Stimulation of DNA Synthesis by Mouse DNA Helicase B in a DNA Replication System Containing Eukaryotic Replication Origins†

Ken Matsumoto,‡,§ Masayuki Seki,|| Chikahide Masutani,⊥ Shusuke Tada,|| Takemi Enomoto,|| and Yukio Ishimi*,‡

Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan, Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, Japan, and Cellular Physiology Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

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ABSTRACT: A number of DNA helicases have been isolated from mammalian cells, but their abilities to stimulate DNA replication accompanied with DNA unwinding have not been addressed so far. We constructed a model DNA replication system using the yeast autonomously replicating sequence (ARS) as the replication origin. In this system, SV40 T antigen as a DNA helicase assembles to the replication origin where the DNA duplex is unwound by torsional stress due to the negative supercoiling of template DNA, which leads to bidirectional DNA replication from the origin. We report here that DNA helicase B isolated from mouse FM3A cells can greatly stimulate DNA synthesis in this replication system in place of SV40 T antigen. DNA synthesis was dependent on the presence of single-stranded DNA binding protein (RP-A), DNA polymerase α/primase from mouse cells, and *Escherichia coli* DNA gyrase. DNA gyrase was required not only at elongation as a DNA swivelase but also at initiation to increase negative superhelical density of template DNA with the assistance of RP-A. A mammalian DNA fragment containing a replication initiation zone upstream of the c-*myc* gene as well as the yeast ARS fragment acted as a *cis*-element in this system using DNA helicase B. Both DNA helicase B and SV40 T antigen have the ability to extensively unwind the template DNA in the presence of RP-A and DNA gyrase, which may be crucial for stimulation of DNA synthesis in this system.

A model for DNA replication has been presented based upon the finding that replication is initiated by the assembly of DNA helicase to the unwound AT-rich region in the replication origin (Bramhill & Kornberg, 1988; Borowiec et al., 1990). In the oriC replication system of *Escherichia coli*, DnaB helicase assembles in the presence of DnaC protein at the AT-rich region in the replication origin where the DNA duplex is unwound by the binding of DnaA protein in negatively supercoiled template DNA. The DnaB protein unwinds the template DNA with the assistance of single-stranded DNA binding protein by moving on lagging strand template DNA in the direction from 5′ to 3′ (Baker et al., 1986; LeBowitz & McMacken, 1986). In the SV40 DNA replication, SV40 T antigen acts not only as an origin binding protein but also as a DNA helicase that unwinds the template DNA by moving on leading strand template DNA in the direction of 3′ to 5′ (Dean et al., 1987; Goetz et al., 1988; Wiekowski et al., 1988). Coupled with the DNA unwinding by T antigen, primase complexed with DNA polymerase α synthesizes primer RNA for the leading and lagging strand DNA synthesis. A multisubunit single-stranded DNA binding protein (RP-A, also called HSSB)[1] that has been identified as an essential host factor in the SV40 DNA replication system is required for the unwinding of template DNA as well as for DNA synthesis (Hurwitz et al., 1990; Tsurimoto et al., 1990; Weinberg et al., 1990).

We constructed a model DNA replication system with the replication origin from *Saccharomyces cerevisiae* using minimal components of the SV40 DNA replication system (Ishimi & Matsumoto, 1993). In this system, SV40 T antigen induces DNA synthesis as a DNA helicase in the presence of RP-A and DNA polymerase α/primase from HeLa cells. T antigen assembles at the replication origin where the DNA duplex is unwound by the torsional stress of negative supercoils to initiate DNA replication from the origin (Ishimi & Matsumoto, 1994). It has been also shown that DNA replication initiates from the center of a human c-*myc* initiation zone in the same system (Ishimi et al., 1994). A number of DNA helicases have been isolated from mammalian cells by monitoring their ability to displace oligonucleotides annealed to single-stranded DNA [reviewed by Thömmes and Hübscher, (1992) and Matson et al. (1994)]. However, their activities to unwind double-stranded circular DNA and to stimulate DNA replication have not been addressed thus far. In the present study, the ability of several mammalian helicases to replace the function of SV40 T antigen in our DNA replication system was examined. Among those tested, only DNA helicase B, which was prepared from mouse FM3A cells (Seki et al., 1986, 1987), greatly stimulated DNA synthesis in this model system.

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* To whom correspondence should be addressed at the Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida, Tokyo 194, Japan. Telephone: 0427 (24) 6266. Fax: 0427 (24) 6317.

‡ Mitsubishi Kasei Institute of Life Sciences.

§ Present address: Laboratory of Molecular Embryology, NICHD, NIH, Bethesda, MD 20892.

|| University of Tokyo.

⊥ The Institute of Physical and Chemical Research (RIKEN).

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[1] Abbreviations: ARS, autonomously replicating sequence; SV40, simian virus 40; T antigen, large tumor antigen; RP-A, replication protein A; ATPase, adenosinetriphosphatase.

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Consistent with these results, a mutant from FM3A cells of which both the cellular DNA synthesis and DNA helicase B activity are temperature-sensitive has been recently isolated (Seki et al., 1995). These *in vitro* and *in vivo* studies strongly suggest that DNA helicase B is involved in cellular DNA replication.

MATERIALS AND METHODS

Construction of Plasmid DNA. ARS1 DNA (YRp7ΔEP, 3.4 kb), which contains a 1 kb fragment from the ARS1 region in pBR322-derived vector, was prepared from *E. coli* as described (Ishimi & Matsumoto, 1993). ARS1 DNAs with a higher negative superhelical density than that of form I DNA were prepared as reported (Ishimi & Matsumoto, 1994). The plasmid ori$^-$ DNA (pBR322ΔEP, 2.5 kb) is a deletion derivative of pBR322, lacking the *Eco*RV−*Pvu*II region (Wobbe et al., 1985). It contains the same sequence as the vector in ARS1 DNA plus the *Eco*RV to *Eco*RI fragment (187 bp) from pBR322 DNA. To construct HMYC DNA (5.3 kb), a 2.8 kb fragment containing the upstream region of the human c-*myc* gene [*Hin*dIII (nucleotide 1) to *Pvu*II (2841) (Gazin et al., 1984)] was inserted into the *Hin*dIII site of ori$^-$ DNA (Ishimi et al., 1994).

Purification of Proteins. DNA polymerase α/primase, topoisomerase I, and RP-A (HSSB) (Ishimi et al., 1988) and DNA-dependent ATPase Q2 (Seki et al., 1994) from HeLa cells; DNA polymerase α/primase from FM3A cells (Takada-Takayama et al., 1990); and *E. coli* DNA gyrase and SV40 T antigen from Sf27 cells infected with recombinant baculovirus (Ishimi & Matsumoto, 1993) were purified as described previously. Purification of RP-A from mouse FM3A cells will be published elsewhere. Briefly, FM3A extracts passed through phosphocellulose were loaded onto a hydroxylapatite column equilibrated with buffer A [30 mM Hepes−KOH, pH 7.5, 0.25 mM EDTA, 10% glycerol, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL antipain, and 0.01 % Triton X-100] plus 0.1 M KCl. Proteins eluted from 80 mM potassium phosphate, pH 7.5, were dialyzed against 0.4 M NaCl in buffer A and then loaded onto single-stranded DNA−cellulose in the same buffer. The proteins that were eluted with 1.5 M NaCl and 50% ethylene glycol in buffer A were dialyzed against 0.1 M NaCl in buffer A, and then separated using a linear NaCl gradient (0.1−0.5 M) on Mono Q. Purified RP-A was dialyzed against 30% glycerol and 25 mM NaCl in buffer A without Triton. DNA helicase B was purified from FM3A cells essentially as described previously (Seki et al., 1986, 1987) by successive chromatographies on first DEAE-cellulose, second DEAE-cellulose, phosphocellulose, single-stranded DNA−cellulose, Q-Sepharose, hydroxylapatite, and FPLC MonoS and MonoQ. DNA-dependent ATPase H1 was purified by column chromatographies as described below by monitoring the DNA-dependent ATPase activity (Seki et al., 1986). HeLa cell extracts (100 mg of protein) (Wobbe et al., 1985) were loaded onto a heparin−agarose column (10 mL) equilibrated with 20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 0.1 mM PMSF, and 0.36 M NaCl. The flow-through fractions were loaded onto a DEAE-cellulose column (10 mL) equilibrated with buffer B (20 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, 20% ethylene glycol, and 0.01% Triton X-100) and 50 mM KCl, and proteins bound to the column were eluted by 0.4 M KCl in buffer B. After dialysis against buffer B plus 50 mM KCl, proteins were loaded onto a phosphocellulose column (2.5 mL) equilibrated with the same buffer. The column was developed by a linear gradient from 50 mM to 0.8 M KCl in buffer B. An ATPase activity eluted at 0.4 M KCl was loaded onto a hydroxylapatite column (2 mL) in buffer B plus 0.35 M KCl, and bound proteins were eluted by buffer B containing 0.1 M instead of 20 mM potassium phosphate. After dialysis against buffer B, proteins were applied to a (carboxylmethyl)cellulose column (0.8 mL) in buffer B containing 50% ethylene glycol, followed by elution by a linear gradient from 0 to 0.3 M KCl. An active fraction recovered at 0.1 M KCl was 5-fold-diluted with 20 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, 10% glycerol, and 30 mM KCl and concentrated by Centricon 30 (Amicon). The purified fraction of ATPase H1 contained 73 and 140 kDa peptides on a sodium dodecyl sulfate−polyacrylamide gel. The protein concentrations of the DNA helicases were 0.10 (SV40 T antigen and DNA-dependent ATPase H1), 0.34 (DNA helicase B), and 0.04 mg/mL (DNA-dependent ATPase Q2).

Replication Conditions. DNA replication was performed as described (Ishimi & Matsumoto, 1993). One hundred nanograms of plasmid DNA was incubated at 37 °C for 1 h with the indicated amounts of DNA polymerase α/primase complex, RP-A and DNA helicase, and DNA gyrase (100 ng of gyrase A and 160 ng of gyrase B) in a 40 μL reaction mixture [40 mM creatine phosphate, pH 7.8, 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 20 μM [α-^{32}P]dCTP [(1−2) × 10^4 cpm/pmol], 200 μM each of CTP, UTP, and GTP, 100 μM each of dATP, dGTP, and dTTP, 0.8 μg of creatine phosphokinase, and 16 μg of bovine serum albumin]. After measurement of the radioactivity in the acid-insoluble fraction, the products were purified and resolved by electrophoresis in a 1.5% agarose gel under denaturing conditions with 30 mM NaOH and 1 mM EDTA or in a 1% agarose gel in Tris−borate−EDTA (TBE) buffer. The gels were dried and visualized by autoradiography.

DNA Helicase Assay. DNA helicase activity was assayed as described previously (Seki et al., 1987). A 17-mer oligonucleotide (5′-GTTTTCCCAGTCACGAC-3′, −40 primer for M13 dideoxy sequencing; USB) was end-labeled with [γ-^{32}P]ATP and annealed with M13 mp19 single-stranded DNA for the helicase substrate. The helicase substrate (0.017 pmol) was incubated at 37 °C for 30 min with DNA helicase and resolved by electrophoresis in a 12% polyacrylamide gel in TBE buffer. The gels were dried and autoradiographed. One unit of DNA helicase is defined as the activity that releases half of the labeled primer from the annealed DNA in the standard conditions. From the experiment in Figure 1A, the concentration of each DNA helicase was determined as 5 units/μL (SV40 T antigen), 33 units/μL (helicase B), 10 units/μL (Q2), and 15 units/μL (H1).

RESULTS

Stimulation of DNA Replication by Mouse DNA Helicase B. Negatively supercoiled plasmid ARS1 DNA was incubated at 37 °C for 1 h with DNA polymerase α/primase and the single-stranded DNA binding protein (RP-A, also called HSSB) from human HeLa cells (referred to as the human system) or mouse FM3A cells (the mouse system) in the

presence of DNA gyrase. DNA gyrase was mainly required at elongation as a swivelase that relieves the torsional stress of positive supercoils accumulated ahead of the replication forks in the human system with SV40 T antigen (Ishimi & Matsumoto, 1994). Three cellular DNA-dependent ATPases in addition to T antigen, which have DNA helicase activities (Figure 1A), were added to this system to test the ability to stimulate DNA replication. These three DNA helicases seem to be different enzymes as judged from their purification procedures (Seki et al., 1986, 1994; and this study). The products of DNA replication were analyzed by alkaline agarose gel electrophoresis (Figure 1B). The addition of SV40 T antigen in both the human and mouse systems significantly stimulated the level of DNA replication. Longer products of about half the length of the template DNA as well as shorter products accumulated in the human system. It has been suggested that these products are synthesized by semidiscontinuous DNA replication (Ishimi & Matsumoto, 1993). In the mouse system, however, the half-length products and a smear of shorter products were synthesized.

The addition of DNA helicase B from mouse FM3A cells (Seki et al., 1986, 1987) stimulated DNA replication in this system. In contrast, DNA-dependent ATPase Q2 (Seki et al., 1994) and ATPase H1 (see Materials and Methods for purification) isolated from HeLa cells did not stimulate DNA replication in either the cognate human or the mouse system (Figure 1B). Among several mammalian DNA helicases tested, only helicase B stimulated DNA replication in place of SV40 T antigen (Figure 1B and data not shown). The products of DNA replication in both the human and mouse systems with DNA helicase B were a smear ranging from 200 to more than 2000 nucleotides. The stimulation of DNA replication by DNA helicase B was greater in the cognate mouse system than in the human system. However, when the amounts of DNA polymerase α/primase from the mouse cells decreased to 0.06 and 0.35 unit, respectively, to make those relatively similar to the amounts of HeLa DNA polymerase α/primase in the human system, the stimulation of DNA replication by helicase B was almost comparable between the two systems (data not shown). These data, together with the results with T antigen (Figure 1B), may suggest that the species specificity of DNA replication is not stringent in this model system. As the amount of helicase B increased, the average size of nascent DNA decreased (Figure 2A), but neither half the length of template DNA (leading strand) nor shorter Okazaki fragments accumulated (see Discussion). In the presence of helicase B, high molecular weight DNAs were detected in the native gel (Figure 2B). When the products were electrophoresed in the native gel after *Dpn*I digestion, most of them remained in the position of the high molecular weight DNA (data not shown), indicating that most of the DNA synthesis occurring in this system is replication-type. Hybridization showed that both strands were synthesized at a similar level in the region (about 500 bp in length) between *EcoR*I and *Sca*I in ARS1 DNA (see Figure 7B) (data not shown). When the mouse DNA polymerase α/primase was titrated from 0.03 and 0.17, 0.06 and 0.35, 0.13 and 0.7, to 0.25 and 1.39 units in the reaction containing DNA helicase B (33 units) and mouse RP-A, the incorporation of deoxyribonucleotides was 3.8, 5.1, 7.7, and 12 pmol, respectively. The average size of synthesized single-stranded DNA gradually decreased as the amount of DNA polymerase α/primase increased, and any

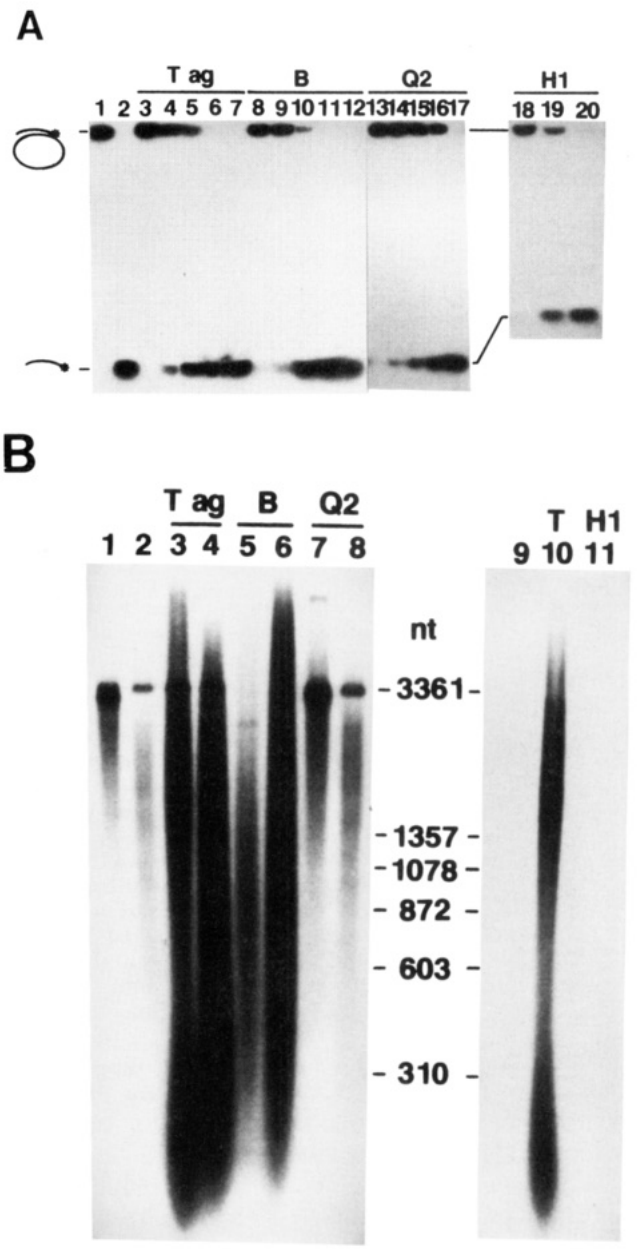

FIGURE 1: DNA helicase B stimulates ARS DNA replication in the mouse system. (A) The helicase activities of SV40 T antigen (0.01, 0.1, 0.2, 0.5, and 1 μL in lanes 3−7, respectively), DNA helicase B (0.01, 0.02, 0.05, 0.1, and 1 μL in lanes 8−12), DNA-dependent ATPase Q2 (0.01, 0.03, 0.06, 0.1, and 1 μL in lanes 13−17), and ATPase H1 (0.01, 0.1, and 1 μL in lanes 18−20) were assayed as described under Materials and Methods. Helicase substrates incubated at 37 °C without enzyme (lane 1) or heat-denatured (lane 2) were electrophoresed in parallel. The positions of end-labeled oligonucleotide annealed with M13 single-stranded DNA and released by DNA helicase are indicated. (B) Negatively supercoiled plasmid DNA containing yeast ARS1 (ARS1 DNA) was replicated with DNA polymerase α/primase (0.015 and 0.32 units, respectively) and RP-A (0.05 μg) from HeLa cells (lanes 1, 3, 5, 7, and 9−11) or with DNA polymerase α/primase (0.25 and 1.39 units, respectively) and RP-A (0.2 μg) from FM3A cells (lanes 2, 4, 6, and 8) in the presence of *E. coli* DNA gyrase. DNA helicases were added to the reaction: SV40 T antigen (25 units, lanes 3, 4, and 10); DNA helicase B (66 units, lanes 5 and 6); DNA-dependent ATPase Q2 (20 units, lanes 7 and 8); and ATPase H1 (30 units, lane 11). The products were resolved by electrophoresis in an alkaline agarose gel. The positions of φX174 DNA fragments digested with *Hae*III and single-stranded linear DNA of the template are indicated. The total nucleotides incorporated were 2.9 (lane 1), 2.5 (lane 2), 14 (lane 3), 22 (lane 4), 5.0 (lane 5), 17 (lane 6), 3.5 (lane 7), 3.3 (lane 8), 1.0 (lane 9), 17 (lane 10), and 1.1 pmol (lane 11).

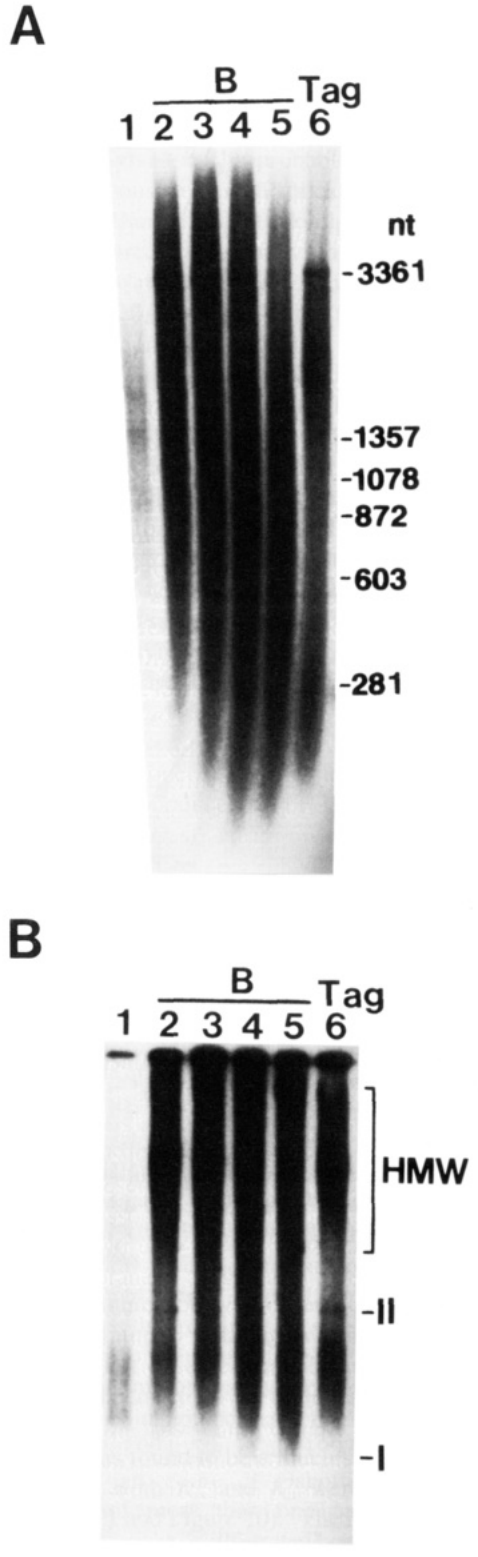

FIGURE 2: Titration of DNA helicase B in the mouse system. Increasing amounts of DNA helicase B (0, 8, 16, 33, and 66 units in lane 1−5, respectively) were added to the reaction mixture that contains DNA polymerase α/primase (0.13 and 0.7 units, respectively) and RP-A from mouse cells in addition to DNA gyrase. DNA replication was performed with 100 ng of ARS1 DNA as the template. Incorporated total nucleotides are 4.5, 12, 15, 16, and 16 pmol in lane 1−5, respectively. Replicated DNAs were analyzed by alkaline (A) or native (B) agarose gel electrophoresis. Lane 6 contains the products synthesized in the human system with SV40 T antigen (25 units, 24 pmol). In (B), positions where forms I and II (nicked circular) and high molecular weight (HMW) DNA migrate are indicated.

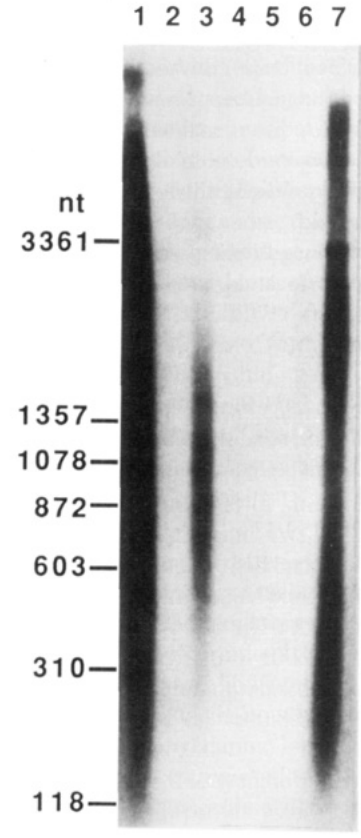

FIGURE 3: Requirements *in trans* for DNA replication in the mouse system with DNA helicase B. DNA replication proceeded using 100 ng of ARS1 DNA with the complete components containing DNA polymerase α/primase (0.13 and 0.7 units), RP-A and DNA helicase B (33 units) from FM3A cells, and DNA gyrase (lane 1). One of the components [DNA gyrase (lane 2), helicase B (lane 3), RP-A (lane 4), and DNA polymerase α/primase (lane 5)] was omitted from the complete reaction. DNA topoisomerase I from HeLa cells (250 units) was added to the complete reaction (lane 6). In lane 7, DNA replication proceeded in the human system with SV40 T antigen (25 units). The products were electrophoresed in an alkaline agarose gel.

distinct products of the leading and the lagging strands were not synthesized in these reactions (data not shown). These results indicate that mouse DNA helicase B stimulates the DNA synthesis in place of T antigen in this model replication system and that the mode of the replication is somehow different from that in the human system with T antigen.

Negative Superhelicity Is Required for DNA Replication. The requirements for the components of the mouse system with DNA helicase B were examined (Figure 3). When mouse RP-A or DNA polymerase α/primase was omitted, quite low or no DNA synthesis was detected. The omission of DNA gyrase from the system resulted in a severe reduction of DNA synthesis, that is in contrast with the ARS1 DNA replication system using SV40 T antigen, where shorter DNAs were synthesized even in the absence of DNA gyrase (Ishimi & Matsumoto, 1993). In the latter system, it has been shown that DNA gyrase mainly acts at the elongation step as a DNA swivelase (Ishimi & Matsumoto, 1994). The finding that DNA topoisomerase I from HeLa cells inhibited DNA replication indicated that the mouse system with DNA helicase B required negative superhelicity of the template DNA. The severe reduction of DNA synthesis by the omission of DNA gyrase raised the possibility that DNA gyrase acts at the initiation step to increase negative

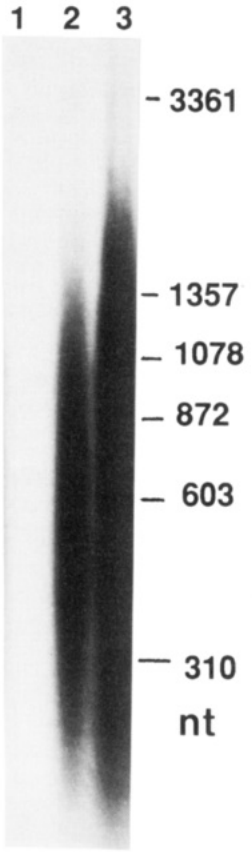

FIGURE 4: Higher superhelical density is required for DNA replication. ARS1 DNAs (100 ng) with higher superhelical density were used as a template in the mouse system that contains DNA polymerase α/primase (0.13 and 0.7 units) and RP-A and helicase B (33 units), and the replication proceeded in the absence of DNA gyrase. The superhelical densities of the template DNAs were −0.06 (form I) (lane 1), −0.098 (lane 2), and −0.128 (lane 3). The products were analyzed by alkaline agarose gel electrophoresis.

superhelicity as well as at the elongation step as a swivelase. We, then, used ARS1 DNA with a higher negative superhelical density than form I as template for the replication that was carried out in the absence of DNA gyrase (Figure 4). In proportion to the negative-superhelical density of the template, DNA synthesis was enhanced, and a density of at least −0.1 was required for DNA synthesis in this system with helicase B. Only shorter DNAs were synthesized in this system, which is consistent with the idea that the replication fork movement is blocked by the torsional stress due to positive supercoils in the absence of DNA gyrase. These results suggest that DNA gyrase is required for ARS DNA replication in this system at the initiation step as well as at the elongation step.

It has been found that SV40 T antigen can extensively unwind the template DNA containing the SV40 origin of DNA replication in the presence of DNA topoisomerase I and human RP-A to generate a highly negatively supercoiled DNA called form U (Dean et al., 1987). When the ARS1 DNA was incubated with DNA gyrase and RP-A under the replication conditions, some of the DNA migrated faster than form I DNA in an agarose gel containing chloroquine (Figure 5), suggesting that unwinding of the template DNA proceeded in this reaction and as a result the DNA became more negatively supercoiled than form I DNA (see Discussion and Figure 9). Both RP-A and gyrase were required for this reaction. The DNAs that migrated faster than relaxed

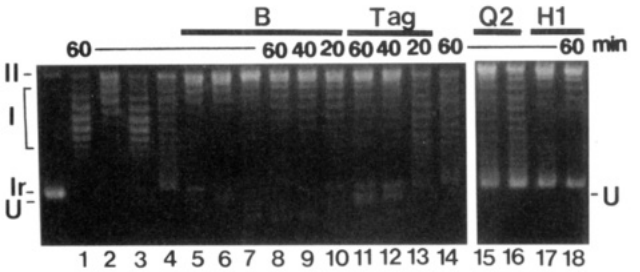

FIGURE 5: Extensive unwinding occurs in the presence of DNA helicase B. ARS1 DNA (form I, 100 ng) was incubated for 1 h at 37 °C in the absence of replication proteins under replication conditions without nucleotides (lane 1). The DNA was incubated with DNA gyrase (lane 2), with human RP-A (80 ng) (lane 3), or with both gyrase and RP-A (lanes 4 and 14). Increasing amounts of helicase B (8, 16, 32, and 66 units in lanes 5−8 and 66 units in lanes 8−10), SV40 T antigen (30 units in lanes 11−13), ATPase Q2 (20 and 40 units in lanes 15 and 16), and ATPase H1 (20 and 40 units in lanes 17 and 18) were added to the reaction in the presence of gyrase and RP-A and incubated for the indicated times. Purified DNAs were electrophoresed in a 1% agarose gel in TBE buffer containing 4.3 μM chloroquine. DNA was visualized by staining with ethidium bromide. Relaxed circular (Ir) DNA was also electrophoresed in parallel, and positions of forms I, II, and U are indicated.

circular DNA (form Ir) were detected by incubating the template DNA with T antigen in the presence of RP-A and gyrase, which are probably highly supercoiled form U DNA (Dean et al., 1987). Addition of helicase B in place of the T antigen also resulted in the appearance of form U-like DNAs. As the amount of helicase B or the duration of incubation increased, DNAs which migrate faster than form U-like DNAs appeared, which may be more highly supercoiled forms than form U DNA. These highly unwound DNAs were not detected in the presence of ATPase Q2 or ATPase H1, although the DNAs which migrated faster than form I DNA but slower than the relaxed DNA increased. These results suggest that DNA helicase B assembles to the unwound origin that is formed by the torsional stress of negative supercoils and unwinds the template DNA to stimulate DNA synthesis.

DNA Replication Is Dependent on the Presence of Origins. When the initiation site was determined in the mouse system with helicase B (Figure 6A,B), the fragments near the B domain of ARS1 formed a peak within the short labeling period (2.5 and 5 min), but the peak was broader than that observed in the human system using T antigen (Ishimi & Matsumoto, 1993). During the incubation for 30 min, all fragments were almost evenly labeled. In the absence of helicase B, DNA synthesis was greatly reduced, but the fragments near the B domain were labeled during incubation for 30 min. These results indicate that DNA helicase B greatly stimulates DNA replication that has been basically initiated near the ARS1 region in the absence of helicase B. To examine the requirement for a replication origin in *cis*, plasmid DNAs with or without the ARS1 region were tested for template activity in the mouse DNA replication system with DNA helicase B (Figure 7). In a replication reaction that contained more template DNA than the standard condition, DNA replication was dependent upon the ARS1 region. We reported that DNA replication is dependent on the ARS1 region when the amount of SV40 T antigen over the template DNA is limiting and that DNA replication initiates from the ARS1 region and proceeds bidirectionally (Ishimi & Matsumoto, 1993).

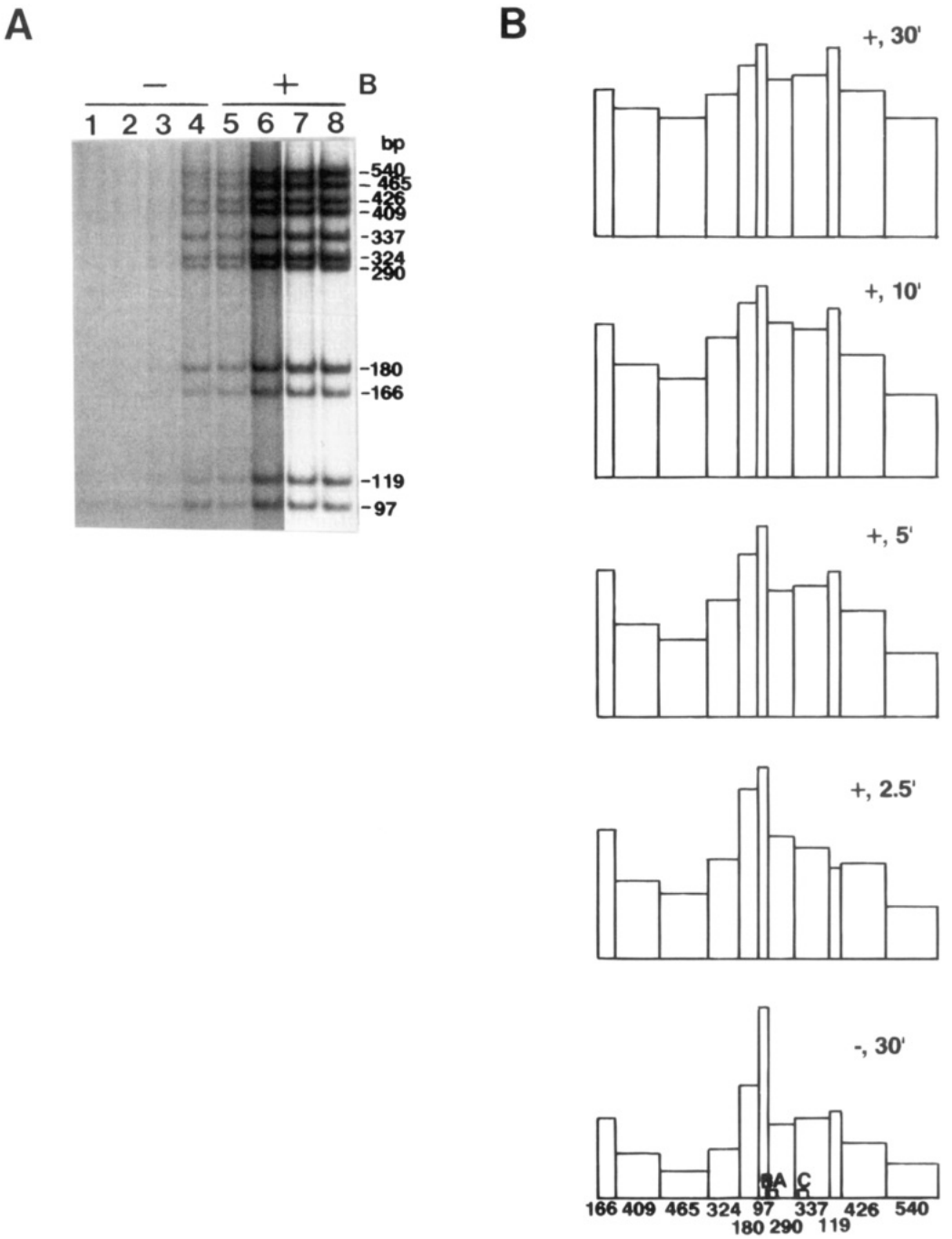

FIGURE 6: Mode of initiation of DNA replication in the system containing helicase B. One hundred nanograms of ARS1 DNA (form I) was incubated in the mouse system without (lanes 1−4) or with (lanes 5−8) helicase B (66 units) for 15 min in the presence of rNTPs and then pulse-labeled by adding dNTPs including [α-^{32}P]dCTP for 2.5 (lanes 1 and 5), 5 (lanes 2 and 6), 10 (lanes 3 and 7), and 30 min (lanes 4 and 8). Replicated DNAs were digested with *Dde*I and *Dra*III and analyzed by 5% polyacrylamide gel electrophoresis. (A) Autoradiograms of the gel are shown, and the size of each fragment is indicated. Half of the reaction was loaded onto the gel in lanes 1−7, and one-sixth of the reaction was in lane 8. Film was exposed for 1 week for lanes 1−6, and for 2 days for lanes 7 and 8. (B) The labeled bands were quantitated, and the radioactivity in the region that does not contain any band was subtracted from them in each lane. The values were divided by the size of each fragment and corrected for base composition. They are presented on a linear map of ARS1 DNA: the highest values observed during the different labeling periods were adjusted to the same level. The A, B, and C domains of ARS1 are indicated by open boxes at the bottom of the figure. Results on the 2.5, 5, 10, and 30 min incubation in the presence of helicase B and the 30 min incubation in the absence of helicase B are shown, as indicated.

These data indicated that the yeast ARS region is required for DNA replication in this system. As one step to establish a cell-free DNA replication system with cellular components, we examined whether DNA from mammalian cells can function as the origin in the mouse system using DNA helicase B. Vassilev and Johnson (1990) identified an initiation zone in the region upstream of the human c-*myc* gene. It has been shown that DNA replication is initiated from the center of the c-*myc* initiation zone in the human system containing T antigen as a DNA helicase (Ishimi et al., 1994). Negatively supercoiled plasmid DNA containing the upstream region of the human c-*myc* gene (HMYC DNA) was shown to be the active template in the mouse system with DNA helicase B (Figure 7, lanes 5 and 6). The level of DNA synthesis of HMYC DNA was much higher than that of ARS1 DNA, when the same amount (by weight) of

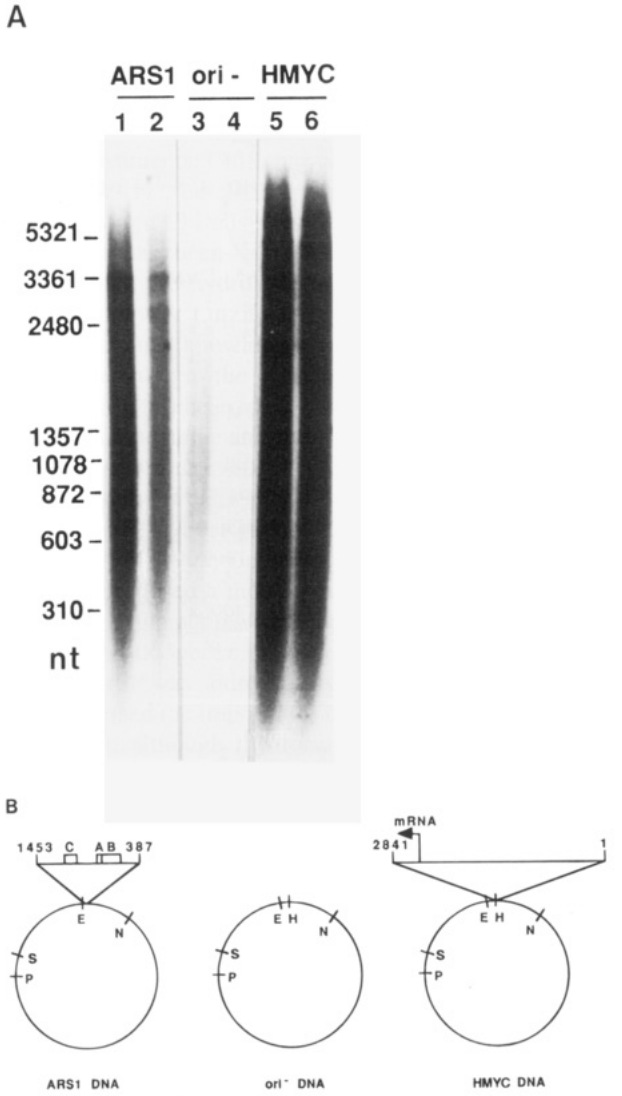

FIGURE 7: DNA replication is dependent on the presence of origins. (A) DNA replication proceeded using various template DNAs (form I) in the mouse system that contains DNA polymerase α/primase (0.13 and 0.7 units), RP-A, helicase B (33 units), and DNA gyrase. The templates were ARS1 DNA (lanes 1 and 2), ori⁻ DNA (lanes 3 and 4), and HMYC DNA (lanes 5 and 6). Two hundred nanograms (lanes 1, 3, and 5) and 300 ng (lanes 2, 4, and 6) of each template were used in 40 μL of the reaction mixture containing a constant amount of replication proteins. (B) The constructs of template DNAs are shown. The A, B, and C domains of ARS1, the P1 transcription start site of the c-*myc* gene, the restriction sites [E (*Eco*RI), H (*Hind*III), N (*Nde*I), P (*Pvu*I), and S (*Sca*I)], and the nucleotide numbers of ARS1 (Tschumper & Carbon, 1980) and the c-*myc* gene (Gazin et al., 1984) are indicated.

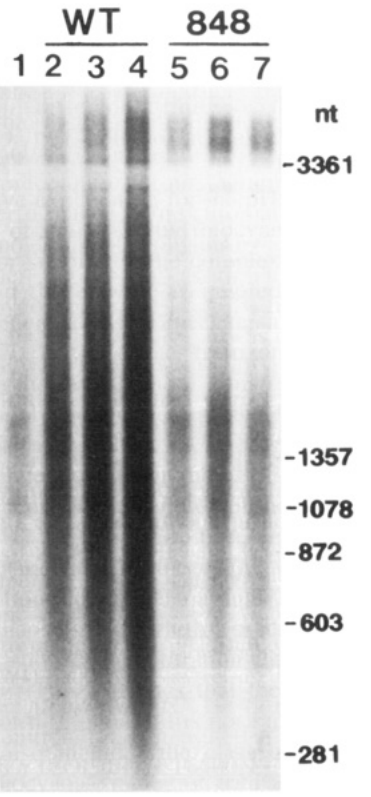

FIGURE 8: DNA helicase B from mutant cells hardly stimulates DNA replication. ARS1 DNA (form I, 100 ng) was incubated with mouse DNA polymerase α/primase (0.25 and 1.39 units), mouse RP-A, and DNA gyrase in the absence (lane 1) or in the presence of helicase B from wild-type cells (5, 10, and 20 units in lanes 2−4) or mutant cells (5, 10, and 20 units in lanes 5−7). After the reactions were incubated for 1 h at 37 °C, purified DNAs were analyzed by alkaline agarose gel electrophoresis. An autoradiogram of the dried gel is shown. Incorporated total nucleotides are 3.0 (lane 1), 6.9 (lane 2), 8.7 (lane 3), 12 (lane 4), 5.2 (lane 5), 5.3 (lane 6), and 4.5 pmol (lane 7).

template DNA was used (lanes 1 vs 5 and 2 vs 6). This indicates that DNA from mammalian cells, which acts as a putative replication origin in human cells, can function as a *cis*-element for supporting DNA replication in the mouse DNA replication system with DNA helicase B.

Helicase B from Mutant Cells Can Hardly Stimulate DNA Replication. We have reported a temperature-sensitive mutant from mouse FM3A cells of which the DNA helicase B activity was also temperature-sensitive (Seki et al., 1995). DNA helicase B was purified from the mutant FT848 cells as from the parental FM3A cells, and the purified helicase B had DNA helicase activity [data not shown and Seki et al., (1995)]. First, we compared the heat-sensitivity of the two helicases B. When the helicases B from wild-type and mutant cells were treated at 40 °C for 20 and 60 min and then added to the mouse replication system, DNA replication in both reactions was similarly inhibited by the heat treatment (data not shown). In this experiment, however, we noticed that the amount of DNA synthesis in the presence of helicase B from the mutant cells was lower than that in the presence of helicase B from the wild-type cells, although the same activity of DNA helicase was included in the two reactions. Then, the activity to stimulate DNA replication was compared between the two helicases B from the wild-type and mutant cells by titrating them in the mouse system (Figure 8). In contrast to helicase B from wild-type cells, DNA replication was not greatly stimulated by helicase B from the mutant cells, indicating that DNA helicase B from the mutant cells is not so potent as helicase B from the wild-type cells in stimulating DNA replication in this system.

DISCUSSION

In bacterial and viral DNA replication systems, DNA helicases, which are encoded by their cognate organisms or their hosts, play important roles in the initiation as well as the elongation of DNA replication (Goetz et al., 1988; Wiekowski et al., 1988; Matson et al., 1990; Seo et al., 1993). Here we showed that DNA helicase B isolated from mouse cells stimulates DNA synthesis in a cell-free DNA replication system. Studies on the human system with SV40 T antigen

as the DNA helicase have revealed that T antigen recognizes the unwound structure of DNA near the replication origin, which is formed by torsional stress due to the negative superhelicity of template DNA, with the assistance of RP-A (Ishimi & Matsumoto, 1994). We speculate from these findings that mammalian DNA helicase B enters the unwound structure near the replication origin by a similar mechanism. Thus, it is possible that DNA helicase B plays a role in the initiation as well as in the elongation of cellular DNA replication. However, the finding that helicase B greatly stimulates DNA replication that has been initiated in the absence of helicase B (Figure 6) suggests that DNA replication is initiated by DNA polymerase α/primase and RP-A from the partially unwound ARS1 region and DNA helicase B plays a major role in the elongation step of DNA synthesis in this system. Recently, we have isolated a temperature-sensitive mutant from mouse FM3A cells of which the DNA helicase B activity was also temperature-sensitive (Seki et al., 1995). In these cells, DNA synthesis decreased upon raising the temperature, suggesting the involvement of helicase B in cellular DNA replication. The results presented in this paper provide biochemical evidence for the involvement of DNA helicase B in cellular DNA replication.

Only helicase B stimulated DNA synthesis in this system among several mammalian DNA helicases tested. The DNA helicases characterized so far displace the annealed oligonucleotide by moving in only one direction with respect to the single-strand template to which they bind. DNA helicase B proceeds from the 5′ to 3′ direction (Seki et al., 1988), which is identical to *E. coli* DnaB helicase (LeBowitz & McMacken, 1986) that is responsible for DNA unwinding in the oriC replication system (Baker et al., 1986) and contrary to SV40 T antigen (Goetz et al., 1988; Wiekowski et al., 1988). This indicates that DNA helicase B loads onto the lagging strand template when it unwinds the DNA duplex at a replication fork. When the ability to induce extensive DNA unwinding was compared among DNA helicase B, ATPase Q2, both of which migrate from the 5′ to the 3′ direction (Seki et al., 1988, 1994), and ATPase H1, DNAs whose mobility was similar to that of highly supercoiled form U DNA (Dean et al., 1987) were detected with helicase B but not with ATPase Q2 and ATPase H1 (Figure 5). Although both ATPase Q2 and ATPase H1 stimulated the DNA unwinding that was induced by RP-A and gyrase, they did not extensively unwind the template DNA. Mouse DNA helicase B has been identified as a DNA-dependent ATPase that was copurified with DNA polymerase α, suggesting its interaction with DNA polymerase α/primase (Watanabe et al., 1982). The finding that the sizes of nascent DNAs decrease in proportion to the amount of helicase B added to the reaction (Figure 2A) may suggest that helicase B assists primer RNA synthesis by DNA polymerase α/primase in this system. Recently, we have shown that helicase B stimulates the primase activity (Saito et al., 1995). It has also been shown that T antigen interacts with DNA polymerase α (Smale & Tjian, 1986; Gannon & Lane, 1987; Dornreiter et al., 1992). Melendy and Stillman (1993) have reported that human RP-A inhibits synthesis by DNA polymerase α/primase on single-strand DNA templates and that T antigen reverses the inhibition, suggesting the interaction of T antigen with RP-A. These abilities of DNA helicase to extensively unwind plasmid DNA and to interact with DNA polymerase α/primase and RP-A may be required for stimulating DNA replication in this system.

The products in the mouse system with DNA helicase B were heterogeneous and had a different profile from those in the human system with SV40 T antigen (Figure 1B) in which distinct leading and lagging strands were synthesized. In the SV40 DNA replication, two replication forks proceed bidirectionally from an origin at a similar speed, and both the leading and lagging strands are synthesized at the forks, coupled with the unwinding of the template DNA. As a result, the leading strands whose size is almost half the length of the template DNA are synthesized. The results with helicase B may indicate that the two replication forks do not proceed at a similar speed from the origin. Another explanation for the results is that the speed of DNA unwinding by helicase B is faster than T antigen, and, thereby, DNA synthesis is not coupled with DNA unwinding in this replication system where DNA polymerase α instead of DNA polymerase δ synthesizes the leading strands (Ishimi et al., 1988; Hurwitz et al., 1990). It has been shown that the speed of the fork movement in cellular DNA replication is about 10 times that in SV40 DNA replication (Edenberg & Huberman, 1975). Furthermore, it has been suggested that the coupling of DNA synthesis with DNA unwinding may not be stringent in eukaryotic cells (Benbow et al., 1992). It is also possible that another DNA helicase(s) is (are) required for proper synthesis of the leading and lagging strands. In the *E. coli* oriC system, the primosome that functions in primer synthesis for Okazaki fragments includes two DNA helicases, DnaB and PriA (n′) (Marians, 1992). It would be important to test these possibilities in a more defined replication system (Hurwitz et al., 1990; Tsurimoto et al., 1990; Weinberg et al., 1990).

In previous studies, we showed that DNA gyrase is required only at the elongation step as a swivelase in the human system with SV40 T antigen (Ishimi & Matsumoto, 1993, 1994). In contrast, DNA gyrase was essential in the mouse system with DNA helicase B when form I plasmid DNA (density: −0.06) was used as the template. DNA gyrase increased the negative superhelical density of plasmid DNA in the presence of RP-A (Figure 5), and DNAs with higher superhelical density were required for the initiation of DNA replication in the absence of DNA gyrase (Figure 4). These results suggest that DNA gyrase plays a role in the initiation by increasing the negative superhelicity of template DNA with the assistance of RP-A in this system (Figure 9), which leads to extensive unwinding of template DNA in the presence of DNA helicase B. DNA helicase B may require a higher superhelical density than T antigen for assembling to the template DNA. With this high superhelical density, a wide region containing ARS1 in the plasmid can be unwound due to the torsional stress. This may explain why the initiation site was broader than that in the human system with T antigen (Figure 6 and Ishimi & Matsumoto, 1993).

Among eukaryotic cells, the essential origin sequence had been identified in *S. cerevisiae*, and a protein complex (ORC) interacting with the sequence has been purified (Bell & Stillman, 1992). Furthermore, a gene (ORC2) coding for a component of the ORC is required for DNA replication in yeast cells (Bell et al., 1993; Micklem et al., 1993). It has been suggested that MCM proteins play important roles in the initiation of DNA replication in *S. cerevisiae* (Yan et

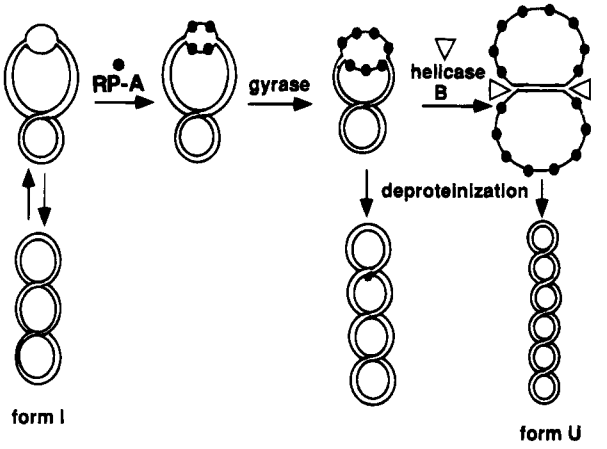

FIGURE 9: Model for DNA unwinding. DNA with a negative-superhelical density (form I) can form two different structures: supercoiled DNA and partially relaxed DNA with an unwound region. RP-A interacts with the unwound single-stranded DNA. DNA unwinding may proceed by introducing negative supercoilings into the partially relaxed DNA with DNA gyrase. The DNA is extensively unwound by DNA helicase B in the presence of RP-A and DNA gyrase. The extensively unwound DNA becomes highly negatively supercoiled (form U) upon removing proteins, although the exact structure of this molecule is not clear.

al., 1993) and in mammalian cells (Kimura et al., 1994) and that they are candidates for the licensing factor (Blow & Laskey, 1988). MCM proteins have the conserved motif of nucleic acid-dependent ATPase, which is also present in viral DNA helicases (Koonin, 1993). Obviously, our replication system lacking these proteins does not faithfully reflect the initiation reactions in yeast cells. In contrast to *S. cerevisiae*, however, neither short DNA sequences that are essential for DNA replication nor initiator protein has been identified in higher eukaryotes, but the regions where initiation of DNA replication occurs have been identified, which are called initiation zones [reviewed by Benbow et al. (1992)]. Replication eye structures were detected by two-dimensional gel electrophoresis in a region over 5 kb in length in the downstream of dihydrofolate reductase gene in Chinese hamster ovary cells (Dijkwell & Hamlin, 1992), in the histone gene repeats in *Drosophila* (Shinomiya & Ina, 1993), and in human rRNA genes (Little et al., 1993). These data suggest that the sequence requirement for the initiation of DNA replication is not stringent in higher eukaryotes and that the changes in higher order structure of DNA may be important for the initiation of DNA replication. In our replication system, DNA replication requires negative supercoiling of template DNA, which results in unwinding of the replication origin (Ishimi & Matsumoto, 1993, 1994). It has been shown that the ease of DNA unwinding is one of the determinants for replication origins (Umek & Kowalski, 1988, 1990; Matsumoto & Ishimi, 1994). In cellular chromosomal DNA, negative superhelical density would be changed by DNA metabolisms such as the progression of transcription (Liu & Wang, 1987) and the displacement of nucleosomes. Several initiation zones (Vassilev & Johnson, 1990; Giacca et al., 1994; Taira et al., 1994) and a replication origin (Kitsberg et al., 1993), which are within 2 kb in length, were identified in the promoter region of several genes. It is possible that high negative-superhelicity, which is required for the assembly of DNA helicase B, is generated by transcription of these genes (Wittig et al., 1992). The role of transcription in initiation of cellular DNA replication has been suggested by the observation that replication and transcription sites are colocalized in human cells (Hassan et al., 1994).

In this study, it has been suggested that this replication system is helpful to distinguish the DNA helicase involved in DNA replication from other DNA helicases and the results provide biochemical evidence for the involvement of DNA helicase B in cellular DNA replication. This replication system consisting of a c-*myc* initiation zone and purified mammalian proteins except *E. coli* DNA gyrase would constitute the first step toward the development of a mammalian cellular DNA replication system.

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