DNA Replication Efficiency Depends on Transcription Factor-Binding Sites

WILLIAM J. TURNER† AND MARY E. WOODWORTH*
Department of Microbiology, Miami University, Oxford, Ohio 45056

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Naturally arising variants of simian virus 40 (SV40), generated by serial passage of the virus at high multiplicities of infection, provide important insight into the role of transcription factor-binding sites in enhancing DNA replication. Although the variants that arise from numerous recombination events are the result of selective pressure to replicate more efficiently than the other variants in the infection, there is no transcription pressure. Therefore, it is interesting that a minimum of two viral Sp1 transcription factor-binding sites are retained and that host AP-1 and NF-1 transcription factor-binding sites are incorporated into the 100-bp regulatory region that maximizes DNA replication in these variants. We cotransfected COS-1 cells (that provide viral large T antigen for DNA replication) to examine the effect of transcription factor-binding sites on the replication of plasmid constructs that contain the SV40 origin of replication (ori). The level of relative replication efficiency (RRE) depends on the number and type of transcription factor-binding sites. Replication increases as the number of transcription factor-binding sites increases within the regulatory region of the variants; AP-1 sites are more effective than NF-1 transcription factor-binding sites. Competition between constructs in transfections magnifies the difference in their RREs. The results indicate that transcription factor-binding sites play an important role in enhancing DNA replication.

Simian virus 40 (SV40) DNA is replicated by host cell machinery (reviewed in references 5 and 11) and one virally encoded protein, large T antigen (T-ag) (6, 45, 50, 56). SV40 DNA replication is bidirectional (7, 17) from a 64-bp core origin of DNA replication (ori) (4, 14, 25, 45, 52). This ori sequence consists of a 15-bp early palindrome, a 27-bp palindrome, and a 17-bp AT-rich region (8). Four pentanucleotides in the central 27-bp palindromic region of ori are binding sites for T-ag (9, 49, 57). Additional regulatory sequences within 180 bp on the late transcription side of ori include three 21-bp repeats (16) and a 72-bp transcriptional enhancer (2, 20, 23, 43). Each 21-bp repeat contains two hexameric GC boxes, which are binding sites for the transcription factor Sp1 (15) and weak binding sites for T-ag (57). The transcriptional enhancer contains a number of protein-binding sites; of these, a binding site for the transcription factor AP-1 is the closest to ori (31, 34, 63).

The involvement of transcription factor-binding sites and the way in which they are arranged within regions that regulate DNA replication are interesting for several reasons. Some arrangements of AP-1 and Sp1 transcription factor-binding sites seem to be favored in evolutionary variants (37). Evolutionary variants arise when SV40 is serially passaged at high multiplicities of infection, whereby recombination leads to new viral species containing deleted and duplicated viral DNA and substituted host DNA. In several SV40 evolutionary variants (ev1101, ev1103, ev1104, and ev1108), the incorporated host sequences that regulate replication contain AP-1 and NF-1 transcription factor-binding sites on the late-transcription side of the SV40 Sp1 sites and ori (26, 54, 62; M. Currier and M. E. Woodworth, unpublished data). The sequence context of an AP-1 transcription factor-binding site influences the effect of the AP-1 site on DNA replication efficiency (54; C. Hodson and M. E. Woodworth, unpublished data; H. Van Buskirk and M. E. Woodworth, unpublished data). Some of the past work involving the effects of Sp1 transcription factor-binding sites did not take the effects of an adjacent AP-1 transcription factor-binding site into account (37, 52). The introduction of synthetic Sp1 transcription factor-binding sites to the ori upstream regulatory region in SV40 allowed measurement of their effects on replication efficiency (35).

Furthermore, transcription factor-binding sites are functional elements of a variety of viral and eukaryotic DNA replication systems. Human papillomavirus type 18 contains a single binding site for the transcription factor Sp1 in its core origin of replication and two AP-1 sites in nearby auxiliary regions, as well as sites for several other transcription factors, both cellular and viral (10). Human papovavirus BK contains, in its enhancer region, two Sp1 sites, each adjacent to a binding site for NF-1 (13). Two sites downstream of the lytic origin of Epstein-Barr virus are protected from DNase I cleavage by Sp1 (22). The B3 regulatory element is essential for ARSI activity in yeast and is bound by the transcription factor Ablp, which enhances origin utilization (41). The autonomously replicating monkey sequence ors8 contains a binding site for the transcription factor Oct-1 (58). B48bs, a sequence that contains a human origin of DNA replication, interacts with the transcription factor USF (59). The RPS14 replication origin of Chinese hamster ovary cells contains an Sp1 site and several AP-1 sites (55). Finally, the involvement of transcription factor-binding sites in increasing the efficiency of DNA replication would lend support to reports that transcription factors play a bifunctional role.

† Present address: Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021.

* Corresponding author. Mailing address: Department of Microbiology, Miami University, 32 Pearson Hall, Oxford, OH 45056. Phone: (513) 529-2724. Fax: (513) 529-2431. E-mail: woodwome@muohio.edu.
role as enhancers of both transcription and replication (3, 11, 39, 44).

The present study investigates the effect of the spatial relationships between binding sites for transcription factors AP-1, NF-1, and Sp1 on the replication efficiency of SV40 ori and the number and combination of transcription factor-binding sites in SV40 regulatory regions and in selected host sequences. The results demonstrate that both host sequences and transcription factor-binding sites in the regulatory regions of SV40 evolutionary variants play a role in determining relative DNA replication efficiency (RRE). The binding sites for the transcription factor AP-1 in evolutionarily selected regulatory regions cooperate with adjacent transcription factor-binding sites to enhance the replication efficiency of ori. The number and type of transcription factor-binding sites, and their distance from ori, modulate their effect on the DNA replication efficiency of SV40 ori-containing plasmids. Competition between constructs also has a significant effect on RRE. The data extend the findings of Sun and Hurley (53), Han and Hurley (28), and Wilderman et al. (61) that suggest a model in which interactions of transcription factors with DNA and replication proteins such as SV40 Tag contort ori to favor initiation of DNA replication.

MATERIALS AND METHODS

Plasmids. The plasmids pOri, pC133, and pC200 (38) contain an insertion of SV40 DNA sequence at the HindIII site of pKP45, a plasmid vector derived from pBR322 by the deletion of nucleotides 675 to 2364, which are poisonous for plasmid replication in mammalian cells (30, 40, 46). The insert in pOri is a 111-bp sequence including the 64-bp SV40 core ori. The 133-bp insert of pC133 contains ori, two GC boxes, and an AP-1 site. The pC200 insert consists of 200 bp, including ori, six GC boxes, and an AP-1 site. Plasmids pOri21, pCN163, pOri2x21, pOri2x21APps, pOri2x21ApD, pOri3x2l, and pOri3GC were constructed by removing the sequences between the ClaI and NcoI restriction endonuclease sites of pC133 and replacing them with complementary oligonucleotides with ClaI and NcoI cohesive ends and containing various reiterations of the AP-1 site and the 21-bp repeat (containing the GC-hexamer-binding sites for Sp1). pC133CN75 contains a 75-bp ClaI/NcoI insert that includes three Sp1-binding sites and 42 bp of host α-satellite DNA. pC133H03 contains 71 bp of the host α-satellite DNA present in evolutionary variant e1103 and was generated by synthesizing oligonucleotides representing α-satellite DNA flanked by ClaI cohesive ends and annealed to form a double-stranded 75-bp fragment and was phosphorylated and ligated into the ClaI site of pC133. The sequence of the insert was 5′-CGATATCAGCACAGTCCATCCTTTTCCCTCAAGAGCCTTTACATGCTGGTGCTCTTGAAGCTTCGACAT-3′, with a CG dinucleotide overhang on the 5′ ends of both strands. Clones in the desired orientation were identified by restriction endonuclease digestion with TfiI and EcoRI. To generate pC133Δ, pC133 was digested with EcoRV and BamHI, deleting a 190-bp fragment of plasmid DNA. Similarly, the 190-bp EcoRV/BamHI fragment of plasmid DNA was deleted in pOriΔ, pC200Δ, pOri2x21Δ, pOri3GCΔ, and pCN163Δ.

Cell culture and transfection. The COS-1 line of African green monkey kidney cells (21) was grown in Eagle’s minimal essential medium and 5% fetal bovine serum (MEM-5) at 37°C under 5% carbon dioxide and 90% humidity. When monolayers of COS-1 cells were 50% confluent, they were cotransfected with 50 ng each of test and control plasmids in the presence of 250 μg of DEAE-dextran/ml in 106 M MEM with 50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.5) as previously described (38). After rocking the cultures every 5 min for 30 min at 37°C, the cells were washed and supplemented with medium containing 100 μM chloroquine diphosphate for 3 h. The chloroquine solution was removed, 5 ml of MEM-5 was added, and the cells were incubated at 37°C.

Analysis of RREs. Cells were lysed 48 h after transfection and Hirt (32) supernatants were prepared. After treatment with proteinase K, extraction with phenol-chloroform (1:1), and precipitation with cold isopropanol, the DNA was linearized with EcoRI and fractionated on a vertical 0.8% agarose gel (40 cm long, 1.5 mm thick) at 3 V/cm for 21 h. The region of interest was excised from the agarose gel and treated for 10 min with 0.25 M HCl, 40 min with denaturation buffer (0.5 M NaOH, 1.5 M NaCl), and 40 min with neutralization buffer (3 M NaCl, 1 M Tris-HCl, pH 5.0). The DNA was transferred by pressure blotter (Stratagen) for 30 min at 75 mm Hg onto a nitrocellulose membrane (BA85; 0.45-μm pore size; Schleicher & Schuell, Inc.) The membrane was incubated with prehybridization buffer at 42°C for 4 h (5× SSC-5× Denhardt’s salt solution [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM NaPO4, pH 6.5, 167 μg of sheared salmon sperm DNA/ml, 50% deionized formamide, 0.3% sodium dodecyl sulfate), hybridized with [α-32P]dCTP-labeled plasmid DNA (108 cpm/50 to 100 ng) for 20 h, and visualized by autoradiography. The radioactive probe was prepared by random oligonucleotide priming and synthesis by the large (Klenow) fragment of E. coli DNA polymerase I in the presence of [α-32P]dCTP. The RREs of the SV40 ori-containing plasmids were quantified by image analysis (NIH Image 1.52, National Institutes of Health [http://rsb.info.nih.gov/nih-image]).

RESULTS

Comparison of single and dual AP-1:Sp1:Sp1 motifs. One way to define sequences that are important for efficient DNA replication is to look for sequences common to genomes that replicate efficiently. Wild-type SV40 and variants of SV40 contain Sp1 transcription factor-binding sites in pairs, while AP-1 sites are found singly. For example, the SV40 evolutionary variant e1104 contains inverted or1s, one with a pair of Sp1 sites and an AP-1 site and the other with two pairs of Sp1 sites and two single AP-1 sites (Fig. 1a). The regulatory region of a passage 40 (p40) variant contains a pair of Sp1 sites and an AP-1 site, which were cloned into the HindIII site of pKP45 to form pC133 (38). The regulatory region of a passage 45 (p45) variant contains two copies of the earlier (p40) variant regulatory region, which had presumably been duplicated during passage. This duplicated regulatory region was ligated between the ClaI and NcoI sites of pC133 to produce pCN163 (Fig. 1a). Input mixtures containing equimolar amounts of pCN163 and pC133A (test and control plasmid DNA, respectively) were used to transfect COS-1 cells. Aliquots of the input mixture and plasmid DNA purified 48 h after transfection were linearized by EcoRI digestion. By 48 h posttransfection, the input plasmid DNA has been degraded (37), and therefore, the ratio between the two bands of replicated pCN163 and pC133A DNA represents the replication efficiency of one relative to the other. Division of this ratio by the ratio of bands produced by electrophoresis of the input mixture compensated for slight differences in the molarities of input DNA. pCN163 containing the later passage variant regulatory region replicated approximately nine times as efficiently as pC133 containing the earlier passage variant regulatory region (Fig. 2). pC133A lacks plasmid sequences from BamHI to EcoRV sites; this 190-bp deletion does not affect its RRE and allows electrophoretic separation from competing constructs. Figure 2 is a typical autoradiogram and representative of the experiments that are described below and summarized in Fig. 3.

Effect of host sequences on RRE. Evolutionary variants of SV40 have upstream auxiliary sequences that enhance DNA replication (37, 54, 62, Currier and Woodward, unpublished). Many variants have incorporated host auxiliary sequences that presumably provide a replicative advantage. The genome of a passage 13 variant, e1103, consists of nine 440-bp repeat units, each containing the SV40 ori and three Sp1 transcription factor-binding sites. Each repeat unit also contains 135 bp of low-copy-number monkey DNA and 143 bp of the 172-bp monkey α-satellite DNA monomer (36, 42). The α-satellite
DNA sequence is highly repeated (48), but its function in the monkey genome is unclear.

The \( \alpha \)-satellite sequence is interesting because it occupies the position of the wild-type upstream auxiliary sequences immediately adjacent to three Sp1 sites and \( \text{ori} \) (Fig. 1b). The first 71 bp of the \( \alpha \)-satellite region of ev1103 occupy the same relative position as the fourth, fifth, and sixth Sp1 sites in wild-type SV40. Because ev1103 is a highly efficient replicator, it was important to determine whether these 71 bp were functionally analogous to the Sp1 transcription factor-binding sites in enhancing replication. A Signal Scan search for mammalian and viral transcription factor-binding sites (47) showed that the 71 bp of \( \alpha \)-satellite DNA contained three binding sites for transcription factor NF-1 but lacked binding sites for AP-1.

\[ \alpha \]-Satellite DNA in the context of pC133. The regulatory region of pC133 includes a pair of Sp1 transcription factor-binding sites and an AP-1 transcription factor-binding site and replicates approximately three and a half times more efficiently than pOri (Fig. 3k). Thus the \( \alpha \)-satellite insert increased the RRE of pC133 by nearly 1.5-fold.

\( \alpha \)-Satellite DNA in the context of three Sp1 sites. In ev1103, \( \alpha \)-satellite DNA is found on the late-transcription side of three binding sites for the transcription factor Sp1 (Fig. 1b). To test the effect of \( \alpha \)-satellite DNA in this, its original context, plasmid constructs (pC133CN75 and pC133GC) containing three Sp1 sites, with or without an additional 42 bp of \( \alpha \)-satellite DNA, respectively, were compared (Fig. 3l). The \( \alpha \)-satellite DNA containing two NF-1 binding sites increased RRE slightly (1.3-fold ± 0.2-fold).

A single AP-1 site in the context of one pair of Sp1 sites. To determine the effect of an AP-1 transcription factor-binding site on RRE, plasmid constructs that are identical except for the AP-1 transcription factor-binding site were required. To generate pOri21, complementary oligonucleotides were synthesized that contained a pair of Sp1 sites (one 21-bp repeat) and the spacing of the original AP-1 region, although four nucleotides were changed to inactivate the AP-1 site (1, 29).
The addition of one pair of Sp1 sites (pOri21) to pOri enhanced replication 2.2 ± 0.5 times (Fig. 3i). However, in four separate experiments, pOri21 replicated only 0.15 ± 0.05 times as well as pC133Δ (Fig. 3b). The data indicate that the AP-1 site increases RRE 6.7-fold in the context of a pair of Sp1 transcription factor-binding sites.

A single AP-1 site in the context of two pairs of Sp1 sites. The regulatory region of pCN163 contains ori plus two pairs of Sp1 sites alternating with two AP-1 transcription factor-binding sites. To determine the effect of a single AP-1 transcription factor-binding site in the context of two pairs of Sp1 sites, variations were constructed by mutation of either one or both of the AP-1 sites in this regulatory region. One contained an intact AP-1 transcription factor-binding site proximal to ori (pOri2x21APp), the second contained an intact AP-1 transcription factor-binding site distal to ori (pOri2x21APd), and in the third (pOri2x21), both AP-1 transcription factor-binding sites were inactivated. The AP-1 sites were inactivated while maintaining the spacing of the original region by changing four nucleotides of the AP-1 site (1, 29). pOri2x21Δ was used to provide a baseline level of replication. In the context of two pairs of Sp1 sites, an AP-1 site proximal to ori (pOri2x21APp) increased RRE 4.5-fold ± 0.2-fold; an AP-1 site distal to ori (pOri2x21APd) increased RRE 4.7-fold ± 0.6-fold (Fig. 3c and d). The data indicate that, regardless of its position, an AP-1 site increases the RRE of a construct containing two pairs of Sp1 sites.

Location of an AP-1 site relative to two pairs of Sp1 sites. The difference in replication between pOri2x21Δ, the baseline control, and either pOri2x21 APp or pOri2x21APd was greater than fourfold, but the variance was too high to judge whether the proximal or distal AP-1 transcription factor-binding site had a greater effect on the RRE of the construct. To reduce experimental variation, pCN163, which contains a regulatory

![Image](http://jvi.asm.org/)

FIG. 3. Summary of data generated by cotransfections of test and control plasmid DNAs. Each construct is depicted schematically. Open arrows represent ori; squares with a central dot represent Sp1 transcription factor-binding sites; squares with an "x" represent AP-1 transcription factor-binding sites; solid bars represent host DNA incorporated into evolutionary variants. Results are presented in terms of the relative DNA replication efficiency (RRE) ± the standard deviation (SD) of the test construct with respect to the competing, control construct (normalized to 1.00). The number of experiments (n) is indicated in parentheses.
region with two pairs of Sp1 transcription factor-binding sites and two intact AP-1 transcription factor-binding sites, was used as a basis for comparison. This construct, being closer in replication efficiency to pOri2x21APp and pOri2x21APd, allowed the determination of their RREs with the benefit of much less variance. pOri2x21APp, with an intact proximal AP-1 site, replicated 0.36 ± 0.05 times as efficiently as pCN163Δ, while pOri2x21APd, with a distal AP-1 site, replicated 0.54 ± 0.02 times as efficiently as pCN163Δ (Fig. 3e and f). That is, the distal AP-1 transcription factor-binding site increased replication efficiency more than the proximal AP-1 transcription factor-binding site.

Addition of AP-1 sites to two pairs of Sp1 sites. The addition of transcription factor-binding sites could affect relative DNA replication efficiency in a number of ways. Each added site could produce an additive increase in replication efficiency, or one of the added sites could be responsible for all of the observed increase in RRE. As shown in Fig. 3c and d, the addition of one AP-1 site to a construct containing two pairs of Sp1 sites increased replication efficiency relative to that of only two pairs of Sp1 sites. Furthermore, pCN163, with two pairs of Sp1 sites and two AP-1 sites, replicates more efficiently than either pOri2x21APp or pOri2x21APd, each of which has two pairs of Sp1 sites but only one AP-1 site (Fig. 3e and f).

A single AP-1 site in the context of three pairs of Sp1 sites. pC200 (38), containing three pairs of Sp1 sites (one pair in each of its three 21-bp repeats) and an AP-1 site, produces the wild-type maximum replication efficiency from ori (38, 52). When the AP-1 site on the late-transcription side of the three Sp1 pairs is deleted from its natural context in the 72-bp transcriptional enhancer, replication efficiency is decreased (27). It was of interest to determine whether the AP-1 site found on the late-transcription side of the three pairs of Sp1 transcription factor-binding sites in wild-type SV40 increases replication in the absence of the remainder of the 72-bp transcriptional enhancer. A pair of complementary oligonucleotides containing the three pairs of Sp1 sites was synthesized without the terminal AP-1 site; the RRE of the new construct, pOri3x21, when compared with that of pC200, was 0.9 ± 0.3 (Fig. 3g). Thus, the presence or absence of the AP-1 site in the context of three pairs of Sp1 sites had little or no effect.

Substitution of two AP-1 sites for two of six Sp1 sites. Substitution of one transcription factor-binding site for another addressed relative effects on replication efficiency. pOri3x21, whose regulatory region contains three pairs of Sp1, and pCN163, in which the third and sixth Sp1 sites proximal to ori were replaced with AP-1 sites, were independently compared with pC200, which contains the wild-type SV40 regulatory region with six Sp1 sites and one AP-1 site. The RRE of pCN163 was 0.9, as was the RRE of pOri3x21 (Fig. 3g and h), or equivalent to that produced by the wild-type regulatory region of pC200. Therefore, the substitution of two AP-1 sites for two of the six Sp1 sites resulted in no significant change in RRE.

DISCUSSION

To gain further insight into the role of transcription factor-binding sites in replication, we analyzed the RREs of various combinations of transcription factor-binding sites.

The regulatory regions of evolutionary variants contribute to the variants’ replicative advantage over wild type. The number, type, and arrangement of transcription factor-binding sites in the regulatory region of variants help determine their replication efficiency. A dramatic ninefold increase in replication occurred when the transcription factor-binding site combination of one AP-1 site and two Sp1 sites was duplicated (as occurs during the naturally arising evolution of variants from serial passage 40 to 45). The ninefold enhancement is significantly greater than the 1.4-fold increase seen when a 71-bp monkey α-satellite DNA sequence (found in an earlier, 13th passage variant, ev1103) containing three NF-1 sites is added to the transcription factor-binding site combination of one AP-1 site and two Sp1 sites. Thus, two regulatory regions, each containing six transcription factor-binding sites, are not necessarily equivalent in their ability to enhance replication; i.e., four Sp1 plus two AP-1 sites (pCN163) are significantly better enhancers of replication than are two Sp1 plus one AP-1 plus three NF-1 sites (pC133H03). Presumably, the AP-1 and Sp1 sites play a more significant role in replication than do the NF-1 sites. The 40th and 45th passage variants (ev1108 and ev1104) incorporated host sequences consisting of both AP-1 and NF-1 transcription factor-binding sites while the 13th passage variant (ev1103) incorporated only NF-1 transcription factor-binding sites. These results are consistent with the assumption that the pressure of serial passage of virus through cells at high multiplicity of infection selects variants of increased replication efficiency and suggests that the number, type, and arrangement of transcription factor-binding sites are important.

The portion of the regulatory region of ev1104 (passage 45) containing two copies of the transcription factor-binding site combination of one pair of Sp1 sites and an AP-1 site produces the same replication efficiency as three pairs of Sp1 transcription factor-binding sites or three pairs of Sp1 sites and an AP-1 transcription factor-binding site (the wild-type regulatory region). The entire variant regulatory region of ev1104 includes two copies of ori in an inverted repeat and a central, host DNA sequence containing one NF-1 transcription factor-binding site and an additional AP-1 transcription factor-binding site (26). Deletion of one or both AP-1 sites from the regulatory region of evolutionary variant ev1104 resulted in decreased replication efficiency (54) and helps explain why AP-1 sites have been conserved in evolutionary variants of SV40.

The AP-1 site has a range of context-dependent effects. An AP-1 site has a greater effect on RRE where there are fewer pairs of Sp1 transcription factor-binding sites present. It should be noted that the configuration of transcription factor-binding sites found in the naturally arising late-passage evolutionary variants ev1104 and ev1108 consists of a transcription factor-binding site combination of a host AP-1 site and a pair of viral Sp1 sites, a configuration that maximizes the impact of the AP-1 site on replication efficiency and perhaps favors the production of this particular configuration of binding sites in serial passage.

Haas et al. (27) made two separate plasmid constructs, each with three contiguous substitution mutations in the AP-1 site of the SV40 72-bp transcriptional enhancer. The enhancer also contains binding sites for several other transcription factors. They used these constructs in the same type of replication
functionally replace an AP-1 site. When compared with a plasmin both wild-type SV40 and variants, and a pair seems to lent to that of a pair of Sp1 sites. Sp1 sites are found in pairs enhancement of replication by a single AP-1 site was equiva-

(pCN163 compared with pC200). In this specific context, the Sp1 sites that constitute the wild-type SV40 regulatory region factors could play a critical role in the activation of DNA transcription (19). The interaction of adjacent bound transcription enhancing unit may apply to DNA replication as well as tran-

scription factor-binding sites form one functional enhanson model of transcriptional activation in which two ad-

obtained (Szymanski and M. E. Woodworth, unpublished data), and suggest that the effect of an AP-1 site on replication might correlate with its distance from ori. The enhanson model of transcriptional activation in which two ad-

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tion (19). The interaction of adjacent bound transcription factors could play a critical role in the activation of DNA replication.

Because both Sp1 and AP-1 sites can function as activators of DNA replication, their relative enhancing activities were investigated. An AP-1 site was substituted for two of the six Sp1 sites that constitute the wild-type SV40 regulatory region (pCN163 compared with pC200). In this specific context, the enhancement of replication by a single AP-1 site was equiva-

tent to that of a pair of Sp1 sites. Sp1 sites are found in pairs in both wild-type SV40 and variants, and a pair seems to functionally replace an AP-1 site. When compared with a plasmid construct (pOri) containing SV40 ori with no additional viral regulatory sequences, the addition of two viral Sp1 transcription factor-binding sites (pOri21) enhances replication and the addition of an AP-1 site to the two viral Sp1 transcription factor-binding sites (pC133) further enhances replication. These data emphasize the importance of both the Sp1 and AP-1 sites. Compe

 Assad et al. | Transcription Factor-Binding Sites and Replication | 5643 | 3.5-fold difference, respectively). Because pOri contains the SV40 ori without any late-transcription-side auxiliary se-

quences, it would seem to provide less competition for transcrip-

tion factors. If there is heightened competition between constructs for scarce protein resources, the better competitor

sequestering more assures its replication. Sequestration of pro-

tein resources would increase the chance that some of the proteins would become limiting. It has been shown that T-ag is the limiting factor in cotransfections (37). Transcription factor-binding sites might also affect replication efficiency by heighten-

ing competition for T-ag. Competition with a construct that lacks transcription factor-binding sites would result in a longer period of time before T-ag would become limiting.

Studies of the dynamics of SV40 DNA replication in vitro have called into question the role of transcription factors. Bullock (5) found no significant difference in replication of SV40 ori with or without auxiliary transcription factor-binding sites. The results of the present study were generated in vivo and extended over 48 h, while the in vitro study was concluded in 20 min. The coactivator for Sp1 is labile at the temperatures used for in vitro replication (18). In addition, their results were generated without competitor constructs. The results argue that transcription factor-binding affinities must be taken into account where competition for resources is a factor in DNA replication. Moreover, the differences between the in vivo and in vitro data suggest that transcription factor-binding sites play an important role in a chromatin environment but not when nucleosomes are absent.

The competitive cotransfection of mammalian cells offers a quantifiable model of phenomena that occur in nature. In infections with DNA viruses, the viral origin of replication must compete with the host origins, which have the advantage of superior numbers early in the infection. A more efficient virus is likely to be a better competitor for initiation factors. Cotransfection experiments might correlate with competition that occurs between host replication origins. Origins in mam-

malian chromosomes have not been associated with any distinctiv
everal sequences, and origins with different sequences may have different affinities for requisite initiation factors. With the exception of T-ag, the initiation factors for the SV40 ori are drawn from the pool of cellular initiation factors. Neoplastic cells escape the conventional controls on DNA synthesis and cell proliferation. Origins of replication in the chromosomes of these cells might have improved ability to gather initiation factors. Competitive replication experiments can lay the groundwork for the study of these phenomena.

A model describing how interactions with transcription fac-

tors affect replication efficiency. The AP-1 site in the enhancer of polyomavirus promotes T-ag-mediated DNA unwinding (24). Transcription factors ZBP-89 and Sp1 stimulate DNA replication of Epstein-Barr virus by tethering viral replication proteins to ori (3). The proline-rich activation domain of NF-1 interacts with histone H3 to activate SV40 DNA replication (44). DNA replication is also enhanced in Saccharomyces cerevisiae by the activation domains of Sp1 and CTF1 (39). In SV40, the binding of Sp1 to three of the weak binding sites for T-ag might increase the efficiency of assembly of the T-ag double hexamer over its strong site II binding sites in the 27-bp palindrome. The T-ag hexamers could separate to begin replication of the DNA or they might remain together as a stali-

py hole, pulling the DNA through as the polymerase replicates it (reviewed in reference 60). To advance the repli-

cative process, any bound Sp1 must be displaced. A bound AP-1 transcription factor nearby could increase the efficiency of replication by affecting the configuration of the Sp1 sites so
that their affinity for Sp1 is reduced. Alternatively, bound transcription factors could increase the local concentration of T-ag by interacting with it directly, perhaps coordinating its arrival at the 27-bp palindromic sequence.

In general, replication efficiency increases with the number of transcription factors present. This is true of the host sequences integrated in evolutionary variants, which at higher passage numbers contain more transcription factor-binding sites. However, the system can become saturated with transcription factor-binding sites, as seen by the decreased enhancement after adding an AP-1 site to greater numbers of Sp1 sites. To explain how an AP-1 site distal to orγ can enhance replication more than a site proximal to ori, it is necessary to consider the effect of the intervening transcription factors bound to the regulatory region. Sp1 changes the conformation of DNA by inducing a bend in the DNA to which it binds (53). Multiple Sp1-binding sites are capable of producing a loop that could bring a distal AP-1 site near enough to interact with T-ag. Multiple Sp1-binding sites are capable of producing a loop that could induce bending of the DNA at which it binds (53). The interaction of SV40 DNA replication with the Jun subunit of AP-1 interacts with SV40 T-ag as it does with polyoma large T-ag (24, 33), AP-1 could facilitate formation of the T-ag hexamers. AP-1 also may interact with the T-ag hexamers, stabilizing the bent conformation imposed upon the DNA by Sp1, inducing distortion of orγ DNA to facilitate initiation of DNA replication. Smelkov and Borowiec (51) demonstrated that the binding of T-ag is strongly dependent on the length of the single-stranded DNA of the replication bubble. An AP-1 transcription factor bound to a site proximal to orγ would be sterically hindered by Sp1 from interaction with T-ag bound at orγ but could enhance replication by further bending the Sp1-induced loop. A construct containing two AP-1 sites, proximal and distal to orγ, replicates better still, probably taking advantage of bending by the proximal site and any T-ag interactions of the distal site. That a single AP-1 site, in the absence of other transcription factor-binding sites, decreased replication efficiency from orγ (Szymanski and Woodworth, unpublished) may be the result of the hypothetical destabilization of T-ag binding at site I proposed in the model above. The elimination of all Sp1 sites would also remove weak T-ag binding at site III.

It is possible that some minimum number of transcription factor-binding sites is required to induce bending, thus making it important that the host DNA that substitutes for three to four Sp1 sites in ev1103, ev1104, and ev1108 provide a long enough stretch of transcription factor-binding sites to induce bending of the DNA. This distortion of the DNA at orγ is probably required to facilitate the initiation of replication. Furthermore, the binding of transcription factors may alter nucleosome phasing and, thereby, enhance replication by facilitating both bending of the DNA and binding of the replication complex. Clearly, nuclear structure plays an essential role in DNA replication in vivo (12). Transcription factors may change the site of initiation of DNA replication and, thereby, change replication efficiency. It is of interest to note that at least one pair of viral Sp1 sites has been retained in all evolutionary variants studied to date. This suggests that not only is the combination of two Sp1 sites and orγ the minimum viral sequence needed for replication, but the spacing between the Sp1 sites and orγ must remain intact. Based on what we now know from the characterization of the regulatory regions that have evolved to give variants a replicative advantage, the term “transcription factors” incompletely describes the functions of these important proteins since they may have been captured from the host to function primarily as part of the replication apparatus rather than as enhancers of transcription.

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