2014–2018 Nucleic Acids Research, 1995, Vol. 23, No. 11 © 1995 Oxford University Press

Stimulation of mouse DNA primase-catalyzed oligoribonucleotide synthesis by mouse DNA helicase B

Akiko Saitoh, Shusuke Tada*, Toshiaki Katada and Takemi Enomoto

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Received January 13, 1995; Revised and Accepted April 18, 1995

ABSTRACT

Many prokaryotic and viral DNA helicases involved in DNA replication stimulate their cognate DNA primase activity. To assess the stimulation of DNA primase activity by mammalian DNA helicases, we analyzed the synthesis of oligoribonucleotides by mouse DNA polymerase α–primase complex on single-stranded circular M13 DNA in the presence of mouse DNA helicase B. DNA helicase B was purified by sequential chromatography through eight columns. When the purified DNA helicase B was applied to a Mono Q column, the stimulatory activity for DNA primase-catalyzed oligoribonucleotide synthesis and DNA helicase and DNA-dependent ATPase activities of DNA helicase B were co-eluted from the column. The synthesis of oligoribonucleotides 5–10 nt in length was markedly stimulated by DNA helicase B. The synthesis of longer species of oligoribonucleotides, which were synthesized at a low level in the absence of DNA helicase B, was inhibited by DNA helicase B. The stimulatory effect of DNA helicase B was marked at low template concentrations and little or no effect was observed at high concentrations. The mouse single-stranded DNA binding protein, replication protein A (RP-A), inhibited the primase activity of the DNA polymerase α–primase complex and DNA helicase B partially reversed the inhibition caused by RP-A.

INTRODUCTION

In all aspects of DNA transactions, including DNA replication, the duplex DNA must be unwound to form single-stranded DNA. The process is catalyzed by a class of enzymes designated DNA helicases, with consumption of energy generated by the hydrolysis of ATP (1).

A cell-free simian virus 40 (SV40) DNA replication system has allowed biochemical investigation of the enzymes and proteins involved in DNA replication in mammalian cells (2–4). However, because the large tumor antigen (T antigen), which is the only virally encoded protein required for this system, functions as a DNA helicase and origin binding protein (5), the DNA helicase and the origin binding protein involved in DNA replication in mammalian cells remains to be identified.

To identify the DNA helicases involved in DNA replication in mammalian cells, we isolated temperature-sensitive mutants defective in DNA replication and found that a mutant designated tsFT848 isolated from mouse FM3A cells had thermolabile DNA helicase activity. Analyses of DNA synthesis in the mutant cells at the restrictive temperature and of the relationship between defective DNA synthesis and thermosensitivity of the DNA-dependent ATPase activity of DNA helicase B indicated that DNA helicase B is involved in DNA replication (6).

DNA helicases involved in DNA replication in prokaryotic and viral systems often interact with their cognate DNA primase physically and functionally. In *Escherichia coli* the replicative DNA helicase, DnaB, is a component of primosomes and DnaB stimulates the DNA primase activity of DnaG. The large form of the T7 gene 4 protein, possessing DNA primase and DNA helicase activity, forms a complex with the small form, possessing only DNA helicase activity, to make an active DNA primase. The bacteriophage T4 gene 41 protein (DNA helicase) forms a complex with the gene 61 protein (DNA primase) and stimulates DNA primase activity (1). The α protein of phage P4 possesses origin recognizing, helicase and primase activities (7). Herpes simplex virus type 1 encodes a DNA helicase–primase that is composed of the products of the UL5, UL8 and UL52 genes (8). In addition, SV40 T antigen physically interacts with DNA polymerase α–primase complex (9) and stimulates the DNA primase activity of the complex (10–12).

In this study we have examined whether mouse DNA helicase B stimulates the DNA primase activity of mouse polymerase α–primase complex using single-stranded circular M13 DNA as a template. In addition, the effect of DNA helicase B on DNA primase activity was also examined in the presence of mouse replication protein A (RP-A).

MATERIALS AND METHODS

Materials

Poly(rA) and oligo(rA)$_{12-18}$ were purchased from Pharmacia. Single-stranded circular M13mp19 DNA (sscM13 DNA) was prepared according to the method of Messing (13). ssDNA–cellulose was prepared as described (14).

* To whom correspondence should be addressed

Purification of helicase B, DNA polymerase α–primase complex and RP-A

DNA helicase B was purified from 1×10^{11} mouse FM3A cells by sequential column chromatography on first DEAE–cellulose (1000 ml), a second DEAE–cellulose (500 ml) and phosphocellulose (180 ml) as described (6). The eluted fractions from the phosphocellulose column were pooled, buffer 2 (20 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, 0.01% Triton X-100, 20% ethylene glycol) was added to adjust the concentration of KCl to 350 mM and the resultant solution loaded onto a Bio-Gel HTP column (80 ml). Proteins were eluted with a linear gradient of 20–150 mM potassium phosphate in buffer 2 containing 350 mM KCl. The active fraction was dialyzed against 75 mM KCl in buffer 2, loaded onto a ssDNA–cellulose column (30 ml) and eluted with buffer 2 containing 500 mM KCl, 1 mM $MgCl_2$, 1 mM ATP. The eluted fraction was dialyzed against 50 mM KCl in buffer 2, loaded onto the first Mono Q HR5/5 column and eluted with a linear gradient of 150–400 mM KCl in buffer 2. The active fraction was dialyzed against buffer 2 (pH 6.8), loaded onto a Mono S HR5/5 column and eluted with a linear gradient of 0–200 mM KCl in buffer 2 (pH 6.8). The active fraction was dialyzed against 100 mM KCl in buffer 2 (pH 6.8), loaded onto the second Mono Q HR5/5 column and eluted with a linear gradient of 100–400 mM KCl in buffer 2. The active fraction was dialyzed against buffer 2 containing 100 mM KCl and 50% glycerol and stored at –80°C.

DNA polymerase α–primase complex was immunoaffinity purified from mouse FM3A cells as described (15). One unit of activity is defined as the amount that catalyzes the incorporation of 1 nmol deoxyribonucleotide monophosphate during 1 h incubation at 37°C.

Multisubunit single-strand DNA binding protein (RP-A) was purified from 1.09×10^{10} FM3A cells by serial column chromatography on phosphocellulose, Bio-Gel HTP, ssDNA–cellulose and Mono Q HR5/5. The protein concentration was determined by the method of Bradford, using bovine γ-globulin as a standard (16).

Analysis of oligoribonucleotide products

Oligoribonucleotide synthesis was performed by the method described previously (17), with a slight modification. Briefly, 4.1 U (DNA polymerase activity) DNA polymerase α–primase was incubated at 37°C for 90 min in 30 μl of a reaction mixture containing 2 mM ATP, 200 μM CTP, 200 μM GTP, 10 μM [α-^{32}P]UTP (148 kBq), 0.2 mg/ml bovine serum albumin and 6 ng sscM13 DNA, unless otherwise specified in the figure legends. The products were resolved by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, 89 mM Tris-borate, pH 8.0, 1 mM Na_3EDTA with size markers prepared with poly(rA) or oligo$(rA)_{12-18}$ as described (18). The radioactive bands were visualized using a Model 250 Molecular Imager (BioRad) or an image analyzer (BAS2000; Fuji Photo Film).

Other methods

DNA-dependent ATPase activity and DNA helicase activity were assayed as described (19,20). One unit of ATPase activity is defined as the amount of enzyme that hydrolyzes 1 nmol ATP/h at 37°C. SDS–PAGE was performed according to the method of Laemmli (21).

RESULTS AND DISCUSSION

Co-purification of DNA primase stimulating activity, DNA helicase and DNA-dependent ATPase activities of DNA helicase B

DNA helicase B was purified from mouse FM3A cells by sequential chromatography through eight columns, as described in Materials and Methods. The aliquot of the purified fraction was applied to a Mono Q column for the experiment shown in Figure 1. The stimulatory activity for DNA primase-catalyzed oligoribonucleotide synthesis, DNA helicase and DNA-dependent ATPase activities of DNA helicase B co-eluted, indicating that DNA helicase B has DNA primase stimulating activity (Fig. 1A). The peak fraction had a specific activity of 8×10^5 U/mg protein and contained a major band of ~130 kDa on SDS–PAGE (Fig. 1B). This fraction was used for the following experiments. The intensity of the 130 kDa band was corelated with the level of DNA helicase activity in the fractions from the final column of the purification steps, indicating that this 130 kDa protein is DNA helicase B. In addition, a cDNA clone isolated by using DNA probes deduced from the partial amino acid sequences of this protein possessed the DNA helicase motifs (data not shown).

Effect of DNA concentration on the stimulation of DNA primase-catalyzed oligoribonucleotide synthesis by DNA helicase B

We measured DNA primase activity by analyzing oligoribonucleotide synthesis by DNA polymerase α–primase complex using sscM13 DNA as a template. We identified and purified a DNA primase stimulating factor, which had RNase H activity, from FM3A cells using a similar system containing 1 μg sscM13 DNA (22). However, we could not detect stimulation of DNA primase activity by DNA helicase B at that concentration of M13 DNA. Thus we examined the effect of DNA helicase B on oligoribonucleotide synthesis by the DNA polymerase α–primase complex by changing the concentration of the M13 DNA. No or little stimulation was observed at 60 ng/assay or higher concentrations of M13 DNA (Fig. 2A and B; compare lanes 7, 9 and 11 with 8, 10 and 12 in Fig. 2A). At a concentration of 6 ng/assay M13 DNA synthesis of oligoribonucleotides 5–10 nt in length was markedly stimulated by DNA helicase B (compare lanes 5 and 6). Oligoribonucleotide synthesis was also stimulated at lower concentrations of M13 DNA (compare lanes 3 and 4). In the absence of sscM13 DNA oligoribonucleotide synthesis was not detected (lanes 1 and 2). These results indicate that DNA helicase B facilitates the interaction of DNA polymerase α–primase complex with the template. We performed further experiments at a M13 DNA concentration of 6 ng/assay.

Analysis of the effects of DNA helicase B on DNA primase-catalyzed oligoribonucleotide synthesis

The DNA helicase B fraction used in this study had no detectable ability to synthesize oligoribonucleotides, even at 10 ng (Fig. 3A, lane 5). The synthesis of oligoribonucleotides 5–10 nt in length was stimulated by DNA helicase B in a dose-dependent manner (Fig. 3A and B). The stimulatory effect was remarkable in the presence of 3 ng of the DNA helicase B fraction. The stimulatory effect of

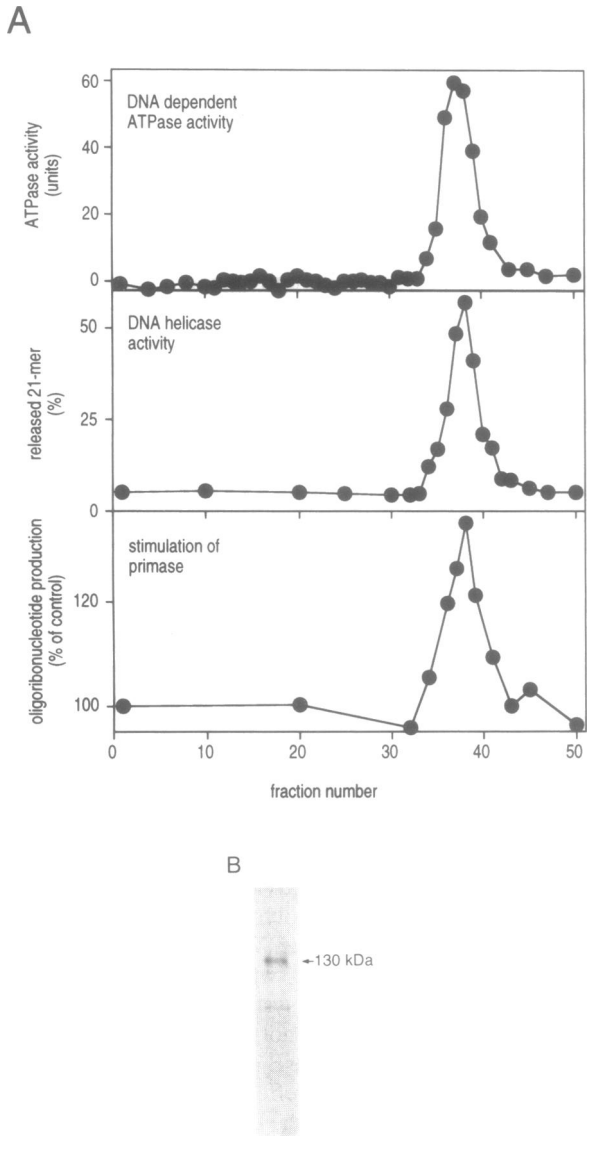

Figure 1. Co-elution of DNA primase stimulating activity and DNA helicase and DNA-dependent ATPase activities from a Mono Q column. (**A**) DNA helicase B purified by eight column chromatographies (2.6×10^4 U) was loaded onto a Mono Q HR5/5 column equilibrated with 50 mM KCl in buffer 2, washed with 150 mM KCl in buffer 2, then eluted with a linear gradient of 150–400 mM KCl in buffer 2. (Top) Aliquots (2 µl) of the eluted fractions were assayed for ATPase activity at 37°C for 15 min. (Middle) Aliquots (5 µl) of 100-fold diluted eluted fractions were assayed for helicase activity at 37°C for 20 min. (Bottom) Aliquots (5 µl) of 1000-fold diluted eluted fractions were incubated at 37°C for 90 min in the standard reaction mixture for primase stimulation assay. Products were analyzed by denaturing PAGE and the radioactivity was quantified with a BioRad Model 250 Molecular Imager. The value obtained from the assay with buffer instead of eluted fractions was defined as 100%. (**B**) The peak fraction of (A) (80 ng) was subjected to SDS–PAGE (12.5%) and silver stained.

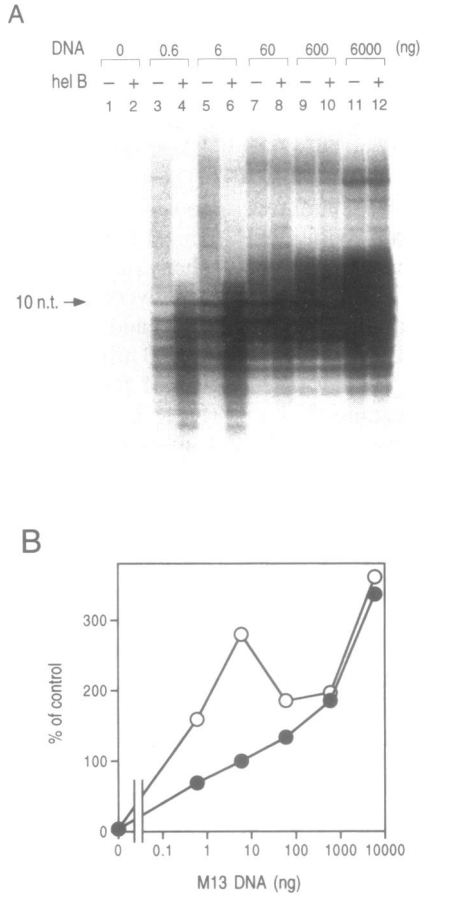

Figure 2. Effect of DNA concentration on stimulation of DNA primase-catalyzed oligoribonucleotide synthesis by DNA helicase B. (**A**) DNA polymerase α–primase was incubated at 37°C for 90 min in reaction mixtures containing none (lanes 1 and 2), 0.6 (lanes 3 and 4), 6 (lanes 5 and 6), 60 (lanes 7 and 8) or 600 ng (lanes 9 and 10) or 6 µg (lanes 11 and 12) sscM13 DNA in the presence or absence of 10 ng DNA helicase B as indicated on the top. The products were analyzed by denaturing PAGE. (**B**) The radioactivity of 5–10 nt oligoribonucleotides in (A) was quantified with an image analyzer (BAS2000; Fuji Photo Film). The results in the presence (○) or absence (●) of DNA helicase B were plotted. The value obtained from the assay under standard conditions (containing 6 ng sscM13 DNA and no DNA helicase B), which was 2.7 fmol oligoribonucleotide synthesized, was defined as 100%.

DNA helicase B was almost saturated at 10 ng/assay. The other mouse DNA helicase, DNA helicase C1 (23), failed to stimulate DNA primase under the same conditions (data not shown).

The synthesis of longer species of oligoribonucleotides, which were produced at low levels in the absence of DNA helicase B (Fig. 3A, lane 6), was inhibited by DNA helicase B (Fig. 3A, lanes 7–10). This effect is also indicated in Figure 2. It has been suggested that the longer species of oligonucleotides are produced by repeated cycles of processive synthesis of a relatively specific size of oligoribonucleotides (17,24). It is unlikely that the increase in the amount of 5–10 nt oligoribonucleotides is mediated by degradation of the longer species of oligoribonucleotide, because the extent of decrease in the amount of longer species was much less than that of the increase in the 5–10 nt oligoribonucleotides (Fig. 3B). In addition, the DNA helicase B fraction had no detectable RNase H activity (data not shown). Thus DNA helicase B stimulates *de novo* oligoribonucleotide synthesis and inhibits repeated synthesis cycles of oligoribonucleotides.

Figure 4 shows the time course of oligoribonucleotide synthesis in the presence and absence of DNA helicase B. In the absence of DNA helicase B the amount of 5–10 nt and longer species of oligoribonucleotides increased with incubation period up to 90 min (lanes 1–4). In the presence of DNA helicase B the stimulated synthesis of 5–10 nt oligoribonucleotides was quite high at all time points tested.

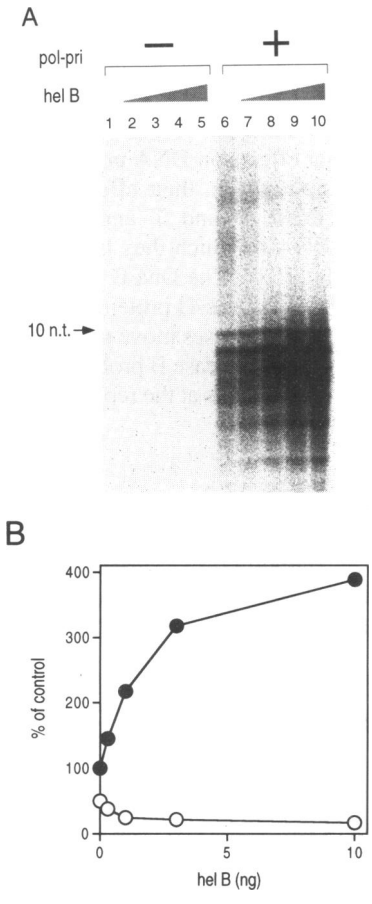

Figure 3. The dose-dependent stimulation of DNA primase-catalyzed oligoribonucleotide synthesis by DNA helicase B. (**A**) The reaction mixtures containing no DNA helicase B (lanes 1 and 6) or 0.3 (lanes 2 and 7), 1 (lanes 3 and 8), 3 (lanes 4 and 9) or 10 ng (lanes 5 and 10) were incubated at 37°C for 90 min with or without DNA polymerase α–primase as indicated on the top. Products were analyzed by denaturing PAGE. (**B**) The oligoribonucleotide products shown in (A) were quantified with an image analyzer (BAS 2000; Fuji Photo Film). The levels of radioactivity of 5–10 nt oligoribonucleotides (○) and the longer products (●) were plotted. The value of 5–10 nt oligoribonucleotides obtained under standard conditions (without DNA helicase B) was defined as 100%.

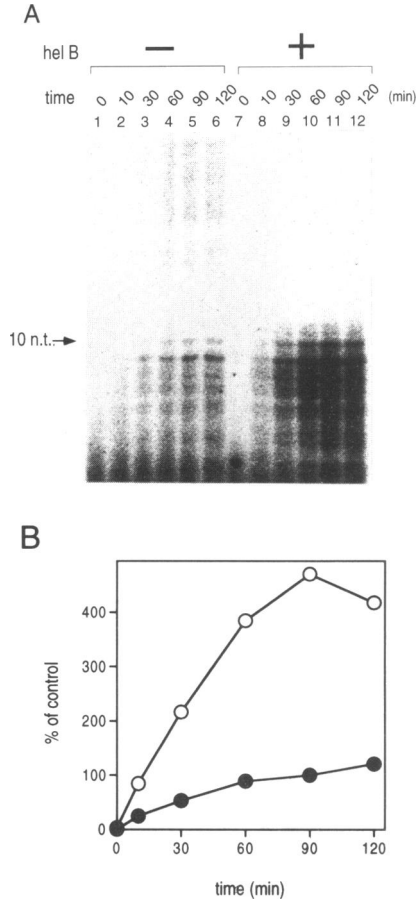

Figure 4. Time course of stimulation of DNA primase activity by DNA helicase B. (**A**) DNA polymerase α–primase was incubated at 37°C in the standard reaction mixture in the presence (lanes 7–12) or absence (lanes 1–6) of 10 ng DNA helicase B. The reactions were stopped at the times indicated on the top. (**B**) The levels of radioactivity of 5–10 nt oligoribonucleotides in (A) were quantified with an image analyzer (BAS2000; Fuji Photo Film). The results in the presence (○) or absence (●) of DNA helicase B were plotted. The value obtained under standard conditions (incubated for 90 min without DNA helicase B) was defined as 100%.

Again, the synthesis of longer oligoribonucleotides was inhibited in the presence of DNA helicase B and the amount of longer species was little increased during the incubation.

The effect of DNA helicase B on the synthesis of oligoribonucleotides in the presence of RP-A

Other studies have shown that RP-A, the ssDNA binding protein in eukaryotic cells, inhibits DNA primase activity using single-stranded circular DNA as a template (10–12). In agreement with these studies, DNA primase-catalyzed oligoribonucleotide synthesis was also inhibited by RP-A in our system (Fig. 5A, compare lanes 1 and 6) and DNA helicase B reversed the inhibition caused by RP-A in a dose-dependent manner (lanes 7–10). The effects of DNA helicase B observed in these experiments were similar to those of SV40 T antigen.

We obtained evidence suggesting that DNA helicase B is involved in DNA replication by analyzing a mutant having a thermolabile DNA helicase B, which shows defects in DNA replication at the non-permissive temperature (6). Similarly to known replicative DNA helicases, mouse DNA helicase B stimulated cognate DNA primase activity 5-fold under certain conditions. To our knowledge this is the first report describing the stimulation of DNA primase by a mammalian DNA helicase.

SV40 T antigen stimulates the DNA primase activity of human DNA polymerase α–primase complex on a ssDNA template and partially reverses inhibition of the primase activity caused by RP-A (10–12). The stimulatory effect was marked at low template and DNA primase concentrations. DNA primase activity was also stimulated by DNA helicase B at low template concentrations. It has been indicated that direct contact of T antigen with DNA polymerase α–primase complex is required for stimulation (9). In addition, T antigen also interacts with RP-A (11). Collins and Kelly have presented a model showing that T antigen facilitates the association of DNA polymerase α–primase complex with the template, whereas RP-A suppresses non-specific priming events (10). Melendy and Stillman have reported the importance of the interaction of T antigen with DNA polymerase α–primase complex and RP-A for primosome assembly during SV40 DNA replication (11).

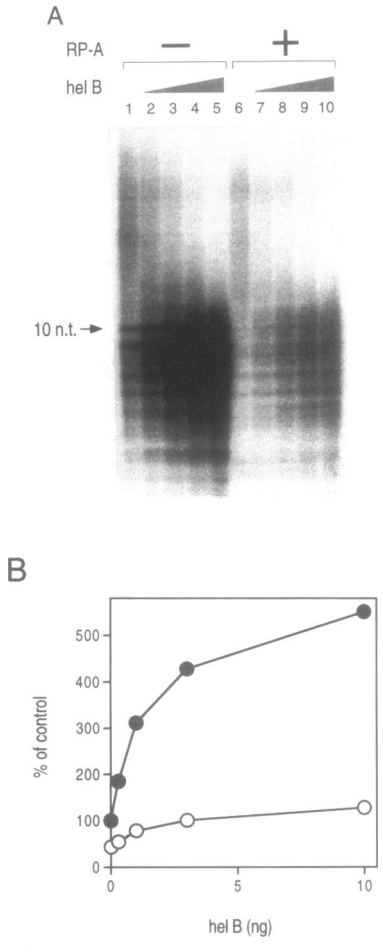

Figure 5. Effect of DNA helicase B on the synthesis of oligoribonucleotides in the presence of RP-A. (**A**) DNA polymerase α–primase was incubated at 37°C for 90 min in the standard reaction mixture containing no DNA helicase B (lanes 1 and 6) or 0.3 (lanes 2 and 7), 1 (lanes 3 and 8), 3 (lanes 4 and 9) or 10 ng (lanes 5 and 10) in the presence (lanes 6–10) or absence (lanes 1–5) of 100 ng RP-A. (**B**) The levels of radioactivity of 5–10 nt oligoribonucleotides in (A) were quantified with an image analyzer (BAS2000; Fuji Photo Film). The results in the presence (○) or absence (●) of RP-A were plotted. The value obtained under standard conditions (without DNA helicase B and RP-A) was defined as 100%.

The use of cell-free systems for studying DNA replication will facilitate understanding the roles of DNA helicase B in DNA replication. A model system that consists of plasmid DNA containing an autonomously replicating sequence (ARS) from *Saccharomyces cerevisiae*, SV40 T antigen, RP-A, DNA polymerase α–primase complex and DNA gyrase has been constructed (25). In this system T antigen acts as a DNA helicase and DNA synthesis starts from the ARS region and proceeds bidirectionally. It is noteworthy that among mammalian DNA helicases so far tested, only DNA helicase B replaced T antigen and functioned in the DNA replication system [Matsumoto,K., Seki,M., Masutani,C., Tada,S., Enomoto,T. and Ishimi,Y. (1995) in press].

The association of DNA polymerase α activity with DNA helicase B was observed through several steps of purification (26). Thus it seems possible that DNA helicase B forms a complex with DNA polymerase α–primase. Although we have not confirmed the direct interaction of DNA helicase B with DNA polymerase α–primase complex and RP-A, it is likely that DNA helicase B stimulates DNA primase activity by facilitating the association of DNA polymerase α–primase complex with the template, as does T antigen, because DNA helicase B and T antigen have similar effects on DNA primase activity.

In spite of the similarity in their effects upon DNA primase activity, DNA helicase B and T antigen move in opposite directions on the DNA to which they bind, namely, 5′→3′ and 3′→5′, respectively (1,20). The Dna B protein of *E.coli*, the T7 gene 4 protein and the T4 gene 41 protein form primosomes at the replication fork. These helicases move on the DNA in the 5′→3′ direction (1). Thus DNA helicase B probably plays a similar role to that of these DNA helicases at the replication fork, residing on the lagging strand template.

ACKNOWLEDGEMENTS

This work was supported by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Matson,S.W. and Kaiser-Rogers,K.A. (1990) *Annu. Rev. Biochem.*, **59**, 289–329.
2 Kelly,T.J. (1988) *J. Biol. Chem.*, **263**, 17889–17892.
3 Stillman,B (1989) *Annu. Rev. Cell Biol.*, **5**, 197–245.
4 Hurwitz,J., Dean,F.B., Kwong,A.D. and Lee,S.-H. (1990) *J. Biol. Chem.*, **265**, 18043–18046.
5 Borowiec,J.A., Dean,F.B., Bullock,P.A. and Hurwitz,J. (1990) *Cell*, **60**, 181–184.
6 Seki,M., Kohda,T., Yano,T., Tada,S., Yanagisawa,J., Eki,T., Ui,M. and Enomoto,T. (1995) *Mol. Cell. Biol.*, **15**, 165–172.
7 Ziegelin,G., Scherzinger,E., Lurz,R. and Lanka,E. (1993) *EMBO J.*, **12**, 3703–3708.
8 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. USA*, **86**, 2186–2189.
9 Dornreiter,I., Höss,A., Arthur,A.K. and Fanning,E. (1990) *EMBO J.*, **9**, 3329–3336.
10 Collins,K.L. and Kelly,T.J. (1991) *Mol. Cell. Biol.*, **11**, 2108–2115.
11 Melendy,T. and Stillman,B. (1993) *J. Biol. Chem.*, **268**, 3389–3395.
12 Schneider,C., Weißhart,K., Guarino,L.A., Dornreiter,I. and Fanning,E. (1994) *Mol. Cell. Biol.*, **14**, 3176–3185.
13 Messing,J. (1983) *Methods Enzymol.*, **101**, 20–78.
14 Tanuma,S., Enomoto,T. and Yamada,M. (1980) *Cell Struct. Funct.*, **5**, 27–37.
15 Takada-Takayama,R., Tada,S., Hanaoka,F. and Ui,M. (1990) *Biochem. Biophys. Res. Commun.*, **170**, 589–595.
16 Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
17 Suzuki,M., Enomoto,T., Masutani,C., Hanaoka,F., Yamada,M. and Ui,M. (1989) *J. Biol. Chem.*, **264**, 10065–10071.
18 Yagura,T., Kozu,T. and Seno,T. (1982) *J. Biol. Chem.*, **257**, 11121–11127.
19 Seki,M., Enomoto,T., Watanabe,Y., Tawaragi,Y., Kawasaki,K., Hanaoka,F. and Yamada,M. (1986) *Biochemistry*, **25**, 3239–3245.
20 Seki,M., Enomoto,T., Yanagisawa,J., Hanaoka,F. and Ui,M. (1988) *Biochemistry*, **27**, 1766–1771.
21 Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
22 Masutani,C., Enomoto,T., Suzuki,M., Hanaoka,F. and Ui,M. (1990) *J. Biol. Chem.*, **265**, 10210–10216.
23 Yanagisawa,J., Seki,M., Kohda,T., Enomoto,T. and Ui,M. (1992) *J. Biol. Chem.*, **267**, 3644–3649.
24 Lehman,I.R. and Kaguni,L.S. (1989) *J. Biol. Chem.*, **264**, 4265–4268.
25 Ishimi,Y. and Matsumoto,K. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5399–5403.
26 Watanabe,Y., Nagata,K., Tawaragi,Y., Enomoto,T., Hanaoka,F. and Yamada,M. (1982) *FEBS Lett.*, **149**, 44–46.