A *cis*-Acting Sequence Promotes Removal of Simian Virus 40 DNA from the Replication Pool

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Pulse-labeled simian virus 40 (SV40) DNA is removed from the pool of molecules available for replication (i.e., it ceases to reenter replication) a few hours after synthesis. We studied this cessation of reentry with mutants containing different deletions in the structural genes of SV40. The DNAs of two independent deletion mutants, *dl*-1007 (24% deletion) and *dl*-1003 (8% deletion), were used as templates for further DNA synthesis (i.e., they reentered replication) to a greater extent than was wild-type DNA. The alteration in reentry kinetics was not because the DNAs were smaller; other deletion mutations that were from 76 to 85% of the length of wild-type DNA (*dl*-BE and *dl*-1133 with a deletion in the late region and *F8dl* with a deletion in the early region) did not reenter replication to a greater extent than the wild type did. Cotransfection experiments showed that the mutant phenotypes of *dl*-1007 and *dl*-1003 were poorly complemented, if at all, by the wild type. Thus, we propose that there is a *cis*-acting sequence located in the *Hind*III E fragment of SV40, not present in either of these mutants, that promotes the efficient removal of DNA from the replication pathway.

Newly synthesized papovavirus DNA is used as a template for further DNA synthesis (i.e., it reenters replication) for a limited period of time (5, 16, 17). The time course of removal of this DNA from the pool of molecules available for replication correlates with its progression into the maturation (encapsidation) pathway (23). This pathway is clearly one way to remove DNA from the replication pool. This mechanism, however, can be nonspecific (1, 9, 11); even cellular DNA is packaged (12, 13, 22, 24). Garber et al. (3) have suggested that the removal of DNA from the replication pathway occurs before encapsidation. They studied the DNA of a simian virus 40 (SV40) mutant (*ts*BC11) that could not progress into the maturation pathway because of a mutation in the VP1 gene. The amount of viral DNA accumulating in a cell infected with this mutant was similar to that seen in a wild-type-infected cell (3).

We characterized the behavior of viral DNA carrying different deletions in the late region of SV40 (i.e., the region coding for structural proteins) by determining reentry kinetics. If there were an active preencapsidation mechanism determining the fate of newly synthesized DNA, some deletion mutants might lack factors critical to the removal of DNA and therefore show more extensive reentry. The data reported here suggest that a preencapsidation step does exist and is specific: a sequence of SV40 DNA lying within the *Hind*III E fragment is required for the efficient removal of DNA from the pool of molecules available for replication.

MATERIALS AND METHODS

Cells, viruses and DNA. BSC-1 cells (obtained from M. Singer, National Institutes of Health) and CV-1 cells (obtained from S. Weissman, Yale University), two permanent cell lines derived from African green monkey kidneys, were grown in Dulbecco modified Eagle medium (DME) containing 10% calf serum and gentamicin (50 µg/ml). COS-1 cells (obtained from Y. Gluzman, Cold Spring Harbor Laboratories [4]) were grown in DME containing 10% fetal calf serum

and gentamicin (50 µg/ml). SV40 wild type (wt776) was plaque purified three times and propagated at a low input multiplicity (0.05 PFU per cell) in BSC-1 cells. *F8dl*, an early-region deletion mutant provided by K. Danna and L. Sompayrac (University of Colorado) (21), was propagated at a low multiplicity of infection in COS-1 cells. Virus stocks were prepared from infected cells by three cycles of freezing and thawing and low-speed centrifugation. The stocks were stored at -20°C.

The regions of the genome which are absent in the deletion mutants we studied are shown in Fig. 1. The density of the mutant virions was nearly the same as that of wild-type virions, making it difficult to prepare pure mutant stocks without contamination by the wild type. Therefore, viral DNAs were prepared from recombinant DNA. SV40 wild-type strain wtB3 (the same as wt776) was provided via D. Nathans, Johns Hopkins University and *dl*-1007 and *dl*-1133 were provided by J. Pipas, University of Pittsburgh. They were cloned into pBR327 (a deletion derivative of pBR322) (19). A Hirt supernatant (7) containing both *dl*-1003 and wtB3 DNAs was provided by D. Nathans. *dl*-1003 DNA was isolated and cloned into pBR327 at the *Bam*HI site. The construction of *dl*-BE, which has a deletion from 0.00 to 0.14 map unit, was described elsewhere (H.-T. Wang, Ph.D. thesis, Indiana University, Indianapolis, 1984). After amplification in bacteria, the SV40 DNA was excised from the plasmid. Approximately 80% of the SV40 linear DNA was converted to form I or form II DNA with T4 DNA ligase. The DNA concentration was determined by comparing serial dilutions of mutant and wtB3 DNAs on the same gel with known quantities of lambda DNA cut with *Hind*III, which gives six fragments covering a 10-fold range in quantity for comparison. Each experiment was repeated several times with different DNA preparations.

Transfection of CV-1 cells with viral DNA. The protocol of Sompayrac and Danna was used for DNA transfection (20). CV-1 cells were seeded onto 10-cm dishes and transfected with viral DNA 24 to 48 h after they reached confluency. The cells were washed twice with serum-free DME and incubated with 6 ml of a transfection mixture containing 1 µg of SV40 DNA in DME with 0.05 M Tris hydrochloride (pH 7.3)

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FIG. 1. Physical map of viral functions and the deletion mutants used in this study relative to *Hind*III restriction sites. Top, Sequences deleted in *dl*-1003 (10), *dl*-1007 (10), *dl*-BE (Wang, Ph.D. thesis), and *dl*-1133 (15). A sequence insertion (142 base pairs [bp] of host DNA) in F8*dl* DNA is shown as a crosshatched box (21). Bottom, Coding regions for the early and late proteins. Ori, SV40 origin of replication. Since SV40 DNA is circular, the *Bam*HI ends should actually be joined. For reference, the *Eco*RI site is defined as the 0.00 map unit position, and *Bam*HI is located at 0.14 map unit.

and DEAE-dextran (200 μ g/ml) at 37°C in a CO₂ incubator for 7.5 h. For cotransfection experiments, 1 μ g of each DNA was used. After each transfection, cells were washed twice with serum-free DME and fed with DME supplemented with 5% calf serum. Approximately 10% of the cells were transfected by this procedure as determined by T-antigen immunofluorescence.

Analyses of viral DNA. At 48 h after transfection, the cells were labeled for 35 min with 300 μ Ci of [³H]thymidine (specific activity, 40 to 60 Ci/mmol) in 1 ml of DME containing 2% calf serum and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0). The [³H]thymidine was chased for 30 min with DME containing 1 μ M thymidine, 2% calf serum, and 25 mM HEPES and then with 5-bromodeoxyuridine (BUdR) chase medium (50 μ M BUdR, 20 μ M 5-fluorodeoxyuridine, 9 μ M deoxycytidine, 2% serum, and 25 mM HEPES in DME [17]). After being chased for various lengths of time, the DNA was extracted by the method of Hirt (7). For infection experiments, confluent BSC-1 cells were infected with SV40 and F8*dl* virions, each at an input multiplicity of 4 PFU per cell. Pulse-chase conditions were identical except that the experiment was initiated at 36 h postinfection and the cells were labeled for 20 min with 150 μ Ci of [³H]thymidine in 1 ml of DME. The Hirt supernatant was used to determine the kinetics of reentry and the percentage of each species of viral DNA present.

In cells transfected with only one type of DNA, form I DNA was purified on an alkaline sucrose gradient as described previously (23). Neutralized form I DNA with BUdR substitution in one strand (HL DNA) was separated from unsubstituted form I DNA (LL DNA) by equilibrium density centrifugation in CsCl (23). The percentage of ³H-labeled HL form I DNA [HL DNA cpm/(HL DNA cpm + LL DNA cpm)] was determined.

In coinfection and cotransfection experiments, Hirt supernatants were applied directly to CsCl gradients to determine the percentage of ³H-labeled HL DNA in each sample. Fractions were dripped from the bottom of the centrifuge tubes. Fractions containing HL and LL DNA were pooled separately and then dialyzed against 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA overnight to remove the CsCl. The DNA was then extracted with phenol-chloroform and precipitated with ethanol. The DNA mixture was digested

by the appropriate restriction enzyme(s) and subjected to 0.8% agarose slab gel electrophoresis to separate wild-type linear DNA from mutant linear DNA. In some experiments, different species of DNA were separated by gel electrophoresis without restriction enzyme cleavage. DNA was recovered from the agarose gel, and the radioactivity was determined (18). The percentages of wild-type (wt) and deletion mutant (dm) DNAs were determined by the following formulas: %wt = [wt cpm/(wt cpm + dm cpm)] $\times$ 100, and %dm = [dm cpm/(wt cpm + dm cpm)] $\times$ 100, respectively. The percentage of HL wild-type DNA in cotransfection experiments was obtained by the formula: %HL wt = {(total cpm of HL DNA $\times$ %wt DNA in HL)/[(total cpm of HL DNA $\times$ %wt DNA in HL) + (total cpm of LL DNA $\times$ %wt in LL)]} $\times$ 100. The percentage of HL deletion mutant DNA was obtained in a similar manner.

RESULTS

A deletion in the late region alters the reentry kinetics of viral DNA. The rate and extent to which newly synthesized SV40 DNA is used as a template for further DNA synthesis (reenters replication) can be determined by pulse-labeling the cells with [³H]thymidine and then chasing with BUdR. ³H-labeled HL DNA (DNA with BUdR substitution in one strand) represents DNA that replicated during the short ³H pulse and reentered replication during the chase with BUdR. We have previously demonstrated a temporal relationship between the removal of DNA from the pool of molecules available for replication (cessation of reentry) and the progression of this same DNA along the maturation pathway (23). If a viral protein is responsible for the cessation of reentry, then in the absence of this protein continuous reentry would be expected. Therefore, the kinetics of reentry of a large-deletion mutant, *dl*-1007, were determined. *dl*-1007 DNA was deleted from 0.83 to 0.07 map unit on the wild-type genome, completely removing the VP3 coding sequence and leaving only the N-terminal coding sequence for VP2 and the C-terminal coding sequence for VP1 (Fig. 1).

CV-1 cells were transfected with wtB3 or *dl*-1007 DNA. At 48 h posttransfection, the cultures were pulse labeled with [³H]thymidine and chased with BUdR as described

FIG. 2. Reentry kinetics of newly synthesized DNA in cells transfected with *dl*-1007 (X) or wtB3 (Δ) DNA. At 48 h posttransfection, cells were pulse labeled with [³H]thymidine and then chased with BUdR chase medium as described in the text. The trichloroacetic acid-precipitable material from a 40- μ l portion of the Hirt supernatant before the addition of BUdR contained 28,878 or 13,383 incorporated into *dl*-1007 or wtB3 DNA, respectively.

FIG. 3. Reentry kinetics of *dl*-1007 and wtB3 DNAs in a cotransfection experiment. Cells were cotransfected with *dl*-1007 and wtB3 DNAs, and at 48 h the standard pulse-chase protocol was initiated. Hirt supernatants were centrifuged in CsCl density gradients and analyzed as described in the text. Approximately 60% of the [3 H]DNA in the Hirt supernatant was *dl*-1007 DNA.

above. HL and LL DNAs were separated by CsCl density equilibrium gradient centrifugation. The percentage of HL DNA was plotted versus the duration of BUdR chase, showing the reentry kinetics of the pulse-labeled DNA into replication (Fig. 2). In cultures transfected with wtB3 DNA, reentry of wild-type DNA ceased as previously described (23). In contrast, the *dl*-1007 DNA continued to reenter replication (i.e., 3 H-labeled HL DNA continued to accumulate).

Even in the presence of wild-type structural proteins, *dl*-1007 DNA continued to reenter replication. The continuous reentry of *dl*-1007 DNA may have been due to either the missing structural protein(s) or the deleted DNA sequence. Cotransfection experiments with wild-type DNA were performed to test these alternatives. Three groups of CV-1 cells were used; two were transfected with either wtB3 or *dl*-1007 DNA, and the third was transfected with both wtB3 and *dl*-1007 DNAs. At 48 h posttransfection, the kinetics of reentry were determined. In singly transfected cells, *dl*-1007 DNA continued to reenter replication after wtB3 DNA had ceased to reenter (data not shown but similar to data in Fig. 2). In the cotransfected cells, the presence of wild-type proteins did not eliminate the continued reentry of *dl*-1007 DNA (Fig. 3).

These data suggest that *dl*-1007 is poorly complemented by wild type. To determine whether genome size (*dl*-1007 DNA was only 76% of the length of wild-type DNA) was relevant to the continued reentry, the replication of F8*dl* DNA was analyzed. This DNA lacks the genetic information from 0.17 to 0.46 map unit, which encodes the carboxyl half of the T antigen (21), and is comparable in size to *dl*-1007. It cannot replicate by itself in CV-1 cells; thus, the cells were cotransfected with F8*dl* and wt776 DNAs, and the reentry kinetics of newly synthesized DNA were followed. The reentry kinetics for F8*dl* DNA were the same as those for wild-type DNA alone (Fig. 4); that is, F8*dl* DNA was removed from the pool of molecules available for replication with the same kinetics as wild-type DNA.

In cotransfection experiments, approximately 10% of the cell population was transfected. It could therefore be argued that individual cells were not cotransfected with *dl*-1007 and wt776. Since F8*dl* DNA will only replicate in the presence of wt776, the kinetics of reentry of F8*dl* DNA indicated its presence in the same cells as wt776 and validated the cotransfection protocol. Because the amount of 3 H-labeled

F8*dl* DNA was about 30% of that of wt776 DNA, it could be argued that only 30% of the cells were cotransfected. To test this, the cells were infected with F8*dl* and wt776 virions at an input multiplicity sufficient to ensure that 90% of the cells would be coinfecting. Again, the reentry kinetics of F8*dl* and wt776 DNAs were very similar (data not shown), and even under these conditions, F8*dl* DNA only incorporated 30% as much [3 H]thymidine as did wt776 DNA.

Effect of other deletions within the *dl*-1007 deletion region on reentry kinetics. These results suggest that the continued reentry seen with *dl*-1007 DNA was not due to the size of the molecule. They suggest that there is a specific DNA sequence located between 0.83 and 0.07 map unit which is required for the removal of DNA from the pool of molecules available for replication. To localize the putative *cis*-acting element that promotes removal from the replication pool, smaller-deletion mutants were analyzed. Mutants *dl*-BE and *dl*-1133 lack 14% of the genome (from 0.00 to 0.14 map unit) and 7% of the genome (from 0.79 to 0.86 map unit), respectively (see above and Fig. 1). The reentry kinetics of these mutants were determined (Fig. 5). Less reentry was found with either *dl*-BE or *dl*-1133 DNA than with wild-type DNA (Fig. 5). However, both *dl*-BE and *dl*-1133 DNAs ceased to reenter replication at about the same time as did wild-type DNA.

A third deletion mutant lacking a sequence within this region did show continued reentry. The DNA of *dl*-1003 lacks the *Hind*III E fragment located between 0.86 and 0.95 map unit (10), partially removing the VP2 and VP3 common coding sequence (Fig. 1). In single-transfection experiments, *dl*-1003 DNA continued to reenter replication (data not shown but similar to data shown in Fig. 2 for *dl*-1007), whereas wtB3 DNA, as expected, ceased to reenter. In a typical experiment, after 8 h in BUdR chase medium, 38% of the 3 H-labeled wtB3 DNA had reentered. Over the same period, 55% of the 3 H-labeled *dl*-1003 DNA had reentered. Even in the presence of wild-type proteins in cotransfection experiments, *dl*-1003 DNA continued to reenter replication (Fig. 6), showing that *dl*-1003, like *dl*-1007, was poorly complemented by wtB3.

DISCUSSION

To determine what factors are critical for the removal of SV40 DNA from the replication pathway, we analyzed the

FIG. 4. Reentry kinetics of F8*dl* and wt776 DNAs after cotransfection. Cells were cotransfected with F8*dl* and wt776 DNAs, and at 48 h the standard pulse-chase protocol was initiated. The percentages of HL F8*dl* DNA and HL wt776 DNA were determined as described in the text. The trichloroacetic acid-precipitable material from a 40- μ l portion of the Hirt supernatant contained 5,646 cpm and 12,517 cpm incorporated into F8*dl* and wt776, respectively.

FIG. 5. Reentry kinetics of *dl*-BE and *dl*-1133 compared with those of wtB3. Cells were transfected with either *dl*-BE (A) or *dl*-1133 (B). Parallel cultures were transfected with wtB3. (A) The trichloroacetic acid-precipitable material from a 40- μ l portion of the Hirt supernatant contained 1,925 or 3,723 cpm incorporated into *dl*-BE or wtB3 DNA, respectively. (B) The trichloroacetic acid-precipitable material from a 40- μ l portion of the supernatant contained 4,774 or 14,828 cpm incorporated into *dl*-1133 or wtB3 DNA, respectively.

effect of deletion mutations on reentry kinetics. We had previously shown that newly synthesized DNA ceased to be used as a template for replication after a few hours and that this cessation correlated temporally with progression of the DNA into the maturation pathway. If this removal were, in fact, due to a viral structural protein, then reentry should continue in the absence of the protein. Therefore, we determined the reentry kinetics for a mutant (*dl*-1007) with a large deletion in the late region. This mutant DNA remained in the replication pool longer than did wild-type DNA in cells transfected in parallel (Fig. 2). Moreover, this DNA remained in the pool of molecules available for replication even in cells cotransfected with wild-type DNA (Fig. 3). In such cells, the structural proteins were available, and wild-type DNA ceased to reenter replication, whereas the deletion mutant DNA did not. These data indicate that there is an intrinsic difference between the *dl*-1007 and wild-type DNA molecules.

Analysis of a deletion of comparable size in the early region (mutant *F8dl*) showed that this difference was not merely an effect of size (Fig. 4). The fact that *F8dl*, *dl*-BE, and *dl*-1133 DNAs (74, 86, and 93% of the length of wild-type DNA, respectively) were all removed from the pool of molecules available for replication supports the argument that size per se is not the determining factor.

Since the size of the deletion was not critical to reentry and the altered reentry phenotype was not complemented by wild type, we postulate that there is a sequence present in the late side of the SV40 genome which promotes removal of DNA from the replication pathway. Continued reentry was

seen with two independent deletion mutants, *dl*-1007 and *dl*-1003 (Fig. 3 and 6). Thus, we propose that the "removal sequence" is located in the *Hind*III E fragment of SV40, the fragment deleted from both *dl*-1003 and *dl*-1007.

We have previously proposed that the fate of newly synthesized DNA is determined by whether it interacts with the proteins required for the initiation of replication (such as T antigen) or with structural proteins and by the relative abundance and affinity of these proteins for the template (Wang, Ph.D. thesis). The additional data presented here suggest that there are two independent regions where proteins interact with SV40 DNA to regulate DNA replication. The initiation proteins act at the origin (see Fig. 1), and proteins involved in removing DNA from the replication pathway act in the *Hind*III-E region. Furthermore, the results with *dl*-BE and *dl*-1133 (Fig. 5) suggest that when the removal sequence is present but other neighboring sequences are absent, the rate of removal is increased. By contrast, the deletion of DNA from a distal region (as in *F8dl*) had no effect on the reentry kinetics (Fig. 4). Thus, neighboring sequences may directly or indirectly interact with the removal sequence in the modulation of reentry kinetics.

Whether the proteins that interact with the removal sequence are viral or cellular in origin remains to be determined. At least portions of the coding sequences for VP1, VP2, and VP3, and the entire coding sequence for the agnoprotein (8), were present in all of our experiments in which removal was observed (i.e., with wtB3, *F8dl*, *dl*-1133, and *dl*-BE). Therefore, these viral products remain candidates for the removal of DNA from the replication pool.

In the past, *dl*-1007 stocks were prepared by coinfection with helper virus. Therefore, the sequence lying in the *Hind*III E fragment is not required for packaging. Because many different DNA constructions, including cellular DNAs (e.g., evolutionary variants and pseudovirions), can be packaged, this mechanism for the control of reentry appears to be nonspecific. Its only requirement appears to be that molecules must be at least 70% as large as the wild type (2, 6, 14). The removal sequence indicated by our data is required for the efficient removal of DNA from the replication pathway by a preencapsidation mechanism. Thus, with *F8dl* DNA, which is a relatively small molecule (74% of the size of wild-type DNA) but contains the removal sequence, 30% of the total extractable [3 H]DNA was *F8dl* DNA, but

FIG. 6. Reentry kinetics of *dl*-1003 (X) and wtB3 (Δ) DNAs after cotransfection. The percentages of HL *dl*-1003 DNA and HL wtB3 DNA were determined as described in the text. Approximately 80% of the trichloroacetic acid-precipitable DNA was *dl*-1003 DNA.

only 20% of the previrion-virion (maturation) complexes (23) contained F8*dl* DNA (data not shown). By contrast, in cells cotransfected with *dl*-1007 and wtB3 DNAs, approximately 60% of the [³H]thymidine was incorporated into *dl*-1007 DNA, but only 10% of the previrion-virion complexes contained *dl*-1007 DNA (data not shown). Therefore, although small size (74%) may be a slight disadvantage in packaging, the absence of the sequence deleted in *dl*-1007 confers a much greater disadvantage. This suggests that the preencapsidation mechanism, which is dependent on a specific removal sequence, facilitates entry into the maturation pathway.

An analysis of the behavior of wtB3 DNA in the presence of *dl*-1003 DNA (Fig. 6) raises the question of whether this mutant actually inhibits wtB3 replication and causes premature removal. When the number of counts incorporated into DNA during the pulse was analyzed, it appeared that wtB3 DNA in the presence of *dl*-1003 DNA incorporated only one-third of the counts it incorporated in the absence of *dl*-1003 DNA. The most straightforward explanation would be that the relative proportion of *dl*-1003 DNA molecules increases with time, since *dl*-1003 DNA is not removed from the pool of molecules available for replication as rapidly as wtB3 DNA is. Since both sets of molecules have identical origin regions, *dl*-1003 would have an advantage for the initiation machinery due to the number of molecules of it that are present. Why this inhibition of wtB3 replication is more evident with *dl*-1003 than with *dl*-1007 is unclear.

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