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Biochemistry

Model system for DNA replication of a plasmid DNA containing the autonomously replicating sequence from *Saccharomyces cerevisiae*

(yeast/negative supercoiling/unwinding)

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ABSTRACT **A negatively supercoiled plasmid DNA containing autonomously replicating sequence (ARS) 1 from *Saccharomyces cerevisiae* was replicated with the proteins required for simian virus 40 DNA replication. The proteins included simian virus 40 large tumor antigen as a DNA helicase, DNA polymerase α·primase, and the multisubunit human single-stranded DNA-binding protein from HeLa cells; DNA gyrase from *Escherichia coli*, which relaxes positive but not negative supercoils, was included as a "swivelase." DNA replication started from the ARS region, proceeded bidirectionally with the synthesis of leading and lagging strands, and resulted in the synthesis of up to 10% of the input DNA in 1 h. The addition of HeLa DNA topoisomerase I, which relaxes both positive and negative supercoils, to this system inhibited DNA replication, suggesting that negative supercoiling of the template DNA is required for initiation. These results suggest that DNA replication starts from the ARS region where the DNA duplex is unwound by torsional stress; this unwound region can be recognized by a DNA helicase with the assistance of the multisubunit human single-stranded DNA-binding protein.**

Among eukaryotes, the autonomously replicating sequence (ARS) of *Saccharomyces cerevisiae* is the only example of a well-defined chromosomal replication origin that functions in plasmids (1–3). It has been shown by two-dimensional gel electrophoresis analysis that the origin of DNA replication and the ARS element are closely mapped (4, 5). The core consensus sequence of ARS consists of 11 bp (5′-$^{T}_{A}$TTTA$^{CG}_{TA}$TTT$^{T}_{A}$-3′) that are essential for the origin function *in vivo* (6–8), and flanking regions of the core consensus are required for efficient and stable replication of the DNA-containing ARS (9–11). ARS1 is derived from the replication origin of chromosome IV, and it has been shown to function as an origin of replication of the chromosome as well as an extrachromosomal DNA element (5, 12). ARS1 consists of three domains, a core consensus of 11 bp (as described above; A domain), a flanking B domain of about 100 bp that is located to the 3′ side of the T-rich strand of the core consensus, and a C domain of about 80 bp that is located 200 bp 5′ from the A domain (on the opposite side of the B domain, which places the A domain in the center) (10, 13). The B domain contains three nearly matched consensus sequences and one ABF1 binding site (14). Deletion of the B or C domain causes at least a 10-fold or a 2- to 3-fold increase in the rate of plasmid loss, respectively (10). *In vitro* systems that supported ARS DNA replication with yeast extracts have been reported (15–17), but these systems were not in general use due to their low efficiency of replication.

Recently, the mechanism of simian virus 40 (SV40) DNA replication has been elucidated with *in vitro* systems (18–20). SV40 large tumor antigen (T antigen), binds to the origin of replication and unwinds the DNA with the assistance of the multisubunit human single-stranded DNA-binding protein (HSSB) and a DNA topoisomerase. DNA primase complexed with DNA polymerase α (DNA polymerase α·primase) then synthesizes primer RNAs in the unwound origin DNA, which are extended with deoxyribonucleotides. HSSB and DNA polymerase α·primase from HeLa cells and T antigen have been shown to interact with each other in the process of primer RNA synthesis (21, 22). Both leading and lagging strands are synthesized with progression of the fork, which is driven by the DNA helicase activity of T antigen and DNA topoisomerase I or II as a "swivelase," which relaxes positive supercoils accumulated ahead of the replication fork. Topoisomerases I and II, which relax both positive and negative supercoils, stimulated SV40 DNA replication with purified proteins (23, 24), suggesting that negative supercoiling of template DNA is not required for initiation of DNA replication. In the replication of a plasmid containing the *Escherichia coli* chromosomal origin of replication (oriC), however, negative supercoiling of template DNA was required for initiation as well as the binding of the dnaA initiator protein (25). Negative supercoiling facilitates both binding of the dnaA protein to the dnaA boxes and the unwinding of the A+T-rich 13-mer sequences adjacent to the dnaA boxes (26). Negative supercoiling alone can induce unwinding of the 13-mer sequence in the absence of dnaA protein under particular conditions (27). DNA gyrase, which relaxes positive but not negative supercoils in the presence of ATP, is essential for initiation by introducing negative supercoils into the template DNA and also for elongation as a swivelase.

In this report, we have developed a system for DNA replication of a plasmid DNA containing ARS1 by using proteins required for SV40 DNA replication. DNA gyrase was used as a swivelase instead of DNA topoisomerases from HeLa cells to keep the template DNA negatively supercoiled. The results show that negative supercoiling of template DNA is sufficient for inducing initiation of replication from ARS1 region in this system.

MATERIALS AND METHODS

Preparation of Template DNA. A negatively supercoiled DNA, YRp7ΔEP (3.4 kbp), was used as a template for DNA replication. For the construction of the DNA, the region containing the 5′ half of *TRP1* gene and the tetracycline-resistance gene was deleted from YRp7 DNA (28) by digestion with *Eco*RV and *Pvu* II, followed by religation. *E. coli* strain HB101 transformed by this plasmid was cultured until late logarithmic phase in the absence of chloramphenicol. After lysis of *E. coli* by the alkali/SDS method, the plasmid DNA was purified by CsCl centrifugation (29). A mutant

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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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DNA lacking the ARS1 origin (Δ760–942) was constructed by digestion of YRp7ΔEP DNA with *Stu* I at nucleotide position 829 (see Fig. 1), followed by deletion of DNA around this site and religation. pBR322ΔEP DNA (2.5 kbp) and ori$^+$ DNA (2.8 kbp) were constructed as reported (30). pBR322ΔEP DNA contains the same sequence as the vector DNA in YRp7ΔEP DNA plus the *Eco*RV–*Eco*RI fragment (187 bp) from pBR322 DNA.

Preparation of DNA Gyrase. DNA gyrases A and B were prepared from *E. coli* by the method of Mizuuchi *et al.* (31) with several modifications that overproduce these two subunits. Gyrase A was purified from *E. coli* strain RW1053 [*recA* Δ(*gal attλ bio*)] transformed by plasmid pMK90, which contains the structural gene encoding the A subunit. After the third step of purification by DEAE-Sepharose column chromatography, the eluted gyrase A was loaded onto a hydroxylapatite column equilibrated with 25 mM potassium phosphate, pH 6.9/1 mM 2-mercaptoethanol/10% (vol/vol) glycerol, instead of loading it onto the valine-Sepharose column (31). Gyrase A was eluted from the hydroxylapatite column with 0.2 M potassium phosphate. Gyrase B was prepared from RW1053 cells transformed by plasmid pYK512, which contains the gene coding for the B subunit (a generous gift from H. Ikeda, Institute of Medical Science, University of Tokyo). After DEAE-Sepharose column chromatography, gyrase B was finally purified by hydroxylapatite column chromatography using the method described above for gyrase A. Relaxed pBR322 DNA (0.1 μg) became negatively supercoiled after incubation at 37°C for 30 min in the presence of both 6 ng of gyrase A and 20 ng of gyrase B, under the conditions described for replication.

Preparation of Other Replication Proteins. SV40 T antigen was purified from recombinant baculovirus-infected Sf27 cells as reported, except that the Superose 6 column chromatography step was omitted (32). The DNA polymerase α·primase complex, HSSB, and topoisomerase I were purified from HeLa cells as reported (23).

Replication Assay. The same conditions used for measuring SV40 DNA replication (23) were used for the replication of YRp7ΔEP DNA. Reaction mixtures (40 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.7), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 200 μM CTP, 200 μM UTP, 200 μM GTP, 100 μM dATP, 100 μM dGTP, 100 μM dTTP, 20 μM [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 μg of SV40 T antigen, 0.2–0.5 μg of HSSB, DNA polymerase α·primase complex (0.1 and 0.3 unit, respectively), 20 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 mixture. The amount of replication proteins required for maximum incorporation was almost identical to those used in the cognate system for SV40 DNA replication. Reactions were carried out at 37°C for 1 h, and then the acid-insoluble radioactivity was measured. After purification, replicated DNA was analyzed by 1.5% agarose gel electrophoresis in 30 mM NaOH and 1 mM EDTA or by 5% polyacrylamide gel electrophoresis in TBE buffer after digestion with *Dde* I and *Dra* III.

RESULTS

DNA Gyrase Stimulates Replication of Plasmid DNA Containing ARS1. Negatively supercoiled YRp7ΔEP DNA (3.4 kbp), which contains ARS1 from *S. cerevisiae*, was used as a template for *in vitro* DNA replication. The DNA contains about 1.0 kbp of yeast DNA, including a perfectly matched ARS consensus sequence (A domain) and the flanking B and C domains (Fig. 1). DNA polymerase α·primase and HSSB from HeLa cells and SV40 T antigen, which are the minimal components of the SV40 DNA replication system (monopolymerase system) (19), were used to replicate the plasmid DNA. It has been shown that leading strands can be synthesized with DNA polymerase α instead of DNA polymerase δ holoenzyme complex (23). SV40 T antigen was expected to function as a DNA helicase in this system. During DNA replication of circular DNA, positive supercoils accumulate ahead of the replication fork, which must be relieved for fork progression. DNA gyrase from *E. coli* was used as a swivelase instead of DNA topoisomerases I and II from HeLa cells to keep the template DNA negatively supercoiled.

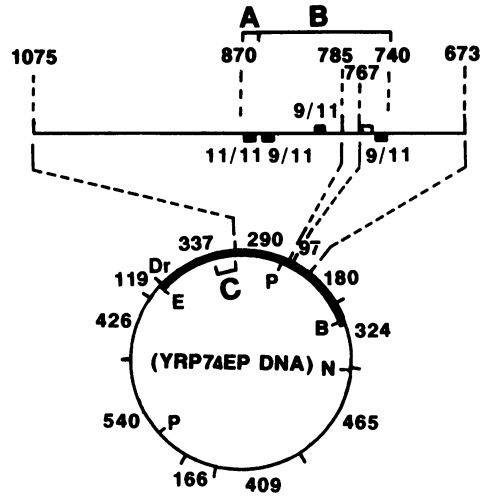

FIG. 1. Structure and restriction map of YRp7ΔEP DNA. Eleven *Dde* I restriction sites and one *Dra* III restriction site (Dr) of YRp7ΔEP DNA and the sizes of digested fragments are indicated outside the plasmid DNA (circle). *S. cerevisiae* DNA is indicated by the thick line, and other restriction sites are indicated (E, *Eco*RI; P, *Pst* I; B, *Bst*XI; N, *Nde* I). An enlargement of the origin region is depicted above the plasmid and numbered (28). Solid boxes indicate sequences homologous to the core consensus; the number below or above the box indicates the number of base pairs that match the 11-bp consensus sequence. 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. The open box indicates the position of the ABF1 binding site. Regions indicated by A, B, and C correspond to the three domains of ARS1.

The effect of DNA gyrase on this system was examined, and the products formed were analyzed by alkaline agarose gel electrophoresis (Fig. 2). In the absence of DNA gyrase, small amounts of short DNAs, 200–600 nt in length, were synthesized. In the presence of both subunits of DNA gyrase (A and B), DNA replication was greatly enhanced, and long DNAs, whose sizes reached half the template DNA (1.7 kb), were synthesized. Incorporation measurements indicated that up to 10% of input DNA was replicated within 1 h in the presence of both gyrase A and gyrase B, an amount comparable to the SV40 DNA replication in the same system containing topoisomerase I instead of gyrase (Fig. 3). The proteins required for this synthesis were examined (Fig. 3). DNA replication was dependent on the presence of all four proteins. Almost no replication occurred in the absence of T antigen or DNA polymerase α·primase as judged by alkaline agarose gel electrophoresis. Low levels of DNA chains shorter than 1000 nt were synthesized without HSSB. When topoisomerase I from HeLa cells was added to the reaction containing the four proteins, DNA replication was markedly reduced. After the reaction, ethidium bromide staining indicated that the bulk of the template DNA was relaxed in the presence of both DNA gyrase and topoisomerase I (data not shown). Similar protein requirements were observed when DNA replication reactions were carried out in the dipolymerase system (containing both DNA polymerases α and δ) instead of the monopolymerase system (data not shown). In

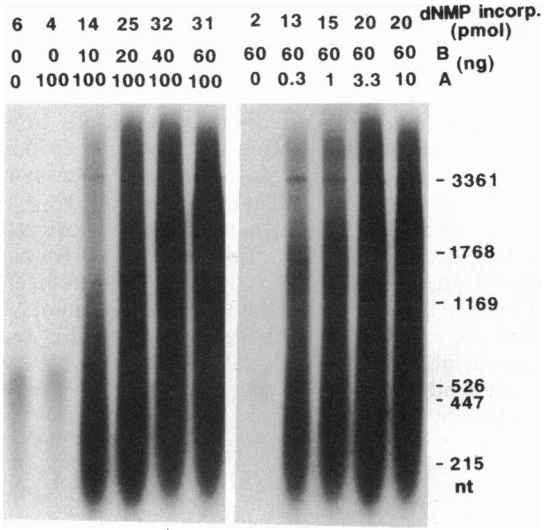

FIG. 2. DNA gyrase stimulates replication of plasmid DNA containing ARS1. YRp7ΔEP DNA, which contains ARS1, was incubated with various amounts of gyrase A and/or gyrase B at 37°C for 1 h in the presence of SV40 T antigen, HSSB, and DNA polymerase α·primase; labeled DNA products were analyzed by alkaline agarose gel electrophoresis. The amount of gyrase A and B protein added to the reaction mixtures and the total nucleotide incorporation are shown above the gel. SV40 DNA fragments digested with *Hin*dIII were used as marker DNAs.

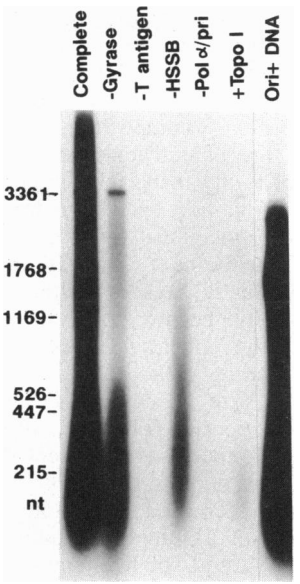

FIG. 3. DNA replication depends on all four proteins, and the addition of DNA topoisomerase I inhibits the replication. One of the four proteins in the complete system was omitted or topoisomerase I was added to the system, as indicated, and the reaction mixture was incubated for 1 h. Ori+ DNA containing the SV40 replication origin was incubated in the presence of T antigen, DNA polymerase α·primase, HSSB, and topoisomerase I for 1 h. The replicated DNAs were analyzed by alkaline agarose gel electrophoresis. Total nucleotide incorporation was 20 pmol (complete), 7 pmol (−Gyrase), <1 pmol [−T antigen or −polymerase α·primase (−Polα/pri)], 4 pmol (−HSSB), 2 pmol [+topoisomerase I (+Topo I)], and 22 pmol (Ori+ DNA).

the dipolymerase system, DNA replication was dependent on the presence of DNA polymerase α·primase. These results indicate that negatively supercoiled DNA containing ARS1 can be efficiently replicated with the purified proteins required for SV40 DNA replication in the presence of DNA gyrase.

DNA Replication Initiates in the ARS Region and Then Proceeds Bidirectionally. To determine where initiation of DNA synthesis occurred in this system, the reactions were pulse-labeled for various periods after preincubation for 15 min in the presence of the four proteins. Replication products were then analyzed by polyacrylamide gel electrophoresis

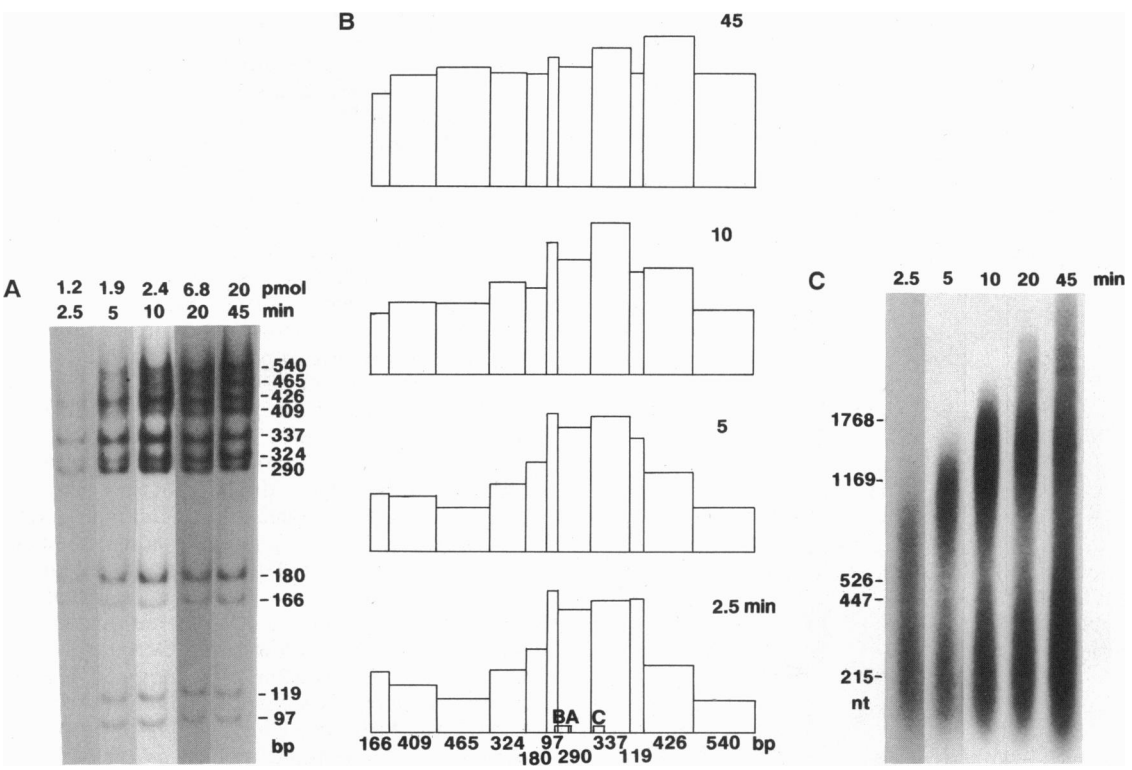

FIG. 4. Replication initiates from the ARS region and proceeds bidirectionally with the synthesis of leading and lagging strands. The complete reaction containing all replication proteins was incubated for 15 min in the presence of rNTPs and then pulse-labeled by adding dNTPs including [α-^{32}P]dCTP for various periods indicated above the gel. 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 (see Fig. 1). Total nucleotide incorporation is shown above the gel. (*B*) The labeled bands were quantitated and the values were divided by the size of each fragment and corrected for base composition. These values, except for the values at 20 min, are presented on a linear map of YRp7ΔEP DNA; the highest values observed during the different labeling periods were adjusted to the same level. The A, B, and C domains are indicated by open boxes at the bottom of the figure. (*C*) The products before digestion with restriction enzymes were analyzed by alkaline agarose gel electrophoresis.

after digestion with *Dde* I and *Dra* III. After incubation for 2.5 and 5 min, two fragments of 290 and 337 bp were preferentially labeled (Fig. 4*A*). When the ^{32}P content of each DNA fragment was quantitated (the value was divided by the length of the fragment and corrected for base composition), two fragments of 97 and 119 bp, which are located adjacent to the 290- and 337-bp fragments, were found to be more highly labeled than the other fragments (Fig. 4*B*). These four fragments formed a peak of the label where the A, B, and C domains of ARS1 are located. Over the next 5- and 10-min incubation periods, the peak gradually became flattened. After 45 min, labeling of the fragments became proportional to the size of each fragment. When the replicated DNAs were analyzed under denaturing conditions without restriction enzyme digestion, the maximum size of nascent DNAs gradually increased with the labeling period (Fig. 4*C*). After a 5-min incubation, two major DNA species were detected: one was short DNA of about 300 nt, and the other was the longer DNA whose size gradually increased. Most likely, these products were synthesized in a semidiscontinuous manner from lagging and leading strands of the template, respectively. Similar products synthesized from SV40 DNA in the same system (Fig. 3) were shown to be lagging- and leading-strand products (23). These data indicate that DNA replication is initiated from the ARS region and proceeds bidirectionally in a semidiscontinuous mode.

DNA Replication Is Dependent on the Presence of ARS1. To determine the role of ARS1 in this replication system, DNAs depleted of ARS1 were used as the template for DNA replication (Fig. 5). Under standard conditions containing 100 ng of template DNA, pBR322ΔEP DNA depleted of all yeast DNA supported replication poorly. However, DNA depleted

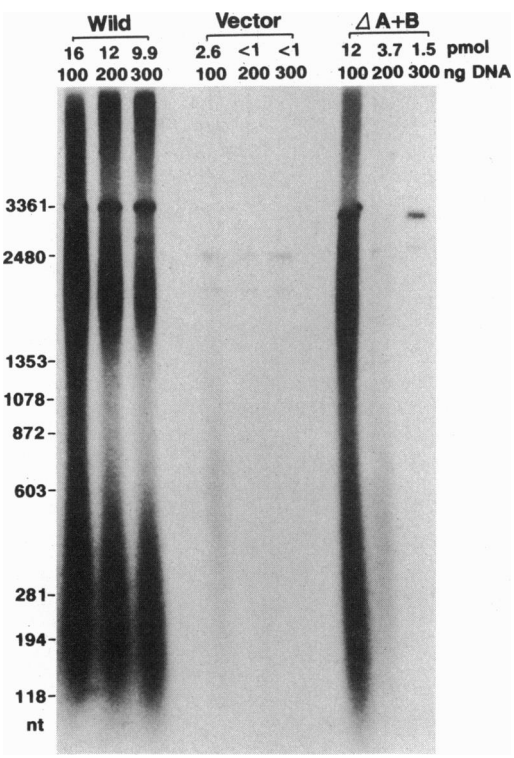

FIG. 5. ARS1 is required for the DNA replication. pBR322ΔEP DNA (vector), a mutant DNA depleted of the ARS1 origin (ΔA+B), and wild-type (Wild) DNA were replicated in the complete system, and the products were analyzed by alkaline agarose gel electrophoresis. The amount of template DNA added to the reaction (40 μl) was varied from 100 to 300 ng. Template DNAs used and nucleotide incorporation are indicated above the gel. φX174 DNA fragments digested with *Hae* III were used as marker DNAs.

of both the A domain and part of the B domain served as a template as efficiently as wild-type DNA. In the presence of this mutant DNA, DNA replication was initiated from residual yeast DNA (data not shown). As the concentration of template DNA in the reaction was increased, however, replication of the mutant DNA was drastically reduced. In contrast, there was only a slight decrease in DNA synthesis with wild-type DNA. These data suggest that the A and B domains of ARS1 are essential in the presence of excess DNA which can act as a nonspecific competitor. Among the replication proteins added, the amount of T antigen was found limiting in the presence of 300 ng of the mutant DNA; doubling the amount of T antigen added to this reaction relieved the inhibition (data not shown). Similar observations have been made in the SV40 core origin-dependent unwinding reaction by T antigen (33).

DISCUSSION

A circular duplex DNA containing ARS1 from *S. cerevisiae* was replicated with purified proteins. In this system, T antigen, HSSB, and DNA polymerase α-primase, which support SV40 DNA replication, and DNA gyrase from *E. coli* were used to replicate the DNA. DNA replication was initiated from the ARS1 region and proceeded bidirectionally, resulting in the synthesis of leading and lagging strands. This contrasts with SV40 and polyoma virus DNA replication systems where species specificity is stringently controlled by the origin sequence.

We have shown that replication from ARS is strongly stimulated by DNA gyrase, which can increase negative superhelicity and relaxes positive supercoiling in circular duplex DNA. DNA topoisomerase I, which relaxes both positive and negative supercoils, inhibited ARS-dependent replication in reactions containing gyrase (Fig. 3). These results suggest that the initiation of replication from the ARS region requires negative supercoiling of template. When the ARS1 template DNA was incubated with only DNA gyrase under the replication conditions used, the negative superhelical density of the DNA, examined by agarose gel electrophoresis of the DNA in the presence of chloroquine, did not increase (data not shown). These results suggest that gyrase mainly stimulates the elongation phase of ARS DNA replication by relaxing positive supercoils that accumulate ahead of the replication fork. Requirement of negative supercoiling of the template for initiation of replication was also observed in the oriC replication system (25) but not in the SV40 system (23, 24).

ARS1 consists of an 11-bp core consensus sequence (A domain) and flanking A+T-rich domains B and C. Almost all ARSs have a DNA structure similar to that of ARS1 except for the absence of the C domain (9): they contain an essential core consensus sequence and a flanking region (B domain) located on the 3′ side of T-rich strand of the core sequence. Umek and Kowalski (34) found that the flanking region of the origin in the 2-μm plasmid was unwound and became sensitive to mung bean nuclease (which recognizes single-stranded regions) when the DNA was negatively supercoiled. There is a correlation between the efficiency of DNA replication *in vivo* and ease of DNA unwinding in the flanking region of the histone H4 ARS (35). Umek *et al.* (36) proposed a model of initiation of DNA replication in *S. cerevisiae* in which an initiator protein recognizes the core consensus sequence. This interaction results in the unwinding of the flanking region called DNA unwinding element, which can be recognized by replication proteins including a DNA helicase. In the present *in vitro* system, the ARS region should be unwound by the torsional stress of negative supercoiling, which can be recognized by T antigen with the assistance of HSSB. Under the replication conditions used, P1 nuclease, a nuclease that

recognizes single-stranded regions in duplex DNA, digested a region inside the vector sequence (promoter of the RNA1 gene) in YRp7ΔEP DNA (data not shown). These results suggest that the ARS region may contain other features, in addition to the DNA unwinding element, that can be recognized by replication proteins. Several 4- of 5-bp matches of GAGGC, which is the T-antigen binding sequence, are present in the ARS region, and these sequences may have an important role in increasing specificity of initiation. However, we have shown that another DNA helicase, the E1 protein of bovine papilloma virus, replaced SV40 T antigen in this system; this finding suggests that T antigen simply acts as a DNA helicase (data not shown). Compared with the P1 nuclease-sensitive site in the vector, the A+T-rich region in ARS is longer and contains many T-rich stretches. These features may be important for initiation of replication, since the region would be stably unwound by torsional stress, and T antigen (37) and HSSB (38) can preferentially bind to the resultant single-stranded region containing T-rich stretches.

Initiator proteins of DNA replication have not been identified in *S. cerevisiae*. Recently, Bell and Stillman (39) identified an origin recognition complex, which binds specifically to the double-stranded consensus sequence. Such a factor may play a crucial role in unwinding of the ARS region, increasing the specificity of the origin function. On the other hand, there are several observations indicating that DNA replication in other eukaryotic cells is not initiated from a fixed point in the DNA (40) but starts randomly from a broad region of DNA (41). These results suggest that changes in the higher order structure of DNA might be important for the initiation of eukaryotic DNA replication. The data presented here that negative supercoiling is sufficient to induce initiation of ARS1 DNA replication lends experimental support for this view. Finally, the system described here should be helpful in identifying cellular DNA helicase(s) involved in DNA replication and could provide a clue to the development of an *in vitro* system for ARS DNA replication with yeast proteins.

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