Loading of a DNA Helicase on the DNA Unwinding Element in the Yeast Replication Origin: Mechanism of DNA Replication in a Model System†

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ABSTRACT: We found that initiation of DNA replication occurs from the region containing the yeast autonomously replicating sequence 1 (ARS1), by incubating negatively supercoiled plasmid DNA with the proteins required for SV40 DNA replication in addition to DNA gyrase (Ishimi, Y., & Matsumoto, K. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 5399–5403). Here, the mechanism of DNA replication and the roles of the replication proteins in this model system were analyzed. Both SV40 T antigen as a DNA helicase and multisubunit human single-stranded DNA binding protein (HSSB) (also called RP-A) were required for the initial step of DNA synthesis. Furthermore, it has been shown that T antigen plays an essential role in the initiation of DNA replication from the ARS region in this system. The digestion of negatively supercoiled DNA with the single-strand-specific nuclease P1 revealed that regions containing A, B, and C domains of ARS1 can be unwound under the conditions used for DNA replication. Footprinting with $KMnO_4$ indicated that T antigen interacted with the unwound B domain where initiation of DNA replication mainly occurred. When circular DNAs of different negative-superhelical densities were replicated in the absence of DNA gyrase, short fragments were synthesized from the ARS region in proportion to its density and they were elongated by addition of HeLa topoisomerase I, which inhibits the initiation of DNA replication in this system. These results suggested that T antigen loads on the B domain of ARS1, where the DNA duplex is destabilized by the torsional stress of negative supercoiling, to form a preinitiation complex with the assistance of HSSB, and that DNA gyrase plays a major role at the elongation step of DNA synthesis as a swivelase. The role of T antigen in this system is consistent with the model of ARS DNA replication, in which a DNA helicase enters the ARS region through the 3′ flanking AT-rich region (B domain) of the consensus sequence.

Studies on bacterial and viral DNA replication systems have presented a model for the initiation of DNA replication (Bramhill & Kornberg, 1988; Borowiec et al., 1990). First, an initiator protein binds to a specific sequence in the replication origin, which causes melting of the DNA duplex in the flanking AT-rich region. Next, a DNA helicase assembled to the melted region starts unwinding the template DNA with the assistance of single-stranded DNA binding protein. SV40 T antigen is an initiator protein that also acts as a DNA helicase in SV40 DNA replication.[1] In the *Escherichia coli* oriC replication system, dnaA protein bound to the origin unwinds the flanking AT-rich region, and dnaB protein that has DNA helicase activity assembles at the region in the presence of dnaC protein. DNA replication occurs only when template DNA is negatively supercoiled by DNA gyrase (Funnel et al., 1986), since the negative supercoiling of template DNA facilitates the assembly of dnaA protein at the origin (Fuller & Kornberg, 1983) and it is required for the unwinding of the AT-rich region (Kowalski & Eddy, 1989).

In *Saccharomyces cerevisiae*, the replication origin consists of a core consensus sequence of 11-bp 5′-(T/A)TTTA(C/T)(G/A)TTT(T/A)-3′ (Broach et al., 1983; Kearsey, 1984; Van Houten & Newlon, 1990) and a flanking region of about 100 bp that is located adjacently to the 3′ side of the T-rich strand of the consensus sequence (reviewed by Umek et al., 1989; Newlon, 1988). Circular DNA containing the origin, which is called the autonomously replicating sequence (ARS), can be replicated as an extrachromosomal DNA element (Hsiao & Carbon, 1979; Stinchcomb et al., 1979). The finding that point mutations of the core consensus sequence abolish the ARS function (Kearsey, 1984; Van Houten & Newlon, 1990) suggests the existence of a protein that recognizes the consensus sequence. Recently, Bell and Stillman (1992) have identified the origin recognition complex that binds specifically to the double-stranded consensus sequence. The 3′ flanking region, characterized by an AT-rich sequence, contains several stretches that are homologous with the consensus sequence (Palzkill & Newlon, 1988). Kowalski and co-workers (Umek & Kowalski, 1988, 1990; Natale et al., 1992) found a correlation between the efficiency of DNA replication *in vivo* and the ease of DNA unwinding in the 3′ flanking region of several ARS, and they called it the DNA unwinding element. One model postulated from these findings is that the binding of an initiator protein to the consensus sequence, which results in the unwinding of DNA duplex in the flanking region, leads to the initiation of DNA replication (Umek et al., 1989). However, an initiator protein with such activity has not yet been identified. It is not known whether the negative supercoiling of template DNA is required for initiation, since the mechanism of initiation has not been characterized in an *in vitro* replication system.

Recently, we showed that DNA replication initiates from the ARS region and proceeds bidirectionally when negatively supercoiled DNA is incubated with the proteins of a DNA helicase (SV40 T antigen), multisubunit single-stranded DNA binding protein (HSSB), DNA polymerase α–primase, and

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[1] Abbreviations: ARS, autonomously replicating sequence; SV40, simian virus 40; T antigen, large tumor antigen; HSSB, the multisubunit human single-stranded DNA binding protein.

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a topoisomerase (DNA gyrase) (Ishimi & Matsumoto, 1993). Here, we analyzed the mechanism by which initiation of DNA replication occurs from the ARS region. The results show that T antigen plays a crucial role in initiation from the ARS region by recognizing the unwound 3′ flanking region that is formed by the torsional stress of negative supercoiling.

MATERIALS AND METHODS

Preparation of Plasmid DNA. YRp7ΔEP DNA (3.4 kb), a deletion derivative of YRp7 DNA (Struhl et al., 1979), contains the 1.0-kbp *Eco*RI–*Eco*RV fragment of yeast ARS1 DNA cloned into the *Eco*RI and *Pvu*II sites of pBR322 DNA. Negatively supercoiled plasmid DNA (form I) was prepared using alkali/SDS, from *E. coli* strain HB101 transformed by the plasmid as described previously (Ishimi and Matsumoto, 1993). The decrease in the linking number (ΔLk) of the DNA was determined by means of two-dimensional gel electrophoresis (Peck & Wang, 1983), and the negative-superhelical density was calculated using the formula $\Delta Lk/Lk_0$, where Lk_0 is the linking number of the relaxed DNA. Circular DNAs with a different superhelical density were prepared essentially as described by Singleton and Wells (1982). YRp7ΔEP DNA was incubated at 37 °C for 30 min with HeLa topoisomerase I in a reaction mixture containing 10 mM Tris-HCl (pH 7.9), 200 mM NaCl, 0.2 mM EDTA, 0.05 mM dithiothreitol, 0.5% glycerol, and 0–7 μM ethidium bromide. Ethidium bromide and topoisomerase were removed by two extractions with phenol/chloroform, followed by two with ether; then the DNA was precipitated with ethanol.

Preparation of Replication Proteins. SV40 T antigen was purified from recombinant baculovirus infected Sf27 cells; DNA polymerase α–primase complex, HSSB, and topoisomerase I were from HeLa cells, and the A and B subunits of DNA gyrase were from *E. coli* which overproduces each of these subunits, as described previously (Ishimi & Matsumoto, 1993).

Replication Assay. The conditions under which SV40 DNA replication (Ishimi et al., 1988) were measured were used for measurement of YRp7ΔEP DNA replication. The reaction mixture (40 μL) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 200 μM each of CTP, UTP, and GTP, 100 μM each of dATP, dGTP, and dTTP, 20 μM of [α-^{32}P]dCTP (1–2 × 10^4 cpm/pmol), 0.1 μg of DNA, 0.8 μg of creatine phosphokinase, 16 μg of bovine serum albumin, 0.3–0.5 μg of SV40 T antigen, 0.06–0.2 μg of HSSB, DNA polymerase α–primase complex (0.1 and 0.3 unit, respectively), 100 ng of gyrase A, and 90 ng of gyrase B. In specified experiments, DNA topoisomerase I (250 units) from HeLa cells was added to the reaction. Reactions proceeded at 37 °C for the indicated times, and then acid-insoluble radioactivity was measured. After purification, replicated DNA was analyzed by 5% polyacrylamide gel electrophoresis in TBE buffer after digestion with *Dde*I and *Dra*III, or by 1.5% agarose gel electrophoresis in 30 mM NaOH and 1 mM EDTA.

Nuclease P1 Digestion. The 1453-bp *Eco*RI fragment containing the TRP1–ARS1 region from *S. cerevisiae* was isolated from YRp7 DNA (Struhl et al., 1979) and end-labeled with T4 polynucleotide kinase. The labeled DNA was circularized with T4 DNA ligase, and the self-ligated product was purified from an agarose gel. The minicircle DNA was incubated with topoisomerase I in the absence or presence of 3 μM ethidium bromide under the conditions described in the above section for relaxed or negatively supercoiled DNA, respectively. The resultant circular DNA (40 ng) was digested with nuclease P1 (BRL) at 37 °C for 15 min in 40 μL of a reaction mixture containing 40 mM creatine phosphate (pH 7.8), 4 mM ATP, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 16 μg of bovine serum albumin, and 0.8 μg of creatine phosphokinase. The mixture was digested with proteinase K and extracted with phenol/chloroform, and the DNA was precipitated with ethanol. The products were digested with *Dra*III and separated on a 1.5% agarose gel containing 30 mM NaOH and 1 mM EDTA. The dried gel was visualized by autoradiography.

$KMnO_4$ Footprinting. $KMnO_4$ footprinting was performed as described by Borowiec and Hurwitz (1988). Supercoiled (form I) or relaxed YRp7ΔEP DNA (0.1 μg) was incubated at 37 °C for 15 min with 0.48 μg of SV40 T antigen or 0.05 μg of HSSB in 40 μL of a reaction mixture containing 40 mM creatine phosphate (pH 7.8), 0.8 μg of creatine phosphokinase, 4 mM ATP, 7 mM $MgCl_2$, and 0.5 mM dithiothreitol. Four microliters of 60 mM $KMnO_4$ (final 6 mM) was added to the reaction mixture. After incubation at 37 °C for 4 min, the reaction was stopped with β-mercaptoethanol (final 1 M). The mixture was desalted by centrifugation through a G-50 gel filtration column. The DNA was linearized by digestion with *Eco*RI and *Hin*dIII and denatured by NaOH. The resultant single-stranded DNA was analyzed by primer extension using a ^{32}P-end-labeled primer (5′-GATGCGCT-TAGATTAAATGG-3′, nucleotides 664-683 in ARS1). The primers were elongated with DNA polymerase (Sequenase Ver 2.0, USB) in the presence of single-stranded DNA binding protein from *E. coli*.

RESULTS

T Antigen Plays an Essential Role in Initiation of DNA Replication from the ARS Region. ARS1 consists of three domains, an A domain consisting of a consensus sequence of 11 bp, a flanking B domain of about 100 bp that is located to the 3′-side of the T-rich strand of the consensus sequence, and a C domain of about 80 bp that is located 200 bp away from the A domain on the opposite side of the B domain (Celniker et al., 1984; Newlon, 1988) (Figure 1). Each of the B and C domains contains three sequences similar to the consensus sequence, and the B domain includes a binding site for a transcription factor, ABF1 (Diffley & Stillman, 1988). Deletion of the B or C domain causes at least a 10- or 2–3-fold increase in the rate of plasmid loss, respectively (Newlon, 1988). Marahrens and Stillman (1992), having examined *in vivo* replication activity of linker substitution mutants, have recently reported that the B domain of ARS1 is defined by multiple functional elements (B1, B2, and B3), as indicated in Figure 5.

Efficient DNA replication occurred from the ARS region when negatively supercoiled plasmid DNA containing ARS1 was incubated with the minimum components required for SV40 DNA replication (T antigen, HSSB, and DNA polymerase α–primase) which constitute the monopolymerase system (Hurwitz et al., 1990) in the presence of DNA gyrase from *E. coli* (Ishimi & Matsumoto, 1993). The roles of these components in the initial step of DNA synthesis were examined. After these mixtures were incubated for 15 min (preincubation), deoxyribonucleotides including [α-^{32}P]dCTP were added, and they were further incubated for indicated periods. DNA synthesis increased linearly, and about 5% of the template DNA was replicated during a 30-min incubation (Figure 2). When the T antigen was omitted during the preincubation and then added together with deoxyribonucleotides, there was a lag period of DNA synthesis (about 10

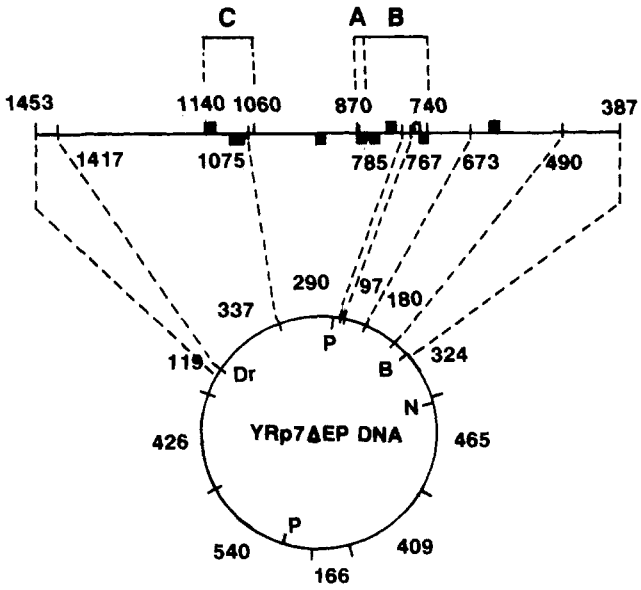

FIGURE 1: Structure and restriction map of YRp7ΔEP DNA. Eleven *Dde*I restriction sites and one *Dra*III restriction site (Dr) of YRp7ΔEP DNA and the sizes of digested fragments are indicated above the plasmid DNA (circle). Other restriction sites are also indicated (P (*Pst*I), B (*Bst*XI), and N (*Nde*I)). The DNA from *S. cerevisiae* enlarged is shown near the top of the figure and numbered (Struhl et al., 1979). Closed boxes indicate the sequences homologous with the core consensus. The position of the box below or above the line indicates the position of the T-rich strand in the upper or lower strand of the sequence, respectively. An open box on the line indicates the position of the ABF1 binding site. Regions indicated by A, B, and C correspond to the three domains of ARS1.

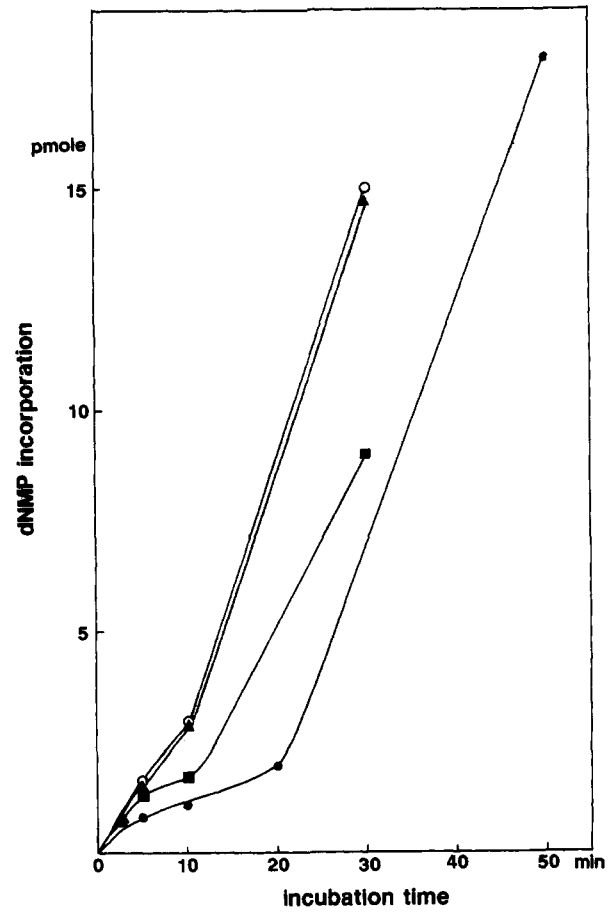

FIGURE 2: The absence of T antigen or HSSB during the initial period of incubation results in the appearance of a lag period before DNA synthesis starts. Negatively supercoiled YRp7ΔEP DNA was incubated with T antigen, HSSB, DNA polymerase α–primase, and DNA gyrase in the presence of ribonucleotides (○). After 15 min, deoxyribonucleotides including [α-^{32}P]dCTP were added to the reaction (time 0) and the incubation was continued. Acid-insoluble radioactivity was measured at the indicated times. The time course of DNA synthesis was also measured in the reaction where T antigen (●), gyrase (▲), or HSSB (■) was omitted during the incubation for 15 min (preincubation) and added at the same time as the deoxyribonucleotides. The amount of DNA synthesis is indicated as total nucleotides incorporated.

min). In the same experiment, the absence of HSSB during the preincubation also resulted in a lag period, whereas the rate of DNA synthesis was not affected by the absence of DNA gyrase.

To determine the mode of DNA replication in these reactions, the replicated DNAs were analyzed by gel electrophoresis after digestion with restriction enzymes (Figure 3A). In the reaction where DNA gyrase was omitted during the preincubation, fragments in the ARS region were heavily labeled during a short period (2.5 min) and all the fragments were evenly labeled after 30 min of incubation (Figure 3B). This result, which is comparable to that obtained from the reaction containing all components (Ishimi & Matsumoto, 1993), suggests that DNA replication initiates from the ARS region and proceeds bidirectionally. A similar mode of DNA replication was observed even when HSSB was absent during the preincubation, although a 166-bp fragment located opposite to the origin in the plasmid was heavily labeled at 2.5 min. When T antigen was absent during the preincubation, however, almost all fragments were labeled evenly from the initial stage of DNA synthesis. These results indicate that interaction of both T antigen and HSSB with the ARS region is required for the initial step of DNA synthesis and, furthermore, that T antigen plays an essential role in the initiation of DNA replication from the ARS region.

T Antigen Interacts with the Unwound B Domain. The finding that negative supercoiling of template DNA is required for the initiation of DNA replication in this system (Ishimi & Matsumoto, 1993) suggests that the DNA duplex in the ARS region is unwound by torsional stress of supercoils. To confirm this notion, the single-strand-specific nuclease P1 was used to detect the unwound region in the template DNA. Only the most sensitive site to the nuclease can be detected in this assay, since introduction of a single nick to supercoiled DNA results in elimination of the torsional stress required for DNA unwinding. The digestion proceeded under the same conditions as those used for DNA replication. Although nuclease P1 recognized a specific locus of YRp7ΔEP DNA, it was mapped to the promoter region of the RNA1 gene in the vector DNA, but not to the ARS region (data not shown). When negatively supercoiled DNA composed of yeast DNA alone was digested with the nuclease, however, DNAs in the region containing the A and B domains (nucleotides 785-915) and those in the region containing the C domain (nucleotides 1065-1245) were preferentially digested (Figure 4). These digestions were not observed in relaxed DNA. The results indicate that the DNA duplex in the three domains of ARS1 is structurally unstable, so that it can be unwound by torsional stress of the negative supercoil, although the RNA1 locus is more sensitive to nuclease P1 than the ARS region in YRp7ΔEP DNA.

Next, the interaction of T antigen and HSSB with the ARS1 region was examined by footprinting with $KMnO_4$. Thymidine residues in the destabilized DNA duplex can be modified with $KMnO_4$. Modification was carried out under the replication conditions, and the modified sites were determined by primer extension (Figure 5). Several bands appeared without

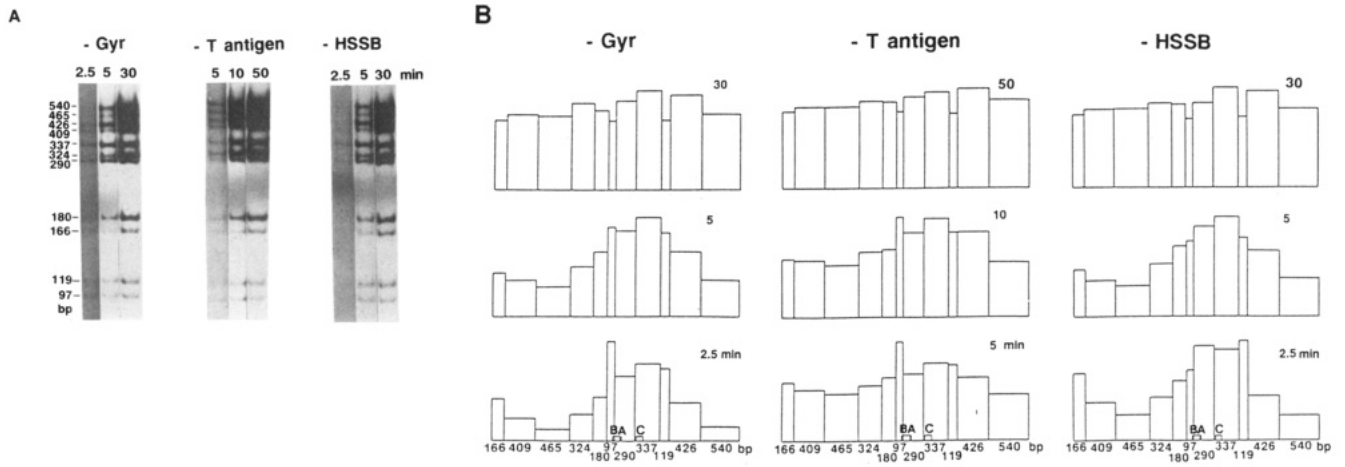

FIGURE 3: The presence of T antigen during the initial period of incubation is essential for the initiation of DNA replication from the ARS region. (A) Replicated DNAs from the reactions in Figure 2 were digested with *Dde*I and *Dra*III and analyzed by polyacrylamide gel electrophoresis. Half of the DNA from each reaction was digested with the nucleases, but $^1/_{10}$ of the DNA was digested from the reactions where incubation time was over 30 min. The gel was dried and visualized by autoradiography. Film was usually exposed for 2 days, except that, for the reactions 5 and 10 min (–T antigen) and 2.5 and 5 min (–HSSB), it was exposed for 2 weeks. The sizes of the digested fragments are shown on the left side of the gel, and proteins omitted during the preincubation, as well as the incubation periods, are indicated at the top. (B) The radioactivity of the bands was quantitated using a Bio-Image Analyzer (BAS2000, Fuji), and the value was divided by the size of each fragment and corrected for base composition. These values are presented on a linear map of YRp7ΔEP; 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 boxes at the bottom of the figure.

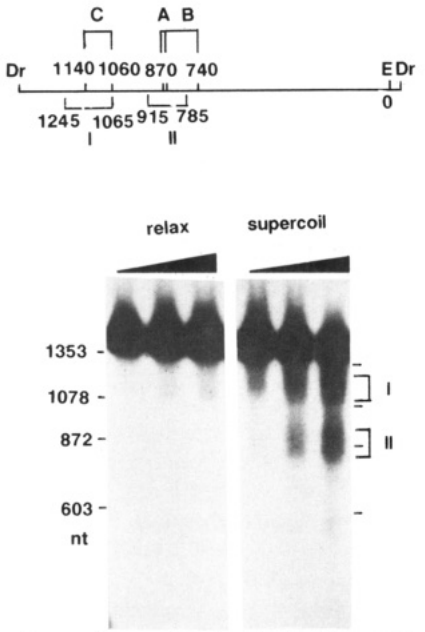

FIGURE 4: The regions containing the A, B, and C domains are unwound in negatively supercoiled DNA. A yeast-derived, 1.4-kbp fragment of linear DNA, which was isolated from YRp7 DNA (Struhl et al., 1979) by digestion with *Eco*RI and labeled at the 5′-ends, was circularized by self-ligation. Negative supercoiling was introduced into the circular DNA as described in Materials and Methods. These supercoiled and relaxed DNAs were digested with increasing amounts of nuclease P1 (0.03, 0.1, and 0.3 unit) as indicated by triangles under the conditions used for DNA replication. After digestion with *Dra*III, the DNA was analyzed by agarose gel electrophoresis under denaturing conditions. The sizes of the bands (I and II) were determined by comparing their mobilities with marker DNAs of ϕX174 DNA fragments digested with *Hae*III. The digested regions estimated from the sizes of the bands are indicated at the top where locations of the A, B, and C domains are mapped on the linear DNA; E (*Eco*RI), Dr (*Dra*III).

modification, which is due to the nonspecific arrest of *E. coli* DNA polymerase at the extension step. When either negatively supercoiled or relaxed DNA was treated with $KMnO_4$, many bands appeared from the entire ARS region. These bands were not changed even when the relaxed DNA had been incubated with T antigen or HSSB prior to the modification. In negatively supercoiled DNA, however, incubation with T antigen resulted in the appearance of additional bands in the B1 and B2 domains, as well as in the region between domains B2 and B3. The presence of HSSB in addition to T antigen did not affect the modification pattern with T antigen itself. The region containing the C domain was also examined by $KMnO_4$ footprinting, but neither T antigen nor HSSB affected the sensitivity to $KMnO_4$ (data not shown). These results suggest that T antigen interacts with the B domain, whose DNA duplex can be easily unwound by torsional stress of negative supercoiling.

DNA Replication Initiated Near the B Domain Can Be Elongated in the Presence of Topoisomerase I. The roles of the negative supercoil of the template DNA and DNA gyrase in this replication system were examined. Plasmid DNAs with a different negative-superhelical density were prepared, and their ability to serve as a template in the absence of DNA gyrase was examined (Figure 6A,B). DNA synthesis increased in proportion to its negative-superhelical density. Density of at least –0.06 (lane 5) was required to initiate DNA replication in this system; this value is almost the same as that of form I DNA, which is usually used for DNA replication. Products shorter than 700 nucleotides were mainly synthesized, which must be due to the arrest of replication fork movement by the torsional stress of positive supercoils accumulated ahead of the forks (Ishimi et al., 1992). In the presence of DNA gyrase, DNA synthesis was enhanced and longer DNAs were synthesized within 30 min of incubation using form I DNA as a template (Figure 6C, lane 1). After the reaction was incubated for 30 min in the absence of DNA gyrase, topoisomerase I from HeLa cells was added to the reaction and the incubation was continued for 15 min (lane 3). DNA replication occurred at a level similar to that in the reaction containing DNA gyrase, and longer leading strands, which accumulated around half the size of template DNA, were synthesized.

To determine where DNA replication initiates in the reaction without DNA gyrase, the reactions were pulse-labeled for various periods after a 15-min incubation and the products

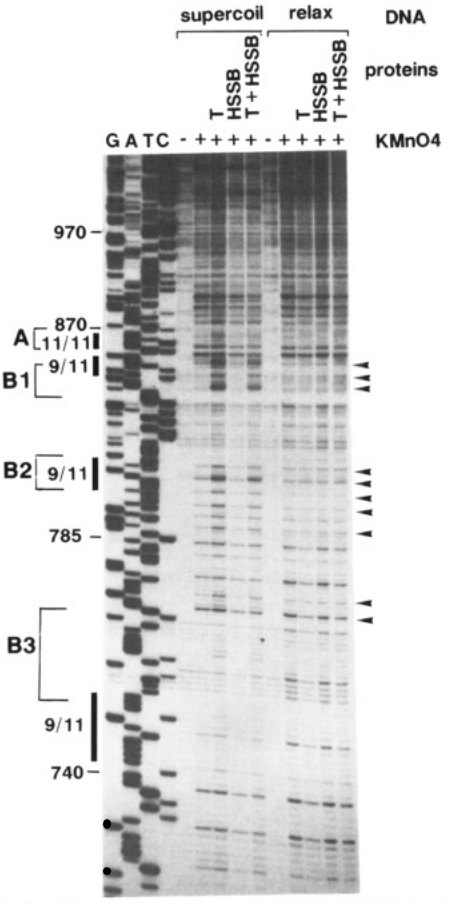

FIGURE 5: T antigen induces distortion of the DNA duplex in the B domain. Supercoiled or relaxed YRp7ΔEP DNA was incubated for 15 min in the absence or presence of T antigen and HSSB under the conditions used for DNA replication. $KMnO_4$ was added to the reaction, and the incubation was continued for 4 min. The sites modified with $KMnO_4$ were determined by primer extension. As a control, primer extension was performed without modification. The structure of the DNA, the proteins added to the reaction, and treatment with $KMnO_4$ are indicated at the top of the gel. Locations of the consensus sequence (11/11), the 9/11 matches of the consensus, A, B1, B2, and B3 domains (Marahrens & Stillman, 1992), and nucleotide numbers are indicated. These were determined by dideoxy sequencing using the same primer that was used for the primer extension (G, A, T, and C). Arrowheads indicate the modified sites that appeared in the presence of T antigen in supercoiled DNA.

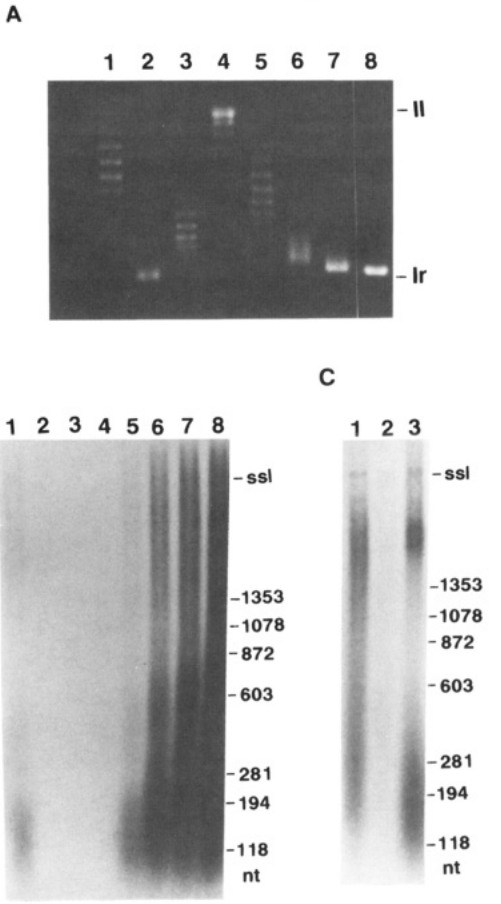

FIGURE 6: Initiation of ARS DNA replication is dependent on the negative-superhelical density of DNA. YRp7ΔEP DNAs with different negative-superhelical densities were prepared as described in Materials and Methods. (A) The DNAs were analyzed by agarose gel electrophoresis in the presence of 7 μM chloroquine and visualized by staining with ethidium bromide. The superhelical density of the DNA was –0.060 (lane 1, form I DNA), 0 (lane 2), –0.019 (lane 3), –0.045 (lane 4), –0.065 (lane 5), –0.082 (lane 6), –0.098 (lane 7), and –0.128 (lane 8). The positions of form Ir (relaxed DNA) and form II (nicked circular DNA) are indicated. (B) These DNAs were replicated for 30 min without DNA gyrase, and purified DNAs were analyzed in an alkaline agarose gel. (C) Form I DNA (superhelical density, –0.060) was incubated with (lane 1) or without (lane 2) DNA gyrase for 30 min. After the reaction without DNA gyrase was incubated for 30 min, topoisomerase I from HeLa cells was added to the reaction, and it was further incubated for 15 min (lane 3). Replicated DNAs were analyzed by alkaline agarose gel electrophoresis. Positions of single-stranded linear DNA (ssl) and ϕX174 DNA fragments digested with *Hae*III as marker DNAs are shown.

were analyzed after digestion with restriction enzymes (Figure 7A). When the extent of fragment labeling was normalized, the fragments in the ARS region formed a peak. Among them, the 97-bp fragment was 2–8-fold more labeled than the other fragments after a 5-min incubation, although the bias for shorter fragments must be considered in this condition (Figure 6B). Selective labeling of the 97-bp fragment was unchanged during 10- and 20-min incubations. The 97-bp fragment, which includes a sequence homologous to the consensus sequence and an ABF1 binding site, is located at the edge of the B domain of ARS1. Next, progression of DNA replication after the addition of topoisomerase I was examined. Reactions were pulse-labeled for various periods in the presence of topoisomerase I after they had been continuously labeled for 10 min in the absence of any topoisomerases (Figure 7B). The 119-, 180-, 290-, and 337-bp fragments were labeled, in addition to the 97-bp fragment, by 2.5 min after the addition of topoisomerase I, and almost all fragments were labeled evenly at 10 min. These results indicate that (1) a negative supercoil of template DNA is required for the initiation of DNA replication that mainly occurs near the B domain of ARS1 and (2) topoisomerase I, which inhibits the initiation of DNA replication by relaxing negative supercoils (Ishimi & Matsumoto, 1993), stimulates DNA replication at an elongation step of DNA synthesis, most probably by relaxing positive supercoils accumulated ahead of replication forks.

DISCUSSION

The mechanism of DNA replication and the roles of components in the model system for ARS DNA replication were analyzed, and they are shown in Figure 8. Circular DNA with a decreased linking number can form two distinct structures: one is a supercoiled DNA, and the other is a partially relaxed DNA with an unwound region. Equilibrium would be present between these structures under certain conditions. Umek and Kowalski (1990) have shown that localized DNA unwinding occurs in an ARS element above a threshold level of negative supercoiling, which was dem-

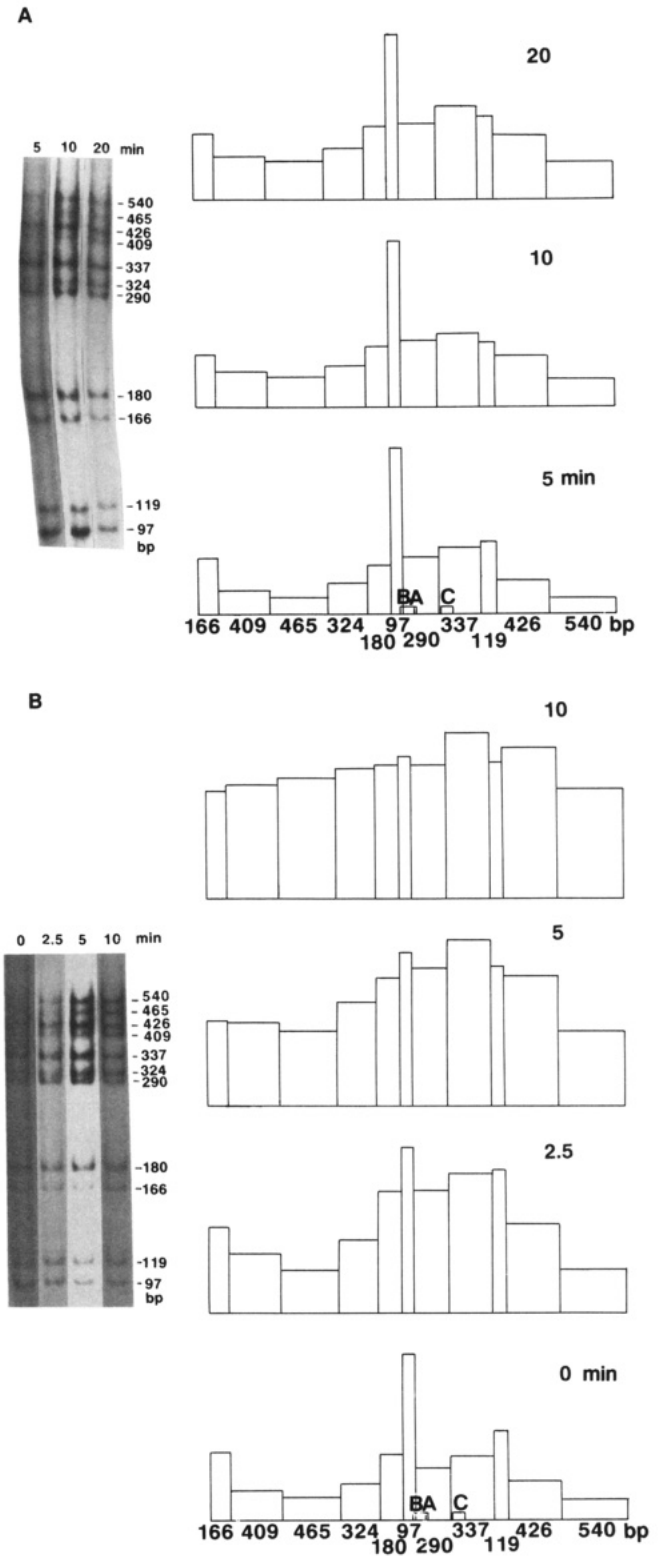

FIGURE 7: DNA replication initiates near the flanking B domain in the absence of DNA gyrase and proceeds bidirectionally after the addition of topoisomerase I. (A) Reactions without DNA gyrase were incubated for 15 min and then labeled for indicated periods by adding dNTPs including [α-^{32}P]dCTP. (B) Reactions without DNA gyrase were incubated for 15 min and then labeled for 10 min. HeLa topoisomerase I was added to the reaction, and labeling was continued for the indicated periods. The replicated DNAs were analyzed by 5% polyacrylamide gel electrophoresis after digestion with *Dde*I and *Dra*III and autoradiographed. The specific activity of each band was calculated as described in Figure 3B.

onstrated by the two-dimensional gel electrophoresis of plasmid topoisomers containing the ARS element. We have shown that the regions containing the three domains of ARS1 can be easily unwound under replication conditions (Figure 4). In agreement with this, we found that the three domains of ARS1 in negatively supercoiled YRp7ΔEP DNA were digested with single-strand-specific mung bean nuclease in the presence of HSSB under low ionic conditions (K. Matsumoto and Y. Ishimi, manuscript in preparation). HSSB stabilized the unwound structure of the ARS region through its preferential binding to T-rich regions. It has been shown that T antigen interacts with the B domain of ARS1 (Figure 5). Since additional modifications with $KMnO_4$ in the presence of T antigen were detected in negatively supercoiled DNA, but not in relaxed DNA, it is plausible that T antigen loads on the B domain where the DNA duplex is unwound by the torsional stress of negative supercoil. Consistent with this, it has been shown that T antigen binds to single-stranded DNA with much higher affinity than to double-stranded DNA (Spillman et al., 1979). T antigen binds to double-stranded DNA containing GAGGC sequences in the SV40 replication origin with relatively high affinity, but no GAGGC sequence is found in the ARS1 region. The finding that T antigen can load on a circular duplex DNA that does not contain an SV40 replication origin has also been reported by Wold et al. (1987), who showed that T antigen unwinds pUC DNA under low ionic conditions.

The formation of a preinitiation complex of T antigen and HSSB at the ARS1 region may be the rate-limiting step for initiation of DNA replication in this system. These complexes of T antigen and HSSB should facilitate assembly of DNA polymerase α–primase to the ARS region through protein–protein interactions (Dornreiter et al., 1992; Matsumoto et al., 1990; Melendy & Stillman, 1993). This model for initiation of DNA replication can explain why both T antigen and HSSB are required for the initial step of DNA synthesis and why T antigen plays a crucial role in the initiation of DNA synthesis from the ARS region. Initiation of DNA replication occurred from the ARS region in the absence of DNA gyrase, and elongation of DNA synthesis was stimulated by topoisomerase I that relaxes both positive and negative supercoils (Figure 7). These results suggest that DNA gyrase is required for this DNA replication system as a swivelase that relaxes positive supercoils accumulated ahead of replication forks. This conclusion is supported by the observation that the absence of DNA gyrase during the preincubation period did not result in a significant delay of DNA synthesis (Figure 2).

Under isotonic replication conditions in the presence of magnesium ions, the promoter region of the RNA1 gene in the vector portion of template DNA was preferentially digested with nuclease P1 (data not shown). This finding is consistent with the results of Sheflin and Kowalski (1985), who found that the promoter region of RNA1 gene was digested with mung bean nuclease under low ionic conditions containing magnesium ions at 37 °C. When negatively supercoiled yeast DNA was incubated with nuclease P1, however, the regions containing the three domains of ARS1 were digested (Figure 4). The following possibilities can be raised from these results: the RNA1 locus is more easily unwound than the ARS region and only a small population of plasmid DNA has the unwound structure in the ARS region, which is due to the presence of a hierarchy in DNA unwinding (Kowalski et al., 1988), or the ARS region is unwound in almost all molecules but the RNA1 locus is more sensitive to the nuclease because of the formation of a secondary structure (Sheflin & Kowalski, 1985). Further analysis is required to verify this directly.

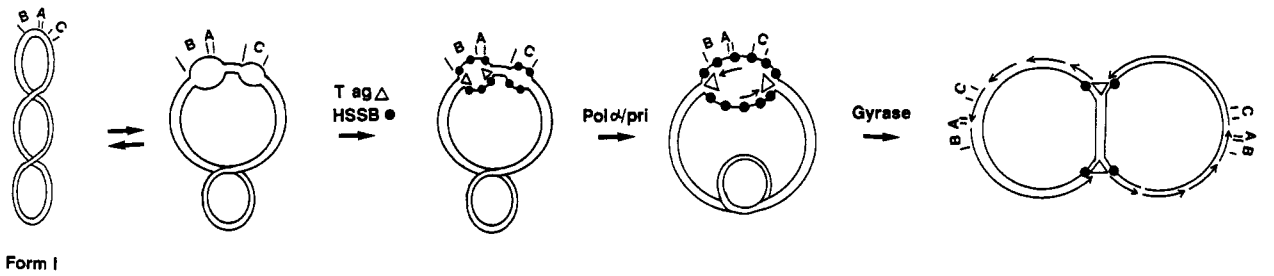

FIGURE 8: Model for ARS DNA replication. DNA with a negative-superhelical density can form two different structures: supercoiled and partially relaxed DNA with an unwound region. Unwinding at regions A and B and at region C can occur within the same molecules, as shown. An alternative possibility is that some molecules are unwound at regions A and B and others are unwound at region C. T antigen interacts with the unwound B domain and HSSB binds to single-stranded DNA in the unwound region to form the preinitiation complex of DNA replication. DNA polymerase α–primase, assembled at the complex by interacting with T antigen and HSSB, synthesizes primer RNA for leading strand synthesis. Both leading and lagging strands are synthesized as the unwinding of template DNA proceeds by means of the DNA helicase activity of T antigen. DNA gyrase stimulates DNA replication by relieving the torsional stress of positive supercoils accumulated ahead of replication forks.

DNA replication was initiated from the ARS, but not from the promoter region of the RNA1 gene that is localized in the 409-bp fragment (see Figure 1). These results suggest that T antigen specifically loads on the ARS region in this system. This raises the issue of the mechanism by which T antigen recognizes the B domain of ARS1. Compared with the promoter region of RNA1 in the vector, the AT-rich region in the B domain is longer and contains many T-rich stretches. These features may be important for recognition by T antigen since the region would be stably unwound by torsional stress and T antigen can preferentially bind to the resultant single-stranded region containing T-rich stretches (Giacherio & Hager, 1979). It is also possible that several 4- of 5-base pair matches of GAGGC in the B domain, which is the T antigen binding site, play an important role in the binding of T antigen. Cellular DNA helicases whose ATPase activities are dependent on the presence of poly(dT) have been identified (Tawaragi et al., 1984). The role of T antigen in this ARS DNA replication system may be replaced by these DNA helicases. We found that E1 protein of bovine papilloma virus, which has DNA helicase activity (Seo et al., 1993), can take over the role of T antigen in this system (unpublished results). These two proteins, each of which recognizes distinct sequences in double-stranded DNA, share a similar structure in the central part of the protein (Clertant & Seif, 1984). It has been shown that T antigen interacts with DNA polymerase α–primase through the central part of the largest subunit of DNA polymerase α (Fanning, 1992). Thus, these characteristics of T antigen may be important for inducing DNA replication from the ARS region.

Footprinting with $KMnO_4$ showed that T antigen binds to the B domain (Figure 5), where the DNA duplex can be easily unwound by torsional stress (Figure 4). This finding is consistent with the model by Umek et al. (1989), since the 3′ flanking region called the DNA unwinding element is postulated to be an entry site of a replication protein such as a DNA helicase. Marahrens and Stillman (1992) recently reported that the B domain of ARS1 is defined by multiple functional elements. The sites modified by $KMnO_4$ in the presence of T antigen matched the B1 and B2 elements. Mutations in these elements may affect the unwinding property of the B domain which is required for the entry of a DNA helicase. In this replication system, negative supercoiling of template DNA is a driving force of DNA unwinding in the ARS region. Negative supercoils can be generated by several DNA dynamics in chromosome. Displacement of one nucleosome from DNA can produce one negative supercoil (Simpson et al., 1985), and transcription also creates negative supercoils in an upstream region of the transcribed gene (Liu & Wang, 1987). A factor which can introduce negative supercoils into DNA in the presence of topoisomerase II has been identified in the silkworm (Ohta & Hirose, 1990). A consensus sequence of topoisomerase II binding sites resembles the ARS consensus sequence (Amati & Gasser, 1988). It is possible that the ARS1 region where nucleosome structure is deficient (Thoma et al., 1984) is specially accessible to the torsional stress of the negative supercoil generated by these mechanisms.

Finally, on the basis of this model system, a cognate system for ARS DNA replication can be set up with yeast proteins. In the present system, however, elongation of DNA synthesis was carried out solely by DNA polymerase α, bypassing the normal requirement for DNA polymerase δ and accessory factors (Hurwitz et al., 1990; Tsurimoto et al., 1990; Weinberg et al., 1990), and a point mutation of the core consensus sequence that abolishes ARS activity *in vivo* could not inhibit replication activity (unpublished results). It is plausible that a factor(s) which recognizes the consensus sequence has an important role in the determination of the specificity of DNA replication *in vivo*. The origin recognition complex that has been identified by Bell and Stillman (1992) may be the factor. Similarly to the oriC replication system, both the factor that binds to the consensus sequence and negative superhelicity around the origin region may be required for the initiation of DNA replication in *S. cerevisiae*.

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