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Functional Interactions of the Simian Virus 40 Core Origin of Replication with Flanking Regulatory Sequences

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We constructed a matched set of plasmids to investigate the interactions of essential core sequences of the simian virus 40 replication origin with flanking regulatory sequences. Deletions of either T-antigen-binding region I or the 21-base-pair repeated promoter elements reduced replication to 50 to 70% of wild-type levels. The simultaneous deletion of both regions decreased replication to less than 5% of wild-type levels. Thus, the double deletion greatly amplified the defects of the single deletions. We conclude that region I and the 21-base-pair repeats have related rather than independent functions in DNA synthesis. Insertion of a synthetic region I or the adenovirus 2 major late promoter at the late side of isolated core sequences in place of the 21-base-pair repeats failed to restore replication. In contrast, insertion of a single 72-base-pair enhancer element stimulated replication of the core origin more than fivefold. Thus, three distinct regulatory elements appear to facilitate core DNA replication by related mechanisms. Flanking sequences have only a small direct effect on T-antigen binding to naked core DNA. Possible mechanisms of action include the regulation of transcription or of chromatin structure.

Simian virus 40 (SV40) is an attractive model for eucaryotic DNA replication (5, 28). A single viral protein, T antigen, binds to a unique origin of replication to initiate each round of viral DNA synthesis. Intracellular viral nucleoprotein closely resembles cellular chromatin, and events in the initiation and elongation of viral DNA synthesis mimic events in the replication of cellular chromosomes (15). The recent establishment of cell-free systems for complete cycles of viral DNA replication provides a means for the identification of cellular replication proteins (20, 21, 25). Hence, a complete mutational analysis of the SV40 origin of replication promises a better understanding of protein-DNA interactions and events that occur within the origin.

Previous studies have established the approximate boundaries of the smallest origin that would support detectable levels of replication. We refer to these sequences as the core origin. Deletion analyses at either end of the origin locate the limits of the core between nucleotides 5208 and 5222 at the early end and nucleotides 26 and 30 at the late end (1, 7, 8, 10, 16, 17, 26). DNA regions flanking both ends of the core origin increase the efficiency of DNA replication. These ancillary regions consist of the high-affinity T-antigen-binding region I at the early end of the core and the 21-base-pair (bp) repeated promoter elements at the late end of the core. Estimates of DNA replication after single deletions of either ancillary region vary widely. They range from 20 to 70% of wild-type levels after deletion of region I (8, 17) and from 10 to 100% of wild-type levels after deletion of promoter elements (16, 19). These variations probably reflect differences both in the context of surrounding DNA and in assay conditions.

We constructed a matched set of plasmids to investigate further the interaction of essential core sequences with flanking regulatory sequences that facilitate core function. We compared the effects of single and double deletions of ancillary regions on replication efficiency under standard conditions at a variety of times after transfection. Although single deletions of region I or the 21-bp repeats delayed and reduced DNA synthesis, maximal levels of replication were greater than 50% of wild-type levels. In contrast, the simultaneous deletion of both regions drastically reduced but did not eliminate core function. Thus, well-defined sequences at either end of the core origin have related rather than independent functions in facilitating DNA replication. Furthermore, replacement of the 21-bp repeats by a single 72-bp repeat of the SV40 enhancer restored replication efficiency. Finally, our results establish the autonomy of a small core origin in the complete absence of adjacent SV40 sequences.

MATERIALS AND METHODS

Plasmid constructions. The common pOR vehicle was derived from pML2 (22) by deletion of the small fragment from *Eco*RI to *Acc*I after conversion of the *Acc*I site to a *Hin*dIII site. This segment served as an acceptor for SV40 origin restriction fragments. The pOR4 and pOR3 plasmids contain SV40 sequences 5171 to 160 between the SV40 *Hin*dIII and *Bst*NI sites and an *Eco*RI linker at the *Bst*NI end. Plasmids pOR2 and pOR1 include SV40 sequences 5171 to 39 between the SV40 *Hin*dIII and *Nco*I sites and a 23-bp polylinker (5′-GTCGACCGGATCCCCGGGAATTC-3′) with *Sal*I, *Bam*HI, *Sma*I, *Xma*I, and *Eco*RI sites. Plasmids pOR4 and pOR2 were derived from SV40 wild-type strain 776 (28), and plasmids pOR3 and pOR1 were derived from SV40 mutant cs-1097 (8), which has a deletion of sequences 5178 to 5208.

Four variants of pOR1 were also constructed. Mutant pOR1-SVI has a 19-bp synthetic T-antigen-binding region I corresponding to SV40 nucleotides 5186-5209 (24a) inserted at the *Bam*HI site of the polylinker. Plasmid pOR1-Ad2 contains the major late promoter within adenovirus 2 sequences 5815 and 6035 between *Xho*I and *Eco*RI linkers inserted at the *Sal*I and *Eco*RI sites of the polylinker. Mutant pOR1-TATA has an AA to CG substitution at SV40 nucleotides 17 and 18 of the TATA box. It was constructed by replacing the *Bgl*I-*Nco*I fragment of pOR1 with the corresponding restriction fragment of mutant CW44ML obtained from B. K. Wasylyk et al. (29). Plasmid pOR1-Enh contains

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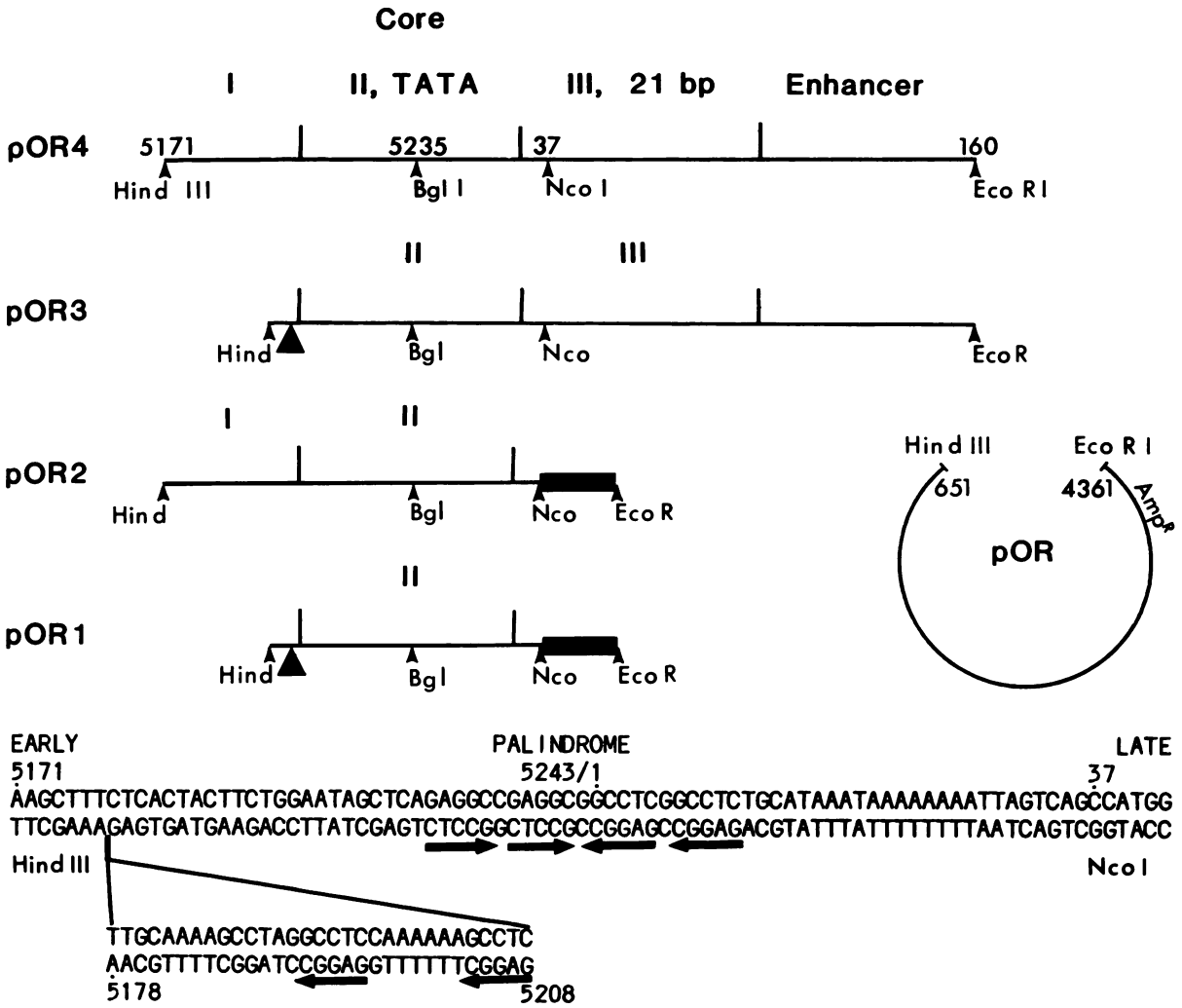

FIG. 1. Matched set of SV40 origin mutants in plasmid pOR. Relevant viral sequences and restriction sites are shown. The triangles in pOR3 and pOR1 indicate an internal deletion of SV40 sequences 5178 to 5208. The heavy bars in pOR2 and pOR1 represent a 23-bp polylinker. The arrowheads below the sequences locate recognition pentanucleotides for T antigen. Constructions were confirmed by dideoxy -sequencing.

SV40 enhancer sequences 107 to 182 inserted between the *Bam*HI and *Eco*RI sites of the pOR1 polylinker.

DNA replication. The assay for DNA replication in COS-1 cells has been previously described (3). Transfection of 0.5 μg of plasmid DNA per 75-cm^2 flask of cells falls within the limits of a linear dose-response curve for replication under our conditions. Transfected cells were maintained continuously at 36°C. To insure equivalent transfection efficiencies with each plasmid preparation, DNAs were purified twice by equilibrium centrifugation in CsCl gradients containing ethidium bromide. The input DNAs were quantitated both by measuring the optical density at 260 nM and by densitometry of form I DNA separated in 1% agarose gels and stained with ethidium bromide.

DNase footprinting. The DNase I footprinting assay has been described in detail previously (4). In the present study, the *Eco*RI-*Hin*dIII origin fragment was labeled on the 3′ end of the early strand at the *Eco*RI site. T antigen was bound to DNA in 100 μl of 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES) [pH 7]–0.1 mM EDTA–50 to 100 mM NaCl. After binding for 60 min at 0°C or for 10 min at 37°C, 5 μl of DNase I in 20 mM $MgCl_2$–10 mM $CaCl_2$ was added for 5 min at the same temperature as that used in the binding reaction. The DNase I concentrations at 0 and 37°C were 0.125 and 0.0005 U per reaction, respectively.

RESULTS

Deletion of flanking regions from the core origin. We constructed a matched set of four plasmids to quantitate the effects of adjacent regulatory sequences on the efficiency of core origin replication (Fig. 1). The wild-type SV40 origin in pOR4 contains all known origin components and includes T-antigen-binding region I, the core origin that overlaps T-antigen-binding region II, three 21-bp repeats of the early promoter that overlap T-antigen-binding region III, and part of the SV40 enhancer. Previous studies have shown that the SV40 origin is independent of enhancer function (1, 10, 19) in the presence of the 21-bp sequences. Clone pOR3 has a deletion of T-antigen-binding region I; clone pOR2 has a deletion of the early promoter region; and clone pOR1 has a deletion of both regions.

We measured the replication efficiency of the clones by transfection of cloned DNA into COS-1 cells that constitutively expressed SV40 T antigen. After incubation at 36°C,

we extracted DNA at daily intervals. The DNA was cut with *Mbo*I to distinguish methylated input DNA from unmethylated progeny DNA and analyzed by gel electrophoresis, blotting, and hybridization with radiolabeled pBR322 DNA. We used *Mbo*I fragments that were common to all of the clones to quantitate the amount of progeny DNA by densitometry of autoradiograms. The residual input DNA in the autoradiograms served as an internal control for the amount of plasmid DNA added to each sample. The polylinkers present in pOR1 and pOR2 had no effect on replication (data not shown). Yields at later times after infection are not shown because levels of progeny DNA decreased, presumably as a result of cell death and detachment caused by plasmid DNA replication.

All deletion clones replicated more slowly and to lower levels than did wild-type pOR4. Fig. 2A summarizes the course of DNA replication during the 3 days after transfection, and Fig. 2B shows an example of the gel blot analysis of progeny DNA yields 72 h after transfection. The delay in replication may account for the variations in estimates of levels of DNA replication reported in previous studies for similar deletions. At maximal levels of replication, origins with deletions in T-antigen-binding region I replicated approximately 70% as well as wild-type origins. This efficiency of replication is similar to that of authentic whole viral DNA with the identical deletion (8). Deletion of the 21-bp repeats reduced replication to 50 to 60% of wild-type levels. In contrast to the single deletions, the simultaneous deletion of region I and the 21-bp repeats caused a more drastic inhibition of DNA replication, to less than 5% of wild-type levels. Thus, the double deletion reduced DNA replication to levels that were at least sevenfold lower than the product of the percentages of wild-type replication for the two single deletions (shown by the dotted line in Fig. 2A). We obtained the same results with three different batches of input DNA. This unexpected finding indicates that the two regions either share redundant functions or cooperate in the same function. In both cases, nonhomologous sequences adjacent to either end of the core origin would have at least one related function in the initiation of DNA replication.

Rearrangement of regions flanking the core origin. To investigate the role of T-antigen-binding region I in replication, we inserted a 19-bp synthetic region I with *Bam*HI overhangs into the *Bam*HI site of the polylinker of pOR1 (Fig. 3A). The synthetic segment corresponds to SV40 sequences 5191 to 5209, which contain two recognition pentanucleotides and a vital 7-bp spacer (Fig. 1). These signals are sufficient to direct the high-affinity binding of T antigen (24a). The insertion placed region I 50 bp to the late side of the center of the core origin rather than 34 bp to the early side, at its usual position. Relative to the core, the polarity of the synthetic binding sequences was the same as that of the natural binding sequences in wild-type pOR4 (Fig. 3B). The rearrangement failed to increase DNA replication above pOR1 levels. Thus, region I must be located either on the early side of the origin or at a precise distance from the core to facilitate the initiation of DNA synthesis.

Substitution of a foreign promoter for the 21-bp repeats. To examine the role of promoter functions in replication, we substituted sequences within and adjacent to the core origin (Fig. 3A). Substitution of CG for AA sequences at nucleotides 17 to 18 of the TATA box reduced the replication of pOR1 to approximately 10% of core levels (Fig. 3B). Wasylyk et al. (29) showed that this mutation causes approximately a 20-fold reduction in transcription starting downstream from TATA sequences, with no significant change in

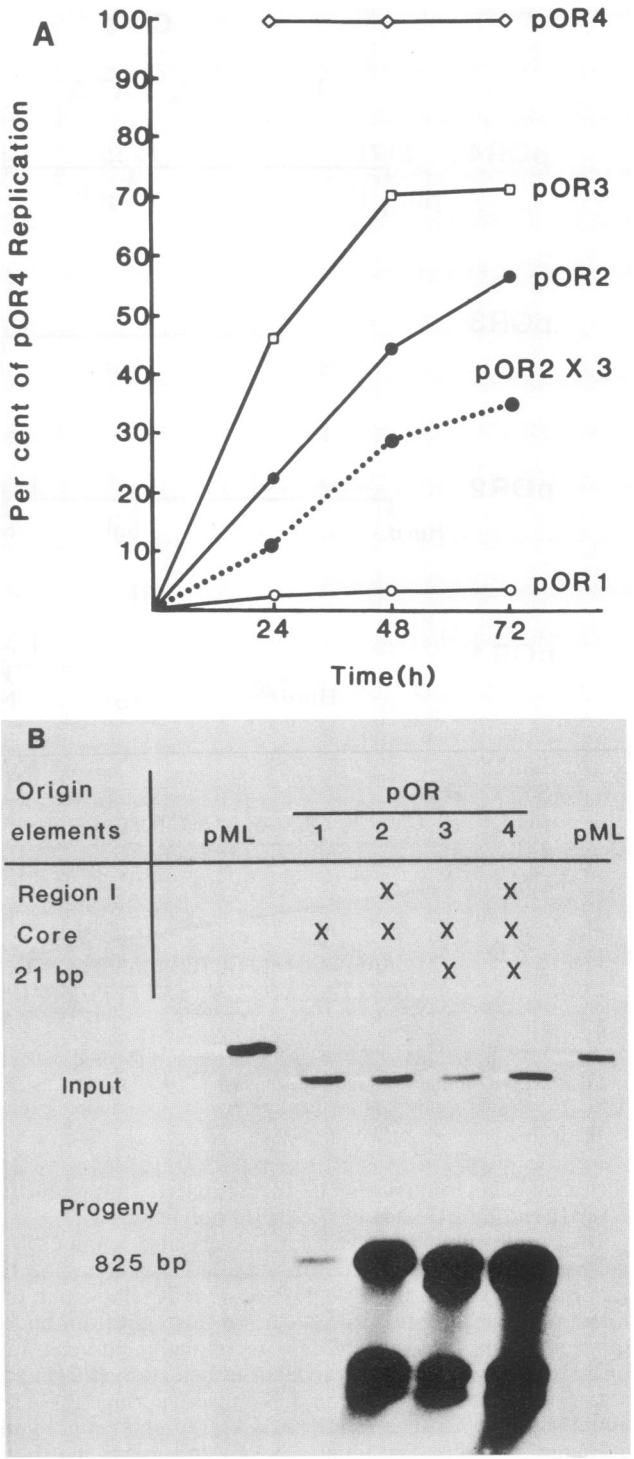

FIG. 2. Replication of wild-type and mutant origins in COS-1 cells. At various times after transfection, DNA was extracted, cut with *Mbo*I, separated by agarose gel electrophoresis, transferred to GeneScreen, and hybridized with nick-translated pBR322 DNA. (A) Quantitative summary of levels of accumulated progeny DNA at various times after transfection. The dotted line shows the level of replication expected for the double deletion of pOR2 and pOR3 if the two deletions affected independent functions. It is the product of the percentages of wild-type replication for the two single deletions. (B) Autoradiogram (4-h exposure) of input and progeny DNAs extracted 3 days after transfection. Input plasmids are identified at the top of the gel. X indicates the presence of the origin elements.

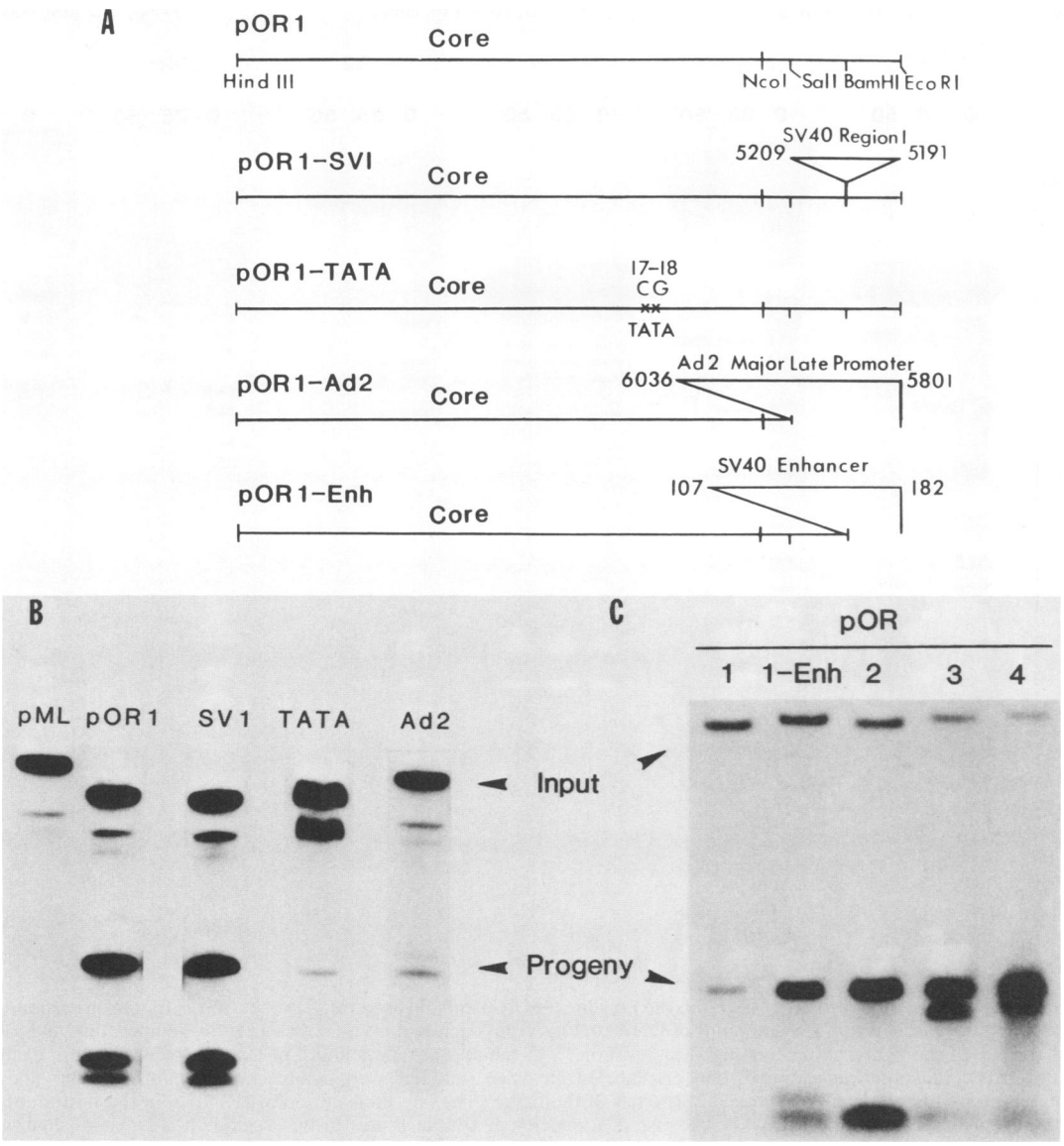

FIG. 3. Replication of the core origin in artificial sequence contexts. DNA was extracted from COS-1 cells 72 h after transfection, and assays were performed as described in the legend to Fig. 2. (A) Summary of the origin constructions tested. (B) Autoradiogram (24-h exposure) of input and progeny DNAs; input plasmids are identified at the top of the gel. (C) Autoradiogram (1-h exposure) of input and progeny DNAs; input plasmids are identified at the top of the gel. See Fig. 1 and 3A for a description of the input plasmids.

the amount of RNA initiating from upstream sites in vivo, and reduces DNA replication about fivefold in COS-1 cells. Because nucleotides 17 to 18 are components of both the TATA sequence and the core origin, the cause of the decrease in replication is ambiguous. The mutation could affect origin function either by altering transcription or by a mechanism independent of transcription. Nevertheless, it is significant that all promoter elements can be deleted or mutated without completely abolishing DNA synthesis. If transcription is essential for the activation of replication, then transcripts originating from foreign sequences in the plasmid may be capable of replacing natural promoter function in the replication process. To test this possibility, we inserted the strong adenovirus 2 major promoter between the *Eco*RI and *Sal*I sites of the polylinker (Fig. 3A). In this construction, the TATA sequences of the adenovirus promoter are located 40 bp upstream from the natural TATA box in the core origin. The substituted promoter inhibited replication 5- to 10-fold as compared with that of pOR1 (Fig. 3B). We do not know how the adenovirus sequences inhibit the SV40 origin. Nevertheless, the inhibition emphasizes the need to use matched plasmids to compare mutant origins.

Substitution of a 72-bp repeat for the 21-bp repeats. Although the SV40 enhancer elements are not required for replication in the presence of the 21-bp repeats (1, 10, 19), the enhancers of the related polyomavirus have been implicated in DNA replication (6, 23). To test the function of the SV40 enhancer in the absence of the 21-bp repeats, we inserted it between the *Bam*HI and *Eco*RI sites of the pOR1 polylinker (Fig. 3A). The insertion places the enhancer in its usual orientation 13 bp to the late side of the *Nco*I site of the core origin. Thus, the 72-bp element is located at the same site as the SV40 T-antigen-binding region I rearrangement discussed previously but 13 bp farther upstream from the

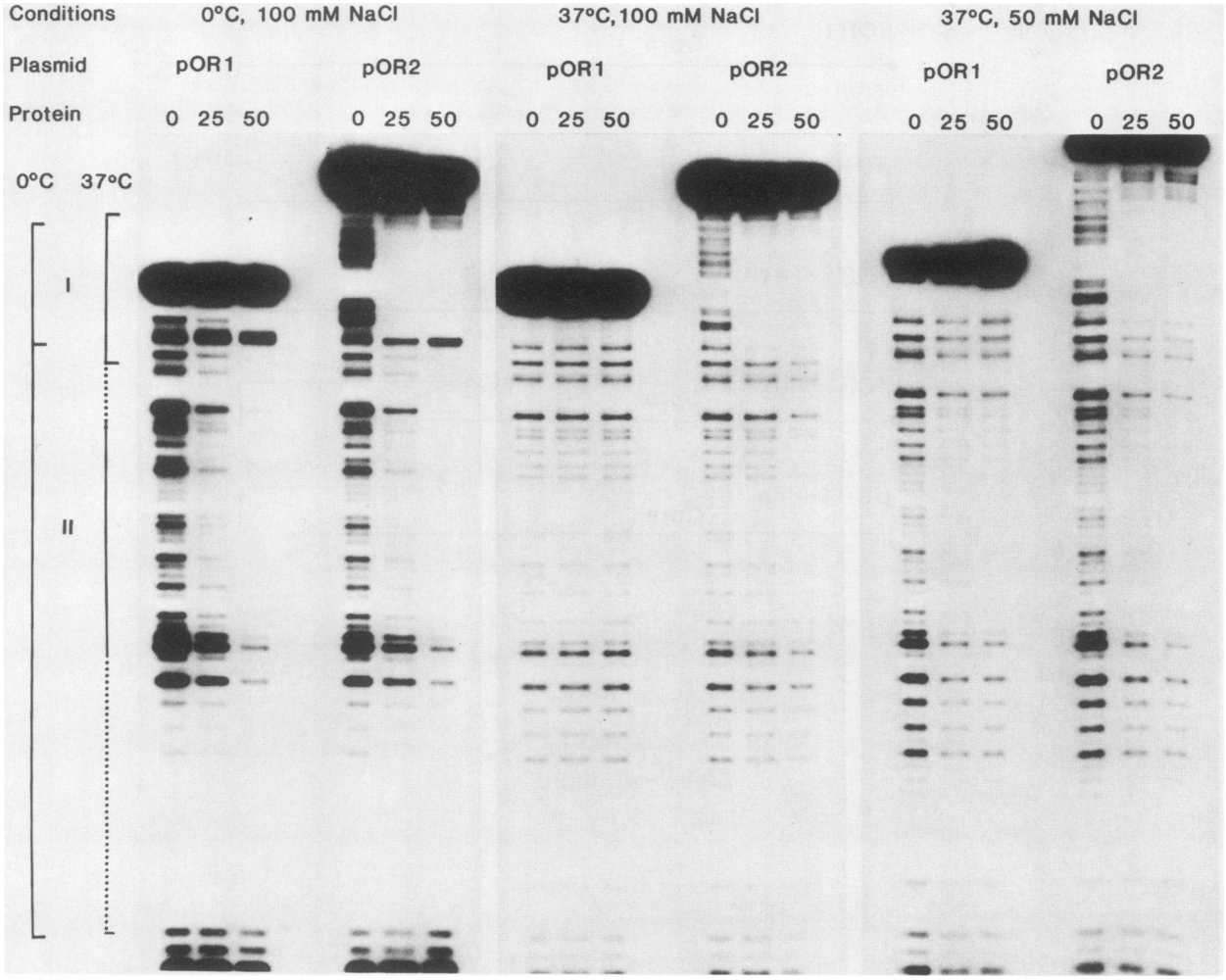

FIG. 4. Binding of T antigen to core origin DNA in the presence (pOR2) and absence (pOR1) of region I. Increasing amounts (nanograms) of protein (T-antigen) were added to a fixed amount of DNA (0.1 ng) from each mutant under a variety of temperature and ionic conditions, as indicated at the top of the figure. After binding at either 0 or 37°C, the appropriate amount of DNase I was added to each sample at the same temperature. The reaction was stopped, and end-labeled cleavage products were analyzed in denaturing gels. The boundaries of DNase-protected regions I and II are summarized at the left of the figure. The late limit of region II shown at the bottom of the figure was difficult to define by using the footprinting assay because of a paucity of DNase I sites in that region. It was established with a fragment survival assay in the presence of excess DNase I (27).

usual position of the 21-bp elements. The 72-bp enhancer sequence stimulated replication 5-fold above core levels, or approximately half as well as the 21-bp repeats in pOR3 (Fig. 3C). The successful interchange of 21- and 72-bp functions suggests that they have a related role in replication.

Effects of flanking regions on T-antigen binding to the core origin. We also examined the effects of adjacent sequences on the binding of T antigen to region II within the core origin. Although binding to region II is independent of regions I and III at low temperatures (17, 27), recent findings suggest that region II binding interactions may be altered at 37°C (9). Figure 4 shows DNase footprints of T-antigen binding to the origin under a variety of conditions. In contrast to the findings of Dixon and Nathans (9), T antigen bound to region I with a greater affinity than to region II at 37°C. We do not know the reason for this difference. At 0°C, binding to region II was equivalent in the presence (pOR2) or absence (pOR1) of region I. At 37°C, the presence of region I enhanced binding to the core to a small extent in both 50 and 100 mM NaCl. Interestingly, temperature determined the extent of DNase I protection by T antigen. At 37°C, region I widened, and region II became more focused than at lower temperatures. T-antigen binding to region III had no effect on core binding under any conditions used (data not shown). Thus, the slight enhancement of core binding by region I at 37°C may explain the slight decrease in DNA replication in the absence of region I, but it does not explain the drastic impairment after the deletion of both region I and the 21-bp repeats.

DISCUSSION

Deletions beyond limits on either side of the T-antigen-binding palindrome of the SV40 origin abolish DNA replication altogether (10, 24). We define these essential sequences as the core origin. In contrast, complete deletions of either T-antigen-binding region I or the 21-bp repeats reduce but do not eliminate replication. Thus, these regions flanking the early and late ends of the core origin have a facilitating rather than an obligatory function. If the two regions performed independent functions in replication, their simultaneous de-

letion would result in a level of replication equivalent to the product of the percentages of wild-type replication for the two individual deletions. Because the level resulting from the combined defect is far less than this level, we conclude that the flanking regions have at least one related function. Furthermore, the ability of the 72-bp enhancer element to replace the 21-bp repeats in replication suggests that it also shares a related function with region I and the 21-bp repeats.

The three distinct regulatory regions that facilitate core origin function are important components of the SV40 promoter. The 21- and 72-bp repeats bind positive regulatory proteins, whereas region I binds the T-antigen repressor (4, 9, 13, 14, 17, 24, 24a, 30). Together, the regions regulate the level of transcription and determine the initiation sites for early mRNA (10, 12–14, 30). These findings suggest a possible role for transcription in the initiation of DNA replication. However, this idea is difficult to reconcile with a number of findings at present. The insertion of the adenovirus 2 major late promoter upstream from the origin inhibits DNA replication rather than facilitates it. Furthermore, DNA replication continues, although at lower levels, after deletion or mutation of all known elements of the early promoter-operator region, including the TATA sequences within the core origin. Finally, Li and Kelly showed that α-amanitin does not inhibit SV40 DNA synthesis significantly in vitro (20). Thus, the quantity of transcription does not correlate well with the levels of replication. Nevertheless, it remains possible that low levels of transcription are sufficient for replication. A further possible requirement for the correct initiation of transcription might explain the role of region I in replication as well as the inhibition of replication by a foreign promoter.

SV40 T-antigen-binding region I, the 21-bp repeats, and the 72-bp repeat share a second common feature. Each region binds regulatory proteins that may facilitate events within the core origin (4, 13, 30). The facilitation could reflect a direct interaction between proteins. For example, T antigen binds to region I in a position that may allow contact with T antigen bound to the core origin. Indeed, the presence of region I increased slightly the binding of T antigen to the core at 37°C in vitro. Because T antigen covers only the center palindrome of the origin at 37°C, the binding of T antigen to region I or of the positive regulatory protein Sp1 to the 21-bp repeats (13) could also directly facilitate the binding of cellular proteins to the adjacent core region. Alternatively, the tight binding of proteins on either side of the origin may prevent the formation of nucleosomes within the core DNA (2, 11, 16, 18) and indirectly increase the access of viral or cellular proteins to the open core region of viral chromatin. If proteins bound to flanking regions do organize chromatin to allow replication, then the location and nature of bound proteins must be critical because substitution of enhancer, but not region I, sequences for the 21-bp repeats restores replication function.

Although generally considered part of a noncoding regulatory region, the SV40 origin contains an open reading frame for 23 amino acids between nucleotides 5191 and 16 (28, 29). It is not known whether this potential peptide exists or would have any function in replication. Mutant pOR1 would remove the 21- and 72-bp repeats of the promoter for the peptide and would also change its reading frame through the deletion of region I sequences. Thus, both deletions would reduce the function of a single gene product.

The SV40 regulatory sequences are organized for compactness and efficiency. The early and late promoters overlap each other as well as the origin of replication. A number of viral and cellular regulatory proteins recognize sequences in region I, the core origin, the 21-bp repeats, and the enhancers (4, 9, 13, 14, 17, 24, 24a, 30). These proteins exclude nucleosomes directly and perhaps at a distance (2, 11, 16). We show here that three distinct regions separated by over 70 bp cooperate in a common function that facilitates replication. The intricacies of this integrated control region present a challenge and an opportunity for the analysis of the interactions among eucaryotic control mechanisms. The isolation of a small, autonomous core origin suggests that the SV40 control region can be dissected into individual components for a detailed genetic analysis.

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ADDENDUM IN PROOF

J. J. Li, K . Peden, R. Dixon, and T. J. Kelly also have shown that, in the absence of the 21-bp repeats, the SV40 enhancers stimulate DNA replication in vivo but not in vitro (personal communication).

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