

DNA Replication from Initiation Zones of Mammalian Cells in a Model System

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We reported that DNA replication initiates from the region containing an autonomously replicating sequence from *Saccharomyces cerevisiae* when negatively supercoiled plasmid DNA is incubated with the proteins required for simian virus 40 DNA replication (Y. Ishimi and K. Matsumoto, Proc. Natl. Acad. Sci. USA 90:5399–5403, 1993). In this study, the DNAs containing initiation zones from mammalian cells were replicated in this model system. When negatively supercoiled DNA containing an initiation zone (2 kb) upstream of the human *c-myc* gene was incubated with simian virus 40 T antigen as a DNA helicase, HSSB (also called replication protein A), and DNA polymerase α -primase complex isolated from HeLa cells, DNA replication was specifically initiated from the center of the initiation zone, which was elongated bidirectionally in the presence of a DNA swivelase. Without HSSB, the level of DNA synthesis was significantly reduced and the localized initiation could not be detected, indicating that HSSB plays an essential role in the initiation of DNA replication. The digestion of negatively supercoiled template DNA with a single-strand-specific nuclease revealed that HSSB stimulated DNA unwinding in the center of the initiation zone where the DNA duplex is relatively unstable. In contrast, DNA replication started from a broad region of an initiation zone downstream of the dihydrofolate reductase gene from chinese hamster ovary cells, but the center of the region was mapped near the origin of bidirectional DNA replication. These results suggested that this system mimics a fundamental process of initiation of eukaryotic DNA replication. The mechanism of initiation is discussed.

Both specific sequences in the origin that are within a few hundred base pairs in length and an initiator protein that interacts with the sequences are essential for initiation of DNA replication in prokaryotes and eukaryotic viruses (6, 11). Binding of the initiator protein to the sequences results in the unwinding of an AT-rich region in the origin, which is recognized by replication proteins, including DNA helicase. Similarly, an essential origin sequence has been found in *Saccharomyces cerevisiae* (10), and a protein complex that binds to the sequence has been identified (3), but the sequences essential for DNA replication have not been found in higher eukaryotes, including *Schizosaccharomyces pombe*. An increasing number of the regions in the chromosome where the initiation of DNA replication occurs in higher eukaryotes have been mapped, and they are called initiation zones (reviewed in reference 4). Vassilev and Johnson (40) have identified an initiation zone of 2 kb in size upstream of the human *c-myc* gene by origin mapping, using PCR amplification of nascent DNA. Consistent with this, it has been reported that the upstream region of the *c-myc* gene has activity by which the plasmid DNA containing the region can be replicated as an extrachromosomal DNA element (19, 31). Among the most extensively studied initiation zones is one located downstream of the dihydrofolate reductase (DHFR) gene from chinese hamster ovary (CHO) cells. Heintz and Hamlin (17) have found early-labeled restriction fragments in the amplicon including the DHFR gene in pulse-labeling studies of synchronized cells. Origin mapping with PCR has shown that an initiation of DNA replication occurs within a region of 2 kb that is localized in the restriction

fragments (41). Furthermore, Burhans et al. (7) have identified a specific initiation site (0.5 kb in size) inside the region by examining the transition point of leading and lagging strands, which is called the origin of bidirectional DNA replication (OBR). In contrast, Hamlin and coworkers (12, 42) have reported that replication eye structures can be detected in the region 55 kb in length downstream of the DHFR gene by two-dimensional gel electrophoresis. These results, which are inconsistent and discussed elsewhere (11, 27), suggest that the mechanism by which initiation of DNA replication occurs in higher eukaryotic cells is more complicated than that in prokaryotic cells.

The *in vitro* system is useful for analyzing the mechanism of DNA replication, but a means of studying cellular DNA replication with cell extracts has not been established. The simian virus 40 (SV40) DNA replication system has provided some understanding of eukaryotic DNA replication, since all the replication proteins required for the viral DNA replication except for SV40 T antigen are derived from the host cells. Binding of T antigen to the specific sequence in the origin induces the melting of the DNA in the flanking regions (5), followed by the unwinding of the template DNA in both directions by its DNA helicase activity (43). HSSB (the multi-subunit human single-stranded DNA-binding protein), which was identified as an essential cellular factor in SV40 DNA replication, assists the DNA unwinding by stabilizing the unwound structure, and it is also involved in DNA synthesis by interacting with T antigen and DNA polymerase α -primase (13, 30, 32). Although a factor equivalent to T antigen has not been found in eukaryotic cells, these findings suggest that both a DNA helicase(s) and HSSB play crucial roles in the initiation of DNA replication.

Recently, we showed that DNA replication was initiated from a yeast origin in a system in which negatively supercoiled DNA was incubated with the proteins required for SV40 DNA replication in the presence of DNA gyrase (21). In this system,

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both SV40 T antigen as a DNA helicase and HSSB interacted with the origin region where the DNA is unwound with the torsional stress of negative supercoils to initiate DNA replication in the presence of DNA polymerase α -primase (22, 29). DNA gyrase is required for the elongation step, as a DNA swivelase that relaxes positive supercoils accumulated ahead of the replication forks. Here, we describe the replication of DNAs containing initiation zones from mammalian cells in this system. The results suggested that DNA replication is initiated from the center of the *c-myc* initiation zone where the DNA is unwound by the torsional stress of negative supercoils, and HSSB plays an essential role in this reaction.

MATERIALS AND METHODS

Preparation of plasmid DNA. HMYC DNA (5.3 kb) contains the 2.8-kb fragment from *Hind*III (nucleotide 1) to *Pvu*II (nucleotide 2845) upstream of the human *c-myc* gene (16) cloned into the *Hind*III site of pBR322 Δ EP DNA (45). Plasmid pX24 (7.7 kb) contains a 5-kb *Xba*I fragment downstream of the DHFR gene (8) that is cloned into the *Xba*I site of pUC13 DNA. Negatively supercoiled plasmid DNA (form I) was extracted by using alkali-sodium dodecyl sulfate (SDS) from *Escherichia coli* HB101 transformed by the plasmid as described previously (21). Relaxed HMYC DNA was prepared by incubating the supercoiled DNA with HeLa topoisomerase I as previously reported (22).

Preparation of replication proteins. SV40 T antigen was purified from Sf27 cells infected with recombinant baculovirus. The DNA polymerase α -primase complex, HSSB, and topoisomerases I and II were prepared 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 (21).

Replication assay. The conditions under which DNA replication of plasmid DNA containing ARS1 was measured (21) were used for replication of HMYC and pX24 DNA. The reaction mixture (40 μ l) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8); 7 mM MgCl₂; 0.5 mM dithiothreitol; 4 mM ATP, 200 μ M (each) CTP, UTP, and GTP; 100 μ M (each) dATP, dGTP, and dTTP; 20 μ M [α -³²P]dCTP (1×10^4 to 2×10^4 cpm/pmol); 0.1 μ g of DNA; 0.8 μ g of creatine phosphokinase; 16 μ g of bovine serum albumin; 0.5 μ g of SV40 T antigen; 0.08 μ g of HSSB; DNA polymerase α -primase complex (0.1 and 0.3 U, respectively); 100 ng of gyrase A; and 160 ng of gyrase B. Reactions proceeded 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 1% agarose gel in Tris-borate-EDTA (TBE) buffer. To analyze the mode of replication, the DNA was incubated for 15 min in the presence of the proteins and then DNA synthesis was started by adding deoxyribonucleotides including [α -³²P]dCTP. Reactions proceeded for the indicated times, and replicated DNAs were analyzed by 5% polyacrylamide gel electrophoresis in TBE buffer after digestion with restriction enzymes. After the gel was dried, the replicated DNA was visualized by autoradiography.

Mung bean nuclease digestion. Negatively supercoiled HMYC DNA (500 ng) was incubated with or without HSSB in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA (9 μ l) at 37°C for 12 min, and then mung bean nuclease (1 μ l; 50 U) was added to the mixture and then incubated for 60 min (29). After purified DNA was digested with a restriction enzyme, the 5' ends of the DNA were labeled with T4 polynucleotide kinase and

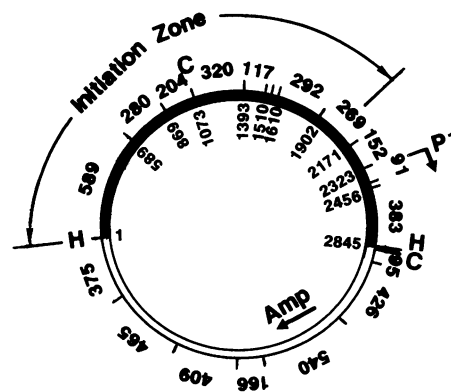


FIG. 1. Restriction map of plasmid HMYC DNA containing a *c-myc* initiation zone. *Dde*I sites are shown by short vertical lines on the circular DNA and other restriction sites (H, *Hind*III; C, *Cla*I) are indicated. The sizes (in base pairs) of the fragments digested with *Dde*I, *Hind*III, and *Cla*I are shown outside the circle. The filled portion in the plasmid indicates the DNA from the human *c-myc* gene and is numbered as reported elsewhere (16). The major transcription initiation site of the *c-myc* gene (P1), a replication initiation zone (40), and the ampicillin resistance gene are indicated.

[γ -³²P]ATP. DNA was analyzed by alkaline agarose gel electrophoresis.

Stability of DNA duplex. The free energy required for DNA strand separation under the specific conditions used in the nuclease hypersensitive assay was calculated by using the computer program developed by Natale et al. (35). The window size was 100 bp.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence in Fig. 5 is K01908.

RESULTS

Replication of plasmid DNA containing an initiation zone upstream of the human *c-myc* gene. An initiation zone of 2 kb has been mapped upstream of the human *c-myc* gene by analyzing newly synthesized DNA in vivo (40). A fragment (2.8 kb) containing the initiation zone was cloned into vector DNA based on pBR322 DNA (Fig. 1), and the resultant negatively supercoiled plasmid DNA (HMYC DNA) was replicated in the reaction mixture containing SV40 T antigen (as a DNA helicase), HSSB and DNA polymerase α -primase prepared from HeLa cells that constitutes the monopolymerase system (18) in addition to DNA gyrase from *E. coli*. The replication conditions were the same as those used for the replication of the DNA containing the autonomously replicating sequence 1 (ARS1) from *S. cerevisiae* (21). About 5% of the input DNA was replicated for 1 h in this system (Fig. 2). Under denaturing conditions, a shorter (100- to 200-base) and a longer DNA that was about half the template DNA in length were detected, each of which most probably corresponded to the lagging and leading strands, respectively (Fig. 2A) (20). High-molecular-weight DNAs were synthesized in this system, which were analyzed by native agarose gel electrophoresis (Fig. 2B). They may have contained late-Cairns-type molecules and catenated dimers (23). Incubation of the replicated DNA with topoisomerase II from HeLa cells resulted in the appearance of form II DNA, suggesting that the catenated dimers are synthesized in this system.

When T antigen was omitted from the reaction mixture, a small amount of longer DNA was synthesized (Fig. 2A), which

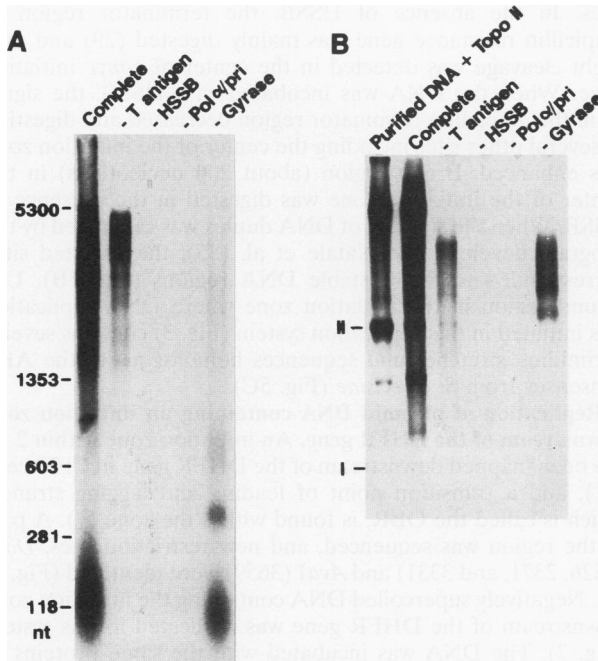


FIG. 2. Replication of HMYC DNA with purified proteins. Negatively supercoiled HMYC DNA (5.3 kb) was incubated at 37°C for 1 h with T antigen, HSSB, DNA polymerase α -primase (Pol α /pri) and DNA gyrase (Complete). One of the proteins that is indicated at the top was omitted from the mixture, and the reaction proceeded as described above. The amounts of incorporated deoxyribonucleotides were 17 pmol (Complete), 3.9 pmol (-T antigen), 2.6 pmol (-HSSB), less than 1 pmol (-Pol α /pri), and 6.2 pmol (-Gyrase). Purified DNAs were analyzed by alkaline (A) or native (B) agarose gel electrophoresis. Autoradiograms of the dried gel are shown. The products from the complete system were treated with HeLa topoisomerase II and electrophoresed in panel B (purified DNA + Topo II). *Hae*III digests of ϕ X174 DNA were electrophoresed as marker DNAs in panel A. The positions of form I and II DNA are indicated in panel B. nt, nucleotide.

is in contrast to the replication of ARS1 DNA, in which almost no DNA synthesis was detected (21). In the absence of HSSB, a low level of DNA synthesis was detected but products could not be seen in this gel because of heterogeneity. Only shorter DNAs were synthesized without DNA gyrase, which is consistent with the idea that the replication fork movement is blocked by the torsional stress of positive supercoils accumulated ahead of the replication forks. These results indicate that the initiation of DNA replication occurs by incubating negatively supercoiled DNA containing the *c-myc* initiation zone with the proteins, T antigen, HSSB, and DNA polymerase α -primase, which is elongated semidiscontinuously in the presence of DNA gyrase.

DNA replication initiates from the center of the initiation zone. To analyze the mode of DNA replication in this system, the reaction was pulse-labeled with or without DNA gyrase and chased in the presence of DNA gyrase. The chase was performed to adjust the size of products synthesized during the various labeling periods. The replicated DNAs were analyzed by gel electrophoresis after digestion with restriction enzymes. In the absence of DNA gyrase, when replication fork movement was blocked, a 204-bp fragment was heavily labeled within 10 min (Fig. 3A). When the label was quantified, divided by the size of each fragment, and corrected for base

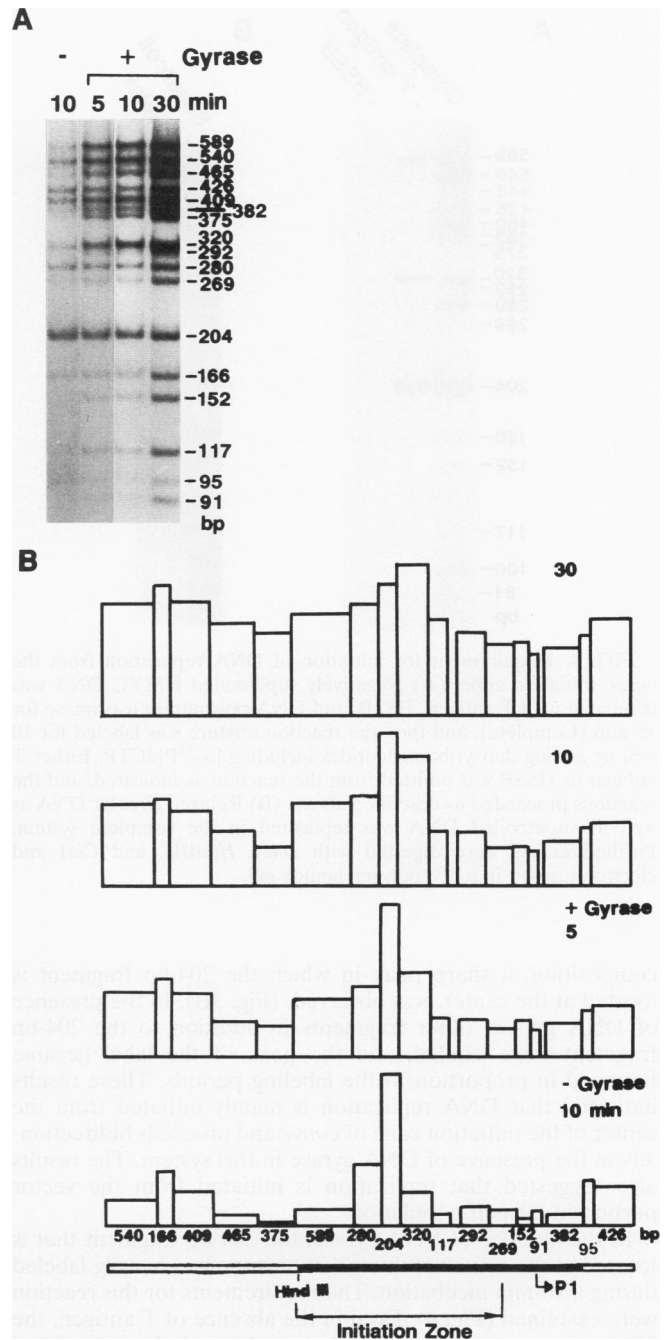


FIG. 3. DNA replication initiates from a specific locus in the *c-myc* initiation zone. (A) Reaction mixtures (the same as for Fig. 2) with or without DNA gyrase were incubated for 15 min, and then DNA synthesis was started by adding deoxyribonucleotides, including [α - 32 P]dCTP. After being labeled for the indicated periods, the reactions except for the 30-min incubation were chased for 20 min in the presence of DNA gyrase by adding a 100-fold excess of nonlabeled dCTP. Purified DNAs were digested with *Dde*I, *Cla*I, and *Hind*III and analyzed in a 5% polyacrylamide gel. Half of the DNA from each reaction mixture and 1/8 of that from the 30-min incubation mixture were electrophoresed, and the gel was exposed to film (Kodak XAR) for 3 days. Photographs were taken while the intensities of the 204-bp bands from different reactions were similar. (B) The radioactivities of the bands in panel A were measured, and then the values were divided by the size of each fragment and corrected for the base composition. These values are presented on a linear map of HMYC DNA: the highest values observed during the different periods were adjusted to the same level.

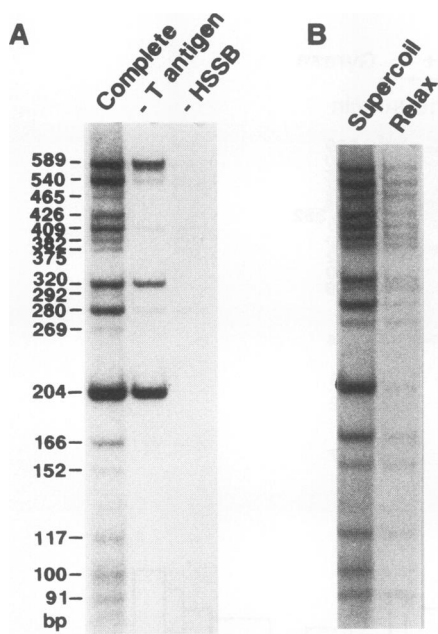


FIG. 4. Requirement for initiation of DNA replication from the *c-myc* initiation zone. (A) Negatively supercoiled HMYC DNA was incubated with T antigen, HSSB, and DNA polymerase α -primase for 15 min (Complete), and then the reaction mixture was labeled for 10 min by adding deoxyribonucleotides including [α - 32 P]dCTP. Either T antigen or HSSB was omitted from the reaction as indicated, and the reactions proceeded as described above. (B) Relaxed circular DNA as well as supercoiled DNA was replicated in the complete system. Purified DNAs were digested with *Dde*I, *Hind*III, and *Cla*I and electrophoresed in a 5% polyacrylamide gel.

composition, a sharp peak in which the 204-bp fragment is located at the center, was observed. (Fig. 3B). In the presence of DNA gyrase, other fragments in addition to the 204-bp fragment were labeled, and the peak of the label became flattened in proportion to the labeling periods. These results indicated that DNA replication is mainly initiated from the center of the initiation zone of *c-myc* and proceeds bidirectionally in the presence of DNA gyrase in this system. The results also suggested that replication is initiated from the vector portion in a small population.

In the absence of DNA gyrase, the 204-bp fragment that is located at the center of the initiation zone was heavily labeled during a 10-min incubation. The requirements for this reaction were examined (Fig. 4). Even in the absence of T antigen, the 204-bp fragment was heavily labeled, although the intensity of the band decreased. Without HSSB, however, DNA synthesis decreased and specific labeling of the 204-bp fragment was undetectable. When relaxed instead of supercoiled DNA was used as the template, DNA synthesis decreased and specific labeling of the 204-bp fragment was undetectable. These results indicated that HSSB plays an essential role in the initiation of DNA replication from a specific locus in the initiation zone in negatively supercoiled DNA.

Mung bean nuclease digestion of HMYC DNA. DNA unwinding of negatively supercoiled HMYC DNA was examined by using single-strand-specific mung bean nuclease (Fig. 5A and B). The DNA was incubated with or without HSSB prior to the digestion, and the digested sites were determined by treating the DNA with a single-cut restriction enzyme, *Kpn*I or *Sma*I, and calculating the distance from each of the restriction

sites. In the absence of HSSB, the terminator region of ampicillin resistance gene was mainly digested (29) and only slight cleavage was detected in the center of *c-myc* initiation zone. When the DNA was incubated with HSSB, the signal from the ampicillin terminator region decreased and digestion at several other sites including the center of the initiation zone was enhanced. Broad region (about 200 nucleotides) in the center of the initiation zone was digested in the presence of HSSB. When the stability of DNA duplex was calculated by the program developed by Natale et al. (35), the digested sites corresponded to the unstable DNA regions (Fig. 5B). Unwound region in the initiation zone where DNA replication was initiated in this replication system (Fig. 3) contains several pyrimidine stretches and sequences homologous to the ARS consensus from *S. cerevisiae* (Fig. 5C).

Replication of plasmid DNA containing an initiation zone downstream of the DHFR gene. An initiation zone within 2 kb has been mapped downstream of the DHFR gene in CHO cells (41), and a transition point of leading and lagging strands, which is called the OBR, is found within the zone (7). A part of the region was sequenced, and new restriction sites, *Dde*I (1626, 2371, and 3331) and *Ava*I (3659) were identified (Fig. 6) (8). Negatively supercoiled DNA containing the initiation zone downstream of the DHFR gene was replicated in this system (Fig. 7). The DNA was incubated with the same proteins as used for the replication of the *c-myc* initiation zone. Specifically labeled fragments were undetectable during a short period in either the presence or the absence of DNA gyrase, which is contrast to the results with the *c-myc* initiation zone. When the radioactivities of the fragments was quantified, however, the labeling was not random within the short (2.5-min) incubation and the fragments from the initiation zone were more heavily labeled than those from vector DNA. These fragments formed a peak whose center was mapped near the OBR. Similar results were obtained in the reaction in which the labeling proceeded for 5 min in the absence of DNA gyrase. After a long (45-min) incubation, the peak became flattened. These results indicate that DNA replication initiates from a broad region of the DHFR initiation zone but that the initiation occurs more frequently from the region containing the OBR in this system.

DISCUSSION

We showed that DNA replication was initiated from a specific site in the *c-myc* initiation zone by incubating negatively supercoiled plasmid DNA with HSSB, T antigen, and DNA polymerase α -primase (Fig. 3 and 4). The initiation site is located at the center of the initiation zone, where the probability of initiation occurring in vivo is the highest in the zone (40), suggesting that this in vitro system mimics a fundamental process of initiation of DNA replication in vivo. HSSB, which stimulates DNA unwinding in the center of the zone (Fig. 5), plays a crucial role in the initiation of DNA replication (Fig. 4). SV40 T antigen enhanced the DNA replication, and negative supercoiling of template DNA was required for the initiation of DNA replication. These results suggest that DNA replication is initiated by the binding of HSSB, T antigen, and DNA polymerase α -primase to the center of the initiation zone, where the DNA is unwound by the torsional stress of negative supercoils. A similar mechanism of initiation was observed when plasmid DNA containing ARS1 from *S. cerevisiae* was replicated in the same system (21). The DNAs in the three domains (A, B, and C) of ARS1 were easily unwound by the torsional stress of negative supercoils, which was recognized by T antigen, HSSB, and DNA poly-

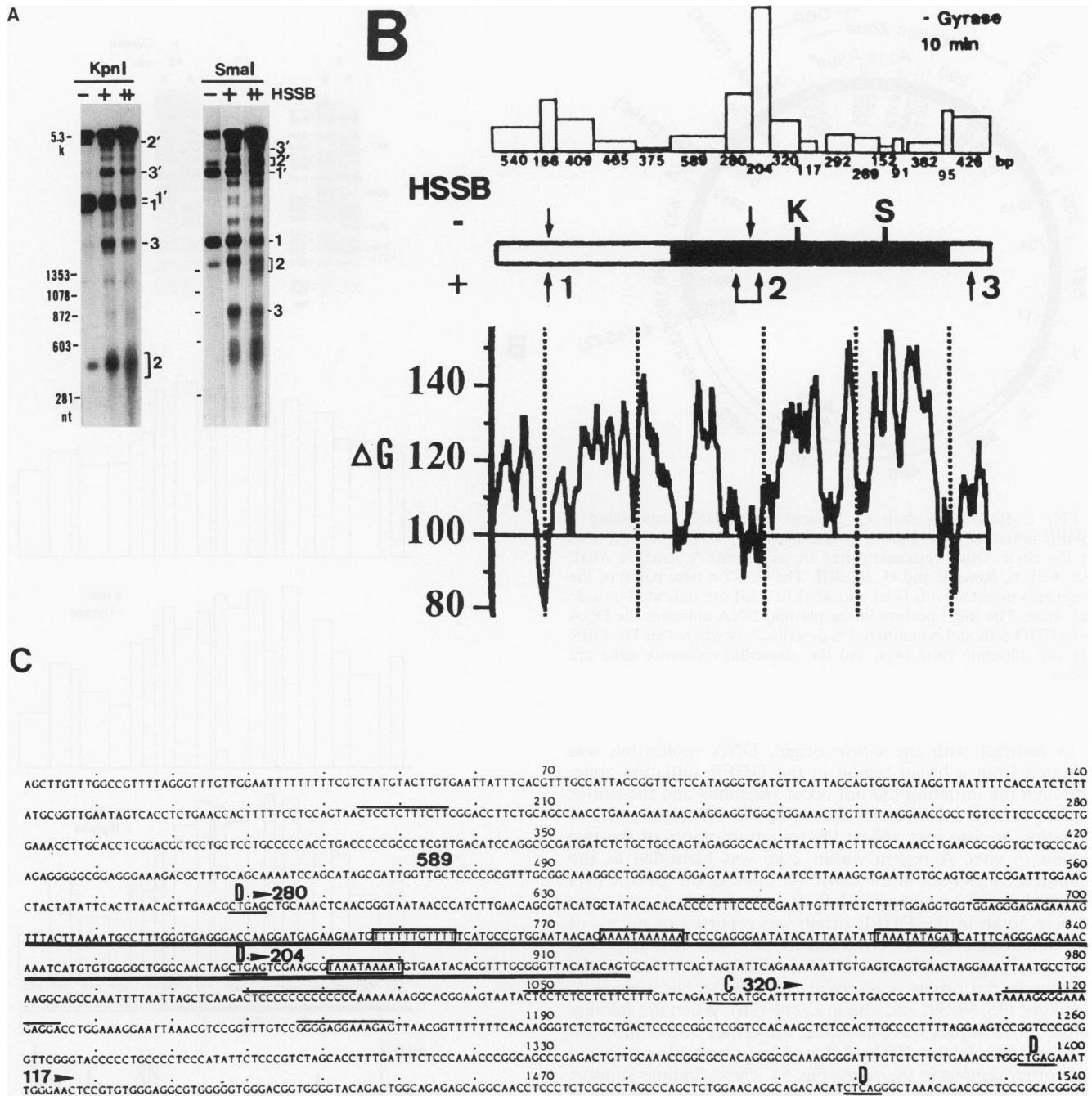


FIG. 5. Mung bean nuclease digestion of HMYC DNA. (A) Negatively supercoiled HMYC DNA (500 ng) was incubated without or with (+, 270 ng; ++, 540 ng) HSSB and then digested with mung bean nuclease. After DNA was digested with *SmaI* or *KpnI*, it was labeled at the 5' ends and electrophoresed in an alkaline agarose gel. DNA was visualized by autoradiography. Hypersensitive sites are numbered (paired). (B) The hypersensitive sites were mapped by calculating the distance from each of the restriction sites. Restriction sites (K and S) and sites hypersensitive to the nuclease (1, 2, and 3) detected in the absence or presence of HSSB are indicated on the linear map of HMYC DNA. The fragment labeling in the replication system when the reaction proceeded for 10 min without DNA gyrase (Fig. 3B) is at the top of the figure. DNA duplex stability of HMYC DNA is shown at the bottom (35). (C) Nucleotide sequence around the center of the *c-myc* initiation zone is shown. Pyrimidine stretches (overlined), sequences of 10-of-11 matches to the ARS consensus sequence (boxed), restriction sites (D, *DdeI*; C, *Clal*), and sizes of restriction fragments are indicated. A region digested with the nuclease in the presence of HSSB is underlined.

merase α -primase (22, 29). The requirement for T antigen is different between the two systems of *c-myc* origin and ARS1, in that T antigen is essential for replication from the ARS1 region. The reason for the difference remains to be resolved.

We found that DNA helicase B isolated from mouse cells in place of T antigen stimulated the DNA replication of HMYC DNA in a system that included SSB and DNA polymerase α -primase from mouse cells (29a).

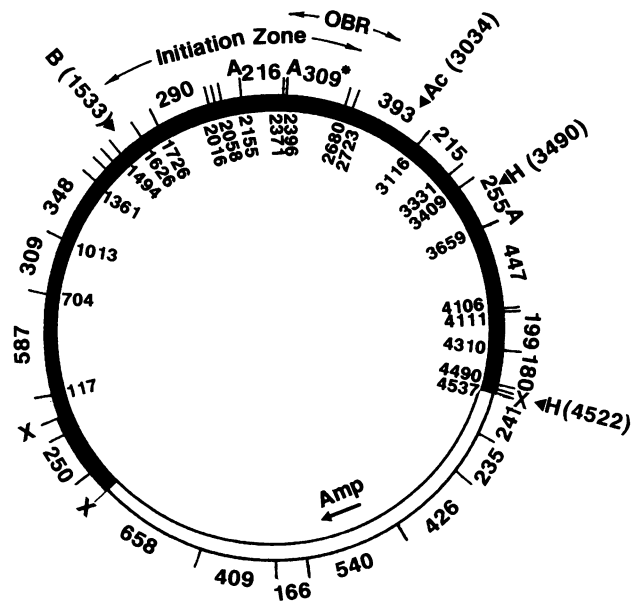


FIG. 6. Restriction map of plasmid pX24 DNA containing a DHFR initiation zone. *DdeI* sites are indicated by short vertical lines on the circle. Other restriction sites are as follows: A, *AvaI*; X, *XbaI*; Ac, *AccI*; B, *BamHI*; and H, *HindIII*. The sizes (in base pairs) of the fragments digested with *DdeI* and *XbaI* or *AvaI* are indicated outside the circle. The filled portion in the plasmid DNA indicates the DNA from CHO cells and is numbered as described elsewhere (8). The OBR (7), the initiation zone (41), and the ampicillin resistance gene are shown.

In contrast with the *c-myc* origin, DNA replication was initiated from a broad region in the DHFR initiation zone, although the initiation did not occur randomly and the center of the peak was located near the OBR. The different modes of initiation in vitro may reflect the initiation modes of the two origins in vivo. A region within 2 kb was identified as the initiation zones both in the *c-myc* (40) and in the DHFR (41) regions by origin mapping with PCR, but a long initiation zone of over 50 kb in the DHFR origin was reported by means of two-dimensional gel electrophoresis (12, 42). Kowalski and coworkers reported that the DNA unwinding element (DUE) present in the origin is essential for DNA replication in *S. cerevisiae* (35, 38, 39) and also in *E. coli* (26). When the stability of the DNA duplex was calculated, the center of the initiation zone of *c-myc* (about 300 bp in length) was unstable compared with other regions in the zone (Fig. 5). These findings support the mechanism of initiation that is proposed above. The DUE was detected by digesting negatively supercoiled DNA with a single-strand-specific mung bean nuclease (Fig. 5). The sites digested with the mung bean nuclease were mapped not only in the center of the initiation zone but also in the vector portion of HMYC DNA (Fig. 5A and B), and the initiation of DNA replication occurred mainly from the initiation zone. These specificities may be generated by the difference in the length of the DUE and the base compositions. The DUE within the *c-myc* origin is longer and contains several T and pyrimidine stretches (Fig. 5C). These features may be important for the establishment of a preinitiation complex, since both DNA polymerase α -primase and HSSB, which play essential roles in the initiation of DNA replication in this system, bind preferentially to the single-stranded DNA containing pyrimidine-rich sequences (4, 24). Consistent with this notion, DNA unwinding

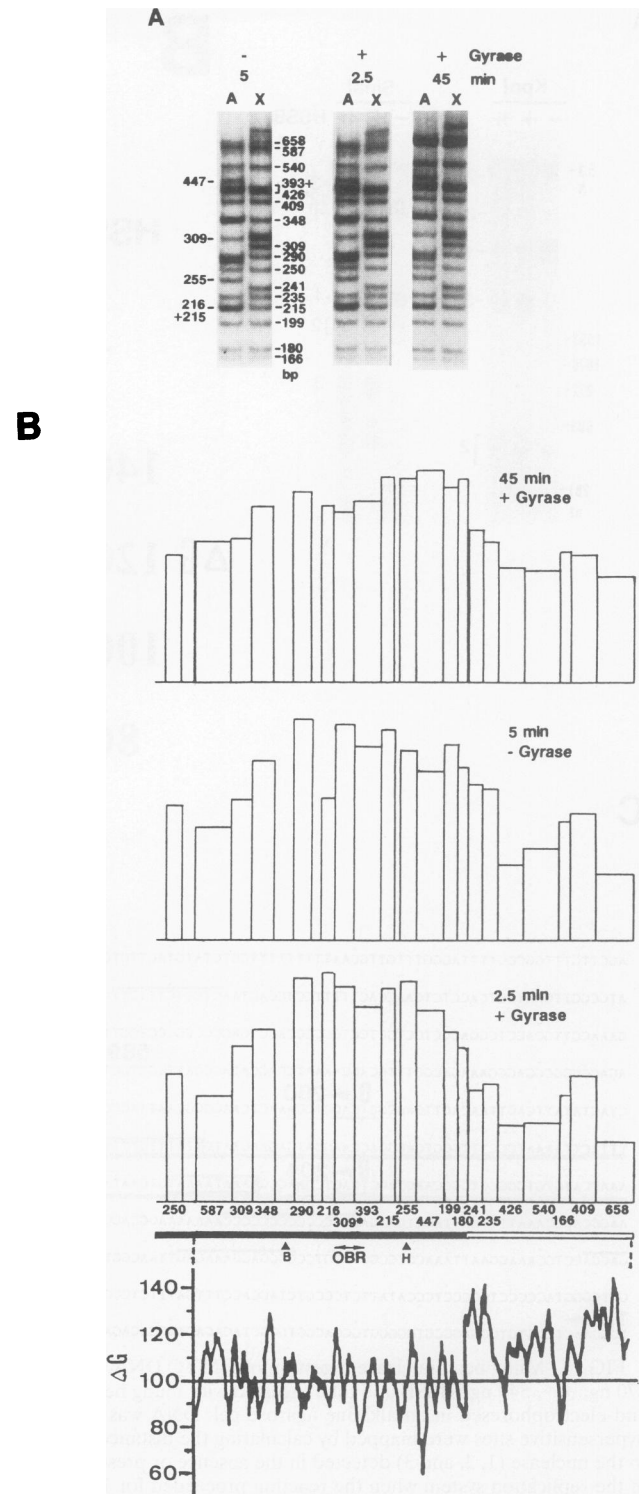


FIG. 7. Replication of pX24 DNA containing a DHFR initiation zone. (A) Negatively supercoiled DNA was incubated for 15 min with T antigen, HSSB, and DNA polymerase α -primase with or without DNA gyrase. DNA synthesis was started by adding deoxyribonucleotides, including [α - 32 P]dCTP, and the incubation was continued for the indicated periods. The reactions were chased for 30 min (2.5- and 5-min pulses) or 15 min (45-min pulse) in the presence of DNA gyrase. Purified DNAs were digested with *DdeI* and *AvaI* (left lanes; A) or *DdeI* and *XbaI* (right lanes; X) and then analyzed by 5% polyacrylamide gel electrophoresis. Half of the DNA from the 2.5- and 5-min

in the center of the initiation zone was stimulated by HSSB (Fig. 5A), suggesting the interaction of HSSB with the region. The role of HSSB in the initiation of cellular DNA replication is also suggested by the fact that the *Xenopus* homolog of HSSB colocalizes with the nuclear prereplication centers poised for DNA synthesis in *Xenopus* eggs (1). Although the DNA near the OBR is easy to unwind, several other regions in the DHFR initiation zone are also easy to unwind (free energy of unwinding is less than 100 kcal) (Fig. 7), which is different from the *c-myc* initiation zone, in which only one region near its center is easy to unwind (Fig. 5). It is possible that a broad initiation from the DHFR initiation zone in this replication system is brought about by these features of the DNA.

Initiation proteins in prokaryotes and eukaryotic viruses were identified. Recently, ORC, which binds specifically to the origin from *S. cerevisiae*, was identified (3), and its involvement in cellular DNA replication was reported (2, 33). Neither essential sequences nor initiator protein has been identified in higher eukaryotes, although a fragment of 2 kb in the human β -globin gene domain that is essential for origin function has been identified (25). The use of replication origins in higher eukaryotes is regulated during different developmental stages (9). Multiple initiation sites of DNA replication can be detected in the region over 50 kb downstream of the DHFR gene in CHO cells (12, 42), in the histone gene repeats in *Drosophila melanogaster* cells (36), and in human rRNA genes (27a). These observations suggest that the requirement of the specific sequence for initiation of DNA replication is not stringent in higher eukaryotes. In this replication system, both the presence of the DUE, which contains pyrimidine-rich sequences, and the negative supercoiling of DNA are important factors for initiation of DNA replication. This mechanism may be consistent with the low sequence requirement for initiation of DNA replication. The chromosomal DNA may be under the torsional stress of supercoils, since it forms loop structures and is topologically constrained (34). Negative supercoiling can be generated by several DNA dynamics. It has been shown that negative supercoiling is generated at the promoter region of RNA transcription because of the unwinding of DNA during transcription (28). Propagation of the torsional stress upstream of the *c-myc* gene during transcription has been suggested by the Z DNA formation of the segments in a transcription system, using isolated nuclei (44). Therefore, *c-myc* transcription may be required for the initiation of DNA replication in vivo in the *c-myc* initiation zone. It is also possible that transcription factors bound to the upstream region of the *c-myc* gene are involved in the initiation of DNA replication by

incubations and 1/8 of that from the 45-min incubation were electrophoresed. The digested fragments were identified from their sizes and also by examining their sensitivities to other restriction enzymes. (B) Radioactivities of bands indicated were measured, and the values were divided by the size of each fragment and corrected for base composition. The radioactivities of the fragments of 166, 188, 199, 215, 235, 241, 250, 290, 309*, 348, 409, 540, 587, and 658 bp were determined from *DdeI* and *XbaI* digestion in panel A, and those of the fragments of 216, 255, 309, and 447 bp were from *DdeI* and *AvaI* digestion. The radioactivity of the 216-bp fragment was determined by subtracting that of the 215-bp fragment in *DdeI* and *XbaI* digestion from that of 215- and 216-bp fragments in *DdeI* and *AvaI* digestion. The radioactivities of the 393- and 426-bp fragments were determined by combining the results of *DdeI* and *AccI* digestion (data not shown). Specific radioactivity of the 250-bp fragment, of which the nucleotide sequence has not been determined, was calculated by estimating its GC content as 50%. These values are presented on a linear map of pX24 DNA. The DNA duplex stability of pX24 DNA is shown at the bottom.

fixing torsional stress generated by transcription. Correlation between gene expression and replication was found in the human β -globin domain (25).

This study showed that DNA replication initiates from the center of the initiation zone in vitro in the absence of initiator proteins in a system consisting of purified replication proteins and negatively supercoiled DNA. We used here a monopolymerase, instead of the dipolymerase system, for SV40 DNA replication, bypassing the requirements for the DNA polymerase δ , PCNA, and RF-C proteins (14, 37, 42a). The role of DNA polymerase α -primase in the initiation of cellular DNA replication has been demonstrated by examining the phenotypes of temperature-sensitive mutants (15). The fact that only DNA polymerase α complexes with primase supports this notion. However, it would be important to set up a dipolymerase system as well as a system with crude extracts for *c-myc* DNA replication. It may be more important to establish a chromatin replication system, since it is likely that nuclear structures including chromatin play important roles in increasing the specificity of initiation of DNA replication in eukaryotic cells (11).

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