Prereplicative Events Involving Simian Virus 40 DNA in Permissive Cells

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Simian virus 40 DNA molecules were found to be unable to replicate for 9 h after infection, even in cells that were already replicating the DNA of preinfecting simian virus 40; after 9 h, the ability of the DNA to replicate began to rise sharply. The kinetics of activation indicated that each DNA molecule undergoes a series of slow consecutive reactions, not involving T-antigen, before it can replicate. These pre-replicative molecular transformations probably involve configurational changes; their nature and their relation to the initiation of viral DNA synthesis is discussed. Observation of the replicative behavior of one viral DNA in the presence of another was made possible by the use of two different mutants with distinguishable DNAs: a viable deletion mutant containing DNA insensitive to *TaqI* restriction enzyme was used to provide viral functions required for replication, and a *tsA* mutant with *TaqI*-sensitive DNA was introduced at various times as a probe to determine the ability of the DNA to replicate under different conditions.

When simian virus 40 (SV40) infects permissive monkey cells, the virus is transported to the nucleus and uncoated (2, 8); early transcription of the DNA takes place (19), and eventually, after the appearance of T-antigen and the cellular events it induces, the viral DNA begins to replicate. It is probable that the viral DNA binds T-antigen before it replicates, since T-antigen is required for the initiation of each round of replication (17) and has been shown in vitro to bind to SV40 DNA at the origin (18). There has been no evidence as to whether SV40 DNA participates in any other pre-replicative events. This is the subject of the present report.

We employed superinfection to examine pre-replicative events that are not normally rate determining: the first-infecting virus was used to provide T-antigen, which sets the time scale for viral DNA replication; the replicative capacity of the DNA of the second-infecting virus was then studied after the normally rate-determining events had already occurred and replication of the first viral DNA was under way. This study was made possible by the use of viruses with distinguishable DNAs: the first-infecting virus contains a *TaqI*-resistant deletion which is absent from the superinfecting virus. We found that the DNA of the superinfecting virus must undergo a series of transformations before it is able to replicate, resulting in a long lag period followed by the gradual acquisition of replicative capacity (defined as the ability to replicate when

in the presence of all factors that participate directly in the replication process).

MATERIALS AND METHODS

Viruses and infection procedure. SV40 *dl2112* (4), containing a deletion of the *TaqI* restriction site, and *tsA58* (originally isolated by P. Tegtmeyer) were used separately or in mixtures to infect growing, 50% confluent CV1-P cells (approximately 2×10^6 cells per 9-cm petri dish). The cells were grown at 37°C and infected as previously described (B. H. Rosenberg, J. F. Deutsch, and G. E. Ungers, *J. Virol. Methods*, in press). When cells were infected with the two viruses at different times, the infection procedures were identical. The time of infection was considered to be the start of the 1.5-h exposure period. Infection and all subsequent procedures were carried out at 41°C, which is nonpermissive for the *tsA58* virus. The multiplicity of infection (MOI) of the *dl* virus was in all cases 1.7 PFU per cell or higher, usually above 3; therefore, essentially all cells of the culture were infected. The MOI of the *tsA* virus was also usually above 3. MOIs were calculated from cell counts and virus titers at 41°C; the titer of *tsA58* virus at 41°C was calculated from the 32°C titer by use of a multiplication factor of 3 as determined with *dl2112* virus. Input ratios of *tsA* and *dl* viruses were calculated from the MOIs. After each infection, the cells were incubated at 41°C in fresh medium containing 5% fetal calf serum and antibiotics. Under these conditions, with growing cells, infection proceeds fairly synchronously and is complete by about 50 h after *dl* infection, when cell death becomes widespread.

Inhibition of DNA synthesis with FUDR. At 9.5 h after *dl* virus infection, fluorodeoxyuridine (FUDR) was

added to the medium at a final concentration of 10^{-4} M (0.1 ml of 0.01 M FUDR per petri dish containing 10 ml of medium). In the experiments shown in Fig. 1B, incubation at 41°C was continued for 6 h (X) or 10 h (Y). The medium containing FUDR was then replaced by fresh 41°C medium containing 5 µg of thymidine per ml. At this time the untreated control had been synthesizing viral DNA for 1 h (X) or 5 h (Y). After the removal of FUDR there was a 3-h delay, generally observed under these conditions, before DNA synthesis started in the treated cells. Thus, there was an overall delay of 4 h (X) or 8 h (Y) in the onset of viral DNA replication, as indicated in the lower part of Figure 1B. The rate of replication rose faster than normal to the maximum rate after FUDR treatment, indicating that the cells of the culture initiated replication more synchronously as a result of the increased accumulation of T-antigen that takes place when the initiation of DNA synthesis is inhibited. Controls, mock treated with FUDR and treated with thymidine, were identical to untreated cultures in all respects except that thymidine uniformly depressed the uptake of [3 H]thymidine during pulse labeling by about 50%, even though the cells were washed with 3 ml of fresh medium before labeling. Cultures treated with FUDR, however, took up nearly twice as much [3 H]thymidine as untreated cultures when pulse labeled, although the maximum rate of viral DNA synthesis was the same as in untreated control cultures (as shown by staining rather than counting the DNA). Controls treated with FUDR for only 45 min at 9.5 h after *dl* infection, too early and brief a treatment to delay viral DNA replication, behaved like the untreated control; thus, the effects shown in Fig. 1B are related to replication delay and not to FUDR per se. FUDR treatment had no effect on the ratio of *tsA* to *dl* DNA replicating after simultaneous infection.

Determination of the rate of viral DNA synthesis. Infected cultures in 9-cm petri dishes were pulse labeled for 1 h at various times by replacing the medium with 3 ml of fresh medium containing [3 H]thymidine (53 Ci/mmol) at 50 µCi/ml. After labeling, SV40 DNA was selectively extracted from the cells by the Hirt procedure (7) to yield a 0.5-ml extract, of which 25-µl portions were precipitated with trichloroacetic acid on Whatman GF/B glass filters, washed with trichloroacetic acid and counted in a toluene-based scintillation cocktail. Maximum rates of synthesis were reached at 25 to 40 h postinfection with *dl* virus, in all cases, depending on MOI; the maximum rate corresponded to approximately 100,000 cpm per h per 25 µl in all cases. Mock-infected cells, treated in the same way, gave about 2,000 cpm; all rates were corrected by subtracting these control values.

Determination of the percentage of *tsA* among replicating DNA molecules. The ratio of *tsA* to *dl* DNA among replicating molecules at various times was determined by pulse labeling the progeny and analyzing the radioactivity ratio after separating *tsA* from *dl* DNA by *TaqI* treatment and gel electrophoresis. The pulse-labeled Hirt extracts obtained for determining the rate of synthesis (see above) were made 0.025 M in EDTA and treated with 0.4 mg of pronase per ml for 1 h at 37°C. Samples were precipitated with 3 volumes of cold absolute ethanol, 1 h at -20°C, and dissolved in 100 µl of 0.01 M Tris (pH 8.2)-0.005 M EDTA. For treatment with *TaqI* restriction enzyme, NaCl was

added to a final concentration of 0.05 M, and bovine serum albumin, to 500 µg/ml; the sample was incubated for 3 min at 65°C and then made 0.01 M in MgCl₂; 2 U of *TaqI* (New England Biolabs) was added, and incubation was continued at 65°C for 15 min. The reaction was stopped by placing the mixture in ice and adding EDTA to 0.02 M. The sample (about 136 µl) was made about 8% in sucrose and layered on 1.4% agarose columns for gel electrophoresis. The electrophoresis procedure and gel staining, slicing, and counting have been described elsewhere (Rosenberg, Deutsch, and Ungers, in press). The *tsA* DNA is cleaved once by *TaqI*, producing a linear molecule (form III); *dl* DNA, in which the *TaqI* restriction site is deleted, remains circular (forms I and II). We have demonstrated that the *TaqI* assay is completely specific under the conditions used. Electrophoretic separation of these forms after *TaqI* treatment has been demonstrated elsewhere (A. Rinaldy, M. Lockhart, J. F. Deutsch, G. E. Ungers, and B. H. Rosenberg, *Mutat. Res.*, in press).

RESULTS

Growing cultures of CV1 cells were infected at zero time with a high multiplicity of SV40 containing a viable deletion (*dl*2112) as a marker and were incubated at 41°C. At various times postinfection, the cells were superinfected with a second virus containing a *tsA* mutation (*tsA*58), and the time at which each viral DNA began to replicate was determined. Replicating *tsA* and *dl* DNA were assayed (see Materials and Methods) by pulse labeling progeny DNA, extracting the mixed viral DNA, treating it with the restriction enzyme *TaqI* (to which *dl*2112 DNA is resistant, since the restriction site is deleted), separating the resulting linear (*tsA*) DNA from circular *dl* DNA by gel electrophoresis, and measuring the radioactivity to determine the relative amounts of labeled *tsA* and *dl* DNA progeny synthesized at a given time. In this system all viral functions necessary for replication were supplied by the *dl* virus, which entered the cells first; the *tsA* virus was incapable of supplying these functions at 41°C, as shown in control experiments without prior *dl* virus infection (data not shown). Thus, it was possible to study the kinetics of *tsA* DNA replication in cells at different stages of preparation for viral DNA synthesis.

When cells were infected with *tsA* virus just at the start of *dl* viral DNA replication, there was almost no *tsA* DNA replication for 9 h, although *dl* DNA replication proceeded normally. This is shown in Fig. 1A, where the rate of total viral DNA synthesis is shown at the bottom and the percentage of *tsA* molecules among all replicating DNA molecules is shown at the top. Superinfection with *tsA* took place at 11 h after *dl* infection; 9 h later (indicated by the arrow at the top of Fig. 1), *tsA* DNA replication began to rise sharply, and in time the percentage of *tsA* mole-

FIG. 1. Superinfection of SV40 *dl2112*-infected cells with SV40 *tsA58* at 41°C. The rate of total viral DNA synthesis, determined by 1-h pulse labeling (see Materials and Methods), is given as a percentage of the maximum rate measured in each case. The percentage of *tsA* DNA among all replicating DNA molecules was measured by analyzing the percentage of *tsA* progeny DNA in the pulse-labeled samples (see Materials and Methods). Infection with *dl2112* virus was at 0 time in all cases. All operations were carried out at 41°C. The arrows at the top of each part of the figure indicate 9 h after *tsA* infection. (A) Infection with *tsA58* virus (MOI = 14.4) at 11 h after *dl* infection (MOI = 9.9); about 60% of the total input virus is *tsA*. (B) Infection with *tsA* virus at 6 h after *dl* infection. Closed circles: *tsA* MOI = 72, *dl* MOI = 33; about 69% of the input virus is *tsA*. Open circles: *tsA* MOI = 3.6, *dl* MOI = 1.7; about 69% *tsA*. X, Y: Similar to open circles except infected cells were treated with FUDR (see Materials and Methods) for 6 h or 10 h, respectively, during the bracketed periods, starting 9.5 h after *dl* infection (about 5 h before the onset of viral DNA replication in untreated cells); after the removal of FUDR, there was an additional delay of 3 h in each case, before the cells were able to synthesize DNA. As a result, the onset of replication was delayed a total of 4 or 8 h, respectively (compared to lower curve with open circles), as indicated in the lower part of the figure (the complete "rate of synthesis" curves for X and Y have been omitted to avoid crowding the figure). The earliest X and Y points shown in the upper curve correspond to the beginning of replication, in each case. (C) Infection with *tsA* virus (MOI = 7.2) at 4 h after *dl* MOI = 3.3) infection; about 69% of the input virus is *tsA*. The dashed curve is an extrapolation to times preceding viral DNA replication.

cules in the replicating fraction leveled at the input percentage, about 60% in this case (*tsA* MOI = 14.4 PFU per cell, *dl* MOI = 9.9 PFU per cell). Analogous results were obtained with different MOIs (7.2 for *tsA*, 3.3 for *dl*, i.e., about 69% *tsA*). These results indicate that for the first 9 h after infection most *tsA* DNA molecules are unable to replicate, even in cells that are replicating *dl* DNA. (The low level of *tsA* DNA that appears to be able to replicate during the 9-h lag [Fig. 1A and B, upper] may be at least in part an artifact, since we sometimes observed up to 9% of the total DNA in linear form without *TaqI* treatment; this would appear to be *tsA* DNA in the *TaqI* assay used.) Since it has been demonstrated that SV40 reaches the nucleus and becomes uncoated and sensitive to enzymes by 4 to 6 h after infection (2, 8), some additional transformation must be necessary before individual DNA molecules can replicate. The sharpness of the transition from incapacity to increasing replicative capacity implies that consecutive events are involved.

The results were similar when superinfection took place earlier. In Fig. 1B, the closed circles

show that when superinfection occurred at 6 h after infection there was still no *tsA* DNA replication until 9 h after *tsA* infection, although *dl* DNA replication began before that. The open circles show a similar experiment with a considerably lower *dl* MOI (1.7 rather than 33), resulting in a later onset of viral DNA synthesis; in this case, *tsA* replication started at the same time as *dl* replication, but still at 9 h after *tsA* infection; the initial low percentage of *tsA* among replicating DNA molecules rose steadily to the input value, 69%, in both experiments. Similar results were obtained with superinfection at 6 h when a wide range of *tsA* input ratios and MOIs was used (see Table 1). The fact that the replicating ratio regularly reached the input ratio indicates that when cells are treated at separate times (under identical conditions) with two different viruses, uptake of the two viruses is identical. (This fact also confirms that, as the rate of viral DNA synthesis increases with time, the number of cells engaged in viral DNA replication also increases [as implied by the data of Graessmann et al. {6} on the rate at which cells reach the T-antigen threshold]; otherwise, mag-

TABLE 1. Superinfection with *tsA58* virus 6 h after *dl2112* virus infection at 41°C

MOI of <i>tsA</i> virus (PFU/cell)	MOI of <i>dl</i> virus (PFU/cell)	% <i>tsA</i> input ^a	% <i>tsA</i> in replicating fraction ^b		Start of <i>tsA</i> viral replication (h after <i>tsA</i> infection)
			Initial	Final	
0.66	3	18	3	16	9
7.2	3.3	69	5	70	9
28.8	3.3	90	15	90	8.5
72	33	69	10	71	9
3.6	1.7	69	7.5	78	9

^a Calculated from the MOIs of *tsA* and *dl* viruses.

^b The percent *tsA* among replicating SV40 DNA molecules was measured at the start of viral DNA replication and late in replication, as in Fig. 1.

nification of the number of *dl* templates by prior replication in most cells of the culture would prevent the replicating fraction of the late-infecting *tsA* virus from reaching the input ratio.)

With *tsA* superinfection at a still earlier time, 4 h after *dl* infection, the *tsA* DNA started to attain replicative capacity *before* either virus started to replicate (Fig. 1C). In this experiment, when replication started, 25% of the replicating DNA was *tsA*; this was more than one-third of the input (69% *tsA*). Unfortunately, it is impossible to measure directly the replicative capacity of *tsA* DNA before there is any viral DNA replication, but extrapolation of the curve (dashed line) indicates that, again, the rise in *tsA* replicative capacity started at about 9 h after *tsA* infection. Thus, the length of the lag is not related to the state of the cell with respect to viral DNA replication or to the length of exposure of the cell to *dl* virus before entry of the *tsA* virus.

To confirm that the 9-h lag was independent of viral DNA replication itself, we used FUDR to inhibit DNA synthesis in cells superinfected with *tsA* at 6 h after *dl*. Results representative of many experiments are shown in Fig. 1B (Xs and Ys). FUDR was added before the onset of replication (as measured in the untreated control [open circles, Fig. 1B]) and was later removed to produce a delay of 4 h (X) or 8 h (Y) in viral DNA replication (see lower curves, Fig. 1B). (Details of the FUDR treatment are given in Materials and Methods.) The end of the 9-h lag occurred while FUDR was present, in both cases. As can be seen in Fig. 1B, upper curve, the earliest replicating samples contained 47% *tsA* (X, at 13 h after *tsA* infection) or 60% *tsA* (Y, at 17 h), much more than the untreated control (open circles, less than 10% *tsA* at 9 h after *tsA* infection), because of the additional 4 to 8 h between *tsA* infection and replication. These results show that the replicative capacity of *tsA* DNA rises along a fixed time curve, regardless of whether viral DNA synthesis actually takes place. The upper curve is therefore independent

of replication itself and represents the kinetics with which SV40 DNA achieves the ability to replicate.

These kinetics are also independent of the MOIs of both viruses in the system, as already indicated (Table 1). The MOI of the *dl* virus, which provides T-antigen, determined the time at which viral DNA replication started and the speed with which the maximum rate of synthesis was reached, as illustrated in Fig. 1B, lower curves (open and closed circles); the MOI of the *tsA* virus had no effect on these parameters at 41°C. Neither MOI affected the maximum rate of DNA synthesis, which was the same within less than a factor of two in all these experiments and was therefore independent of the number of template molecules initially present.

To summarize, these results show that when SV40-infected cells are superinfected with a distinguishable mutant, it can be demonstrated that the DNA of the superinfecting virus is unable to replicate for 9 h, after which the replicative capacity of that DNA increases from a value near zero to a maximum which reflects the normal titer of the virus. The same pattern is followed whether or not viral DNA replication actually occurs and whether or not there is sufficient T-antigen or cellular factors present to permit replication.

In Fig. 1, parts A, B, and C show that with progressively earlier times of superinfection the rise in replicative capacity of the superinfecting *tsA* virus also shifted to earlier times; these data predict that when *tsA* infection takes place at zero time, simultaneous with *dl* infection, the replicative capacity of the *tsA* DNA will start to rise at 9 h and will still be considerably less than maximal at the start of replication (11 to 15 h after *dl* infection, in Fig. 1, depending on MOI). The same may also be true of simultaneously infecting *dl* DNA, which in this case has no more time than the *tsA* DNA to prepare for replication. If the two mutant DNAs increase in replicative capacity at the same rate, they will increase in parallel when infection is simulta-

TABLE 2. Simultaneous infection with *tsA* and *dl*2112 viruses at 41°C

% <i>tsA</i>		Time of early replication (h after <i>dl</i> infection) ^c
Among infecting viruses ^a	Among early replicating DNA molecules ^b	
30	30	12
69	68	13
90	89	12

^a Percent *tsA* virus in the inoculum, calculated from the titers of the input *tsA* and *dl* viruses. The *tsA* input was varied while the *dl* input was held constant at MOI = 3.3.

^b Replication of *tsA* viral DNA was measured early in viral DNA replication (see next column) by using gel electrophoresis to resolve pulse-labeled progeny *tsA* and *dl* DNAs after treatment with *TaqI*, as in Fig. 1 (see Materials and Methods).

^c Viral DNA replication was measured as in Fig. 1. The times shown correspond to the earliest measurable replicative activity.

neous, and when replication starts, they will replicate at the input ratio regardless of whether they have achieved full replicative capacity. Experiments with simultaneous infection of *tsA* and *dl* viruses, summarized in Table 2, confirm that the fraction of *tsA* among the earliest-replicating DNA molecules corresponds to the input ratio of *tsA* and *dl* viruses. Thus, *tsA* and *dl* DNAs behave in the same way, at least when infection is simultaneous.

DISCUSSION

The results presented here show that the DNA of superinfecting SV40 is unable to replicate until about 9 h after infection, even if the DNA of earlier-infecting SV40 is already replicating and all the necessary replication factors are therefore present. What accounts for this period of replicative incapacity? It is known that SV40 viruses reach the cell nucleus about 2 h after infection (2, 8, 16); the DNA is completely uncoated and accessible to enzymes by 4 to 6 h after infection (2, 8). This still leaves a period of several hours before the viral DNA begins to increase in replicative capacity. It is possible that superinfecting viruses are somehow delayed in transport or uncoating. However, the data show that with the same infection procedure the same amount of virus is taken up and uncoated, whether it is the first or second virus to which the cell is exposed, because ultimately the initial and superinfecting viruses replicate in the ratio of their input MOIs. Furthermore, since the results are the same regardless of the MOI of either virus and do not change with the time of superinfection, it seems unlikely that the replication lag is due to saturation of a transport or uncoating mechanism. More likely, it reflects subsequent events in which the uncoated viral DNA must participate prior to its replication—a requirement which superinfection with a distinguishable virus has enabled us to detect.

The long, flat lag period of 9 h followed by a

sharp transition to increasing replicative capacity constitutes kinetic evidence that a number of sequential reactions with low rate constants must take place before an entering DNA molecule can replicate. No single reaction, however slow, could account for these kinetics. A single autocatalytic reaction can be ruled out in view of the fact that the *tsA* kinetics were unchanged even in the presence of an excess of preinfecting *dl* molecules (Table 1).

Since similar kinetics were obtained regardless of the timing of infection with respect to the replication of other SV40 DNA molecules in the same cell, the concentrations of replication factors (other than any that may be constantly present in the cell) cannot be relevant and the reactions in question probably consist of changes in the template molecules themselves rather than of interactions with T-antigen or cellular replication factors. Two kinds of molecular change come immediately to mind: changes in the configuration or protein content of the SV40 chromosome and insertion or removal of superhelical turns in the DNA. There may also be other possibilities. A plausible sequence of reactions that could account for the observed kinetics is discussed below. The sequence involves nucleosome disruption and DNA configurational change at the origin of the chromosome, resulting in supercoiling which is then removed in a stepwise fashion to stabilize a configuration that can then bind the required number of T-antigen molecules, alone or in combination with other factors necessary for the initiation of replication.

Intracellular SV40 DNA is present, both before and during replication, in the form of nucleohistone. Loss of histones from the origin region, for example, might be a prerequisite for replication. In this regard, a rather large fraction (25%) of SV40 chromosomes isolated during replication contain a nucleosome-free region at 0.67 to 0.75 map units (9); only a small fraction

of SV40 chromosomes have a lower protein content (higher density) than normal at 4 to 6 h postinfection, just after uncoating (2). An impetus for nucleosomal protein loss might arise from the existence of alternate low-energy configurations of the origin region (15), which contains various palindromic, symmetrical, and repeated sequences (10, 13).

Sensitivity of SV40 DNA to cellular DNA swivel (nicking-rejoining) enzymes can be induced by configurational changes such as those accompanying loss of nucleosomal proteins (5, 20). A change in supercoiling brought about in this way could account for the fact that a heterogeneous, highly supercoiled form of SV40 DNA (76S in alkali) has been observed to increase in amount before the start of viral DNA replication (2). Mammalian swivel enzymes remove one positive or negative superhelical turn at a time (11); changes in supercoiling involving a number of turns would therefore require a series of consecutive reactions.

Chromosome alterations such as these could stabilize a template configuration required for the efficient binding of T-antigen or other factors necessary for the initiation of replication. Tight binding of the factor(s) might drive the overall reaction sequence to completion. Although T-antigen can bind specifically in vitro to isolated, nonsupercoiled SV40 DNA (18), the required DNA configuration may occur with much lower probability in the unaltered viral nucleoprotein. Supercoiling has been shown to play an important role in the specific binding of proteins to DNA (3, 14).

The replicative capacity of SV40 DNA starts to rise at about 9 h after infection, several hours before replication normally starts. A later event must therefore normally serve as the final trigger for replication. Graessmann et al. (6) have shown that when individual cells reach a threshold concentration of T-antigen they can start to replicate SV40 DNA (as deduced from capsid synthesis). (The concentration of T-antigen necessary to induce the cellular conditions necessary for replication is significantly lower [6].) The data (Fig. 7 of reference 6) show that there is a sharp onset of viral DNA replication with respect to T-antigen concentration, which implies that T-antigen participates in a series of consecutive reactions before replication can begin. A simple interpretation is that all three T-antigen binding sites at the SV40 DNA origin (1, 18) must be occupied to trigger replication; depending on the binding constants, this requirement could yield the normally observed relationship of viral DNA replication to T-antigen concentration. Our data indicate that this triggering event cannot take place until the template has achieved "replicative capacity," which is prob-

ably equivalent to attaining the proper configuration for T-antigen binding.

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