Cell, Vol. 59, 541–551, November 3, 1989, Copyright © 1989 by Cell Press

Transcriptional Activator Nuclear Factor I Stimulates the Replication of SV40 Minichromosomes In Vivo and In Vitro

Linzhao Cheng and Thomas J. Kelly
Department of Molecular Biology and Genetics
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Summary

SV40 DNA replication in vivo is greatly stimulated by *cis*-acting transcriptional elements. We studied a model viral chromosome containing a single binding site for the cellular transcriptional activator, nuclear factor I (NF-I/CTF), located adjacent to the replication origin. The presence of the NF-I recognition site increased replication efficiency over 20-fold in vivo. Purified NF-I had little effect on the replication efficiency in the standard SV40 cell-free system when the template was introduced as naked DNA. However, NF-I specifically prevented the repression of DNA replication that occurred when the template was preassembled into chromatin. Our data support a model in which the binding of a transcriptional activator perturbs the local distribution of nucleosomes, thereby increasing the accessibility of the origin region.

Introduction

Simian virus 40 (SV40) has proven to be an exceptionally useful model for studies of mammalian DNA replication (Campbell, 1986; Kelly, 1988; Challberg and Kelly, 1989). The SV40 genome is a circular duplex DNA molecule containing a single origin of DNA replication. In the nucleus of the host cell, where SV40 DNA replication takes place, the viral genome is complexed with histones to form a minichromosome that is similar in structure to cellular chromatin. Replication requires a single SV40-encoded protein, T antigen, but is otherwise dependent upon cellular replication proteins. The development of an efficient cell-free system for SV40 DNA replication (Li and Kelly, 1984) has provided a means to identify and characterize a number of such cellular proteins.

The core origin of DNA replication consists of a 65 bp segment of the SV40 genome that contains binding sites for the SV40 large T antigen and a 17 bp AT-rich region. The core origin is clearly sufficient to support initiation of SV40 DNA replication both in vivo and in vitro. However, sequence elements outside of the core origin can dramatically affect the efficiency of initiation in vivo. The largest effects have been seen with elements previously associated with activation of transcription, such as the SV40 enhancers or the binding sites for the transcriptional factor Sp1. The presence of these sequence elements adjacent to the replication origin increases the efficiency of DNA replication in vivo at least 10-fold (Bergsma et al., 1982; Li et al., 1986; Lee-Chen and Woodworth-Gutai, 1986; DeLucia et al., 1986; Hertz and Mertz, 1986).

Activation of DNA replication by enhancers and other transcriptional elements is not limited to SV40 and in fact appears to be a quite general feature of the replication of eukaryotic viruses. The phenomenon was first seen with polyoma virus (de Villiers, 1984), but has since been observed for adenovirus, Epstein-Barr virus, and bovine papillomavirus, as well as SV40 (Jones et al., 1987; Reisman et al., 1985; Lusky and Botchan, 1986). A number of possible explanations for the stimulatory effects of the transcriptional elements have been suggested, but the underlying molecular mechanisms remain unclear (Kelly, 1988; DePamphilis, 1988).

To understand the mechanism by which the SV40 transcriptional elements activate DNA replication in vivo, it would be useful to duplicate the effect in vitro. However, in previous studies we and others have observed little effect of such elements on replication efficiency in the standard cell-free system (Stillman et al., 1985; Li et al., 1986). One possible reason for this discrepancy is that the template for DNA replication in vivo, unlike that in vitro, is the chromatin-like SV40 minichromosome. Therefore, we have studied the mechanism of action of transcriptional elements in a novel cell-free system in which the DNA template is assembled into chromatin prior to DNA replication. To simplify these studies we made use of a template that contains a single binding site for nuclear factor I (NF-I) located adjacent to the core SV40 origin of DNA replication. NF-I is a cellular site-specific DNA binding protein originally identified as a stimulatory factor for adenovirus DNA replication and subsequently shown to be identical to the cellular transcription factor CTF (CCAAT transcription factor) (Nagata et al., 1983; Rawlins et al., 1984; Rosenfeld and Kelly, 1986; Jones et al., 1987; Santoro et al., 1988).

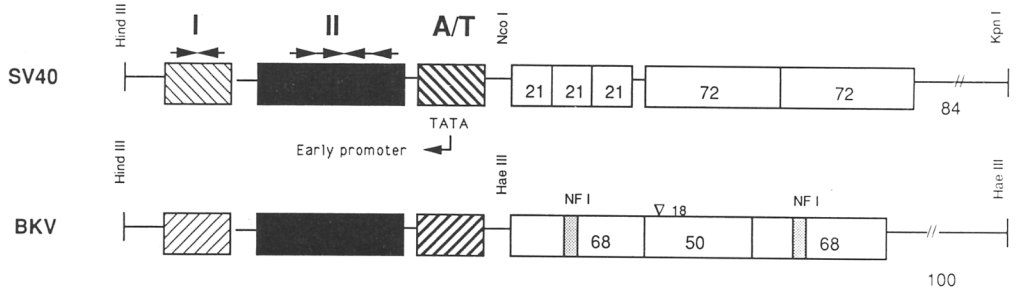
The presence of the NF-I recognition site in this template increased the efficiency of DNA replication over 20-fold in vivo. Purified NF-I had no detectable effect on the replication efficiency of the same template in the standard cell-free system. However, preincubation with NF-I specifically prevented the repression of DNA replication that otherwise occurred when the template was assembled into chromatin prior to initiation of DNA synthesis. Thus, in the chromatin replication system, SV40 minichromosomes containing the NF-I recognition site replicated much more efficiently than minchromosomes lacking the NF-I site. Our data are consistent with a model in which the binding of NF-I (or other transcriptional activators) perturbs the local distribution of nucleosomes, thereby increasing the accessibility of the origin region to initiation factors.

Results

The BKV Enhancer Can Substitute for the SV40 Transcriptional Elements in Stimulating DNA Replication

It has previously been shown that the presence of the SV40 transcriptional elements adjacent to the minimal origin increases the efficiency of SV40 DNA replication in

A.



B.

NF-I

CACAGGGAGGAGCTGCTTACCCATGGAATGCAGCCAAACCATGACCTCAGGAAGGAAAGTGCATGACT

CCATGGAATGCAGCCAAACCAT

Figure 1. Organization of Regulatory Regions of SV40 and BKV

(A) Functionally important elements of DNA replication and transcription of the two papovaviruses are outlined in boxes. Shading of the boxes indicates the degree of similarity of DNA sequences of the counterpart elements of the two viruses. The replication origins of the two viruses are very similar in nucleotide sequence (I and II indicate T antigen binding sites and A/T indicates the AT-rich region). The BKV (Dunlop strain) transcriptional enhancers consist of three copies of a 68 bp repeat sequence (18 bp deletion in the center copy). The SV40 transcriptional elements (21 bp and 72 bp repeats) are unrelated to the BKV enhancers. The locations of the NF-I binding sites in the BKV enhancers are also indicated.

(B) DNA sequence of the 68 bp repeat of the BKV enhancer. The consensus recognition sequence of NF-I is underlined. Shown below is the DNA sequence of the 22 bp segment used as a single NF-I binding site in this report.

vivo at least 10-fold (Bergsma et al., 1982; Li et al., 1986; Lee-Chen and Woodworth-Gutai, 1986; DeLucia et al., 1986; Hertz and Mertz, 1986). Analysis of deletion mutations has revealed that either the 21 bp GC-rich repeats (Sp1 binding sites) or the 72 bp SV40 enhancers can stimulate DNA replication in the absence of the other element. The stimulatory effect requires that the transcriptional elements be located in *cis* to the minimal origin, but is otherwise independent of experimental conditions such as transfection method, amount of input DNA, time period of DNA replication, or presence of competing DNA templates (Li et al., 1986). To further examine the specificity of the effect, we investigated whether the SV40 transcriptional elements could be replaced by those derived from another virus.

The human papovavirus BKV resembles SV40 in many ways, including the general organization of its genome (Howley, 1980). The minimal origins of replication of the two viruses are almost identical in sequence, but the adjacent transcriptional regulatory elements are essentially unrelated (Figure 1A). The commonly studied Dunlop strain of BKV contains three copies of a 68 bp sequence (18 bp in the central copy are deleted) that act as transcriptional enhancers. It has been demonstrated that the 260 bp segment containing all three repeats can activate transcription from homologous and heterologous promoters in a distance- and orientation-independent fashion (Rosenthal et al., 1983; Grinnell et al., 1986). The enhancer is also required for optimal BKV DNA replication in vivo (Deyerle et al., 1989).

We constructed plasmid templates containing the minimal SV40 origin of DNA replication and transcriptional elements derived either from SV40 or from BKV. In all cases the transcriptional elements were inserted immediately adjacent to the AT-rich region of the SV40 origin. The constructs were introduced into cultured cells together with a common reference plasmid, pKP.HNO, which contained only the SV40 origin. After incubation for 48 hr, the transfected cells were lysed, and episomal DNA was isolated. The DNA was digested with DpnI to eliminate unreplicated input DNA and with EcoRI to linearize the products of replication. The replicated DNA was fractionated by agarose gel electrophoresis, and the ratio of the test plasmid DNA to the reference plasmid DNA was determined by hybridization. This ratio, normalized to the ratio of the input DNA molecules, is defined as the relative replication efficiency of the test plasmid.

Figure 2 shows the results of experiments in which the various plasmid constructs were introduced into cells at a 1:20 ratio to the reference plasmid. The cells employed in these experiments included one simian (COS-1) and two human (SV-1 and SV-G) cell lines that constitutively produce the SV40 T antigen (Gluzman, 1981; Major and Matsumura, 1984; Major et al., 1985). In all of the cell lines studied, the relative replication efficiencies of the templates containing transcriptional elements in *cis* (lanes 2, 3, and 4) were significantly greater than that of the control template (pUC.HNO) lacking such elements (lane 1). Quantitative estimates of the replication efficiencies are summarized in Table 1.

The magnitudes of the stimulation by the SV40 enhancers and GC-rich repeats in COS-1 cells were similar to those reported previously (Li et al., 1986; Lee-Chen and Woodworth-Gutai, 1986; DeLucia et al., 1986; Hertz and

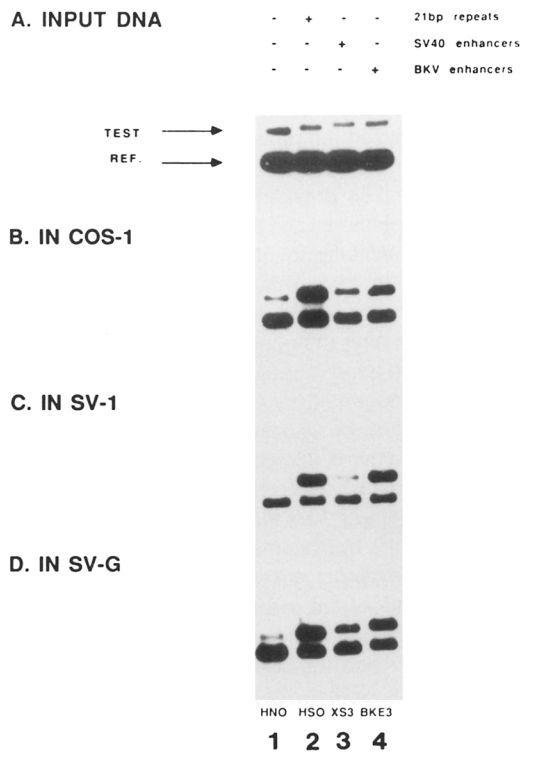


Figure 2. Effect of Transcriptional Elements on SV40 DNA Replication In Vivo

Test plasmids pUC.HNO (lane 1), pUC.HSO (lane 2), pUC.XS3 (lane 3), and pUC.BKE3 (lane 4) were cotransfected into the indicated cell lines together with the reference template pKP.HNO. All plasmids (both test and reference) contained the identical minimal origin of SV40 replication. The presence or absence of additional transcriptional elements in each of the test plasmids is indicated at the top of the figure. The recipient cell lines were COS-1 simian cells (B), SV-1 human cells (C), and SV-G human cells (D). After 48 hr DNA was isolated from the transfected cells and digested with DpnI to remove unreplicated DNA. Following linearization with EcoRI, the DNA was fractionated by 1% agarose gel electrophoresis and hybridized to a radioactive DNA probe containing the SV40 origin.

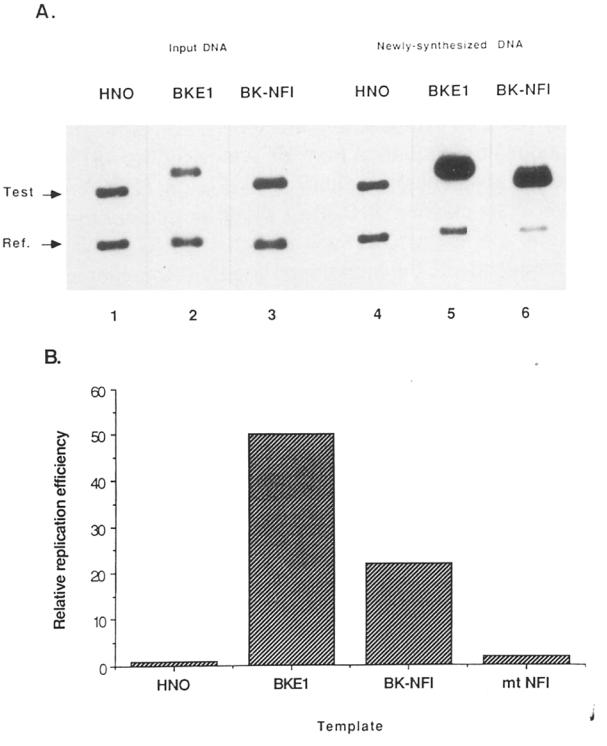


Figure 3. The NF-I Binding Site Stimulates SV40 DNA Replication in SV-G Cells

(A) In vivo replication of SV40 templates containing the NF-I recognition site. The experimental procedure here is similar to that described in Figure 2, except that equimolar quantities of test and reference templates (30 fmol of each) were used in each transfection. The control template pUC.HNO (lanes 1 and 4) contains the SV40 origin of DNA replication but lacks the SV40 transcriptional elements. Plasmid pUC.BKE1 (lanes 2 and 5) contains one copy of the 68 bp repeat of the BKV enhancer in addition to the SV40 origin. Plasmid pUC.BK-NFI (lanes 3 and 6) contains a 22 bp synthetic NF-I binding site in addition to the SV40 origin (see Figure 1 for DNA sequence).

(B) Quantitation of relative replication efficiencies of templates in SV-G cells. The figure summarizes data from two separate experiments. The template mt NFI contains a double-base substitution in the 22 bp segment ofpUC.BK-NFI so that the sequence of the NF-I site is changed from TAA . . . GCCAA to TAA . . . GGGAA.

Mertz, 1986). The SV40 transcriptional elements also stimulated DNA replication 10- to 70-fold in human cells. The BKV enhancer stimulated DNA replication at least 10-fold in all three cell types. Thus, the observed stimulation was independent of the origin of the transcriptional elements (SV40 vs. BKV), the species of the host cell (simian vs. human), and the tissue of the host cell (kidney vs. glial).

Table 1. The Relative Replication Efficiency of SV40 Templates in Three Cell Lines

	Transcriptional Elements		Relative Replication Efficiency		
Template	SV40	BKV	COS-1	SV-1	SV-G
HNO	–	–	1	1	1
HSO	21 bp repeats	–	22	68	30
XS3	72 bp repeats	–	6	10	10
BKE3	–	68 bp repeats	10	61	27

COS-1 = SV40-transformed CV1 (monkey kidney cell), SV-1 = SV40-transformed HEK (human embryonic kidney cell), and SV-G = SV40-transformed human fetal glial cell.

A Single Synthetic NF-I Site Enhances SV40 DNA Replication In Vivo

To simplify analysis of the mechanism by which transcriptional elements stimulate DNA replication, we sought to define the smallest sequence element required for the stimulatory effect. The BKV enhancer contains a TGG-A . . . GCCAA sequence motif that is located near the center of the 68 bp repeat of the Dunlop strain and is conserved in all other BKV strains (Nowock et al., 1985). This motif is recognized by the cellular site-specific DNA binding protein NF-I, also known as transcription factor CTF (Rosenfeld and Kelly, 1986; Jones et al., 1987; Santoro et al., 1988).

Genetic studies have demonstrated that an intact NF-I

recognition site is required for maximal stimulation of transcription by the BKV enhancer (Deyerle and Subramari, 1988). To test whether the NF-I binding site within the BKV enhancer contributes to the stimulation of DNA replication, a 22 bp synthetic oligonucleotide containing the NF-I recognition sequence from BKV (see Figure 1B) was inserted adjacent to the minimal SV40 origin of DNA replication in the plasmid pUC.HNO, which lacks other transcriptional elements. This new construct (pUC.BK-NFI) was introduced into the human cell line SV-G together with the reference plasmid pKP.HNO as before.

For purposes of comparison, a plasmid containing a single complete copy of the BKV enhancer (pUC.BKE1) was also tested. As shown in Figure 3, the presence of a single copy of the NF-I recognition sequence increased replication efficiency more than 20-fold over the control plasmid (pKP.HNO) lacking transcriptional elements. Since the presence of a complete copy of the BKV enhancer increased replication efficiciency about 50-fold, it is evident that that the NF-I site is sufficient to account for most, if not all, of the observed stimulatory effect of the BKV enhancer.

To confirm that the NF-I recognition sequence in the synthetic 22 bp insert is responsible for the stimulation of DNA replication, we studied a mutant construct (mt NFI) containing a double-base subsitution in which the sequence TGGA . . . GCCAA was changed to TGGA . . . GGGAA. We have previously shown that either of these two base substitutions completely abolishes specific DNA binding of purified NF-I (Rosenfeld et al., 1987). The mutant template displayed greatly reduced replication efficiency in vivo relative to the templates containing the wild-type NF-I recognition site (Figure 3B).

Thus, our data strongly suggest that NF-I is responsible for activating DNA replication of these templates in SV-G cells. Although NF-I appears to be a relatively abundant protein in most cells, we cannot of course rule out the possibility that another protein that recognizes a similar sequence motif might be involved. Other experiments (Del Vecchio et al., 1989; L. C. and T. J. K., unpublished data) suggest that the BKV enhancer contains an element(s) in addition to the NF-I site that can independently stimulate DNA replication, so the complete BKV enhancer may be functionally redundant with respect to DNA replication.

We also analyzed the replication of templates containing or lacking the synthetic NF-I site in the standard SV40 cell-free system. As previously observed with the SV40 transcriptional elements, the presence of the NF-I recognition site had essentially no effect on the efficiency of DNA replication in vitro. Moreover, the addition of NF-I protein at a sufficient concentration to fully saturate the recognition site did not alter the observed replication efficiency. The NF-I protein used in these experiments was purified to apparent homogeneity by recognition site affinity chromatography as previously described (Rosenfeld and Kelly, 1986) and was active in stimulating adenovirus DNA replication in vitro (data not shown).

Numerous attempts were made to demonstrate stimulation by NF-I in vitro by systematically altering the reaction conditions. For example, we tested the effects of varying the ionic strength of the reaction buffer, the ratio of the concentrations of T antigen and cell extract, and the order of addition of NF-I and T antigen. Under no circumstances was an effect of NF-I or its recognition site observed. These studies led us to the conclusion that stimulation of DNA replication by NF-I may require additional factors present in vivo, but not in vitro.

Development of a Cell-Free System for Studying the Replication of Chromatin Templates In Vitro

One major difference between the in vitro and in vivo replication reactions is the nature of the DNA templates. In vivo, the template is organized into a chromatin structure referred to as the SV40 minichromosome. In contrast, little if any chromatin assembly occurs in vitro under the usual assay conditions. To study the requirements for replication of chromatin templates in vitro, we developed a two-stage chromatin assembly/DNA replication system. In this system the DNA template is initially incubated with a chromatin assembly system derived from Xenopus oocytes. Fol-

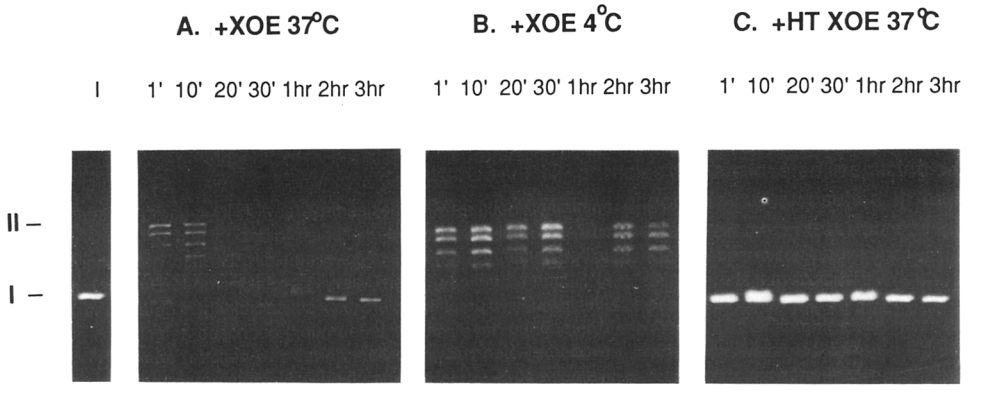


Figure 4. Topological Status of DNA after Chromatin Assembly

Standard assembly reactions containing plasmid pUC.BK-NFI and Xenopus oocyte extract (XOE) were incubated for the indicated times and then deproteinized. After degradation of RNA with RNAase A, the DNA was analyzed by electrophoresis in a 1% agarose gel. (A) Assembly reaction incubated at 37°C. (B) Assembly reaction incubated at 4°C. (C) Assembly reaction incubated at 37°C with heat-treated (65°C for 5 min) oocyte extract.

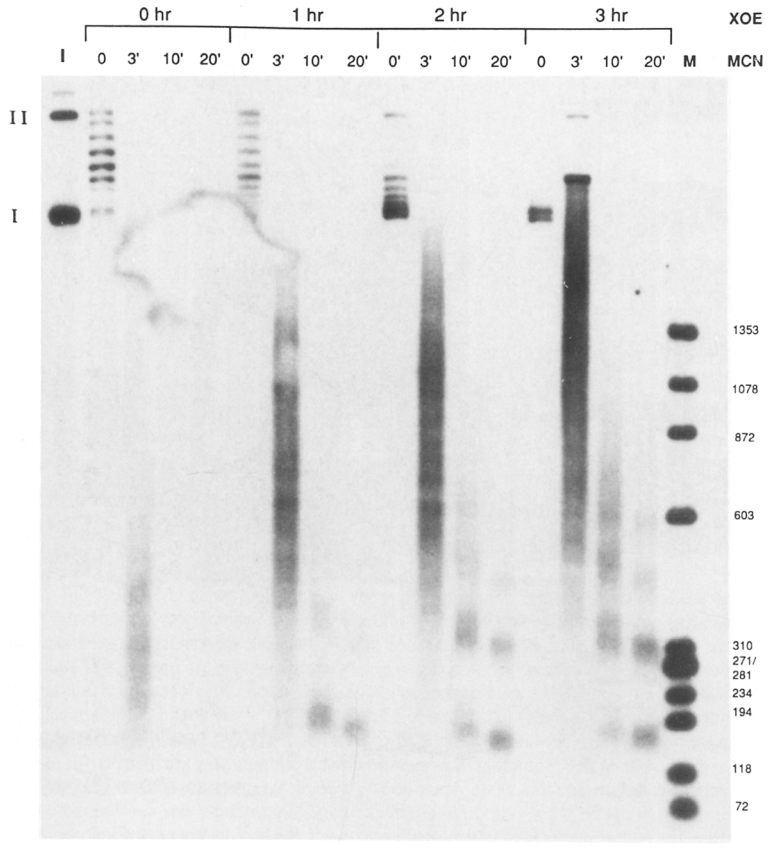


Figure 5. Analysis of Assembled Chromatin by Micrococcal Nuclease Digestion

Plasmid pUC.BK-NFI was incubated with Xenopus oocyte extract (XOE) for 0, 1, 2, and 3 hr at 37°C and then digested with 10 U of micrococcal nuclease (MCN) at 23°C for the indicated times. After removal of proteins, the resulting DNA was subjected to electrophoresis on a 1.8% agarose gel, transferred to a nylon membrane, and visualized by hybridization to ^{32}P-labeled input DNA. The status of DNA before (0′) and after (3′, 10′, 20′) nuclease digestion is shown. The marker (M) DNA fragments were obtained by digesting φX174 DNA with HaeIII, followed by labeling with [γ-^{32}P] ATP at the 5′ ends using T4 polynucleotide kinase.

lowing chromatin assembly, the proteins and cofactors required for SV40 DNA replication are added, and DNA synthesis is allowed to proceed for a fixed period of time.

The kinetics of chromatin assembly during the first stage of the reaction were analyzed by gel electrophoresis and nuclease protection experiments. Plasmid pUC.BK-NFI, containing both the minimal SV40 origin and the NF-I recognition sequence, was incubated with the Xenopus oocyte extract for various periods of time and subjected to gel electrophoresis following deproteinization (Figure 4A). The supercoiled input plasmid (I) was rapidly relaxed during the first few minutes of the incubation and then progressively resupercoiled in a slow process lasting 1 to 3 hr. This two-phase reaction has been observed previously both in vivo, following injection of supercoiled DNA into Xenopus oocytes, and in vitro, following incubation of supercoiled DNA with oocyte extracts as in the present case (Laskey et al., 1977; Glikin et al., 1984; Ryoji and Worcel, 1984).

The rapid early phase of the reaction has been attributed to the action of a topoisomerase activity in Xenopus oocytes. The slow phase has been correlated with the assembly of the DNA into nucleosomes and the removal of the resulting superhelical tension by the endogenous topoisomerase. After deproteinization the number of negative supercoils reflects the extent of chromatin assembly. As previously reported, chromatin asssembly did not occur at 4°C, although the DNA was efficiently relaxed under these conditions (Figure 4B). If the oocyte extract was heated to 65°C prior to the incubation, the DNA remained fully supercoiled (Figure 4C). Adding purified topoisomerase I to such reaction mixtures resulted in DNA relaxation, but chromatin assembly still did not occur (data not shown), indicating that other heat-labile proteins are required.

Assembly of the template into chromatin was also assayed by micrococcal nuclease digestion (Figure 5). After incubation in the assembly reaction mixture for various periods of time, samples were adjusted to 3 mM $CaCl_2$ and digested with micrococcal nuclease for 0, 3, 10, or 20 min. The products of digestion were then fractionated by electrophoresis. As before, the supercoiled input DNA was initially relaxed and then slowly resupercoiled. After 3 hr of assembly, essentially all of the DNA was in a highly supercoiled form. We were able to resolve 13 distinct topoisomers between this supercoiled form and the most relaxed form observed at early times during assembly. Since the average change in the linking number corresponding to the assembly of a single nucleosome is approximately one, and the size of the template was 2772 bp, the nucleosomes in the final product were spaced approximately 200 bp apart on average, as reported previously.

A similar picture emerged from analysis of the products of micrococcal nuclease digestion of the assembled chromatin. At time 0 of assembly the DNA was very sensitive to nuclease digestion and yielded only small oligonucleotides of random sizes. After 1 hr of assembly a major limit

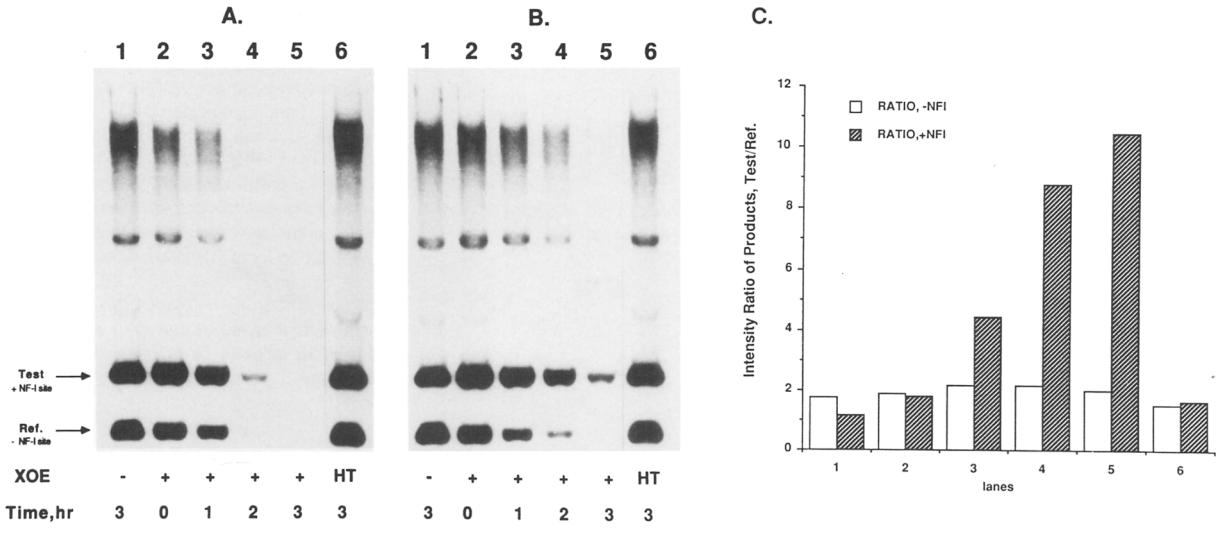


Figure 6. In Vitro Replication of Minichromosomes Assembled in the Presence or Absence of NF-I

(A) Replication of templates assembled into chromatin in the absence of NF-I protein. Equimolar quantities of the test plasmid pUC.BK-NFI (SV40 origin plus NF-I site) and the reference plasmid pKP.HNO (SV40 origin alone) were first incubated in the chromatin assembly system for 0, 1, 2, or 3 hr as indicated. The resulting templates were then tested for their ability to support DNA replication in vitro. All replication reactions were allowed to proceed for 1 hr. The products of DNA replication were deproteinized and subjected to 1% agarose gel electrophoresis in the presence of ethidium bromide. Under these conditions the various topoisomers of test and reference DNAs were compressed into single bands. The locations of unit-length circular molecules of the test and reference templates are indicated by arrows on the left. In the reaction of lane 1, the extraction buffer substituted for the Xenopus oocyte extract. In the reaction of lane 6 heat-treated oocyte extract (65°C for 5 min) was used.

(B) DNA replication of templates assembled into chromatin in the presence of NF-I protein. The experimental procedure was identical to (A), except that purified NF-I protein was added to assembly reaction mixtures before addition of Xenopus oocyte extract.

(C) Quantification of relative replication efficiencies. The incorporation of [^{32}P]dCMP into unit-length circular DNA products was determined by assaying bands excised from dried gels (A) and (B) above. The data are plotted as the ratio of the incorporation into the test plasmid to the incorporation into the reference DNA plasmid.

product of about 150 bp was observed, together with a smaller amount of a product about twice this size. By 3 hr a ladder containing at least six discrete species spaced 150–200 bp apart was observed, consistent with the hypothesis that the assembled chromatin consisted of closely spaced nucleosomes. The addition of NF-I to the assembly reaction mixture had no detectable effect on the pattern of products generated by micrococcal nuclease digestion (data not shown), indicating that the factor does not have a global effect on the chromatin assembly process.

Assembly of the Template into Chromatin Reduces the Efficiency of DNA Replication In Vitro

Figure 6A shows the effect of the chromatin assembly step on the efficiency of the subsequent DNA replication reaction. Two of the templates previously analyzed in vivo were used in this experiment. Both templates contained the SV40 minimal origin, but one (pUC.BK-NFI) also contained the synthetic NF-I recognition site adjacent to the origin, while the other (pKP.HNO) did not and served as an internal reference. The two templates were incubated with the chromatin assembly system for various periods of time and then assayed for their ability to support SV40 origin–dependent DNA replication under standard in vitro replication conditions.

We found that preincubation with the chromatin assembly system significantly reduced the efficiency of replication of both DNA templates. Moreover, the extent of the reduction in replication efficiency was strongly dependent upon the length of the preincubation. Preincubation for 0 hr had no detectable effect on replication efficiency (Figure 6A, lane 2 vs. lane 1). However, preincubation for 2–3 hr almost completely abolished DNA replication (lanes 4–5). Both templates were affected equally, since the molar ratio of the two newly synthesized products was independent of preincubation time and was the same as the molar ratio of the input DNA templates (approximately one).

Several lines of evidence indicated that the observed reduction in DNA replication efficiency following preincubation with Xenopus oocyte extract was due to assembly of the template into chromatin. First, the time of preincubation required to abolish DNA replication was the same as the time required to complete chromatin assembly (see above). Second, the inhibitory effect of preincubation was *cis*-acting, since naked DNA added to the DNA replication reaction mixture after 3 hr of assembly was replicated normally (data not shown). Third, heated Xenopus extract, which is deficient in chromatin assembly, was also ineffective in reducing replication efficiency (Figure 6A, lane 6). Finally, when the preincubation was carried out at 4°C rather than 37°C the replication efficiency was not affected, consistent with the observation that the chromatin assembly reaction requires an elevated temperature (see above). We also verified that the DNA synthesis observed

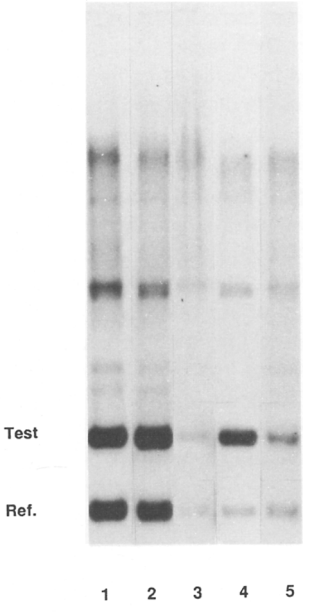


Figure 7. Requirement for NF-I during Chromatin Assembly

The test and reference plasmids were incubated in the chromatin assembly system for 2 hr in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of NF-I. The times of chromatin assembly were 0 hr (lanes 1 and 2) and 2 hr (lanes 3–5). The resulting templates were then tested for their ability to support SV40 DNA replication in vitro as described in the legend of Figure 6. In the reaction of lane 5, NF-I protein was added to the reaction after chromatin assembly, but before DNA replication was initiated.

in the chromatin replication system was dependent upon T antigen and the viral origin of DNA replication, indicating that the presence of Xenopus oocyte extract does not alter the specificity of the reaction.

NF-I Stimulates the Replication of Chromatin Templates Containing the NF-I Recognition Site

When the chromatin assembly reaction was carried out in the presence of purified NF-I, dramatically different results were obtained (Figure 6B). After 1–3 hr of assembly the template containing the NF-I recognition site replicated with significantly greater efficiency than the reference template lacking the NF-I site. In control experiments the presence of NF-I had no effect on the replication efficiency of either template when the preincubation was carried out with heat-treated Xenopus extract or with extraction buffer alone (Figure 6B). Similarly, NF-I had no effect when the preincubations were performed at 4°C (data not shown).

To quantify the relative replication activities of the two templates in the presence and absence of NF-I, the appropriate bands were excised from the gel and assayed for radioactivity (Figure 6C). For each reaction the ratio of the incorporation of [^{32}P]dCMP into the two templates was determined. In the standard replication reaction lacking both NF-I and the Xenopus assembly system (lane 1), the incorporation ratio was about 1.5:1, consistent with the fact that the template containing the NF-I site is 50% larger than the template lacking the NF-I site. Approximately the same incorporation ratio was observed when chromatin assembly was carried out in the absence of NF-I. However, in the presence of NF-I the incorporation ratio increased from 1.5:1 after 0 hr of preincubation to 10.5:1 after 3 hr of preincubation. These results indicate that the binding of NF-I can greatly reduce the inhibitory effect of chromatin assembly on DNA replication.

Figure 7 shows the effect of altering the time of addition of NF-I on the efficiency of replication of chromatin templates. As before, when purified NF-I was added prior to the chromatin assembly reaction, the template containing the NF-I site replicated much more efficiently than the template lacking the NF-I site (lane 4). In contrast, when NF-I was added after assembly the difference in replication efficiency of the two templates was significantly smaller (lane 5 vs. lane 4). Thus, for maximal effect the NF-I protein must be present throughout the assembly reaction. This finding is consistent with the hypothesis that the binding of NF-I increases replication efficiency by perturbing the assembly process. Since NF-I does not appear to have global effects on chromatin organization (see above), it seems likely that the protein alters chromatin structure in the neigborhood of its binding site near the origin.

Discussion

In eukaryotic cells the natural template for DNA replication is a nucleoprotein complex, called chromatin, that contains histones and other DNA binding proteins. As an approach to understanding the requirements for replication of chromatin templates, we have developed a model system in which plasmids containing the SV40 origin of DNA replication are assembled into chromatin and then used as templates for DNA replication in vitro. We have made use of this system to analyze the mechanism of activation of DNA replication by enhancers and other transcriptional elements.

To simplify the analysis we constructed a plasmid template in which the normal SV40 transcriptional elements were replaced by a single binding site for the transcriptional activator protein, NF-I. Our data indicate that assembly of the template into chromatin strongly represses DNA replication. However, if NF-I is present during the assembly process, chromatin-mediated repression is significantly reduced.

This effect is not observed with templates that lack the NF-I recognition site, indicating that relief of repression requires specific NF-I binding. Moreover, NF-I is unable to reverse the repression of DNA replication if added after the template has been assembled into chromatin, suggesting that the binding of NF-I and histones are mutually exclusive. Finally, in the absence of chromatin assembly NF-I has no detectable effect on the efficiency of DNA replication in vitro whether or not the template contains an NF-I recognition site.

To explain these observations we propose, first, that the binding of a regulatory protein to a site on the DNA precludes the packaging of that site into a nucleosome,

and second, that the resulting boundary constraint perturbs the local distribution of nucleosomes, so that nearby segments of DNA have a significantly reduced probability of being packaged into chromatin. Thus, the adjacent DNA, in this case the SV40 origin of DNA replication, is rendered more accessible to initiation factors.

In its simplest form this model suggests that transcription factors activate SV40 DNA replication by an indirect mechanism involving changes in chromatin structure, rather than a more direct mechanism involving interaction with the replication machinery. Although the latter alternative cannot be completely ruled out, it seems quite unlikely in the present case since NF-I has no effect on the extent of replication of naked DNA templates containing the SV40 origin. It is also clear that the stimulation of DNA replication by NF-I is not a result of increased transcription through the origin region, since the degree of stimulation is unaffected by the RNA polymerase inhibitor α-amanitin at a concentration of 100 μg/ml (data not shown).

The model that we propose makes a number of specific predictions that can now be tested in the chromatin replication system. For example, we expect that increasing the distance between the NF-I binding site and the origin by a few hundred base pairs will dramatically reduce the stimulatory effect of the protein on DNA replication. We also expect that a variety of site-specific DNA binding proteins (e.g., Sp1, Ap-1, etc.) may be capable of stimulating SV40 DNA replication when their binding sites are placed next to the minimal origin. Finally, it may be possible to demonstrate by direct analysis that the binding of NF-I modifies the spatial distribution of nucleosomes in the vicinity of the replication origin.

Theoretical analyses suggest that a simple stochastic model for nucleosome assembly is sufficient to account for the magnitude of the effects that we have observed. We have carried out computer simulations of the chromatin assembly process and have also applied the recent analytic treatment of Kornberg and Stryer (1988). In both approaches it was assumed that nucleosomes are formed at random on DNA, that nucleosomes occupy about 160 bp of DNA and do not overlap with one another, and that a DNA segment associated with a regulatory protein (e.g., NF-I) cannot be packaged into a nucleosome.

The calculations reveal that the presence of a regulatory protein significantly perturbs the average local distribution of nucleosomes and that the perturbation is propagated over several hundred base pairs. As one moves away from the boundary imposed by the protein, the probability that a given segment of DNA is packaged into a nucleosome fluctuates in a periodic fashion and gradually approaches the probability for bulk chromatin. Assuming that the average density of nucleosomes is similar to that in HeLa cell chromatin, the presence of a single sequence-specific DNA binding protein can increase the probability that the adjacent 100 bp are not packaged into a nucleosome by a factor of about 10. This value is consistent with the observed stimulatory effect of NF-I on SV40 DNA replication in vitro.

Previous in vivo studies have provided considerable support for the concept that transcriptional elements can induce a nucleosome-free region in the vicinity of the SV40 origin of DNA replication. Such a region was originally detected by virtue of its hypersensitivity to various endonucleases (Scott and Wigmore, 1978; Varshavsky et al., 1978) and was subsequently observed directly in the electron microscope (Saragosti et al, 1980). The nucleosome-free region includes the minimal origin of DNA replication, the GC-rich repeats (Sp1 binding sites), and the SV40 enhancers. It is present in about 25% of SV40 minichromosomes isolated from infected cells (Saragosti et al., 1980; Sogo et al., 1986).

Several studies indicate that the genetic determinants of the nucleosome-free region lie outside of the minimal origin in the adjacent transcriptional elements (Fromm and Berg, 1983; Jongstra et al., 1984; Gerard et al., 1985; Innis and Scott, 1984). Analysis of mutants in which the transcriptional elements are translocated to other sites in the SV40 genome suggests that the GC-rich repeats and the SV40 enhancers are each independently capable of inducing a nuclease-hypersensitive site. This finding is of interest since we and others have demonstrated that either of these two genetic elements can activate SV40 DNA replication in vivo when located adjacent to the minimal origin.

When the transcriptional elements are moved more than 90 bp from the minimal origin, the efficiency of SV40 DNA replication is reduced at least 10-fold, coincident with a reduction in the accessibility of the origin to various endonucleases (Innis and Scott, 1984). While these data do not definitively prove that the establishment of an altered chromatin structure is a prerequisite for efficient initiation of SV40 DNA replication at the origin, they are certainly consistent with such a model.

We have carried out several experiments to determine whether the requirement for NF-I in the chromatin replication system can be bypassed by preincubation of the DNA template with the viral initiator protein, T antigen. These experiments were inconclusive for technical reasons. When T antigen was present during the assembly incubation, the templates were inactivated for subsequent DNA replication. The inactivation was not dependent upon the presence of the Xenopus oocyte extract and could not be reversed by adding fresh T antigen. Although we do not yet completely understand this phenomenon, one possible explanation is that prolonged incubation leads to the formation of nonproductive T antigen–DNA complexes.

We have also observed that the ability of NF-I to prevent repression of DNA replication is reduced upon prolonged incubation of the template in the chromatin assembly system (see Figure 6). This may indicate that under the conditions of the in vitro experiments nucleosomes can eventually displace NF-I. In vivo, we expect that the outcome of the competition between regulatory factors and nucleosomes will be determined by the complex interplay of several elements, including the number of recognition sites for the factor(s), the effective intracellular concentrations of the factor(s) and histones, and the relative affinities of factor(s) and histones for the DNA.

Although this report provides evidence that the binding of regulatory factors can prevent chromatin-mediated re-

pression of DNA replication, similar effects have been previously described for transcription. For example, assembly of the Xenopus 5S ribosomal RNA gene into chromatin represses transcription by RNA polymerase III in vitro (Bogenhagen et al., 1982; Gottesfeld and Bloomer, 1982). The observed repression can be prevented by preincubation of the template with the sequence-specific DNA binding protein TFIIIA and other transcriptional factors.

Similarly, transcription of the adenovirus major late promoter in vitro is strongly inhibited by chromatin assembly, but the inhibition can be largely prevented by preincubation with TFIID, the TATA box binding factor (Workman and Roeder, 1987). In both these cases templates that have been completely assembled into chromatin are refractory to activation by addition of the appropriate factors.

These data have been interpreted as indicating that the formation of a transcription complex and the formation of a nucleosome at the same site are mutually exclusive events and that both kinds of nucleoprotein structures are relatively stable. Recently, Workman et al. (1988) have reported that the immediate-early protein (IE) of pseudorabies virus activates transcription from the major late promoter by potentiating the binding of TFIID to the TATA box during chromatin assembly. The IE protein itself is not required for transcription per se and has no effect on the efficiency of transcription in the absence of histones. These observations are similar to those reported here, in that IE, like NF-I, may function mainly to enhance the ability of initiation factors to compete effectively with histones for binding to DNA. However, future work is required to determine whether IE and NF-I act by similar mechanisms.

In vitro studies of eukaryotic promoters have also provided evidence that the binding of specific regulatory proteins can increase the accessibility of the adjacent DNA as assayed by nuclease sensitivity. When chromatin is assembled onto the adult β-globin gene in the presence of partially purified protein factors from chicken erythrocytes, the 5′-flanking region displays a hypersensitive site resembling that observed within the nucleus. No such site is observed in the absence of the factors or if the factors are added after nucleosome assembly (Emerson et al., 1985). Thus, it is possible that gene activation by modulation of chromatin organization may be a regulatory mechanism common to both transcription and DNA replication in mammalian cells.

While the nucleosome exclusion mechanism described above most likely represents the major mechanism for activation of SV40 DNA replication by transcription factors, different mechanisms of activation may be operative in other viral systems. For example, initiation of adenovirus DNA replication is highly dependent upon the binding of NF-I to a recognition site adjacent to the minimal origin. However, in this case the activation of DNA synthesis by NF-I (at least 50-fold) can be observed in a purified cell-free system that lacks histones (Rosenfeld et al., 1987). Although the precise mechanism of action of NF-I in the adenovirus system is not yet clear, it seems likely that the protein functions to stabilize a specific initiation complex by direct protein–protein interactions with other replication proteins. Thus, the same regulatory protein may be able to activate DNA replication by both direct and indirect mechanisms.

It seems likely that the requirement for an open chromatin structure in the vicinity of replication origins is not unique to viral chromosomes, but may extend to cellular chromosomes as well. The chromosomal copy of the yeast origin ARS1 has recently been shown to contain a nucleosome-free region that includes most of the sequences thought to be important for origin function (Thomas and Simpson, 1985; Thomas, 1986; Lohr and Torchia, 1988). Fine structure mapping has revealed the presence of several proteins bound to specific sites within this region. Such a structure could clearly facilitate access of initiation factors to the origin during the S phase of the cell cycle. In higher eukaryotes where the number of active origins can change dramatically during development (Blumenthal et al., 1973), it is possible that factors that modify the local distribution of nucleosomes may play a role in regulating the efficiency of origin utilization.

Experimental Procedures

DNA Templates

The reference plasmid pKP.HNO, containing the SV40 origin but lacking transcriptional elements, was constructed by inserting the segment of SV40 from nucleotide 5171 (HindIII site) to 37 (NcoI site) between the corresponding sites of the pBR322 derivative pKP55 (1.8 kb; Li et al., 1986). The remaining plasmids were constructed by inserting the appropriate fragments into the polylinker region of the larger vector pUC19 (2.8 kb). Plasmid pUC.HNO contains the same segment of SV40 DNA as a control template.

Plasmid pUC.HSO contains the segment of SV40 between nucleotides 5171 and 128 (SV40 origin plus GC-rich 21 bp repeats). Plasmid pUC.XS3 contains the SV40 segment between nucleotides 5171 and 294 with a deletion of nucleotides 35 to 108 (SV40 origin plus SV40 enhancers). Plasmid pUC.BKE3, containing the SV40 origin and the BKV enhancers, was made by inserting the 260 bp HaeIII fragment of BKV (Dunlop strain, nucleotides 3453 to 3712) into the NcoI site of pUC.HNO (after filling in the termini with DNA polymerase).

Plasmid pUC.BKE1, containing the SV40 origin and one copy of the BKV enhancer repeat, was constructed by excising the central NcoI fragment of the BKV enhancer (nucleotides 3475 to 3593) from plasmid pUC.BKE3. Plasmid pUC.BK-NFI, containing the SV40 origin and a 22 bp synthetic NF-I binding site, was constructed by inserting the oligonucleotide 5′-CATGGAATGCAGCCAAACCATG-3′ between the NcoI and SacI sites in the polylinker of pUC.HNO. The double-base substitution mutant pUC.mt NFI was made in the same way using the oligonucleotide 5′-CATGGAATGCAGGGAAACCATG-3′.

All the plasmids were propagated as monomers in a Dam^+ E. coli strain, DH5α (BRL), and purified by a standard alkali lysis procedure, phenol and chloroform extraction, and banding twice by CsCl equilibrium gradients.

Tissue Culture Cells

Three SV40 T antigen–producing cells were used: the monkey kidney cell line COS-1 (Gluzman, 1981), the human fetal glial cell line SV-G, and the human embryonic kidney cell line SV-1 (Major and Matsumura, 1984; Major et al., 1985). The latter two cell lines were transformed by the SV40 T antigen by the same method used to generate COS-1 cells. COS-1 cells were grown in DMEM (Gibco) plus 10% fetal bovine serum, whereas MEM (Gibco) plus 20% fetal bovine serum was used for the two human cells.

Assay of Replication In Vivo

Monolayer cells ($2–5 \times 10^5$) in 6 cm dishes were transfected by the DEAE–dextran method, with a mixture of DNA containing an experimental plasmid as well as the internal reference plasmid pKP.HNO. At 40–48 hr after transfection, low molecular weight DNA was isolated

by the Hirt (1967) procedure, and replication was quantified as described (Li et al., 1986). The isolated DNA was digested with DpnI to remove unreplicated input DNA, and with EcoRI to linearize both test and reference plasmids. The digested products were fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with labeled (Feinberg and Vogelstein, 1983) SV40 minimal origin fragment. After autoradiography, the DpnI-resistant bands were cut out and counted in a liquid scintillation spectrometer to calculate the ratio of the two templates.

Preparation of Extracts

Oocyte extracts, capable of in vitro nucleosome assembly, were prepared from Xenopus laevis (large size; Nasco, Fort Atkinson, WI) by the method of Laskey et al. (1977). Ovaries were washed twice in buffer OR2 (825 mM NaCl, 25 mM KCl, 10 mM Na_2HPO_4, 50 mM HEPES, 38 mM NaOH, 1 mM $CaCl_2$, 1 mM $MgCl_2$ [pH 7.8]), and then teased into pieces. The pieces were washed in the same buffer and digested with 0.5% collagenase (type 1A; Sigma). Dispersed oocytes were washed extensively, decanting away small oocytes and somatic tissue. The remaining oocytes (primarily stage 6) were homogenized, and extracts were prepared as described (Laskey et al., 1977; Glikin et al., 1984).

Cytoplasmic extracts required for supporting SV40 replication in vitro were prepared from HeLa (S3) cells as described previously (Wold et al., 1989).

Purification of NF-I and T Antigen

NF-I was purified from nuclear extract of HeLa cells and stored according to the published protocol by Rosenfeld and Kelly (1986). The activity of the preparation was monitored by its ability to bind the DNA fragment containing the adenovirus replication origin. The purified NF-I protein was stored in the standard buffer (25 mM HEPES [pH 7.5], 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.01% NP40, 20% glycerol, 120 mM NaCl) and was active in supporting adenovirus in vitro replication.

SV40 T antigen was purified from HeLa cells infected with the recombinant adenovirus vector R284 by immunoaffinity chromatography as described previously (Wold et al., 1989). Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Nucleosome Assembly

The standard assembly reaction contained 25 ng of plasmid DNA and 4 μl of Xenopus oocyte extract in a 12 μl reaction mixture. Reactions were incubated at 37°C for 0 to 3 hr. Control reactions were performed by adding oocyte extraction buffer instead of extract proteins, or by incubating with oocyte extracts on ice. Nucleosome assembly was monitored by 1% gel electrophoresis to determine the degree of negative supercoiling after deproteinization and RNA degradation. Nucleosome assembly was also monitored by digesting with micrococcal nuclease (10 U; Boehringer Mannheim Biochemical) at 23°C in the presence of 3 mM $CaCl_2$. After removal of proteins, the resulting DNA was subjected to 1.8% agarose gel electrophoresis, transferred and cross-linked to a nylon membrane (Zetaprobe; Bio-Rad) in the presence of 0.4 M NaOH, and visualized by hybridization to ^{32}P-labeled input DNA.

Chromatin Replication In Vitro

The conditions for replication reactions were as described previously (Wold et al., 1989). The standard 25 μl reaction mixtures contained 30 mM HEPES (pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 40 mM creatine phosphate, 100 μg/ml creatine kinase, 100 μM each of dATP, dGTP, dTTP, 25 μM dCTP with 2.5 μCi (92.5 kBq) [α-^{32}P]dCTP, 75 μg of HeLa cytosol, and 0.20 μg of SV40 T antigen. When DNA replication was coupled to nucleosome assembly, the reaction was modified as following. First, 15 ng of test and 10 ng of reference DNA (about eqimolar quantities) were incubated with NF-I protein (145 ng) or its buffer in the standard NF-I binding buffer (25 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 150 mM NaCl) plus 5 mM ATP in an 8 μl solution for 20 min at room temperature. Second, 4 μl of oocyte extract was added, and nucleosome assembly was allowed to proceed at 37°C for up to 3 hr. Third, the other components required for replication were added into 12 μl of assembly mixture to make up 25 μl of standard reaction as described above.

One hour after initiation, replication reactions were terminated by adding 100 μl of stop solution containing 0.3% SDS and 20 mM EDTA. Proteins were degraded by incubation with 250 μg/ml proteinase K at 37°C for 1 hr. The replication products were collected by precipitation and analyzed in 1% agarose gel in the presence of 0.5 mg/ml ethidium bromide. Gels were dried and exposed to Kodak XAR-5 film with an intensifying screen at −80°C.

Acknowledgments

We would like to thank Dr. Bret Jesse and Hui Zhang for help in preparing Xenopus oocyte extract and Dr. Edward O'Neil for the generous supplement of purified NF-I protein. We are grateful to Drs. David Weinberg, Joachim Li, Marc Wold, and other colleagues in the laboratory for fruitful discussion. This work was supported by grant CA40414-03 from the National Institutes of Health.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 11, 1989; revised September 21, 1989.

References

Bergsma, D. J., Olive, D. M., Hartzell, S. W., and Subramanian, K. N. (1982). Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. USA *79*, 381–385.

Blumenthal, A. B., Krienstein, H. J., and Hogness, D. S. (1973). The units of DNA replication in D. melanogaster chromosomes. Cold Spring Harbor Symp. Quant. Biol. *38*, 205–223.

Bogenhagen, D. F., Wormington, W. M., and Brown, D. D. (1982). Stable transcription complexes of Xenopus 5S RNA genes: a means to maintain the differentiated state. Cell *28*, 413–421.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

Campbell, J. L. (1986). Eukaryotic DNA replication. Annu. Rev. Biochem. *55*, 733–771.

Challberg, M. D., and Kelly, T. J. (1989). Animal virus DNA replication. Annu. Rev. Biochem. *58*, 671–717.

DeLucia, A. L., Deb, S., Partin, K., and Tegtmeyer, P. (1986). Functional interactions of the simian virus 40 core origin of the replication with flanking regulatory sequences. J. Virol. *57*, 138–144.

Del Vecchio, A. M., Steinman, R. A., and Ricciardi, R. P. (1989). An element of the BK virus enhancer required for DNA replication. J. Virol. *63*, 1514–1524.

DePamphilis, M. L. (1988). Transcriptional elements as components of eukaryotic origins of DNA replication. Cell *52*, 635–638.

de Villiers, J., Schaffner, W., Tyndall, C., Lupton, S., and Kamen, R. (1984). Polyoma virus DNA replication requires an enhancer. Nature *312*, 242–246.

Deyerle, K. L., and Subramari, S. (1988). Linker scan analysis of the early regulatory region of human papovavirus BK. J. Virol. *62*, 3378–3387.

Deyerle, K. L., Sajjadi, F. G., and Subramari, S. (1989). Analysis of origin of DNA replication of human papovavirus BK. J. Virol. *63*, 356–365.

Emerson, B. M., Lewis, C. D., and Felsenfeld, G. (1985). Interaction of specific nuclear factors with the nuclease-hypersensitive region of the chicken adult β-globin gene: nature of the binding domain. Cell *41*, 21–30.

Feinberg, A. F., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6–13.

Fromm, M., and Berg, P. (1983). Simian virus 40 early and late-region promoter functions are enhanced by the 72-base-pair repeat inserted at distant locations and inverted orientations. Mol. Cell. Biol. *3*, 991–999.

Gerard, R. D., Montelone, B. A., Walter, C. F., Innis, J. W., and Scott,

W. A. (1985). Role of specific simian virus 40 sequences in nuclease-sensitive structure in viral chromatin. Mol. Cell. Biol. *5*, 52–58.

Glikin, G. C., Ruberti, I., and Worcel, A. (1984). Chromatin assembly in Xenopus oocytes: in vitro studies. Cell *37*, 33–41.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell *23*, 175–182.

Gottesfeld, J., and Bloomer, L. S. (1982). Assembly of transcriptionally active 5S RNA gene chromatin in vitro. Cell *28*, 781–791.

Grinnell, B. W., Berg, D. T., and Walls, J. D. (1986). Activation of the adenovirus and BK virus later promoters: effects of the BK virus enhancer and trans-acting viral early proteins. Mol. Cell. Biol. *6*, 3596–3605.

Hertz, G. Z., and Mertz, J. E. (1986). Bidirectional promoter elements of simian virus 40 are required for efficient replication of viral DNA. Mol. Cell. Biol. *6*, 3513–3520.

Hirt, B. (1967). Seletive extraction of polyoma DNA from infected mouse cultures. J. Mol. Biol. *26*, 365–369.

Howley, P. M. (1980). Molecular biology of SV40 and the human polyomaviruses BK and JC. In Viral Oncology, G. Klein, ed. (New York: Raven Press), pp. 489–550.

Innis, J. W., and Scott, W. A. (1984). DNA replication and chromatin structure of simian virus 40 insertion mutants. Mol. Cell. Biol. *4*, 1499–1507.

Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., and Tjian, R. (1987). A cellular DNA-binding protein that activates eukaryotic transcription and replication. Cell *48*, 79–89.

Jongstra, J., Reudelhuber, T. L., Oudet, P., Benoist, C., Chae, C.-B., Jeltsch, J.-M., and Chambon, P. (1984). Induction of altered chromatin structures and simian virus enhancer and promoter elements. Nature *307*, 708–714.

Kelly, T. J. (1988). SV40 DNA replication. J. Biol. Chem. *263*, 17889–17892.

Kornberg, R. D., and Stryer, L. (1988). Statistical distributions of nucleosomes: nonrandom locations by a stochastic mechanism. Nucl. Acids Res. *16*, 6677–6690.

Laskey, R. A., Mills, A. D., and Morris, N. R. (1977). Assembly of SV40 DNA chromatin in a cell-free system from Xenopus eggs. Cell *10*, 237–243.

Lee-Chen, G. -J., and Woodworth-Gutai, M. (1986). Simian virus 40 DNA replication: functional organization of regulatory elements. Mol. Cell. Biol. *6*, 3086–3093.

Li, J. J., and Kelly, T. J. (1984). Simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA *81*, 6973–6977.

Li, J. J., Peden, K. W. C., Dixon, R. A. F., and Kelly, T. J. (1986). Functional organization of the simian virus 40 origin of DNA replication. Mol. Cell. Biol. *6*, 1117–1128.

Lohr, D., and Torchia, T. (1988). Structure of the chromosomal copy of yeast ARS1. Biochemistry *27*, 3961–3965.

Lusky, M., and Botchan, M. R. (1986). Transient replication of bovine papilloma virus type 1 plasmids: *cis* and *trans* requirements. Proc. Natl. Acad. Sci. USA *83*, 3609–3613.

Major, E. O., and Matsumura, P. (1984). Human embryonic kidney cells: stable transformation with an origin-defective simian virus 40 DNA and use as hosts for human papovavirus replication. Mol. Cell. Biol. *4*, 379–382.

Major, E. O., Miller, A. E., Mourrain, P., Traub, R. G., de Wilt, E., and Sever, J. (1985). Establishment of a line of human fetal glial cells that supports JC virus multiplication. Proc. Natl. Acad. Sci. USA *82*, 1257–1261.

Nagata, K., Guggenheimer, R. A., and Hurwitz, J. (1983). Specific binding of a cellular DNA binding protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA *80*, 6177–6181.

Nowock, J., Borgmeyer, U., Puschel, A. W., Rupp, R. A. W., and Sipple, A. E. (1985). The TGGCA-binding protein binds to the MMTV-LTR, the adenovirus origin of replication, and the BK virus enhancer. Nucl. Acids Res. *13*, 2045–2061.

Rawlins, D. R., Rosenfeld, P. J., Wides, R. J., Challberg, M. D., and Kelly, T. J., Jr. (1984). Structure and function of the adenovirus origin of replication. Cell *37*, 309–319.

Reisman, D., Yates, J., and Sugden, B. (1985). A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting elements. Mol. Cell. Biol. *5*, 1822–1832.

Rosenfeld, P. J., and Kelly, T. J. (1986). Purification of nuclear factor I by DNA recognition affinity chromatography. J. Biol. Chem. *261*, 1398–1408.

Rosenfeld, P. J., O'Neill, E. A., Wides, R. J., and Kelly, T. J. (1987). Sequence-specific interactions between cellular DNA-binding proteins and adenovirus origin of DNA replication. Mol. Cell. Biol. *7*, 875–886.

Rosenthal, N., Kress, M., and Khoury, G. (1983). BK viral enhancer element and a human cellular homolog. Science *222*, 749–755.

Ryoji, M., and Worcel, A. (1984). Chromatin assembly in Xenopus oocytes: in vivo studies. Cell *37*, 21–32.

Santoro, C., Nermod, N., Andrews, P. C., and Tjian, R. (1988). A family of human CCAAT-box-binding proteins active in transcription and replication: cloning and expression of multiple cDNAs. Nature *334*, 218–224.

Saragosti, S., Moyne, G., and Yaniv, M. (1980). Absence of nucleosomes in a fraction of SV40 chromatin between the origin of replication and the region coding for the late leader RNA. Cell *20*, 65–73.

Scott, W. A., and Wigmore, D. J. (1978). Sites in simian virus 40 chromatin which are preferentially cleaved by the endonucleases. Cell *15*, 1511–1518.

Sogo, J. M., Stahl, H., Koller, T., and Knippers, R. (1986). Structure of replicating simian virus 40 minichromosomes. J. Mol. Biol. *189*, 189–204.

Stillman, B., Gerard, R. D., Guggenheimer, R. A., and Gluzman, Y. (1985). T antigen and template requirements for SV40 replication in vitro. EMBO J. *4*, 2933–2939.

Thomas, F. (1986). Protein–DNA interactions and nuclease-sensitive regions on yeast plasmid chromatin. J. Mol. Biol. *190*, 177–190.

Thomas, F., and Simpson, R. T. (1985). Local protein–DNA interactions may determine nucleosome positions on yeast plasmids. Nature *315*, 250–252.

Varshavsky, A. J., Sundin, O. H., and Bohm, M. J. (1978). SV40 viral minichromosome: preferential exposure of the origin of replication as probed by restriction endonucleases. Nucl. Acids Res. *5*, 3469–3477.

Wold, M. S., Weinberg, D. H., Virshup, D. M., Li, J. J., and Kelly, T. J. (1989). Indentification of cellular proteins required for simian virus 40 DNA replication. J. Biol. Chem. *264*, 2801–2809.

Workman, J. L., and Roeder, R. G. (1987). Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II. Cell *51*, 613–622.

Workman, J. L., Abmayr, S. M., Cromlish, W. A., and Roeder, R. G. (1988). Transcriptional regulation by the immediate early protein of pseodorabies virus during in vitro nucleosome assembly. Cell *55*, 211–219.