Isolation and characterization of a processive DNA helicase from the fission yeast *Schizosaccharomyces pombe* that translocates in a 5'-to-3' direction

Changwoo LEE and Yeon-Soo SEO¹

Nucleic Acid Biochemistry Laboratory, Basic Research Center, Samsung Biomedical Research Institute, 50 Ilwon-Dong, Kangnam-Ku, Seoul, 135-230, Korea

We report here the isolation and characterization of a novel DNA helicase from extracts of the fission yeast *Schizosaccharomyces pombe*. The enzyme, called DNA helicase II, also contains an intrinsic DNA-dependent ATPase activity. Both the helicase and ATPase activities co-purified with a 63 kDa polypeptide on an SDS/polyacrylamide gel. The protein has a sedimentation coefficient of 4.8 S and a Stokes radius of 36 Å (3.6 nm); from these data the native molecular mass was calculated to be 65 kDa. The enzyme translocates in a 5'-to-3' direction with respect to the substrate strand to which it is bound. Unwinding reactions carried out in the presence of increasing enzyme showed a sigmoidal curve, suggesting either co-operative interactions between monomers or multimerization of DNA helicase II in the presence of single-stranded DNA and/or ATP. This enzyme favoured adenosine nucleotides (ATP and dATP) as its energy source, but utilized to limited extents GTP, CTP,

dGTP and dCTP. Non-hydrolysable ATP analogues did not support helicase activity. Kinetic analyses showed that the unwinding reaction was rapid, being complete after 50–100 s of incubation. Addition of unlabelled substrates to the helicase reaction after preincubation of the enzyme with substrate did not significantly diminish unwinding. The ATPase activity of DNA helicase II increased proportionally with increasing lengths of single-stranded DNA cofactor. In the presence of circular DNA, ATP hydrolysis continued to increase up to the longest time tested (3 h), whereas it ceased to increase after 5–10 min in the presence of shorter oligonucleotides. The initial rate of ATP hydrolysis during the first 5 min of incubation time was not affected by DNA species used. These data indicate that the enzyme does not dissociate from the single-stranded DNA once it is bound and is therefore highly processive.

INTRODUCTION

DNA replication, repair and recombination are critical processes for living organisms and are generally carried out by complex collections of interacting proteins. One essential component of these so-called molecular machines is a DNA-dependent nucleoside triphosphatase (NTPase). Some NTPases also promote the unwinding of the double-helical DNA, which is one of the most thermodynamically stable biological molecules known [1–3]. These enzymes, called DNA helicases, can transiently abolish the stable helical structure of DNA by harnessing energy derived from the hydrolysis of NTPs. This energy is required to overcome the thermodynamic energy barrier imposed by molecular interactions between the bases of DNA and for the helicase to translocate along the DNA strands to which it is bound. The single-stranded DNA (ssDNA) generated in the helicase reaction is utilized by other enzymes that participate in DNA replication, repair and recombination. The unwinding activity catalysed by a helicase exhibits a specific directionality (5'-to-3' or 3'-to-5') with few exceptions.

DNA helicases have been isolated from a wide variety of sources from bacteria to humans, as well as bacteriophages and animal viruses. The biochemical properties of a number of DNA helicases have been reviewed extensively [1–5]. Since the detection of the first DNA helicase (*Escherichia coli* DNA helicase I) [6], more than 60 distinct DNA helicase activities have been described [2]. Most organisms contain multiple helicases; for example, at least 12 different DNA helicases have been identified in *E. coli* [7]. Eight distinct helicases have been identified in *Saccharomyces cerevisiae* [8], and more than 15 from various types of mammalian

cells, including those from murine, bovine and human sources [4,5,9–14]. The recent advances in genome sequencing projects of many organisms, coupled with computer-assisted comparisons of the amino acid sequence motifs conserved in helicase enzymes [15] have revealed the presence of a large number of putative DNA and RNA helicases. For example, the completed *S. cerevisiae* genome database [16] contains more than 80 open reading frames with conserved helicase sequence motifs. Thus the list of identified DNA helicases should continue to grow as the proteins encoded by these open reading frames are studied biochemically.

The presence of multiple DNA helicases in individual cells probably reflects the variety and complexity of DNA metabolic reactions and the distinct structural template requirements for a given helicase. The precise *in vivo* functions of specific helicases can be best deciphered when both genetic and biochemical approaches are available. The best-characterized helicases are prokaryotic and eukaryotic viral replicative helicases [17–25] and those involved in nucleotide excision repair and basal transcription [26–29]. The replicative helicases of *E. coli*, T7, and T4 phages (DnaB, T7 gene 4 and T4 gene 41 proteins respectively) participate in the movement of the replication fork, and they share similar properties [17–19]. For example, all three bind the lagging-strand template and translocate in the 5'-to-3' direction along with the replication fork. The helicase activities of these proteins are stimulated greatly by the presence of a replication-fork-like structure on the DNA substrate. Simian-virus-40 (SV40) T antigen, bovine papilloma-virus-1 E1, and herpes simplex-virus-1 UL9 are the most extensively characterized eukaryotic viral replicative helicases. Unlike prokaryotic replicative heli-

Abbreviations used: NTPase, nucleoside triphosphatase; ssDNA, single-stranded DNA; ssc, single-stranded circular; SSB, ssDNA-binding protein; RPA, replication protein A; SpRPA, *Schizosaccharomyces pombe* RPA; ATP[S], adenosine 5'-[γ-thio]triphosphate; p[NH]ppA, adenosine 5'-[β,γ-imido]triphosphate; p[CH₂]ppA, adenosine 5'-[β,γ-methylene]triphosphate; DTT, dithiothreitol; SP-, sulphonylpropyl-; SV40, simian virus 40.

¹ To whom correspondence should be addressed (e-mail ysseo@smc.samsung.co.kr).

cases, they all translocate in a 3'-to-5' direction and are capable of unwinding duplex DNA containing their cognate origins of replication [20–24].

In our laboratory we focus on the systematic isolation of DNA helicases from *Schizosaccharomyces pombe* in an effort to identify the elusive eukaryotic helicase(s) involved in cellular DNA replication. The Dna2 protein, a DNA helicase from *S. cerevisiae* identified recently by genetic screening and biochemical studies, has been implicated in eukaryotic cellular DNA replication [30,31]. However, its association with Fen-1, a protein involved in Okazaki fragment maturation, implies that Dna2 is likely to function in lagging-strand synthesis rather than in propagation of replication forks [32]. This observation suggests that the eukaryotic helicase involved in replication-fork movement has not yet been identified. In previous studies we described the isolation of the DNA helicase I from *S. pombe* cells [33]. Cloning of the gene encoding DNA helicase I and sequence homology comparison (J. S. Park and Y.-S. Seo, unpublished work) suggests that the enzyme belongs to the *E. coli* DNA helicase II family, which is involved in DNA repair. In a continuing effort to identify a replicative helicase from eukaryotic cells, we have isolated another novel DNA helicase from fission yeast that displays properties different from those of DNA helicase I. In the present paper we report the purification and characterization of this DNA helicase, termed DNA helicase II.

EXPERIMENTAL

DNA, oligonucleotides and NTPs

Bacteriophage- ϕ X174 single-stranded circular DNA (ssDNA) was purchased from New England Biolabs. M13mp18 ssDNA was prepared as described in [34]. Nucleotides (NTPs, dNTPs and ATP analogues) were obtained from Boehringer-Mannheim, and [α - 32 P]dCTP and [γ - 32 P]ATP (3000 Ci/mmol each) were purchased from Amersham International. Various polynucleotides such as poly(U) and poly(dI·dC) were from Pharmacia Biotech or Life Sciences. Oligonucleotides (24-, 52-, 55-, 73- and 81-mers) were commercially synthesized and electrophoretically purified. The 52-mer sequence (5'-CGA ACA ATT CAG CGG CTT TAA CCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3') is complementary to ϕ X174 DNA at nucleotide positions 703–754 [35] and contains an *Hpa*II restriction site (underlined). The 73-mer (5'-tailed oligonucleotide) contains additional 21 nucleotides (5'-AAT CAT AGA TAG CAT CTC CGT-3') added to the 5' region of the 52-mer, whereas the 81-mer (3'-tailed oligonucleotide) contains an additional 29 nucleotides (5'-TTA CCC CAG TCG CGG AAT ACT ATT TCA AA -3') at the 3' end. The 55-mer (5'-TTT AGA GCT TGA CGG GGA AAG CCG GCG AAC GTG GCG AGA AAG GAA GGG AAG AAA G-3') contains a sequence complementary to M13mp18 at nucleotide positions 5592–5647 [36] and an *Hpa*II restriction site (underlined). The 24-mer sequence (5'-GGG AAA ACA TTA TTA ATG GCG TCG-3') and the 73-mer (5'-tailed oligonucleotide) were used to measure size-dependent ATP hydrolysis by DNA helicase II.

Enzymes and proteins

The following proteins were obtained commercially: *E. coli* ssDNA-binding protein (SSB) and T4 g32 (Pharmacia), restriction enzymes (Promega), the Klenow fragment of *E. coli* DNA polymerase I (Boehringer-Mannheim) and polynucleotide kinase (Bio-Rad). Recombinant *S. pombe* replication protein A (SprPA) was isolated from an *E. coli* SprPA-overproducing

strain [37] with slight modifications of the method described in [33].

Preparation of various DNA helicase substrates

The substrate used throughout to monitor DNA helicases with 5'-to-3' directionality was prepared as follows: the annealing reaction mixture (30 μ l), which consisted of M13mp18 ssDNA (6 pmol), the 55-mer oligonucleotide (90 pmol) and buffer [25 mM Tris/HCl (pH 7.8)/1 mM EDTA/300 mM NaCl] was incubated at 94 °C for 3 min and cooled slowly (–0.5 °C/10 min interval) to 23 °C in a PCR thermocycler (MJ Research, Watertown, MA, U.S.A.). The annealed substrate DNA was labelled with Klenow fragment and [α - 32 P]dCTP (0.15 μ M) in a 60 μ l reaction volume at 23 °C for 30 min, and then supplemented with excess non-radioactively labelled dCTP (200 μ M). The reaction mixture was incubated at 23 °C for an additional 10 min, terminated by adding EDTA to 10 mM, and then filtered through Sepharose CL-4B (1 ml; Pharmacia) to remove un-annealed oligonucleotides and free nucleotides. The recovery (80–90 %) and specific radioactivity (3000–3500 c.p.m./fmol) of the substrate DNA was calculated on the basis of values obtained by liquid-scintillation counting of acid-insoluble precipitates before and after the Sepharose CL-4B fractionation step. The substrate was then digested with *Hpa*II (150 μ l reaction volume; 2 h; 37 °C) to linearize the circular DNA. This substrate contains a partial duplex at each end of the linearized M13 ssDNA with only one end labelled. The labelled fragment can be released only by DNA helicases with 5'-to-3' polarity (cf. Figure 5A below).

ϕ X174 DNA that was annealed to the 52-mer was also prepared as described above and used for standard reactions to characterize the biochemical properties of purified DNA helicase II. The substrate used to measure the maximal length of duplex DNA unwound by the helicase was prepared as described in [33]. Oligonucleotides (5 pmol each) for the 3'-tailed (81-mer) or 5'-tailed (73-mer) substrates were first labelled at their 5' ends with polynucleotide kinase (Bio-Rad) and [γ - 32 P]ATP (10 pmol; 3000 Ci/mmol) (20 μ l reaction volume; 1 h; 37 °C). The labelling efficiency of each oligonucleotide was determined by measuring trichloroacetic acid-precipitable radioactivity in the reaction after incubation. The 5'-labelled oligonucleotides were then annealed to ϕ X174 ssDNA (2 pmol) and purified as described above. The recovery (60–80 %) and specific radioactivity ($\approx$ 2000 c.p.m./fmol) of the substrate preparations were calculated by measuring the ratio of ϕ X174 ssDNA-annealed oligonucleotide to free oligonucleotide using non-denaturing PAGE in 1 $\times$ TBE (89 mM Tris base/89 mM boric acid/2 mM EDTA). The substrate used to determine the directionality of DNA helicase movement was constructed by hybridizing the 52-mer oligonucleotide to ϕ X174 ssDNA as described previously [33].

Helicase assays

The standard reaction mixture (20 μ l) contained 25 mM Tris/HCl (pH 7.5), 2 mM ATP, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 200 μ g/ml BSA and 15 fmol of 32 P-labelled DNA substrate. Because the displacement reaction occurred even at 0 °C (see Figure 6 below), all reaction mixtures were assembled on ice without an energy source, and reactions were initiated by adding ATP to 2 mM. Reactions were incubated for 3 min at 37 °C and terminated with 4 μ l of 6 $\times$ stop solution [60 mM EDTA (pH 8.0)/40 % (w/v) sucrose/0.6 % SDS/0.25 % Bromophenol Blue/0.25 % Xylene Cyanol]. The reaction products were subjected to electrophoresis at 150 V for 60 min through 10 %

(w/v)-polyacrylamide gels containing 0.1% SDS in 0.5 × TBE. The products were analysed and quantitated using a PhosphorImager (Molecular Dynamics).

ATPase assays

DNA-dependent ATPase activity was measured in a reaction mixture (20 µl) containing 25 mM Tris/HCl (pH 7.8), 2 mM DTT, 2 mM MgCl₂, 200 µg/ml BSA, 125 µM non-radioactively labelled ATP (2.5 nmol total), 10 nM [γ -³²P]ATP (0.2 pmol total; > 5000 Ci/mmol) and 50 ng of M13 ssDNA or other polynucleotides as indicated. After incubation at 37 °C for 30 min, an aliquot (1 µl) was spotted on to a polyethyleneimine-cellulose plate (J. T. Baker Co.) and developed with 0.5 M LiCl/1.0 M formic acid. The products were analysed and quantified with the use of a PhosphorImager.

Preparation of *S. pombe* extracts

The wild-type fission yeast (strain 972h⁻) *S. pombe* was inoculated to an A₆₀₀ of 0.05 in 50 litres of YD media (5 g of yeast extract and 30 g of dextrose/litre; Difco), grown in a fermenter (Bio-engineering, Wald, Switzerland) at 30 °C with vigorous aeration to mid-exponential phase (A₆₀₀ ≈ 4.0), and harvested by centrifugation at 4000 rev./min for 10 min in a Beckman HS-2 rotor. Cell pellets (120 g wet weight) were washed twice with cold water and resuspended in 240 ml of suspension buffer [50 mM Hepes/KOH (pH 7.5)/1% DMSO/1 mM EDTA/1 mM EGTA/1 M NaCl/1 mM PMSF/1 mM benzamide/1.5 µg/ml leupeptin and antipain/1.0 µg/ml pepstatin A/1 mM DTT]. The cells were disrupted in a Bead beater chamber (Biospec Products, Bartlesville, OK, U.S.A.) with eight cycles of a 30 s pulse and a 7 min cooling interval. Whole-cell extracts were collected by filtration, and DNA was removed by precipitation with polyethyleneimine (0.1%, v/v). The extracts were then cleared by centrifuging for 1 h at 37000 rev./min in a Beckman 45Ti rotor, and the supernatant was collected, dialysed for 3 h against 2 litres of buffer T [25 mM Tris/HCl (pH 7.5)/1 mM EDTA/10% (v/v) glycerol/1 mM DTT/0.1 mM PMSF/0.15 µg/ml leupeptin and antipain], adjusted to 0.14 M NaCl, and stored at -80 °C until use.

Purification of DNA helicase II

S. pombe cell extract (3.74 mg/ml; 1600 ml) prepared as described above from 600 g of cells, was applied directly to a sulphopropyl (SP)-Sepharose (Pharmacia) XK26 column (2.6 cm × 34 cm, 180 ml) equilibrated with buffer T containing 0.14 M NaCl. The column was washed with the buffer T containing 0.2 M NaCl and eluted with a 1.5 litre linear gradient of 0.2–1.0 M NaCl in buffer T. Helicase activities eluted at 0.4 M NaCl were pooled, dialysed for 3 h against buffer T (4 litres) and adjusted to 0.2 M NaCl. The dialysis residue (0.50 mg/ml, 320 ml) was loaded on to a heparin-Sepharose (Pharmacia) column (1.0 cm × 13 cm, 10 ml) equilibrated with buffer T plus 0.2 M NaCl. After extensive washing (10 column vol.) with buffer T containing 0.2 M NaCl, the column was eluted with a 200 ml linear gradient of 0.2–1.0 M NaCl in buffer T. The helicase activity, which peaked at 0.4 M NaCl, was pooled (0.71 mg/ml, 55 ml) and dialysed for 6 h against buffer T plus 0.1 M NaCl (2 litres). The dialysed protein pool was loaded at a rate of 5 ml/h on to an ssDNA-cellulose (Sigma) column (0.5 cm × 3 cm, 0.6 ml) equilibrated with buffer T containing 0.1 M NaCl. After washing with buffer T containing 0.25 M NaCl (15 ml), the ssDNA column was eluted with a 12 ml linear gradient of 0.05–1.0 M NaCl in buffer T. The helicase activity, which was eluted at 0.7 M NaCl, was pooled (0.029 mg/

ml, 3.5 ml), diluted to 100 mM NaCl, and injected on to an FPLC Resource Q column (1 ml; Pharmacia) equilibrated with buffer T plus 0.1 M NaCl. The column was washed with buffer T containing 0.15 M NaCl and eluted with a 20 ml linear gradient of 0.15–0.8 M NaCl in buffer T. The peak helicase activity, which was eluted at 0.22 M NaCl, was pooled (0.010 mg/ml, 2 ml), concentrated 10-fold (0.2 ml final vol.) using a Biomax-10k (Ultrafree centrifugal filter; Millipore) as recommended by the manufacturer, applied to a glycerol gradient [5 ml, 15–30% (v/v) glycerol in buffer T plus 0.5 M NaCl] and subjected to centrifugation for 24 h at 45000 rev./min in a Beckman SW55 Ti rotor. Fractions (220 µl each) were collected from the bottom of the gradient and assayed for DNA helicase and ATPase activities. The active fractions, which contained > 90% of the total helicase activity, were pooled, supplemented with BSA (final concn. 1 mg/ml) to increase enzyme stability, and stored at -80 °C. Unless otherwise stated, this preparation was used to examine the helicase and ATPase activities associated with the enzyme. For further analysis, an aliquot of the peak glycerol gradient fraction (120 µl) was diluted 2-fold and subjected to a second glycerol-gradient-centrifugation step as described above.

RESULTS

Purification of DNA helicase II

We have reported previously the purification and characterization of a major DNA helicase from *S. pombe*, termed DNA helicase I [33], which translocates in a 3'-to-5' direction. During purification of DNA helicase I, we noticed that the detection of other DNA helicase activities was difficult, primarily owing to the masking of other unwinding activities by the abundant DNA helicase I. This complication limited the detection of low-abundance helicases or those with low specific activity. To avoid this problem, we decided to use a substrate that detects only those helicases that translocate in the 5'-to-3' direction. The substrate, prepared as described in the Experimental section, contains two partial duplexes (one at each end of the linearized M13 ssDNA), but only one end is labelled. The labelled fragment can be released by a helicase with 5'-to-3' polarity, thus allowing the detection of 5'-to-3' DNA helicases only.

The 5'-to-3' helicase activities were not detectable in the very crude extracts. We were able to observe very weak helicase activities with the 5'-to-3' substrate only after the crude extracts were subjected to SP-Sepharose column chromatography (results not shown). This step eliminated most of the protein (> 97%, Table 1) and enriched the 5'-to-3' helicase activity, which revealed

Table 1 Purification of DNA helicase II from *S. pombe*

Fraction	Volume (ml)	[Protein] (mg/ml)	Total (mg)	Total activity (units*)	Specific activity (units/mg)
Crude extracts	1600	3.74	5980	—	—
SP-Sepharose	320	4.5	160	70.8	0.443
Heparin-Sepharose	55	0.71	39	36.4	0.933
ssDNA cellulose	3.5	0.029	0.1	13.9	139
Resource Q (FPLC)	2.0	0.010	0.02	7.2	360
Glycerol gradient†	0.65	0.023	0.015	6	400

* One unit of DNA helicase activity is defined as the amount of enzyme required to displace 1 pmol of duplex DNA substrate in the standard DNA helicase assay described in the Experimental section.

† Protein concentration was estimated on the basis of silver-stained BSA as standard, assuming that BSA and DNA helicase II are stained equally well with silver.

Figure 1 Glycerol-gradient-sedimentation analysis of DNA helicase II

The peak fraction of helicase activity from the first glycerol gradient (0.12 ml) was diluted 2-fold, loaded on to a 15–35% glycerol gradient (5 ml) and centrifuged as described in the Experimental section. Fractions (0.2 ml) were collected from the bottom of the tube. Proteins were analysed by SDS/PAGE, and DNA helicase and ssDNA-dependent ATPase activities were assayed across the glycerol-gradient fractions. (A) SDS/12%-PAGE of the glycerol-gradient fractions (20 μ l) was carried out as described in [49], and the gel was silver-stained. The load ('Load', fraction 15 from the first glycerol gradient) and glycerol-gradient fractions analysed are indicated above the top of the Figure. The following prestained protein molecular size markers ('MW marker'; Bio-Rad) were used: phosphorylase *b* (107 kDa), BSA (76 kDa), ovalbumin (52 kDa), carbonic anhydrase (36.8 kDa), trypsin inhibitor (27.2 kDa) and lysozyme (19 kDa). (B) An autoradiogram of the helicase reaction products. DNA helicase activity was measured in reaction mixtures (20 μ l) that contained 2 μ l of each glycerol-gradient fraction with 15 fmol of substrate (a 52-mer oligonucleotide annealed to ϕ X174 ssDNA) under the conditions described in the Experimental section. The boiled substrate ('Boiled') and substrate-only ('Substrate') controls are indicated above the pertinent lanes. (C) Quantification of DNA helicase activity (●) and ATPase activity (○). ATPase activity was assayed with 2 μ l of each glycerol-gradient fraction in 20 μ l reactions in the presence of 0.1 mM ATP as described in the Experimental section. The sedimentation value of DNA helicase II (4.8 S, open arrow) was determined with reference to marker proteins, which included BSA (4.4 S), aldolase (7.4 S), and catalase (11.2 S) run in a parallel gradient. The sedimentation profiles of the marker proteins were determined with the use of the Bradford protein assay [50].

its presence in fractions that were eluted at a relatively high salt concentration (0.4 M NaCl). When we repeated the helicase assay with the regular substrates used for DNA helicase I isolation [33], we observed that the tailing activity of the major DNA helicase I activity peak occurred where the 5'-to-3' helicase was eluted, thus interfering with the detection of the novel helicase (results not shown). The use of a substrate selective for

5'-to-3' directionality was, therefore, critical for the detection of this new helicase. DNA helicase activity could not be measured reliably in the SP-Sepharose fraction because of the presence of nucleases that degraded the DNA substrates. Nuclease activities were progressively separated from the DNA helicase activity at every stage of purification. The subsequent purification steps included heparin-Sepharose, ssDNA-cellulose, anion-exchange (Resource Q) on FPLC, and glycerol-gradient sedimentation, as summarized in Table 1. Major purification was achieved with the ssDNA-cellulose chromatographic step, which removed more than 99.5% of the contaminating proteins (Table 1). In addition, this step removed virtually all of the residual nuclease activity (results not shown). The helicase activity from this step was eluted at a broad range of salt concentrations (0.5–1.0 M), resulting in a diluted preparation. The Resource Q column was used to further purify and to concentrate the ssDNA cellulose fraction. SDS/PAGE analysis of fractions obtained from the Resource Q step revealed the presence of a 63 kDa polypeptide that appeared to co-migrate with helicase activity (results not shown), although other polypeptides were detected in these fractions. The active fractions from the Resource Q column were pooled, concentrated 10-fold, and subjected to two successive glycerol-gradient-centrifugation steps. SDS/PAGE analysis of the active fractions from the second glycerol gradient also resulted in the detection of a 63 kDa polypeptide (Figure 1A). This polypeptide co-migrated through the glycerol gradient with a single peak of DNA helicase and DNA-dependent ATPase activities (Figures 1B and 1C).

The hydrodynamic properties of DNA helicase II were examined by determining the sedimentation coefficient and Stokes radius of the protein. Glycerol-gradient analysis and gel-filtration chromatography on a Sephacryl S-200 column yielded a sedimentation coefficient of 4.8 S (Figure 1C) and a Stokes radius of 36 Å (3.6 nm) (results not shown) respectively. Assuming a partial specific volume of 0.725 ml/g, DNA helicase II was calculated to have a native molecular mass of 65 kDa [38]. These data suggest that DNA helicase II exists as a monomer in solution.

Enzyme titration and kinetics

As shown in Figure 2, the formation of ssDNA with increasing amounts of enzyme was sigmoidal, implying that DNA helicase II may have to multimerize before it can act as helicase. Below 5 ng of enzyme per reaction, unwinding was barely detectable (< 0.5 fmol). With 20 ng of enzyme, the reaction was saturating, and the amount of substrate unwound was 12 fmol (80% of input substrate), 4–5-fold more than that obtained with 10 ng of enzyme per reaction (Figures 2A and 2B).

On the basis of the enzyme titration curves shown in Figures 2(A) and 2(B), we performed time-course experiments with 15 ng of the enzyme in the reaction (Figures 2C and 2D). The amount of unwound product increased linearly only with short incubation times (up to 50 s). At longer incubation times, helicase activity reached a plateau after 40% of the substrate had been unwound. This experiment demonstrates that the enzyme is remarkably rapid in its rate of duplex DNA unwinding and/or translocation along the template DNA.

Processive unwinding and translocation of DNA helicase II

The rapid kinetics prompted us to examine whether DNA helicase II acts in a processive fashion. We first carried out the following experiments to investigate whether the enzyme is sequestered on the substrate. The addition of more substrate (15 fmol) after a

Figure 2 Influence of increasing amounts of DNA helicase II on and the kinetic analysis of the oligonucleotide displacement reaction

(A) DNA helicase activity was measured in reaction mixtures (20 μ l) that contained various amounts of enzyme with 15 fmol of substrate (a 52-mer oligonucleotide annealed to ϕ X174 sscDNA) and were incubated for 5 min. The amount of DNA helicase II added (ng) is noted at the top of each lane. The boiled substrate ('Boiled') and substrate-alone ('Substrate') controls are indicated. (B) Quantification of helicase activity in (A). (C) kinetic analysis of the unwinding activity of DNA helicase II. The reaction mixture (160 μ l) contained 120 ng of enzyme; aliquots (20 μ l) were withdrawn at the indicated times and subjected to non-denaturing 10%-PAGE as described in the Experimental section. The substrate-alone ('Substrate') control is indicated above the appropriate lane. (D) Quantification of the results in (C).

100 s incubation failed to increase the amount of unwinding products generated (Figure 3A). However, when the reaction was supplemented with additional enzyme (15 ng) after a 100 s incubation (Figure 3A), ssDNA products increased with kinetics strikingly similar to those shown in Figure 2(D). These results indicate that the substrate is not limiting and the substrate that had not reacted is still in an available form. This observation raised two possibilities: (1) the enzyme was tightly bound to and, hence, sequestered on the ϕ X174 sscDNA substrate or (2) the enzyme became inactivated during the reaction.

We first carried out substrate-competition experiments in order to obtain evidence that DNA helicase II is stably sequestered on the substrate (Figure 3B). When reactions containing enzyme (15 ng) and labelled substrate (15 fmol) were supplemented immediately with either a 20-, 40- or 80-fold excess of unlabelled competitor substrate (ϕ X174 sscDNA), an immediate decrease (81, 95 and 97% respectively) in unwinding of the labelled substrate was observed (Figure 3B). However, when competitor DNA was added to the reaction after a 3 min preincubation on ice of the enzyme with labelled substrate, the extent of unwinding of the labelled substrate was not affected significantly (13, 20 and 36% inhibition was observed with a 20-, 40- and 80-fold excess of ϕ X174 sscDNA respectively) (Figure 3B). This observation supports the idea that the helicase is stably bound to a template and unwinds duplex DNA in a processive manner.

Figure 3 Influence of supplemental enzyme and substrate on the formation of ssDNA by DNA helicase II

(A) Standard helicase reactions containing 15 fmol of labelled 52-mer annealed to ϕ X174 sscDNA carried out in triplicate. To one set ($\blacktriangle$), additional enzyme (15 ng) was added at the time indicated (100 s, arrow). To the second set ($\square$), additional substrate (15 fmol) was added after 100 s of incubation. The third set did not receive any supplementation ($\bullet$). The reactions were incubated further as indicated and analysed as described in the Experimental section. (B) The processivity of DNA helicase II was determined by the addition of unlabelled substrate to the reaction mixture containing labelled DNA (15 fmol) and subsaturating amounts (15 ng) of enzyme. Two sets of reaction mixtures were assembled on ice. One set of reaction mixtures was preincubated ($\circ$) in the absence of ATP on ice for 3 min before the addition of unlabelled ϕ X174 sscDNA, and the reactions were started by incubating for 5 min at 37 $^{\circ}$ C in the presence of ATP (2 mM). The second set of reaction mixtures were treated in the same way as described above, except that the unlabelled competitor DNA was present at the beginning of the reaction ($\bullet$). The amounts of unlabelled ϕ X174 sscDNA are indicated.

To examine whether DNA helicase II also translocates along ssDNA in a processive manner, we investigated the effects of various DNA sizes on the ATPase activity of the helicase. If the enzyme translocates processively along the DNA, it should exhibit higher ATPase activity in the presence of long circular DNA (for example, M13 sscDNA) than with short oligonucleotides (for example, a 24-mer or 73-mer). Before performing this experiment we determined the saturating level of each DNA in the reaction. The amount of DNA sufficient to saturate the ATP-hydrolysis reaction of the enzyme was 5 ng (results not shown). In the presence of 5 ng of DNA (either M13 sscDNA, 24-mer or 73-mer) in the reaction, we observed that the amount of ATP hydrolysed was indeed affected by the size of the added DNA (results not shown). In order to confirm this result, we performed

Figure 4 Effect of the length of ssDNA on the ATPase activity of DNA helicase II

(A) Kinetic analysis of polynucleotide-dependent ATPase activity. ATP hydrolysis was measured under the conditions described in the Experimental section. Reaction mixtures contained 15 ng of DNA helicase II in the presence of one of each ssDNA (5 ng) of different sizes (M13 sscDNA, 24-mer, and 73-mer) and were incubated for the period of time indicated at the top of each lane. No-DNA and no-enzyme controls were also included to measure background levels of ATP hydrolysis. (B) Quantification of results obtained in (A). The background value obtained in the absence of enzyme was subtracted from the amount of ATP hydrolysed in reactions containing polynucleotides. Polynucleotide cofactors used are as indicated on the Figure.

a time-course analysis to investigate the influence of DNA sizes on ATP hydrolysis in detail (Figure 4). In the presence of the 24-mer (Figure 4A, lanes 6–10) and 73-mer (Figure 4A, lanes 11–15) oligonucleotides, ATP hydrolysis increased linearly only when the incubation period was short (5 and 10 min respectively) and thereafter no significant increase of ATP hydrolysis was observed (Figure 4B). The amounts of ATP hydrolysed in the presence of the 24-mer and 73-mer oligonucleotides were 230 pmol and 510 pmol respectively after 40 min of incubation (Figure 4B). In striking contrast, ATP hydrolysis increased in a linear fashion for 90 min in the presence of M13 sscDNA (Figure 4A, lanes 1–5; Figure 4B). This result suggests that the enzyme is still actively engaged in translocation along the circular DNA with concomitant hydrolysis of ATP and argues against the possibility that the enzyme is inactivated during the reaction as mentioned above. In fact we observed that ATP hydrolysis was sustained at a constant rate for up to 180 min, resulting in more than

Figure 5 Directionality of DNA helicase II unwinding of duplex DNA

(A) Schematic structure of the linear partial duplex substrate used to measure the polarity of DNA helicase II. Asterisks indicate radioisotopic labels at each 3'-end. (B) Unwinding direction was determined with 15 ng of DNA helicase II and 15 fmol of the substrate shown in (A) under the standard reaction conditions described in the Experimental section. The reaction products were resolved by electrophoresis through a non-denaturing 10%-polyacrylamide gel as described in the Experimental section. DNA helicase I, which translocates in the 3'-to-5' direction, was included as a control (lane 6). The faint band designated as the 52-mer originated from incomplete digestion of the partial duplex of 52-mer-annealed ϕ X174 substrate during substrate preparation. Control reactions that were boiled ('Boiled'), or contained substrate only ('Substrate') and other omissions (—ATP and —MgCl₂) are indicated.

3000 pmol of ATP hydrolysed (results not shown). The initial rate of ATP hydrolysis during the first 5 min of incubation was not affected, regardless of the DNA species used (Figure 4B). This observation suggests that the helicase is capable of circling M13 sscDNA multiple times without dissociating. Taken together, these results demonstrate that DNA helicase II acts in a highly processive fashion in both translocation and unwinding.

Directionality of DNA helicase II

Because we purified DNA helicase II using a substrate that detects only 5'-to-3' helicases, we assumed that it translocates on substrate DNA in a 5'-to-3' direction. In order to confirm this fact and to rule out the potential contamination of DNA helicase I that co-purified with DNA helicase II at the early step of purification (SP-Sepharose), we used a linearized ϕ X174 sscDNA containing a 23-mer oligonucleotide fragment at the 5'-end and a 29-mer at the 3'-end, both of which were labelled (Figure 5A). As shown in Figure 5(A), release of the 29-mer is diagnostic of 5'-to-3' directionality, whereas release of the 23-mer indicates 3'-to-5' directionality. As expected, the 29-mer was

Figure 6 Effect of temperature on the unwinding activity of DNA helicase II

(A) The effect of temperature on formation of ssDNA by DNA helicase II was examined in the standard reaction mixture (20 μ l) containing 15 ng of DNA helicase II and 15 fmol of 52-mer-annealed ϕ X174 sscDNA substrate. Reaction mixtures were assembled on ice, prewarmed for 3 min at the temperature tested, and the reaction started by adding 2 mM ATP. The reaction mixtures were incubated for the periods of time indicated at the top of each lane. For each zero time point, ATP was added after the addition of stop mix. The boiled ('Boiled') and substrate-alone ('Substrate') controls are indicated above the appropriate lanes. (B) Quantification of results obtained from (A). The incubation temperature and incubation period are as indicated on the Figure.

displaced preferentially by DNA helicase II (Figure 5B, lane 5), whereas the 23-mer remained associated with the ϕ X174 sscDNA. In contrast, the 23-mer was unwound preferentially by DNA helicase I, which has 3'-to-5' directionality (Figure 5B, lane 6). The specific release of the 29-mer only by DNA helicase II excludes the presence of DNA helicase I in the enzyme preparation. Release of the 29-mer was dependent upon both ATP and $MgCl_2$ (Figure 5B, lanes 3 and 4).

Use of other substrates

The influence of fork structures in the DNA helicase substrate on helicase activity was investigated. Three different substrates were prepared as described in the Experimental section and tested under standard assay conditions. All three substrates had the same duplex length (52 bp) and the identical sequence, but differed in the presence of non-complementary tails at either the 5'- or 3'-end when hybridized to ϕ X174 sscDNA. The efficiency of unwinding by DNA helicase II was not affected by the

Table 2 Properties of the unwinding activity of DNA helicase II

Additions or omissions	Amount added	Relative activity (%)
Complete*		100†
Add NaCl	0.5, 0.1, 0.2, 0.4 M	103, 109, 43, 19
ADP	0.5, 1.5 mM	98, 91
ATP[S], p[NH]ppA or p[CH ₂]ppA	1.5 mM each	96–97
Omit $MgCl_2$		< 1
Add $MnCl_2$ or $CaCl_2$	1 mM each	< 5
Omit ATP		
Add ADP, ATP[S], p[NH]ppA or p[CH ₂]ppA	1.5 mM each	< 1
Complete		
Add <i>E. coli</i> SSB	0.2, 0.4 μ g	101, 102
T4 gp32	0.2, 0.4 μ g	98, 95
SpRPA	0.2, 0.4 μ g	105, 120
Omit ATP		
Add GTP	0.25, 1.5 mM	2, 12
CTP	0.25, 1.5 mM	< 1, 8
UTP	0.25, 1.5 mM	< 1, < 1
dATP	0.25, 1.5 mM	30, 98
dGTP	0.25, 1.5 mM	2, 5
dCTP	0.25, 1.5 mM	< 1, 10
dTTP	0.25, 1.5 mM	< 1, < 1

* The complete reaction contained DNA helicase II (15 ng) in the standard reaction mixture described in the Experimental section.

† Displacement of 7.5 fmol of DNA substrate is equivalent to 100%.

presence of either the 5'- or 3'-tail on the substrates (results not shown). In order to examine the maximum length of duplex DNA that can be unwound by the helicase, we prepared a substrate with a longer duplex region by extending the annealed oligonucleotide (52-mer) with the Klenow fragment as described in the Experimental section. DNA helicase II was capable of unwinding efficiently up to 250 bp duplex DNA (results not shown).

Influence of temperature on the unwinding activity

When we were carrying out the substrate-challenge experiments above, it was noted that the displacement reaction occurred during preincubation on ice and was dependent on the presence of ATP (results not shown). In agreement with this observation, ATP hydrolysis also occurred when the reaction was carried out on ice (results not shown). We therefore examined the effects of temperature on the unwinding activity of DNA helicase II (Figure 6). The reaction mixtures were assembled on ice in the absence of ATP and preincubated for 3 min at the various temperatures to be tested. The reactions were then supplemented with ATP to 2 mM, and incubation for the indicated time periods immediately followed. For the zero-time controls (Figure 6A, lanes 3, 7 and 11), we first added stop mix and then ATP to the reactions. The reactions at the three different temperatures (0, 15, and 37 °C) all showed very similar kinetics, reaching plateaux after 1 min of incubation with ATP, although the extents of unwinding varied (3.7, 4.2 and 8.3 fmol respectively) (Figure 6B). The time required for the reaction to reach a plateau with preincubation appeared to be somewhat shorter than that (80–100 s) observed when it was omitted (Figure 2). This may reflect the fact that the preincubation eliminated the time required for the helicase to oligomerize on the DNA.

Table 3 Properties of the NTPase activity of DNA helicase II

Additions or omissions	Amount added	Relative activity (%)
Complete*		100†
Add NaCl	0.05, 0.1, 0.2, 0.4 M	109, 89, 45, 18
Omit MgCl ₂		< 1
Omit DNA		< 1
Add <i>E. coli</i> SSB	0.2, 0.4 µg	98, 92
T4 gp32	0.2, 0.4 µg	98, 97
SpRPA	0.2, 0.4 µg	101, 90
Omit ATP		
Add GTP	250 µM	12
CTP	250 µM	4
UTP	250 µM	< 1
dATP	250 µM	86
dGTP	250 µM	11
dCTP	250 µM	3
dTTP	250 µM	ND‡
Omit M13 sscDNA	50 ng	
Add M13 RF1 DNA	50 ng	48
Poly(dT)	50 ng	17
Poly(dI · dC)	50 ng	18
Poly(U)	50 ng	< 1
Boiled tRNA		< 1

* The complete reaction contained DNA helicase II (15 ng) in the standard reaction mixtures described in the Experimental section.

† The amount (700 pmol) of ATP hydrolysed is equivalent to 100%.

‡ ND, not determined.

Requirements of the helicase activity

Helicases utilize NTPs as their energy sources for translocation and unwinding. As expected, the unwinding reaction by DNA helicase II is absolutely dependent on ATP, but no activity was seen with non-hydrolyzable ATP analogues such as ADP, adenosine 5'-[γ-thio]triphosphate (ATP[S]), adenosine 5'-[β,γ-imido]triphosphate (p[NH]ppA) and adenosine 5'-[β,γ-methylene]triphosphate (p[CH₂]ppA) (Table 2). In the presence of 2 mM ATP, DNA helicase activity was not competitively inhibited by the addition (1.5 mM) of these non-hydrolysable ATP analogues (Table 2). Among all eight nucleotides, ATP and dATP supported unwinding activity with equal efficiency (Table 2). GTP, dGTP, CTP, dCTP supported unwinding activity to limited extents (5–12 % of that with ATP at 1.5 mM) (Table 2). Consistent with this observation, all four of them were also hydrolysed to limited extents (3–12 % of that with ATP) by the helicase (Table 3). The enzyme required MgCl₂ for its activity, but it was not active in the presence of MnCl₂ or CaCl₂ (1 mM) (Table 2). The *K_m* of ATP for the helicase reaction was determined to be 0.65 mM (results not shown). The enzyme functioned efficiently over wide ranges of pH from 6.5 to 8.9 (results not shown). Helicase activity was insensitive to 0.1 M NaCl, but was inhibited 57 and 81 % upon addition of 0.2 M and 0.4 M NaCl respectively (Table 2). We also investigated the effects of ssDNA-binding proteins from various sources (for example, *S. pombe* RPA, *E. coli* SSB and T4 gp32) on unwinding by DNA helicase II. We did not observe any significant influence of any of these proteins on helicase activity with the standard oligonucleotide-annealed substrate under the conditions described in the Experimental section (Table 2), although the unwinding of substrate that contained a long duplex region was slightly stimulated (results not shown). Unlike DNA helicase I, DNA helicase II was not stimulated by SpRPA or *E. coli* SSB at low ATP

concentrations (results not shown), which is the most unique property of DNA helicase I [33].

Properties of NTPase activity

As shown in Figure 1(C), both the DNA helicase and DNA-dependent ATPase activities were detected coincidentally in the glycerol-gradient-centrifugation analysis. The DNA-dependent ATPase activity was further characterized using the standard reaction conditions described in the Experimental section. As observed in the DNA helicase assay, ATP hydrolysis required MgCl₂ (Table 3), and other bivalent ions such as Mn²⁺ (as MnCl₂) or Ca²⁺ (as CaCl₂) did not support ATP hydrolysis at 1 mM concentration (results not shown). In keeping with the effects of NaCl on unwinding (Table 2), ssDNA-dependent ATPase activity was inhibited to similar extents at high NaCl concentrations (55 % at 0.2 M and 82 % at 0.4 M NaCl) (Table 3). The ssDNA-dependent ATPase activity was also unaffected by the addition of SpRPA, T4 gp32 or *E. coli* SSB (Table 3). We also examined hydrolysis of other nucleotides by DNA helicase II. The helicase hydrolysed ATP and dATP efficiently (Table 3), in agreement with their ability to support the displacement reaction (Table 2).

As shown in Table 3, several polynucleotides were also examined for their ability to support ATP hydrolysis under standard reaction conditions. Without DNA, the enzyme was virtually inactive (< 1 % ATP hydrolysed). M13 sscDNA supported ATP hydrolysis most effectively. M13 RF1 (double-stranded DNA) was active, but less efficient (48 % of M13 sscDNA) (Table 3). Homopolymers such as poly(dT) and poly(dI · C) weakly supported ATPase activity. In contrast, the synthetic RNA poly(U) did not support ATP hydrolysis at all (Table 3). Moreover, boiled or native yeast tRNA failed to support ATP hydrolysis (Table 3). In agreement with this observation, DNA helicase II lacked any detectable RNA-unwinding activity (results not shown).

DISCUSSION

In the present paper we have described the purification and biochemical characterization of a novel DNA helicase, termed DNA helicase II, using a DNA substrate that allows detection of DNA helicases that translocate in the 5'-to-3' direction. We have demonstrated that DNA helicase II functions in a highly processive fashion, both in unwinding and ATP hydrolysis. The helicase was purified more than 900-fold over the SP-Sepharose fraction (Table 1). The enzyme activity co-purified with a 63 kDa polypeptide that was monomeric in solution.

Eukaryotic DNA helicases that translocate in the same direction as the DNA helicase II include DNA helicase IV from HeLa cells [5], DNA helicase B, C and D from calf thymus [39], and helicases B, C and D [8], DNA helicase III [40] and the HcsB protein [41] from *S. cerevisiae*. Comparison of various other biochemical properties such as preference for nucleotides as energy source, sensitivity to salts, polynucleotide cofactors for ATPase activity and molecular size suggests that the *S. pombe* DNA helicase II in the present study is unlikely to be a functional homologue of any of these 5'-to-3' mammalian [5,39] or *S. cerevisiae* helicases previously described [8,40,41].

DNA helicase II and the Rad3 protein [42] share some properties in common; they both act in a processive manner in the unwinding reaction and translocate in the same direction [43]. Both strongly favour ATP and dATP to support unwinding

[28], although DNA helicase II uses GTP, dGTP, CTP and dCTP to a limited extent (Table 2). However, the two enzymes differ in other respects. Most significantly, they show a great difference in kinetics and extents of product formed in the presence of comparable amounts of enzyme [28,43]. Other notable difference includes the use of bivalent-ion cofactors and the sensitivity to salt. The optimal concentration of MgCl_2 for the Rad3 protein is 5–10 mM, whereas this high concentration was inhibitory to DNA helicase II (optimal concentration, 1–2 mM). In addition, for Rad3, MnCl_2 at 2 mM substituted effectively (70 %) for 5 mM MgCl_2 [28]. In contrast, DNA helicase II was inactive in the presence of 1 mM MnCl_2 (Table 2). The Rad3 ATPase activity was stimulated by 150 mM KCl, whereas addition of this salt concentration was inhibitory to DNA helicase II (Table 3). The molecular size inferred from the *rad15⁺* gene of *S. pombe* (functional homologue of *S. cerevisiae* RAD3) is 90 kDa [44], whereas DNA helicase II is 63 kDa in size. Despite these differences, additional information is required to determine unambiguously whether these two proteins are related functionally or not. Recently, a new helicase, termed DNA helicase A, has been purified from *S. cerevisiae* [45,46]. This enzyme shares features in common with both DNA helicase I [33] and DNA helicase II (the present study) from *S. pombe*. It translocates in the 5'-to-3' direction like DNA helicase II, but is stimulated severalfold by RPA at high ATP concentration (3.4 mM), similarly to DNA helicase I. However, DNA helicase A is not related to DNA helicase I of *S. pombe*, since the two enzymes show no similarity when their amino acid sequences are aligned (J. S. Park and Y.-S. Seo, unpublished work). DNA helicase II resembles DNA helicase A in some biochemical properties, such as polarity and its ability to hydrolyze ATP and dATP preferentially. However, it remains to be shown whether DNA helicase II is related to DNA helicase A, as other comparable biochemical parameters of DNA helicase A have not been described.

Oligonucleotide-displacement reactions showed a highly sigmoidal response to the amounts of DNA helicase II added. This observation suggests that either a co-operative interaction between monomers or an induction of multimerization of DNA helicase II is required for it to function as helicase, as observed for many prokaryotic replicative DNA helicases and SV40 T antigen. This prompted us to examine whether the enzyme is able to form a multimeric complex in the presence of ATP and MgCl_2 , as was demonstrated for SV40 T antigen [47,48]. Active helicase fractions were preincubated with 2 mM ATP and 2 mM MgCl_2 at 37 °C for 5 min and analysed on a glycerol gradient or by a gel filtration (Sephacryl S-200; Pharmacia) to monitor changes in the sedimentation profile or Stokes radius respectively. However, we failed to observe any alterations in the hydrodynamic properties (results not shown). This observation may suggest that multimerization of enzyme is catalysed by interaction of the helicase with DNA.

Another distinctive biochemical property of DNA helicase II is that it was active even at 0 °C. Both duplex unwinding (Figure 6) and ATP hydrolysis (results not shown) occurred when the reaction mixtures were incubated on ice. The relative amounts of ATP hydrolysed (with 15 ng of enzyme in the reaction) after 30 min of incubation at 0 and 15 °C were 9.2 and 38.2 % respectively of the value (980 pmol) obtained at 37 °C (results not shown). The ratio of the degree of duplex unwinding to ATP hydrolysis seemed to be greatest at the lowest temperature tested. Although it is not clear at present, one possible explanation that may account for this observation is that non-productive ATP hydrolysis is suppressed at low temperatures, thereby increasing the average fuel efficiency for duplex unwinding.

Other unique property of the enzyme is that it was unusually rapid in the unwinding reaction, which reached completion after 50–100 s of incubation under standard reaction conditions (Figure 2). The rapid reaction kinetics may indicate that the enzyme translocates rapidly along the ssDNA and/or unwinds the duplex region with great speed. Alternatively, the enzyme may be targeted directly to the duplex junction. At this point we are unable to distinguish between these three possibilities. However, the substrates used in all experiments consisted of excessive ssDNA (> 100-fold) and short partial duplexes to be unwound. It is thus most likely that the rapid kinetics result from the enzyme's ability to translocate rapidly along ssDNA.

At present, the function of DNA helicase II is not known. Its biochemical properties, such as the processive mode of unwinding, rapid translocation along ssDNA, and possible oligomerization, favour the notion that DNA helicase II is involved in DNA replication. Because DNA helicase II translocates in the 5'-to-3' direction, it would move on the lagging strand towards the replication fork. We are examining currently whether this helicase is capable of supporting rolling circle synthesis of DNA, which is indicative of propagating replication forks. We also plan to clone the full-length cDNA and genomic DNA for DNA helicase II in order to clarify its biological function in *S. pombe*. In addition, we hope to identify structurally similar mammalian helicases and use *S. pombe* genetics to test whether they are functionally related to DNA helicase II.

We thank Dr. Y. J. Kim and Dr. H. S. Shin for their critical reading of the manuscript before its submission. This work was supported by a grant (95-B-002) from the Samsung Biomedical Research Institute.

REFERENCES

- Matson, S. W. and Kaiser-Rogers, K. A. (1990) Annu. Rev. Biochem. **59**, 289–329
- Lohman, T. M. and Bjornson, K. P. (1996) Annu. Rev. Biochem. **65**, 169–214
- Thömmes, P. and Hübscher, U. (1992) Chromosoma **101**, 467–473
- West, S. C. (1996) Cell **86**, 177–180
- Tuteja, N. and Tuteja, R. (1996) Nat. Genet. **13**, 11–12
- Abdel-Monem, M., Durwald, H. and Hoffmann-Berling, H. (1976) Eur. J. Biochem. **65**, 441–449
- Matson, S. W., Bean, D. W. and George, J. W. (1994) BioEssays **16**, 13–22
- Li, X., Yoder, B. L. and Burgers, P. M. J. (1992) Chromosoma **102**, S93–S99
- Zhang, S. and Grosse, F. (1991) J. Biol. Chem. **266**, 20483–20490
- Tuteja, N., Tuteja, R., Rahman, K., Kang, L.-Y. and Falaschi, A. (1990) Nucleic Acids Res. **18**, 6785–6792
- Seo, Y. S., Lee, S.-H. and Hurwitz, J. (1991) J. Biol. Chem. **266**, 13161–13170
- Seo, Y. S. and Hurwitz, J. (1993) J. Biol. Chem. **268**, 10282–10295
- Thömmes, P., Ferrari, E., Jessberger, R. and Hübscher, U. (1992) J. Biol. Chem. **267**, 6063–6073
- Seki, M., Yangisawa, J., Kohda, T., Sonoyama, T., Ui, M. and Enomoto, T. (1994) J. Biochem. (Tokyo) **268**, 523–531
- Gorbalenya, A. E. and Koonin, E. V. (1993) Curr. Opin. Struct. Biol. **3**, 419–429
- Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M. et al. (1996) Science **274**, 563–567
- LeBowitz, J. and McMacken, R. (1986) J. Biol. Chem. **261**, 4738–4748
- Matson, S. W., Tabor, S. and Richardson, C. C. (1983) J. Biol. Chem. **258**, 14017–14024
- Venkatesan, M., Silver, L. L. and Nossal, N. G. (1982) J. Biol. Chem. **257**, 12426–12434
- Stahl, H., Dröge, P. and Knippers, R. (1986) EMBO J. **5**, 1939–1944
- Goetz, G. S., Dean, F. B., Hurwitz, J. and Matson, S. W. (1988) J. Biol. Chem. **263**, 383–392
- Seo, Y. S., Müller, F., Lusky, M. and Hurwitz, J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 702–706
- Yang, L., Mohr, I., Fouts, E., Lim, D. A., Nohaile, M. and Botchan, M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5086–5090
- Lee, S.-K. and Lehman, I. R. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 2838–2842
- Crute, J. J., Tsurumi, T., Zhu, L., Weller, S. K., Olivo, P. D., Challberg, M. D., Mocarski, E. S. and Lehman, I. R. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2186–2189

- 26 Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H. J., Chambon, P. and Egly, J.-M. (1993) *Science* **260**, 58–63
- 27 Sung, P., Bailly, V., Weber, C., Thompson, L. H., Prakash, L. and Prakash, S. (1993) *Nature (London)* **365**, 852–855
- 28 Sung, P., Prakash, L., Matson, S. W. and Prakash, S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8951–8955
- 29 Guzder, S. N., Sung, P., Bailly, V., Prakash, L. and Prakash, S. (1994) *Nature (London)* **369**, 568–571
- 30 Budd, M. E. and Campbell, J. L. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7642–7646
- 31 Budd, M. E., Choe, W. C. and Campbell, J. L. (1995) *J. Biol. Chem.* **270**, 26766–26769
- 32 Budd, M. E. and Campbell, J. L. (1997) *Mol. Cell. Biol.* **17**, 2136–2142
- 33 Park, J. S., Choi, E., Lee, S. H., Lee, C. and Seo, Y. S. (1997) *J. Biol. Chem.* **272**, 18910–18919
- 34 Messing, J. (1983) *Methods Enzymol.* **101**, 20–78
- 35 Sanger, F., Coulson, A. R., Friedmann, T., Air, G. M., Barrell, B. G., Brown, N. L., Fiddes, J. C., Hutchison, C. A., Slocombe, P. M. and Smith, M. (1978) *J. Mol. Biol.* **125**, 225–246
- 36 Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* **33**, 103–119
- 37 Ishiai, M., Sanchez, J. P., Amin, A. A., Murakami, Y. and Hurwitz, J. (1996) *J. Biol. Chem.* **271**, 20868–20878
- 38 Siegel, L. M. and Monty, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346–362
- 39 Thömmes, P., Ferrari, E., Jessberger, R. and Hübscher, U. (1992) *J. Biol. Chem.* **267**, 6063–6073
- 40 Shimizu, K. and Sugino, A. (1993) *J. Biol. Chem.* **268**, 9578–9584
- 41 Biswas, E. E., Chen, P. H., Leszyk, J. and Biswas, S. B. (1995) *Biochem. Biophys. Res. Commun.* **206**, 850–856
- 42 Sung, P., Prakash, L., Weber, S. and Prakash, S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6045–6049
- 43 Harosh, I., Naumovski, L. and Friedberg, E. C. (1989) *J. Biol. Chem.* **264**, 20532–20539
- 44 Murray, J. M., Doe, C. L., Schenk, P., Carr, A. M., Lehmann, A. R. and Watts, F. Z. (1992) *Nucleic Acids Res.* **20**, 2673–2678
- 45 Biswas, E. E., Fricke, W. M., Chen, P. H. and Biswas, S. B. (1997) *Biochemistry* **36**, 13277–13284
- 46 Biswas, S. B., Chen, P. H. and Biswas, E. E. (1997) *Biochemistry* **36**, 13270–13276
- 47 Mastrangelo, I. A., Hough, P. V., Wall, J. S., Dodson, M., Dean, F. B. and Hurwitz, J. (1989) *Nature (London)* **338**, 658–662
- 48 Dean, F. B., Borowiec, J. A., Eki, T. and Hurwitz, J. (1992) *J. Biol. Chem.* **267**, 14129–14137
- 49 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- 50 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254