de dichroïsme circulaire (CD) où la petite réduction dans l'hélice α peut être attribuée au changement de structure du motif à doigt de zinc en raison du remplacement de deux acides aminés de cystéine par des asparagines. Le motif à doigt de zinc a ainsi un effet dominant sur la fixation de l'ADN. Nous présumons que certains résidus chargés positivement conservés (Arg^{382} et Lys^{408}) et les résidus hydrophobes exposés aux solvants (Phe^{374} et Phe^{389}) présentés dans ce motif à doigt de zinc pourraient être directement impliqués dans l'identification et la fixation de l'ADN.

Pour d'autres hélicases sans motif à doigt de zinc, telles que PrcA, Rep, UvrD, et l'hélicase de HCV (virus de l'hépatite C), les surfaces impliquées dans la fixation de l'ADN se composent principalement des motifs Ia, IV et V. Plusieurs observations suggèrent que cela est identique pour les autres membres des hélicases de la famille RecQ (Bennett *et al.,* 2004). Ces membres contiennent aussi un domaine HRDC, qui possède les activités de fixation à l'ADN (Liu *et al.,* 1999). Puisque le double mutant étudié semble garder sa structure tridimensionnelle globale, nous avons été surpris de la perte totale de l'activité de fixation à l'ADN alors que les domaines hélicase et HRDC qui ont des propriétés de fixation à l'ADN sont encore présents.

Le motif à doigt de zinc jouerait donc un rôle essentiel dans la fixation de l'ADN, tandis que les domaines hélicase et HRDC peuvent fonctionner comme domaines auxiliaires pour l affinité à l'ADN. Dans un travail non publié, nous avons montré

que le domaine hélicase isolé et le domaine HRDC isolé ont une affinité très faible pour l'ADN simple brin et l'ADN double brin. Il est probable que quand l'ADN est liée au motif à doigt de zinc, le domaine hélicase joue un rôle essentiel dans l'activité de déroulement, tandis que le domaine HRDC peut diriger la spécificité de fixation à l'ADN. Ainsi, il est intéressant de proposer que la coopération entre le motif à doigt de zinc, les domaines hélicase et HRDC détermine la spécificité du substrat d'ADN, la cinétique, et la processivité de l'hélicase. Ces analyses montrent que le motif à doigt de zinc joue un rôle essentiel dans la fixation à l'ADN.

1.2. Le motif à doigt de zinc joue un rôle important dans la stabilité de la structure tertiaire

Les résultats de cette étude et l'analyse structurale tridimensionnelle indiquent que le motif à doigt de zinc est stabilisé par quatre résidus cystéine. Il est important de noter que le changement d'un de ces quatre résidus conservés de cystéine entraine une forte instabilité des protéiness. Ces résultats montrent de manière inattendue, que le motif à doigt de zinc est impliqué dans la stabilité de l'enzyme entière. Il est possible que le motif à doigt de zinc intervienne dans la conformation locale des régions reliant les différents domaines et contribuerait ainsi à la stabilisation de la structure tertiaire de la protéine.

En plus des quatre résidus fortement conservés de cystéine, le motif à doigt de zinc pourrait encore être stabilisé par des interactions très importantes parmi les α-hélices 16, 17 et 18. Il semble que les liaisons hydrogène entre Phe^{374} et Arg^{381} et entre Arg^{381} et Asp^{401} jouent un rôle important en stabilisant le motif à doigt de zinc et donc la structure la protéine. En accord avec ce postulat, les mutations de Arg^{381} ou de Asp^{401} entraine une forte sensibilité à la dégradation par protéolyse. Des résultats semblables ont déjà été observés pour la protéine BLM impliqué dans le syndrome Bloom (Janscak *et al.,* 2003). Le remplacement de Asp^{1064} par Ala, situé à une position équivalente à celle de Asp^{401} de l'hélicase RecQ, réduit sensiblement ses activités hélicase et de fixation à l'ADN, tandis que la protéine possède les mêmes activités que l'enzyme sauvage quand Asp^{1064} est remplacé par Asn.

Ce phénomène est probablement le résultat d 'un changement des propriétés de la chaîne latérale : Asn pourrait probablement établir une liaison hydrogène avec Arg^{1037},

stabilisant la structure de la protéine, à l 'inverse de Ala. Ainsi, les cystéines, C1 à C4, et les liaisons hydrogène entre Phe^{374} et Arg^{381} et entre Arg^{381} et Asp^{401} contribuent à la stabilisation du motif à doigt de zinc et augmentent la stabilité de la structure tertiaire de la protéine.

L'atome de zinc n'apparait pas directement impliqué dans la fixation de l'ADN, les activités ATPase et hélicase puisque son élimination ne change pas son activité enzymatique. Il semble donc que l'ion zinc n'est pas essentiel pour stabiliser la conformation de la protéine, car les hélicases sauvages, en présence ou absence de zinc, montrent ses thermostabilités très semblables entre 25°C et 54 °C. Néanmoins, l'ion zinc pourrait avoir un rôle prépondérant dans la mise en place de la structure secondaire du motif à doigt de zinc pendant le repliement de la protéine. Une fois le motif à doigt de zinc replié correctement en présence de l'ion zinc, sa structure est stable même en son absence. Il y a plusieurs exemples dans lesquels les ions métalliques jouent un rôle important dans la formation des éléments structuraux secondaires, sans être indispensables une fois la structure tertiaire établie (Banci *et al.*, 2003).

1.3. Signification biologique du cluster de cystéine des hélicases de la famille RecQ

L'alignement des séquences en acides aminés de plusieurs hélicases RecQ indique que les résidus cystéine impliqués dans la formation du doigt de zinc sont conservés. Les mutations de ces résidus cystéine de ce motif peuvent avoir des effets dramatiques sur les activités enzymatiques. Deux mutations de l'hélicase BLM qui enntrainent le syndrome de Bloom impliquent deux des quatre résidus conservés Cys^{1036} et la Cys^{1055}, respectivement (Ellis *et al.*, 1995, Foucault *et al.*, 1997). L'analyse *in vitro* prouve que ces mutations suppriment les activités ATPase et hélicase de BLM (Bahr *et al.*, 1998). De plus, les protéines mutantes qui contiennent trois des quatre cystéines modifiées sont très très instables. En outre, même les mutations des résidus situés proches des cystéines conservés, tels que R1038A et D1064A , causent une perte de la fixation à l'ADN et des activités ATPase et hélicase altérées (Coleman, J. E. 1992). Cette région est également essentielle à la fonction *in vitro* de l'hélicase Sgs1 de levure qui est une homologue RecQ (Mullen *et al.*, 2000, Onoda *et al.*, 2000).

Les comparaisons de séquence parmi les ADN hélicases suggèrent que le motif à

doigt de zinc semble être spécifique de la famille RecQ. L'analyse de séquence et les études structurales tridimensionnelles ont prouvé que d'autres ADN hélicases, tels que la protéine 4 du phage T7 (Singleton *et al.,* 2000), Rep (Korolev *et al.*, 1997), PcrA (Velankar *et al.*, 1999), et l'hélicases NS3 du virus de l'hépatite C (Yao *et al.,* 1997), n'emploient pas le motif à doigt de zinc pour la fixation à l'ADN. Notre laboratoire poursuit actuellment cette étude pour confirmer que la spécificité de la fixation à l'ADN régulé par le motif à doigt de zinc serait une caractéristique unique de la famille RecQ .

En résumé, notre étude indique que le motif à doigt de zinc de l'hélicase RecQ joue des rôles importants dans la fixation de l'ADN et l'intégrité fonctionnelle de l'enzyme. Ainsi, l'évolution a choisi un mécanisme complexe pour lier toutes les fonctions de l'enzyme à ce petit sous-domaine. Bien que beaucoup d 'études restent à faire, il apparait que les enzymes RecQ qui présentent un repliement imparfait du motif à doigt de zinc ne peuvent plus assurer leur fonction hélicase.

2. Régulation de la fonction de l'hélicase RECQ5 humaine par le doigt de zinc

Dans cette étude, nous avons caractérisé les activités enzymatiques de trois isoformes RECQ5 humaines. RECQ5 offre un modèle pour étudier la fonction intrinsèque du domaine hélicase de RecQ et sa régulation par le motif à doigt de zinc.

2.1. Le domaine hélicase de RECQ5 humain a une activité intrinsèque élevée qui tend à favoriser l'hybridation de l'ADN.

L'activitédes hélicases de la famille RecQ à favoriser l'hybridation de l'ADN a été découverte la première fois dans le cas de RECQ5β humain (Garcia *et al.*, 2004) puis retrouvée pour BLM, WRN, RECQ1, RECQ4, et drosophile RECQ5b (dmRECQ5b) (Machwe *et al.*, 2005 ; Sharma *et al.,* 2005 ; Cheok *et al.,* 2005 ; Macris *et al.,* 2005). En outre, il a été montré que WRN, BLM et dmRECQ5b peuvent catalyser l'échange de brin d'ADN en combinant les activités du déroulement et de l'hybridation de l'ADN (Machwe *et al.*, 2005). Les régions C-terminales de ces protéines sont impliquées dans ces propriétés d'hybridation de l'ADN (Garcia *et al.*, 2004 ; Machwe *et al.,* 2005 ; Cheok *et al.*, 2005) et de l'échange de brin d'ADN (Machwe *et al.,* 2005). Ce mécanisme de l'hybridation de l'ADN catalysée par l'extrémité C-terminale des hélicases de la famille RecQ reste à étudier. D'autre part,

le domaine hélicase de RecQ, qui possède les motifs fixant l'ADN, est probablement aussi impliqué dans l'hybridation de l'ADN comme nous l'avons montré dans le cas de RECQ5 qui présente une activité intrinsèque élevée à favoriser l'hybridation. Nos résultats indiquent que cette fonction d'hybridation d'ADN catalysée par le domaine hélicase est fortement inhibée par l'ATP, l'ATPγS ou le doigt de zinc de RECQ5β, mais pas l'ADP.

RECQ5α offre un modéle de domaine hélicase natif de RecQ qui permet d'étudier ses activités enzymatiques, en particulier l'hybridation de l'ADN.

Cette propriété du domaine helicase RECQ5 n'est pas surprenante puisque les nucléotides régulent cette activité dans le cas de RECQ5β（Garcia *et al.,* 2004), de BLM (Garcia *et al.,* 2004 ; Machwe *et al.*, 2005 ; Cheok *et al.,* 2005), de WRN (Machwe *et al.*, 2005), et de dmRECQ5b (Machwe *et al.,* 2005) , indiquant l 'implication des domaines helicase de cette famille RecQ. Les études précédentes (Garcia *et al.,* 2004) et nos résultats (Article 2, Fig. 7A, B et Tableau 1) indiquent que RECQ5β catalyse l'hybridation de l'ADN plus efficacement que son fragment C-terminal considéré comme le motif unique impliqué dans l'hybridation de l'ADN (Garcia *et al.*, 2004). Cette observation suggère que le domaine hélicase contribue à l'activité de l'hybridation de l'ADN de RECQ5β sauvage. Troisièmement, dmRECQ5b, qui comprend un domaine hélicase et un doigt de zinc, possède une faible activité à favoriser l'hybridation de l'ADN (Machwe *et al.*, 2005).

L'activité de l'hybridation de l'ADN du domaine hélicase RECQ5 peut être inhibée par la présence du doigt de zinc, puisque RECQ5β^{1-475} possède une activité sensiblement inférieure à RECQ5α. En outre, dmRECQ5b, semblable à RECQ5β^{1-475} humain (Article 2, Fig. 1A), a une activité faible (Machwe *et al.,* 2005). RECQ4 humain, qui ne possède pas de doigt de zinc putatif et d'activité hélicase posséde l'activité d'hybridation de l'ADN (Macris *et al.*, 2005).

2.2. Le motif à doigt de zinc régule les activités enzymatiques de RECQ5

La comparaison quantitative des activités enzymatiques multiples des protéines RECQ5α, RECQ5β et RECQ5γ nous permet de proposer que le motif à doigt de zinc joue un rôle important dans la régulation des activités enzymatiques de RECQ5. La fixation de l'ATP semble être déterminée par les motifs du domaine hélicase qui sont

présents dans chacune des trois isoformes RECQ5. Ainsi, RECQ5α, RECQ5β et RECQ5γ fixent l'ATP avec une affinité semblable. Comme d'autres helicases, les motifs conservés du domaine hélicase des protéines RecQ, à savoir Ia, IV et V, comportent une surface fixant l'ADN (Caruthers et McKay, 2002). En accord avec ces données, RECQ5α, RECQ5β et RECQ5γ fixent l'ADN (Article 2, Tableau 2). Toute fois, l'étude cinétique montre que RECQ5α et RECQ5γ fixent faiblement l'ADN, tandis que RECQ5β fixe l'ADN rapidement et forme un complexe protéine-ADN stable, ce qui suggèrent que le domaine C-terminal contient un autre domaine de fixation à l'ADN. Il a été montré que les domaines RQC et HRDC des hélicases RecQ sont impliqués dans la fixation de l'ADN (Bachrati et Hickson, 2003). Comme le domaine HRDC n'est pas présent dans RECQ5β (Bachrati et Hickson, 2003), le domaine RQC serait le site important de fixation de l'ADN. RECQ5β semble d'autre part ne pas avoir de domaine WH (Garcia *et al.,* 2004). Conformément à cette notion, les mutants portant la délétion C-terminale, RECQ5β$^{1-475}$ et RECQ5β$^{1-662}$ ont une affinité pour l'ADN semblable à RECQ5β. Ces données suggèrent que le doigt de zinc du domaine RQC putatif soit l'élément principal qui augmente la fixation de l'ADN.

Les activités ATPase et hélicase des protéines RECQ5 sont corrélées à leur affinité pour l'ADN. Les deux activités exigent le motif C-terminal de RECQ5β, car RECQ5α et RECQ5γ n'ont aucune activité ou une faible activité ATPase et hélicase. En particulier, le doigt de zinc du domaine RQC est l'élément principal de la fixation à l'ADN, mais il semble supprimer l'activité intrinsèque de l'hybridation de l'ADN du domaine hélicase RECQ5 en augmentant la fixation de l'ADN. D'ailleurs, le doigt de zinc joue également un rôle important dans l'échange des brins d'ADN catalysé par RECQ5β en coordonnant les activités de déroulement et d'hybridation d'ADN (Article 2, Fig. 8A). La mutagenèse dirigée ponctuelle confirme que le doigt de zinc régule les activités enzymatiques du domaine hélicase RECQ5.

2.3. Le motif à doigt de zinc peut réguler les fonctions des hélicases RecQ

Les études de l' hélicase RECQ5 doit nous aider à comprendre la régulation des hélicases RecQ par le motif à doigt de zinc. Le modèle (Article 2, Fig. 8A) proposé du RECQ5 humain peut être utilisé pour les hélicases de la famille RecQ.

BLM a été caractérisé extensivement. Les activités ATPase et hélicase ont été étudiées (Bachrati et Hickson, 2003). Récemment, il a été montré que BLM possède également une activité favorisant l'hybridation de l'ADN et l'échange des brins d'ADN (Machwe *et al.*, 2005 ; Cheok *et al.,* 2005). Plusieurs mutations dans le gène *BLM* sont des mutations non-sens ou des décalages du cadre de lecture qui conduisent à des protéines tronquées. Ces mutations aboutissent une perte des activités ATPase et hélicase. Il a été montré que deux mutations ponctuelle, C1036F et C1055S (Ellis *et al.,* 1995 ; Barakat *et al.*, 2000), sont impliqués dans la formation du doigt de zinc (Bernstein et al., 2003 ;Guo *et al.*, 2005). Les protéines mutantes ont complètement perdu leurs activités enzymatiques (Guo *et al.,* 2005). Ces résultats soutiennent clairement que le doigt de zinc de BLM régule les activités enzymatiques du domaine hélicase (Article 2, Fig. 8B).

WRN est une autre hélicase RecQ humaine extensivement caractérisée. Le fragment contenant un domaine hélicase et un domaine RQC possède une activité hélicase semblable à celle de l'hélicase WRN sauvage (Doherty *et al.,* 2005), mais l'activité diminue quand K1016 du motif à doigt de zinc est remplacé par Ala (Lee *et al.,* 2005). D'ailleurs, quand le domaine RQC est supprimé, l'activité hélicase du fragment diminue dramatiquement (Lee et al., 2005), ce qui suggère que le doigt de zinc de WRN régule les activités enzymatiques (Article 2, Fig. 8C) (Hu *et al.,* 2005).

L'hélicase RECQ1 humaine a récemment été caractérisée. Les activités hélicase et d'hybridation d'ADN ont été également observées pour cette protéine (Sharma *et al.,* 2005). RECQ1 ne contient pas de domaine HRDC, mais semble posséder un domaine RQC (Bachrati et Hickson, 2003). Nous prévoyons donc que le doigt de zinc putatif (Article 2, Fig. 8D) régulerait les activités enzymatiques du domaine hélicase de RECQ1.

En plus du domaine hélicase, RECQ4 humaine ne montre aucune identité évidente avec les autres membres de la famille RecQ et ne contient pas de domaines RQC et HRDC (Article 2, Fig. 8E) (Bachrati et Hickson, 2003). Il a été montré que RECQ4 n'a pas d'activité hélicase, mais catalyse l'hybridation de l'ADN et l'hydrolyse de l'ATP (Macris *et al.,* 2005). Dans ce cas, le doigt de zinc n'est pas disponible pour activer l'activité hélicase et pour supprimer l'activité de l'hybridation de l'ADN du domaine hélicase de RECQ4.

3.Caractéristiques enzymatiques des hélicases SubL et SubS de *Bacillus subtilis* contenant le motif doigt de zinc

Nous avons montré que les protéines SubL et SubS purifiées de *Bacillus subtilis* possèdent les activités helicase et ATPase. La fonction de ces deux protéines est en accord avec le fait qu'elles possèdent un motif à doigt de zinc, car ce motif du domaine RecQ-Ct est nécessaire pour l'activité enzymatique des hélicases RecQ (Liu *et al.,* 2004, Guo *et al.,* 2005). L'activité hélicase de ces protéines est dépendante de la présence d'ATP ou de dATP qui sont hydrolysés pour fournir l'énergie nécessaire au déroulement. L'activité ATPase de l'hélicase RecQ d'*E. coli* est plus fortement stimulée par l'ADN simple brin que par l'ADN double brin (Umezu *et al.,* 1990), l'hélicase SubS est similaire, cependant l'hydrolyse de l'ATP catalysée par la protéine SubL est fortement stimulée par les deux formes d'ADN. Leur activité ATPase est dépendante de la présence du Mg^{++}. Les protéines SubL et SubS montrent une activité ATPase optimale entre pH 7.0 et 8.8.

Nous avons montré que les hélicases SubL et SubS catalysent le déroulement de l'ADN avec la polarité 3' - 5' par rapport au brin sur lequel l'enzyme est fixée. En accord avec cette polarité, il a été montré que SubL et SubS déroulent un substrat d'ADN duplex avec une extrémité 3' sortante mais pas 5' sortante. Ces résultats peuvent montrer qu'une extrémité 3' sortante est nécessaire pour commencer le déroulement de l'ADN duplex catalysé par SubL et SubS. Les caractéristiques du déroulement catalysé par SubL et SubS sont semblables à celles de l'hélicase RecQ d'*E. coli* (Umezu *et al.,* 1990) qui partage l'homologie du domaine hélicase. Dans les autres membres de la famille RecQ, il a été précédemment montré que la protéine RecQL humaine possède une activité de déroulement de l'ADN *in vitro* (Seki *et al.,* 1994a-b, Puranam *et al.,* 1994). Récemment, les activités hélicases des protéines BLM, WRN et RECQ5 (Garcia *et al.,* 2004) (homologues humains de RecQ) ont aussi été montré. Ainsi il semble que tous les membres de la famille RecQ possédent une activité hélicase commune.

Nous avons montré que les protéines SubL et SubS ne sont pas capables de dérouler l'ADN duplex aux bouts francs, contrairement à la protéine RecQ d'*E. coli*, ou d'autres hélicases telles que RecBCD (Harmon et Kowalczykowski, 1998, Lohman et Bjornson, 1996, West, 1996). Bennett et ses collègues (1999) ont montré que Sgs1 de *Saccharomyces cerevisiae*, a besoin d'une extrémité 3' sortante au moins

contenant 3 nucléotides et a employé la jonction entre l'ADN simple brin et l'ADN double brin comme site principal dans la reconnaissance et la fixation de substrat d'ADN. Nos résultats sont en accord avec celui de Sgs1.

En outre, nous avons montré que l'hélicase SubL est capable de dérouler l'ADN duplex avec un flap 5' et de fourche de réplication, ce qui suggère que l'enzyme est capable de fixer une jonction et qu'une extrémité 3' sortante d'ADN n'est pas indispensable pour le déroulement de l'ADN par la protéine SubL. Cette enzyme est aussi capable de dérouler une structure de type X (un modèle pour l'intermédiaire de recombinaison de jonction de Holliday). Cette dernière forme une structure de type X en présence du Mg^{2+} et ne possède donc pas d'ADN simple brin, même à son noyau (Lilley, 1999 ; Lilley et Norman, 1999). Ainsi SubL n'exige pas une extrémité 3' sortante d'ADN pour la fixation.

L'activité de SubL dérouler l'ADN avec un flap 5' est importante du point de vue génétique car cette structure est un intermédiaire de réplication de l'ADN. La taille moyenne du fragment Okazaki (100-150 nucléotides) (Bae *et al.,* 2001) peut être influencée par l'action de l'hélicase SubL sur la structure avec un flap 5'. L'hélicase SubL peut être importante pour la maintenance de la progression de fourche de réplication lorsque la réplication de l'ADN est bloquée pour cause de dommages d'ADN.

Dans cette étude, nous avons également démontré que SubS ne peut pas dérouler l'ADN avec un flap 5', la structure de fourche de réplication, la structure Kappa et la structure de type X. Ces résultats la différencient ceux de la protéine SubL. Il a été montré qu'il y a différence de spécificité de substrat d'ADN entre les hélicase SubS et SubL. Puisqu'il n'y a pas de domaine HRDC dans la séquence d'acides aminés de SubS, l'efficacité du déroulement et l'activité ATPase sont environ 15 fois plus faible que celles de SubL. L'étude précédente indique que le domaine HRDC est nécessaire à la fixation stable à l'ADN (Bernstein et Keck, 2003b). Cette dernière est importante pour le déroulement de l'ADN catalysé par l'hélicase. Récemment, il a été montré que le domaine HRDC de l'hélicase BLM est indispensable pour le déroulement des structures de type double jonctions de Holliday (Wu *et al.,* 2005). Ainsi, la déficience du domaine HRDC dans la protéine SubS peut aboutir à l' impossibilité du déroulement des substrats d'ADN mentionnés ci-dessus.

En utilisant EMSA, il a été montré que l'hélicase SubL possède une affinité plus élevée pour des fourche que l'ADN simple brin, à l'inverse SubS. Ceci peut avoir des

implications dans le mécanisme du déroulement de l'ADN par SubL, car la fixation préférentielle de l'enzyme à cette strucure de fourche pourrait être importante pour l'action de l'hélicase (Lohman et Bjornson, 1996).

Nos données indiquent que la protéine SubL montrent une activité efficace à favoriser l'hybridation de l'ADN, à l'inverse de SubS. La signification biologique de nos résultats ne peut pas être aisément estimée car nous ne possédons pas d'information sur les conséquences phénotypiques des cellules déficientes en protéines SubL et SubS de *Bacillus subtilis*. Les activités biochimiques de SubL suggèrent que l'hélicase puisse être impliquée dans la réparation de l'ADN qui exige une coopération entre le déroulement et l'hybridation de l'ADN. SubL pourrait jouer un rôle dans le cas de blocage de la fourche de réplication par ses activités hélicase et d'hybridation qui pourrait faciliter la régression de fourche. Ainsi, SubL peut dérouler préférentiellement l'ADN avec un flap 5' ou une fourche de réplication. Des études complémentaires sur le rôle *in vivo* des protéines SubL et SubS sont nécessaires pour mieux comprendre la fonction des ADN hélicases de la famille RecQ dans la maintenance de l'intégrité génomique.

REFERENCES BIBLIOGRAPHIQUES

A

Abdel-Monem, M., Durwald, H. & Hoffmann-Berling, H. (1976) Enzymic unwinding of DNA. II. Chain separation by an ATP-dependent DNA unwinding enzyme. Eur. J. Biochem. 65, 441-449.

Ahmad, F., Kaplan, C. D. and Stewart, E. (2002) Helicase activity is only partially required for Schizosaccharomyces pombe Rqh1p function. Yeast 19, 1381–1398

Ajima J, Umezu K, Maki H. (2002). Elevated incidence of loss of heterozygosity (LOH) in an sgs1 mutant of *Saccharomyces cerevisiae*: roles of yeast RecQ helicase in suppression of aneuploidy, interchromosomal rearrangement, and the simultaneous incidence of both events during mitotic growth. Mutat. Res., 9336: 1-16.

Amaratunga, M. & Lohman, T.M. (1993). E. coli rep helicase unwinds DNA by an active mechanism. Biochemistry 32, 6815-6820.

Ayora, S., Weise, F., Mesa, P., Stasiak, A. & Alonso, J.C. (2002) Bacillus subtilis bacteriophage SPP1 hexameric DNA helicase, G40P, interacts with forked DNA. Nucleic Acids Res. 30, 22802289.

B

Bachrati CZ, Hickson ID. (2003) RecQ helicases: suppressors of tumorigenesis and premature aging. Biochem J. Sep 15; 374(Pt 3):577-606.

Bae, S. H., Bae, K. H., Kim, J. A., and Seo, Y. S. (2001) RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes. Nature 412,456-461.

Bahr, A., de Graeve, F., Kedinger, C. and Chatton, B. (1998) Point mutations causing Bloom's syndrome abolish ATPase and DNA helicase activities of the BLM protein. Oncogene 17, 2565–2571

Banci L, Bertini I, Cramaro F, Del Conte R, Viezzoli MS. (2003) Solution structure of Apo Cu, Zn superoxide dismutase: role of metal ions in protein folding. Biochemistry. 42(32):9543-53.

Barakat,A., Ababou,M., Onclercq,R., Dutertre,S., Chadli,E., Hda,N., Benslimane,A., & Amor-Gueret,M. (2000) Identification of a novel BLM missense mutation (2706T>C) in a Moroccan patient with Bloom's syndrome. Hum. Mutat. 15, 584-585.

Bennett RJ, Keck JL. (2004) Structure and function of RecQ DNA helicases. Crit Rev Biochem Mol Biol. 39(2):79-97.

Bennett, R. J., Keck, J. L., and Wang, J. C. (1999) Binding specificity determines polarity of DNA unwinding by the Sgs1 protein of S. cerevisiae. J. Mol. Biol. 289,

235–248

Bennett, R. J., Sharp, J. A. and Wang, J. C. (1998) Purification and characterization of the Sgs1 DNA helicase activity of Saccharomyces cerevisiae. J. Biol. Chem. 273, 9644–9650

Berg, J. M., and Shi, Y. (1996) The galvanization of biology: a growing appreciation for the roles of zinc. Science. 1996 Feb 23;271(5252):1081-5. Science 271, 1081–1085

Bernstein, D. A., and Keck, J. L. (2003b) Domain mapping of Escherichia coli RecQ defines the roles of conserved N- and C-terminal regions in the RecQ family. Nucleic Acids Res. 31, 2778–2785

Bernstein, D. A., Zittel, M. C., and Keck, J. L. (2003a) High-resolution structure of the E.coli RecQ helicase catalytic core .EMBO J. 22, 4910–4921

Betterton MD, Julicher F. (2003) A motor that makes its own track: helicase unwinding of DNA. Phys Rev Lett. 91(25):258103.

Biswas, N., and Weller, S. K. (1999) A Mutation in the C-terminal Putative Zn2+ Finger Motif of UL52 Severely Affects the Biochemical Activities of the HSV-1 Helicase-Primase Subcomplex J. Biol. Chem. 274, 8068-8076

Bjergbaek L, Cobb JA, Gasser SM. (2002) RecQ helicases and genome stability: lessons from model organisms and human disease. Swiss Med Wkly. Aug 10;132(31-32):433-42.

Blander G, Kipnis J, Leal JFM, Yu C-E, Schellenberg GD, Oren M. (1999). Physical and Functional Interaction between p53 and the Werner's Syndrome Protein .J. Biol. Chem., 274: 29463-29469.

Brosh JrRM, Karmakar P, Sommers JA, Yang Q, Wang XW, Spillare EA, Harris CC, Bohr VA. (2001a). p53 Modulates the Exonuclease Activity of Werner Syndrome Protein. J. Biol. Chem., 276: 35093-35102.

Brosh JrRM, Li J-L, Kenny MK, Karow JK, Cooper MP, Kureekattil RP, Hickson ID, Bohr VA. (2000a). Replication Protein A Physically Interacts with the Bloom's Syndrome Protein and Stimulates Its Helicase Activity. J. Biol. Chem., 275: 23500-23508.

Brosh JrRM, Orren DK, Nehlin JO, Ravn PH, Kenny MK, Machwe A, Bohr VA. (1999). Functional and physical interaction between WRN helicase and human replication protein A. J. Biol. Chem., 274: 18341-18350.

Brosh, Jr, R. M., Majumdar, A., Desai, S., Hickson, I. D., Bohr, V. A. and Seidman, M. M. (2001b) Unwinding of a DNA triple helix by the Werner and Bloom syndrome helicases. J. Biol. Chem. 276, 3024–3030

Brosh, R. M., Jr., Waheed, J., and Sommers, J. A. (2002) Biochemical

Characterization of the DNA Substrate Specificity of Werner Syndrome Helicase. J. Biol. Chem. 277, 23236–23245

Bujalowski, W. & Jezewska, M.J. (1995). Interactions of Escherichia coli primary replicative helicase DnaB protein with single-stranded DNA. The nucleic acid does not wrap around the protein hexamer. Biochemistry 34, 85138519.

C

Caruthers,J.M. & McKay,D.B. (2002) Helicase structure and mechanism. Curr. Opin. Struct. Biol. 12, 123-133.

Chakraverty, R. K. & Hickson, I. D. (1999) Defending genome integrity during DNA replication: a proposed role for RecQ family helicases. BioEssays 21, 286-294.

Cheok,C.F., Wu,L., Garcia,P.L., Janscak,P., & Hickson,I.D. (2005) The Bloom's syndrome helicase promotes the annealing of complementary single-stranded DNA. Nucleic Acids Res. 33, 3932-3941.

Cobb, J. A., Bjergbaek, L., Shimada, K., Frei, C., and Gasser, S. M. (2003) DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1 . EMBO J. 22, 4325–4336

Cohen, H. & Sinclair, D. A. (2001). Recombination-mediated lengthening of terminal telomeric repeats requires the Sgs1 DNA helicase. Proc. Natl Acad. Sci. USA 98, 3174-3179

Coleman JE. (1992) Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem.;61:897-946.

Constantinesco, F., Forterre, P., Koonin, E.V., Aravind, L. & Elie, C.A. (2004) . Bipolar DNA helicase gene, herA, clusters with rad50, mre11 and nurA genes in thermophilic archaea. Nucleic Acids Res. 32, 14391447.

Constantinou A, Tarsounas M, Karow JK, Brosh RM, Bohr VA, Hickson ID, West SC. (2000). Werner's syndrome protein (WRN) migrates Holliday junctions and co-localizes with RPA upon replication arrest. EMBO Rep., 1: 80-84.

Courcelle J, Hanawalt PC. (1999). RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated Escherichia coli. Mol. Gen. Genet., 262: 543-551.

Courcelle, J., Donaldson, J. R., Chow, K. H., and Courcelle, C. T. (2003). DNA Damage-Induced Replication Fork Regression and Processing in *Escherichia coli*. Science 299, 1064–1067

Cui S, Arosio D, Doherty KM, Brosh RM Jr, Falaschi A, Vindigni A. (2004) Analysis of the unwinding activity of the dimeric RECQ1 helicase in the presence of human replication protein A. Nucleic Acids Res. ;32(7):2158-70.

Cui, S., Klima, R., Ochem, A. E., Arosio, D., Falaschi, A. and Vindigni, A. (2003) Characterization of the DNA-unwinding activity of human RecQ1, a helicase specifically stimulated by human replication protein A. J. Biol. Chem. 278, 1424–1432

D

Davey S, Han CS, Ramer SA, Klassen JC, Jacobson A, Eisenberger A, Hopkins KM, Lieberman HB, Freyer GA. (1998). Fission yeast rad12+ regulates cell cycle checkpoint control and is homologous to the Bloom's syndrome disease gene. Mol. Cell. Biol., 18: 2721-2728.

Delagoutte, E. & von Hippel, P.H. (2002) Helicase mechanisms and the coupling of helicases within macromolecular machines. Part I. Structures and properties of isolated helicases. Q. Rev. Biophys. 35, 431478.

Denhardt, D.T., Dressler, D.H. & Hathaway, A. (1967) The abortive replication of NX174 DNA in a recombination-deficient mutant of Escherichia coli. Proc. Natl Acad. Sci. USA 57, 813820.

Delagoutte E and von Hippel PH. (2005) Mechanistic studies of the T4 DNA (gp41) replication helicase: functional interactions of the C-terminal Tails of the helicase subunits with the T4 (gp59) helicase loader protein.J Mol Biol. ;347(2):257-75.

Dillingham, M.S., Spies, M. & Kowalczykowski, S.C. (2003) RecBCD enzyme is a bipolar DNA helicase. Nature (London) 423, 893897.

Doherty,K.M., Sommers,J.A., Gray,M.D., Lee,J.W., von Kobbe,C., Thoma,N.H., Kureekattil,R.P., Kenny,M.K., & Brosh,R.M., Jr. (2005) Physical and functional mapping of the replication protein a interaction domain of the werner and bloom syndrome helicases. J. Biol. Chem. 280, 29494-29505.

E

Earnshaw, D.L., Moore, K.J., Greenwood, C.J., Djaballah, H., Jurewicz, A.J., Murray, K.J. & Pope, A.J. (1999). Time-resolved fluorescence energy transfer DNA helicase assays for throughput screening. J. Biomol. Screening 4, 239248.

Ellis NA, German J. (1996). Molecular genetics of Bloom's syndrome. Hum. Mol. Genet., 5: 1457-1463.

Ellis, N.A., Groden, J., Ye, T.Z., Straughen, J., Lennon, D.J., Ciocci, S., Proytcheva, M., and German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. Cell 83:655–666.

F

Faragher RGA, Kill IR, Hunter JAA, Pope FM, Tannock C, Shall S. (1993). The gene responsible for Werner syndrome may be a cell division "counting" gene. Proc. Natl. Acad. Sci. USA, 90: 12030-12034.

Foucault, F., Vaury, C., Barakat, A., Thibout, D., Planchon, P., Jaulin, C., Praz, F., and Amor-Gueret, M. 1997. Characterization of a new BLM mutation associated with a topoisomerase II alpha defect in a patient with Bloom's syndrome. Hum Mol Genet 6:1427–1434

Franchitto, A., and Pichierri, P. (2002) Protecting genomic integrity during DNA replication: correlation between Werner's and Bloom's syndrome gene products and the MRE11 complex. Hum. Mol. Genet. 11, 2447–2453

Frei C, Gasser SM. (2000a). The yeast Sgs1p helicase acts upstream of Rad53p in the DNA replication checkpoint and colocalizes with Rad53p in S-phase-specific foci. Genes Dev., 14: 81-96.

Frei C, Gasser SM. (2000b) RecQ-like helicases: the DNA replication checkpoint connection. J Cell Sci. Aug;113 (Pt 15):2641-6.

Friedberg EC, Walker GC, Siede W. (1995). DNA Repair and Mutagenesis. Washingston DC: ASM Press.

Fry, M. & Loeb, L. A. (1999). Human werner syndrome DNA helicase unwinds tetrahelical structures of the fragile X syndrome repeat sequence d(CGG)n. J. Biol. Chem. 274, 12797-12802

Fukuchi K, Martin GM, Monnat JrRJ. (1989). Mutator phenotype of Werner syndrome is characterized by extensive deletions.Proc. Natl. Acad. Sci. USA, 86: 5893-5897.

Fukuchi K, Tanaka K, Kumahara Y, Marumo K, Pride MB, Martin GM, Monnat JrRJ. (1990). Increased frequency of 6-thioguanine-resistant peripheral blood lymphocytes in Werner syndrome patients. Hum. Genet., 84: 249-252.

Furuichi Y. (2001) Premature aging and predisposition to cancers caused by mutations in RecQ family helicases. Ann N Y Acad Sci. Apr ;928:121-31.

G

Garcia PL, Liu YL, Jiricny J, West SC and Janscak P (2004) Human RECQ5β, a protein with DNA helicase and strand-annealing activities in a single polypeptide. *The EMBO Journal* **23,** 2882–2891

Garkavtsev IV, Kley N, Grigorian IA, Gudkov AV. (2001). The Bloom syndrome protein interacts and cooperates with p53 in regulation of transcription and cell growth control. Oncogene, 20: 8276-8280.

Gorbalenya, A.E., Koonin, E.V.(1993) Helicases : amino acid sequence comparisons and structure-functions relationshipsCurr. Opin. Struct. Biol. 3 :419-429.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. & Blinov, V.M. (1988) A novel superfamily of nucleoside triphosphate-binding motif containing proteins which are

probably involved in duplex unwinding in DNA and RNA replication and recombination. FEBS Lett. 235, 1624.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. & Blinov, V.M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression DNA and RNA genomes. Nucleic Acids Res. 17, 4713-4730.

Goto M. (2000). Werner's syndrome: from clinics to genetics. Clin. Exp. Rheumatol., 18: 760-766.

Gray MD, Shen J-C, Kamath-Loeb AS, Blank A, Sopher BL, Martin GM, Oshima J, Loeb LA. (1997). The Werner syndrome protein is a DNA helicase. Nat. Genet., 17: 100-103.

Groden J, German J. (1992). Bloom's syndrome. XVIII. Hypermutability at a tandem-repeat locus. Hum. Genet., 90: 360-367.

Guo RB, Rigolet P, Zargarian L, Fermandjian S, Xi XG. (2005) Structural and functional characterizations reveal the importance of a zinc binding domain in Bloom's syndrome helicase. Nucleic Acids Res. 33(10):3109-24.

H

Hakem R, Mak TW. (2001). Animal Models of Tumor-Suppressor Genes. Annul. Rev. Genet., 35: 209-241.

Han, H. & Hurley, L. H. (2000). G-quadruplex DNA: a potential target for anti-cancer drug design. Trends Pharmacol. Sci. 21, 136-142

Hanada K, Iwasaki M, Ihashi S, Ikeda H. (2000) UvrA and UvrB suppress illegitimate recombination: synergistic action with RecQ helicase. Proc Natl Acad Sci U S A. 97(11):5989-94.

Harmon FG, DiGate RJ, Kowalczykowski SC. (1999). RecQ helicase and topoisomerase III comprise a novel DNA strand passage function: a conserved mechanism for control of DNA recombination. Mol. Cell, 3: 611-620.

Harmon FG, Kowalczykowski SC. (1998). RecQ helicase, in concert with RecA and SSB proteins, initiates and disrupts DNA recombination. Genes Dev., 12: 1134-1144.

Harmon, F. G. and Kowalczykowski, S. C. (2001) Biochemical characterization of the DNA helicase activity of the *Escherichia coli* RecQ helicase. J. Biol. Chem. 276, 232–243

Harrigan, J. A., Opresko, P. L., von Kobbe, C., Kedar, P. S., Prasad, R., Wilson, S. H., and Bohr, V. A. (2003) The Werner Syndrome Protein Stimulates DNA Polymerase β Strand Displacement Synthesis via Its Helicase Activity. J. Biol. Chem. 278, 22686–22695

Hehman, G.L. & Hauswirth, W.W. (1992) DNA helicase from mammalian

mitochondria. Proc. Natl Acad. Sci. USA 89, 8562-8566.

Hicham Alaoui-Ismaili, M., Gervais, C., Brunette, S., Gouin, G., Hamel, M., Rando, R.F. & Bedard, J. (2000) .A novel high throughput screening assay for HCV NS3 helicase activity. Antiviral Res. 46, 181-193.

Hickson, I. D. (2003). RecQ Helicases: Caretakers of The Genome. Nature Reviews Cancer 3, 169-178

Hodgeman, T.C. (1988) A new superfamily of replicative proteins. Nature (London) 333, 2223.

Hotta, Y. & Stern, H. (1978) DNA unwinding protein from meiotic cells of Lilium. Biochemistry 17, 1872-1880.

Hu,J., Zeng,W., Feng,H., Lin, G., & Xi,X.G. Solution structure of a multifunctional DNA-and protein-binding motif of human Werner syndrome protein. Proc. Natl. Acad. Sci. U. S. A. **(2005, in press)**

Huang, P. et al. (2001). SGS1 is required for telomere elongation in the absence of telomerase. Curr. Biol. 11, 125-129

Huang, S. et al. (1998). The premature ageing syndrome protein, WRN, is a 3'-5' exonuclease. Nature Genet. 20, 114-116

Huang, S., Beresten, S., Li, B., Oshima, J., Ellis, N. A., and Campisi, J. (2000) Characterization of the human and mouse WRN 3'-5' exonuclease. Nucleic Acids Res. 28, 2396–2405

Huber, M. D., Lee, D. C., and Maizels, N. (2002) G4 DNA unwinding by BLM and Sgs1p: substrate specificity and substrate-specific inhibition .Nucleic Acids Res. 30, 3954–3961

Hubscher, U. & Stalder, H.P. (1985) Mammalian DNA helicase. Nucleic Acids Res. 13, 54715483.

I

Ishimi, Y. (1997) A DNA helicase activity is associated with an MCM4-6, and -7 protein complex. J. Biol. Chem. 272, 2450824513.

J

Janscak, P., Garcia, P. L., Hamburger, F., Makuta, Y., Shiraishi, K., Imai, Y., Ikeda, H., and Bickle, T. A. (2003) Characterization and Mutational Analysis of the RecQ Core of the Bloom Syndrome Protein .J. Mol. Biol. 330, 29–42

Johnson, F. B. et al. (2001). The *Saccharomyces cerevisiae* WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase. EMBO J. 20, 905-913

K

Kamath-Loeb AS, Johansson E, Burgers PMJ, Loeb LA. (2000). Functional interaction between the Werner Syndrome protein and DNA polymerase delta. Proc. Natl. Acad. Sci. USA, 97: 4603-4608.

Kamath-Loeb AS, Loeb LA, Johansson E, Burgers PMJ, Fry M. (2001). Interactions between the Werner Syndrome Helicase and DNA Polymerase Specifically Facilitate Copying of Tetraplex and Hairpin Structures of the d(CGG)n Trinucleotide Repeat Sequence. J. Biol. Chem., 276: 16439-16446.

Kamath-Loeb, A. S., Shen, J. C., Loeb, L. A. & Fry, M. (1998). Werner syndrome protein. II. Characterization of the integral 3'-5' DNA exonuclease. J. Biol. Chem. 273, 34145-34150

Kaneko, H. et al. (1997). BLM (the causative gene of Bloom syndrome) protein translocation into the nucleus by a nuclear localization signal. Biochem. Biophys. Res. Commun. 240, 348-353.

Karow JK, Chakraverty RK, Hickson ID. (1997). The Bloom's syndrome gene product is a 3'-5' DNA helicase. J. Biol. Chem., 272: 30611-30614.

Karow JK, Constantinou A, Li JL, West SC, Hickson ID. (2000). The Bloom's syndrome gene product promotes branch migration of Holliday junctions. Proc. Natl. Acad. Sci. USA, 97: 6504-6508.

Karow, J. K., Newman, R. H., Freemont, P. S., and Hickson, I. D. (1999) Oligomeric ring structure of the Bloom's syndrome helicase. Curr. Biol. 9, 597–600

Karow, J. K., Wu, L. & Hickson, I. D. (2000). RecQ family helicases: roles in cancer and aging. Curr. Opin. Genet. Dev. 10, 32-38.

Khakhar RR, Cobb JA, Bjergbaek L, Hickson ID, Gasser SM.(2003) RecQ helicases: multiple roles in genome maintenance.Trends Cell Biol. Sep;13(9):493-501.

Khu YL, Koh E, Lim SP, Tan YH, Brenner S, Lim SG, Hong WJ, Goh PY. (2001) Mutations that affect dimer formation and helicase activity of the hepatitis C virus helicase.J Virol. ;75(1):205-14.

Kim S-h, Kaminker P, Campisi J. (2002). Telomeres, aging and cancer: In search of a happy ending. Oncogene, 21: 503-511.

Kinzler KW, Vogelstein B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature, 386: 761-763.

Kitao S, Lindor NM, Shiratori M, Furuichi Y, Shimamoto A.(1999) Rothmund-thomson syndrome responsible gene, RECQL4: genomic structure and products. Genomics. Nov 1;61(3):268-76.

Korolev S, Hsieh J, Gauss GH, Lohman TM, Waksman G. (1997) Major domain swiveling revealed by the crystal structures of complexes of E. coli Rep helicase bound to single-stranded DNA and ADP. Cell. 22;90(4):635-47.

Kyono, K., Miyashiro, M. & Taguchi, I. (1998). Detection of hepatitis C virus helicase activity using the scintillation proximity assay system. Anal. Biochem. 257, 120-126.

L

Lal, S.K., Giroux, M.J., Brendel, V., Vallejos, C.E. & Hannah, L.C. (2003). The maize genome contains a Helitron insertion. Plant Cell 15, 381-391.

Lee,J.W., Kusumoto,R., Doherty,K.M., Lin,G.X., Zeng,W., Cheng,W.H., von Kobbe,C., Brosh,R.M., Jr., Hu,J.S., & Bohr,V.A. (2005). Modulation of Werner syndrome protein function by a single mutation in the conserved RQC domain. J. Biol. Chem.

Levine AJ. (1997). p53, the cellular gatekeeper for growth and division. Cell, 88: 323-331.

Leon O., Roth M. (2000) Zinc fingers: DNA binding and protein-protein interactions. Biol. Res. vol.33 n.1 Santiago 2000

Lilley,D.M.J. (1999) Structures and interactions of helical junctions in nucleic acids. In Neidle,S. (ed.), *Oxford Handbook of Nucleic Acid Structure*. Oxford

Lilley DM, Norman DG. (1999) The Holliday junction is finally seen with crystal clarity.Nat Struct Biol. 6(10):897-9.

Linder, P., Lasko, P.F., Ashburner, M., Leroy, P., Nielsen, P.J., Nishi, K., Schneir, J. & Slonimski, P.P. (1989). Birth of the DEAD-box. Nature (London) 337, 121122.

Lindor NM, Furuichi Y, Kitao S, Shimamoto A, Arndt C, Jalal S. (2000). Rothmund-Thomson syndrome due to RECQ4 helicase mutations: report and clinical and molecular comparisons with Bloom syndrome and Werner syndrome. Am. J. Med. Genet., 90: 223-228.

Liu, Z., Macias, M. J., Bottomley, M. J., Stier, G., Linge, J. P., Nilges, M., Bork, P. and Sattler, M. (1999). The three-dimensional structure of the HRDC domain and implications for the Werner and Bloom syndrome proteins. Structure 7, 1557–1566

Liu JL, Rigolet P, Dou SX, Wang PY, Xi XG. (2004) The zinc finger motif of Escherichia coli RecQ is implicated in both DNA binding and protein folding. J Biol Chem. 279(41):42794-802.

Lohman, T.M. (1992) *Escherichia coli* DNA helicases: mechanisms of DNA unwinding. Mol. Microbiol. 6, 514.

Lohman TM, Bjornson KP. (1996) Mechanisms of helicase-catalyzed DNA

unwinding. Annu Rev Biochem. 65:169-214.

Lu J, Mullen JR, Brill SJ, Kleff S, Romeo AM, Sternglanz R. (1996). Human homologues of yeast helicase. Nature, 383: 678-679.

Luo G, Santoro IM, McDaniel LD, Nishijima I, Mills M, Youssoufian H, Vogel H, Schultz RA, Bradley A. (2000). Cancer predisposition caused by elevated mitotic recombination in Bloom mice. Nat. Genet., 26: 424-429.

M

Machwe, A., Xiao, L., Groden, J., Matson, S.W., & Orren, D.K. (2005) RecQ family members combine strand pairing and unwinding activities to catalyze strand exchange. J. Biol. Chem. 280, 23397-23407.

Macris,M.A., Krejci,L., Bussen,W., Shimamoto,A., & Sung,P. (2005) Biochemical characterization of the RECQ4 protein, mutated in Rothmund-Thomson syndrome. DNA Repair (Amst).

Maluf NK, Ali JA, Lohman TM. **(2003)** Free Full Text Kinetic mechanism for formation of the active, dimeric UvrD helicase-DNA complex.
J Biol Chem ;278(34):31930-40.

Matson, S.W. (1991) DNA helicases of Escherichia coli. Prog. Nucleic Acids Res. Mol. Biol. 40, 289-326.

Matson, S.W., Tabor, S. & Richardson, C.C. (1983). The gene 4 protein of bacteriophage T7. Characterization of helicase activity. J. Biol. Chem. 258, 14017-14024.

Matsumoto, T., Shimamoto, A., Goto, M. & Furuichi, Y. (1997). Impaired nuclear localization of defective DNA helicases in Werner's syndrome. Nature Genet. 16, 335-336

Mechanic, L.E., Hall, M.C. & Matson, S.W. (1999) *Escherichia coli* DNA helicase II is active as a monomer. J. Biol. Chem. 274, 1228812498.

Miller J, AD McLachlan and A Klug, (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J 4, pp. 1609–1614.

Mohaghegh, P. & Hickson, I. D. (2001). DNA helicase deficiencies associated with cancer predisposition and premature ageing disorders. Hum. Mol. Genet. 10, 741-746.

Mohaghegh, P., Karow, J. K., Brosh, J. R., Jr., Bohr, V. A., and Hickson, I. D. (2001b) The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases .Nucleic Acids Res. 29, 2843–2849

Monnat JrRJ, Hackmann AF, Chiaverotti TA. (1992). Nucleotide sequence analysis of human hypoxanthine phosphoribosyltransferase (HPRT) gene deletions.

Genomics, 13: 777-787.

Morozov, V., Mushegian, A. R., Koonin, E. V. & Bork, P. (1997). A putative nucleic acid-binding domain in Bloom's and Werner's syndrome helicases. Trends Biochem. Sci. 22, 417-418

Morris, P.D., Tackett, A.J., Babb, K., Nanduri, B., Chick, C., Scott, J. & Raney, K.D. (2001) Evidence for a functional monomeric form of the bacteriophage T4 DdA helicase. DdA does not form stable oligomeric structures. J. Biol. Chem. 276, 19691-19698.

Mullen JR, Kaliraman V, Brill SJ. (2000) Bipartite structure of the SGS1 DNA helicase in Saccharomyces cerevisiae. Genetics. 154(3):1101-14.

Murray JM, Lindsay HD, Munday CA, Carr AM. (1997). Role of Schizosaccharomyces pombe RecQ homolog, recombination, and checkpoint genes in UV damage tolerance. Mol. Cell. Biol., 17: 6868-6875.

Murray RK, Granner DK, Mayes PA, Rodwell VW (2000) Harper's Biochemistry, 25, 402-404

Myung K, Datta A, Chen C, Kolodner RD. (2001). SGS1, the *Saccharomyces cerevisiae* homologue of BLM and WRN, suppresses genome instability and homeologous recombination.Nat. Genet., 27: 113-116.

N

Nakagawa, T. and Ogawa, H. (1999). The Saccharomyces cerevisiae MER3 gene, encoding a novel helicase-like protein, is required for crossover control in meiosis. The EMBO Journal, 18, 5714–5723

Nakayama, H., (2002) RecQ family helicases: roles as tumor suppressor proteins. Oncogene 21, 9008 – 9021

Nakayama, H., Nakayama, K., Nakayama, R., Irino, N., Nakayama, Y. & Hanawalt, P.C. (1984) Isolation and genetic characterization of a thymineless death-resistant mutant of *Escherichia coli* K12: identification of a new mutation (rec Q) that blocks the Rec F recombination pathway. Mol. Gen. Genet. 195, 474-480.

Nakayama, K., Irino, N., and Nakayama, H. (1985) The recQ gene of Escherichia coli K12: molecular cloning and isolation of insertion mutants. Mol. Gen. Genet. 200, 266-271

Naqvi, A., Tinsley, E. & Khan, S.A. (2003) Purification and characterization of the PcrA helicase of Bacillus anthracis. J. Bacteriol. 185, 6633-6639.

Neff, N.F., Ellis, N.A., Ye, T.Z., Noonan, J., Huang, K., Sanz, M., and Proytcheva, M. (1999). The DNA helicase activity of BLM is necessary for the correction of the genomic instability of bloom syndrome cells. Mol Biol Cell 10:665–676.

O

Oakley TJ, Hickson ID. (2002). Defending genome integrity during S-phase: putative roles for RecQ helicases and topoisomerase III. DNA Repair, 1: 175-207

Ogilvie VC, Wilson BJ, Nicol SM, Morrice NA, Saunders LR, Barber GN, Fuller-Pace FV. (2003) The highly related DEAD box RNA helicases p68 and p72 exist as heterodimers in cells. Nucleic Acids Res. ;31(5):1470-80.

Onoda F, Seki M, Miyajima A, Enomoto T. (2000). Elevation of sister chromatid exchange in Saccharomyces cerevisiae sgs1 disruptants and the relevance of the disruptants as a system to evaluate mutations in Bloom's syndrome gene. Mutat. Res., 459: 203-209.

Onoda F, Seki M, Miyajima A, Enomoto T. (2001). Involvement of SGS1 in DNA damage-induced heteroallelic recombination that requires RAD52 in Saccharomyces cerevisiae. Mol. Gen. Genet., 264: 702-708.

Opresko, P. L., Cheng, W. H., von Kobbe, C., Harrigan, J. A., and Bohr, V. A. (2003) . Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. Carcinogenesis 24, 791–802

Opresko, P. L., Laine, J. P., Brosh, R. M. Jr, Seidman, M. M. & Bohr, V. A. (2001). Coordinate action of the helicase and 3' to 5' exonuclease of Werner syndrome protein. J. Biol. Chem. 276, 44677-44687.

Opresko, P. L., Cheng, W. H. and Bohr, V. A. (2004) Junction of RecQ Helicase Biochemistry and Human Disease. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* Vol. 279, No. 18, pp. 18099–18102.

Orren, D. K., Theodore, S., and Machwe, A. (2002) The Werner syndrome helicase/exonuclease (WRN) disrupts and degrades D-loops in vitro. Biochemistry 41, 13483–13488

Oshima J, Huang S, Pae C, Campisi J, Schiestl RH. (2002). Lack of WRN results in extensive deletion at nonhomologous joining ends. Cancer Res., 62: 547-551.

Ozsoy, A. Z., Ragonese, H. M., and Matson, S. W. (2003) Analysis of helicase activity and substrate specificity of Drosophila RECQ5 . Nucleic Acids Res. 31, 1554–1564

Özsoy, A. Z., Sekelsky, J. J. and Matson, S. W. (2001) Biochemical characterization of the small isoform of *Drosophila melanogaster* RECQ5 helicase. Nucleic Acids Res. 29, 2986–2993

P

Pham, X.H., Reddy, M.K., Ehtesham, N.Z., Matta, B. & Tuteja, N. (2000) A DNA helicase from Pisum sativum is homologous to translation initiation factor and stimulates topoisomerase I activity. Plant J. 24, 219229.

Poplawski, A., Grabowski, B., Long, S. E. and Kelman, Z. (2001). The Zinc Finger Domain of the Archaeal Minichromosome Maintenance Protein Is Required for Helicase Activity. J. Biol. Chem., Vol. 276, Issue 52, 49371-49377

Prince PR, Emond MJ, Monnat JrRJ. (2001). Loss of Werner syndrome protein function promotes aberrant mitotic recombination. Genes Dev., 15: 933-938.

Puranam, K. L., and Blackshear, P. J. (1994) Cloning and characterization of RECQL, a potential human homologue of the Escherichia coli DNA helicase RecQ. J. Biol. Chem. 269, 29838-29845

R

Raney, K.D., Sowers, L.C., Millar, D.P. & Benkovic, S.J. (1994). A fluorescence-based assay for monitoring helicase activity. Proc. Natl Acad. Sci. USA 91, 66446648.

Rong, S. B., Väliaho, J. and Vihinen, M. (2000) Structural basis of Bloom syndrome (BS) causing mutations in the BLM helicase domain. Mol. Med. 6, 155–164

S

Salk D, Au K, Hoehn H, Martin GM. (1981). Cytogenetics of Werner's syndrome cultured skin fibroblasts: variegated translocation mosaicism. Cytogenet. Cell Genet., 30: 92-107.

Sekelsky JJ, Brodsky MH, Rubin GM, Hawley RS.(1999) Drosophila and human RecQ5 exist in different isoforms generated by alternative splicing. Nucleic Acids Res. Sep 15;27(18):3762-9.

Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., and Yamaoka, T. (1994a) Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12. *Nucleic Acids Res.* **22**, 4566-4573

Seki M, Yanagisawa J, Kohda T, Sonoyama T, Ui M, Enomoto T. (1994b). Purification of two new DNA-dependent adenosine triphosphatases having DNA helicase activity from HeLa cells and comparison of the properties of the two enzymes. J. Biochem. (Tokyo), 115: 523-531.

Sengupta, S., Linke, S. P., Pedeux, R., Yang, Q., Farnsworth, J., Garfield, S. H., Valerie, K., Shay, J. W., Ellis, N. A., Wasylyk, B., and Harris, C. C. (2003) BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination . EMBO J. 22, 1210–1222

Seybert, A., Posthuma, C. C., van Dinten, L.C., Snijder, E.J., Gorbalenya, A. E. , and Ziebuhr, J. (2005). A Complex Zinc Finger Controls the Enzymatic Activities of Nidovirus Helicases. Journal of Virology, 79 (2): 696-704.

Sharma,S., Sommers,J.A., Choudhary,S., Faulkner,J.K., Cui,S., Andreoli,L., Muzzolini,L., Vindigni,A., & Brosh,R.M., Jr. (2005) Biochemical analysis of the DNA unwinding and strand annealing activities catalyzed by human RECQ1. J. Biol. Chem. 280, 28072-28084**.**

Shen J-C, Gray MD, Oshima J, Loeb LA. (1998b). Characterization of Werner syndrome protein DNA helicase activity: directionality, substrate dependence and stimulation by replication protein A. Nucleic Acids Res., 26: 2879-2885.
Shen, J. C. et al. (1998). Werner syndrome protein. I. DNA helicase and DNA exonuclease reside on the same polypeptide. J. Biol. Chem. 273, 34139-34144

Shen, J. C., and Loeb, L. A. (2000) Werner syndrome exonuclease catalyzes structure-dependent degradation of DNA . Nucleic Acids Res. 28, 3260–3268

Shimamoto, A., Nishikawa, K., Kitao, S. & Furuichi, Y. (2000). Human RecQ5, a large isomer of RecQ5 DNA helicase, localizes in the nucleoplasm and interacts with topoisomerases 3 . Nucleic Acids Res. 28, 1647-1655

Siitonen, H.A., Kopra, O., Kaariainen, H., Haravuori, H., Winter, R.M., Saamanen, A.M., Peltonen, L., and Kestila, M. (2003). Molecular defect of RAPADILINO syndrome expands the phenotype spectrum of RECQL diseases. Hum Mol Genet 12:2837–2844.

Singleton, M.R., Sawaya, M.R., Ellenberger, T. & Wigley, D.B. (2000). Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. Cell 101, 589600.

Singleton MR, Wentzell LM, Liu Y, West SC, Wigley DB. (2002b) Structure of the single-strand annealing domain of human RAD52 protein. Proc Natl Acad Sci U S A. 99(21):13492-7.

Singleton MR, Wigley DB. (2002a) Modularity and specialization in superfamily 1 and 2 helicases. J Bacteriol. Apr;184(7):1819-26.

Sivaraja, M., Giordano, H. & Peterson, M.G. (1998). High-throughput screening assay for helicase enzymes. Anal. Biochem. 265, 2227.

Sonoda E, Sasaki MS, Morrison C, Yamaguchi-Iwai Y, Takata M, Takeda S. (1999). Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. Mol. Cell. Biol., 19: 5166-5169.

Soultanas P, Wigley DB. (2000) DNA helicases: 'inching forward'. Curr Opin Struct Biol. Feb;10(1):124-8.

Soultanas, P. & Wigley, D. B. (2001) Unwinding the 'Gordian knot' of helicase action. Trends Biochem. Sci. 26, 47-54.

Spillare EA, Robles AI, Wang XW, Shen J-C, Yu C-E, Schellenberg GD, Harris CC. (1999). p53-mediated apoptosis is attenuated in Werner syndrome cells. Genes

Dev., 13: 1355-1360.

Stahl, H., Droge, P. & Knippers, R. (1986) DNA helicase activity of SV40 large tumor antigen. EMBO J. 5, 19391944.

Stewart E, Chapman CR, Al-Khodairy F, Carr AM, Enoch T. (1997). rqh1+, a fission yeast gene related to the Bloom's and Werner's syndrome genes, is required for reversible S phase arrest. EMBO J., 16: 2682-2692.

Subramanya, H.S., Bird, L.E., Brannigan, J.A. & Wigley, D.B. (1996) Crystal structure of a DExx box DNA helicase. Nature (London) 384, 379383.

Sugino, A., Bung, H.R., Sugino, T., Naumovski, L. & Friedberg, E.C. (1986) A new DNA-dependent ATPase which stimulates yeast DNA polymerase I and has DNA-unwinding activity. J. Biol. Chem. 261, 1174411750.

Sun, H., Bennett, R. J. & Maizels, N. (1999). The Saccharomyces cerevisiae Sgs1 helicase efficiently unwinds G-G paired DNAs. Nucleic Acids Res. 27, 1978-1984

Sun, H., Karow, J. K., Hickson, I. D. & Maizels, N. (1998). The Bloom's syndrome helicase unwinds G4 DNA. J. Biol. Chem. 273, 27587-27592

Suzuki N, Shimamoto A, Imamura O, Kuromitsu J, Kitao S, Goto M, Furuichi Y. (1997). DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. Nucleic Acids Res., 25: 2973-2978.

T

Tachibana A, Tatsumi K, Masui T, Kato T. (1996). Large deletions at the HPRT locus associated with the mutator phenotype in a Bloom's syndrome lymphoblastoid cell line. Mol. Carcinog., 17: 41-47.

Tanner, N.K., Cordin, O., Banroques, J., Doère, M. & Linder, P. (2003) The Q MOTIF: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. Mol. Cell 11, 127138.

Thompson LH, Schild D. (1999). The contribution of homologous recombination in preserving genome integrity in mammalian cells.Biochimie, 81: 87-105.

Tsaneva, I.R., Muller, B. & West, S.C. (1993). RuvA and RuvB proteins of Escherichia coli exhibit DNA helicase activity in vitro. Proc. Natl Acad. Sci. USA 90, 1315-1319.

Tuteja, N. & Tuteja, R. (2004) Prokaryotic and eukaryotic DNA helicases:Essential molecular motor proteins for cellular machinery. Eur. J. Biochem. 271, 1835–1848

Tuteja, N. & Tuteja, R. (1996) DNA helicases: the long unwinding road. Nat. Genet. 13, 1112.

Tuteja, N. (2003) Plant DNA helicases: the long unwinding road. J. Exp. Bot. 54,

22012214.

Tuteja, N., Beven, A.F., Shaw, P.J. & Tuteja, R. (2001) A pea homologue of human DNA helicase I is localized within the dense fibrillar component of the nucleolus and stimulated by phosphorylation with CK2 and cdc2 protein kinases. Plant J. 25, 917.

Tuteja, N., Phan, T.N. & Tewari, K.K. (1996) Purification and characterization of a DNA helicase from pea chloroplast that translocates in the 3'-to-5' direction. Eur. J. Biochem. 238, 5463.

Tuteja, N., Tuteja, R., Rahman, K., Kang, L.-Y. & Falaschi, A. (1990) A DNA helicase from human cells. Nucleic Acids Res. 18, 67856792.

Tuteja, R., Malhotra, P., Song, P., Tuteja, N. & Chauhan, V.S. (2002) Isolation and characterization of eIF-4A homologue from *Plasmodium cynomolgi*. Mol. Biol. Parasitol. 124, 7983.

U

Umezu K, Nakayama H. (1993). RecQ DNA helicase of *Escherichia coli*. Characterization of the helix-unwinding activity with emphasis on the effect of single-stranded DNA-binding protein. J. Mol. Biol., 230: 1145-1150.

Umezu K, Nakayama K, Nakayama H. (1990). *Escherichia coli* RecQ protein is a DNA helicase. Proc. Natl. Acad. Sci. USA, 87: 5363-5367.

V

van Brabant, A. J., Stan, R. & Ellis, N. A. (2000). DNA helicases, genomic instability, and human genetic disease. Annu. Rev. Genomics Hum. Genet. 1, 409-459

van Brabant, A. J., Ye, T., Sanz, M., German, J. L., III, Ellis, N. A., and Holloman, W. K. (2000) Binding and melting of D-loops by the Bloom syndrome helicase. Biochemistry 39, 14617–14625

van Dinten, L. C., van Tol, H., Gorbalenya, A. E., and Snijder, E. J. (2000) The Predicted Metal-Binding Region of the Arterivirus Helicase Protein Is Involved in Subgenomic mRNA Synthesis, Genome Replication, and Virion Biogenesis . J. Virol. 74, 5213-5223

Velankar SS, Soultanas P, Dillingham MS, Subramanya HS, Wigley DB. (1999) Crystal structures of complexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism. Cell. 97(1):75-84.

Venkatesan, M., Silver, I.L. & Nossal, N.G. (1982). Bacteriophage T4 gene 41 protein, required for the synthesis of RNA primers, is also a DNA helicase. J. Biol. Chem. 257, 1242612434.

von Kobbe, C., Thoma, N. H., Czyzewski, B. K., Pavletich, N. P., and Bohr, V. A. (2003) Werner Syndrome Protein Contains Three Structure-specific DNA Binding

Domains. J. Biol. Chem. 278, 52997–53006

W

WANG L, OGBURN CE, WARE CB, LADIGES WC, YOUSSOUFIAN H, MARTIN GM, OSHIMA J. (2000b) Cellular Werner phenotypes in mice expressing a putative dominant-negative human WRN gene.Genetics. Jan ;154(1) :357-62.

Wang W, Seki M, Narita Y, Sonoda E, Takeda S, Yamada K, Masuko T, Katada T, Enomoto T. (2000a). Possible association of BLM in decreasing DNA double strand breaks during DNA replication.EMBO J., 19: 3428-3435.

Wang XW, Tseng A, Ellis NA, Spillare EA, Linke SP, Robles AI, Seker H, Yang Q, Hu P, Beresten S, Bemmels NA, Garfield S, Harris CC. (2001). Functional interaction of p53 and BLM DNA helicase in apoptosis. J. Biol. Chem., 276: 32948-32955.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. (2000). BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures . Genes Dev. 14, 927–939

Watson, J.D. & Crick, F.H.C. (1953) Molecular structure of nucleic acids. Nature (London) 191, 737738.

Watt PM, Hickson ID, Borts RH, Louis EJ. (1996). SGS1, a homologue of the Bloom's and Werner's syndrome genes, is required for maintenance of genome stability in Saccharomyces cerevisiae. Genetics, 144: 935-945.

West, S.C. (1996) DNA helicases: new breeds of translocating motors and molecular pumps. Cell 86, 177180.

Whitby, M.C., Vincent, S.D. & Lloyd, R.G. (1994). Branch migration of Holliday junctions: identification of RecG protein as a junction specific DNA helicase. EMBO J. 13, 52205228.

Wu, L., Chan, K. L., Ralf, C., Bernstein, D. A., Garcia, P.L., Bohr, V. A., Vindigni, A., Janscak, P., Keck, J. L. and Hickson, I.D. (2005) The HRDC domain of BLM is required for the dissolution of double Holliday junctions. *The EMBO Journal* **24,** 2679–2687.

Wu, X. & Maizels, N. (2001). Substrate-specific inhibition of RecQ helicase. Nucleic Acids Res. 29, 1765-1771

X

Xu, H. Q., Deprez, E., Zhang, A. H., Tauc, P., Ladjimi, M. M., Brochon, J. C., Auclair, C., and Xi, X. G. (2003a) The *Escherichia coli* RecQ Helicase Functions as a Monomer* . J. Biol. Chem. 278, 34925–34933

Xu, H.Q., Zhang, A.H., Auclair, C. & Xi, X.G. (2003b). Simultaneously monitoring

DNA binding and helicase-catalyzed DNA unwinding by fluorescence polarization. Nucleic Acids Res. 31,

Y

Yao N, Hesson T, Cable M, Hong Z, Kwong AD, Le HV, Weber PC. (1997) Structure of the hepatitis C virus RNA helicase domain. Nat Struct Biol. 4(6):463-7.

Yarranton, G.T. & Gefter, M.L. (1979) Enzyme catalyzed DNA unwinding: studies on *Escherichia coli* rep protein. Proc. Natl Acad. Sci. USA 76, 16581662.

Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, Martin GM, Mulligan J, Schellenberg GD.(1996) Positional cloning of the Werner's syndrome gene. Science. Apr 12;272(5259):258-62.

Z

Zhang, L., Schwartz, G., O'Donnell, M. & Harrison, R. (2001). Development of a novel helicase assay using electrochemiluminescence. Anal. Biochem. 293, 3137.