A block in initiation of simian virus 40 assembly results in the accumulation of minichromosomes containing an exposed regulatory region
(chromatin structure/nucleosome assembly/DNA replication/temperature-sensitive mutant)

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Communicated by Maxine Singer, December 20, 1985

ABSTRACT The initiation of simian virus 40 assembly is blocked at the nonpermissive temperature in cells infected with the viral capsid protein VP1 mutant tsC219. Greater than 95% of the minichromosomes isolated from these cells are accessible to cleavage by Bgl I and SpH I, which recognize the sequences near the viral replication origin and in the transcription enhancer elements, respectively. The accessibility of the Ori region to Bgl I is considerably reduced when virion assembly is allowed to proceed in tsC219-infected cells at the permissive temperature. A reduced accessibility to Bgl I is also observed for chromatin isolated from cells infected with wt776, the wild-type parental strain of tsC219. For wt776 chromatin, variability to Bgl I sensitivity is observed and this can be correlated to the relative virion-to-chromatin yield. A similar correlation is not apparent for restriction endonucleases that recognize sequences within the coding region of simian virus 40 chromatin. These results, considered together, indicate that, when virion assembly initiation is blocked, nucleosomes are nonrandomly arranged with respect to the viral regulatory sequences. It appears that the open regulatory region in minichromosomes is established during replication and that a protected regulatory region is generated with the onset of virion assembly.

Differences in the structure of chromatin could have profound effects on the regulation of gene expression in eukaryotic cells. Over the past decade, simian virus 40 (SV40) chromatin has served as a paradigm for examining histone-DNA interactions and the structure-function relationship of chromatin. In lytically infected cells, the SV40 DNA is folded by the cellular core histones into nucleosomes during replication to form a minichromosome or SV40 chromatin (1, 2). The minichromosomes serve as templates for expression of the "early" and "late" genes. These genes code for the large tumor (T) antigen, which is required for the initiation of SV40 replication, and for the virion capsid proteins VP1, VP2, and VP3 (reviewed in ref. 3).

Numerous studies have shown that, in lytically infected cells, a population of the minichromosomes is assembled into a specific structure that contains an open region hypersensitive to cleavage by endonucleases (4–7). The nuclease-hypersensitive region includes the replication origin, the transcription promoters and initiation sites of both the early and the late genes, and two copies of the 72-base-pair (bp) transcription enhancer element (4–7). The involvement of DNA replication in establishment of the SV40 open regulatory region has been an intriguing possibility (4, 8) but a controversial one (9, 10). A question related to this controversy has been why only a small fraction of the viral chromatin exhibits this structural discontinuity (9, 10).

To investigate the role of the virion assembly process in the production of SV40 chromatin exhibiting a protected regulatory region, we have examined the restriction endonucleolytic cleavage patterns of minichromosomes isolated from cells infected with the wild-type virus wt776 and with the temperature-sensitive mutant tsC219. A proline-to-serine substitution in VP1 (11) renders tsC219 temperature-sensitive in virus assembly (12, 13); the initiation of virus assembly is blocked in cells incubated at 40°C but not in those incubated at 33°C (12, 13). The studies presented here show that minichromosomes containing an open region accumulate when virus assembly is blocked in tsC219-infected cells. In addition, studies on the structure of chromatin assembled in tsC219-infected cells at 33°C, and on stability of wild-type virions, have shown that the minichromosomes that contain a protected regulatory region either arise at the onset of virion assembly or are the product of virion disassociation.

MATERIALS AND METHODS

Cells, Virus, and Infection. BSC-1 cells were adapted for growth at 40°C and infected at 37°C with wt776 or tsC219 at about 50 plaque-forming units per cell (14). Twelve hours after infection, the cells were shifted to 40°C or 33°C or were allowed to remain at 37°C. They were labeled with 50 μCi of [3H]thymidine (1 Ci = 37 GBq) per plate, approximately 32 hr after infection at the respective temperatures. The cells were harvested 16 hr after labeling and concentrated for nucleoprotein complex isolation as described (14).

Chromatin Isolation and Digestion. The harvested cells were gently Dounce homogenized in buffer B (a hypotonic buffer composed of 10 mM Heps (pH 7.8), 5 mM KCl, and 0.5 mM MgCl2) or in an isotonic buffer (buffer B containing NaCl). After the addition of phenylmethylsulfonyl fluoride, the SV40 nucleoprotein complexes were allowed to diffuse from nuclei by gentle shaking of the homogenate at 4°C. Subsequently, the cellular chromatin was pelleted and the supernatant was fractionated in sucrose gradients [5–31.5% sucrose made up in 0.05 M Tris (pH 7.5)] using a Beckman SW41 rotor (14). Sucrose gradient fractions corresponding to the “light” and “heavy” regions of the 75S chromatin peak were pooled, adjusted to 30 mM Tris, pH 7.5/6 mM MgCl2/5 mM 2-mercaptoethanol/50 mM NaCl and then digested at 33°C for 30 min in the presence of excess restriction endonuclease. As an internal control, exogenous unlabeled plasmid DNA (0.5 μg) was added to the digestion mixtures to ensure completeness of the reaction.

Gel Electrophoresis and Fluorography. Samples were concentrated by ethanol precipitation and then suspended in TE buffer (10 mM Tris-Cl, pH 8.0/0.1 mM EDTA) containing 1% NaDodSO4. Immediately prior to electrophoresis in a 1% agarose gel, a 1:40 dilution of 32P-labeled SV40 DNA was added to each sample.

Abbreviation: SV40, simian virus 40.
agarose gel, the samples were heated at 45°C for 20 min to
deproteinize the DNA. Electrophoresis was carried out as
described (12). The gels were first stained with ethidium
bromide, to examine the digestion pattern of the controls, and
then processed for fluorography (12). The extent of chromatin
cleavage was determined by calculating the percentage of
linear DNA detected in densitometric scans of underexposed
fluorograms.

RESULTS

SV40 Assembly in tsC219-Infected Cells. Previous studies
have indicated that both the 75S chromatin and the 220S
virions accumulate at 33°C in cells infected with tsC219 (13,
15). Virion assembly is drastically reduced in mutant-infected
cells at 37°C and is totally blocked at 40°C (12, 15). This block
in assembly results in the accumulation of minichromosomes
that contain a full complement of the core histones but only a
trace amount, if any, of the capsid proteins (12, 13).

Restriction Endonuclease Digestion Pattern of tsC219 Chromatin. A map of SV40 DNA depicting the locations of the
coding and regulatory sequences and of the cleavage sites of
the restriction enzymes used to examine the structure of
tsC219 chromatin as a function of the extent of virion
assembly is shown in Fig. 1. The minichromosomes were
isolated 48 hr after infection from cells continuously labeled
with [3H]thymidine for 16 hr at 40°C, 37°C, or 33°C. The
chromatin isolation procedure involved the sedimentation of
total cellular extracts, including the cytoplasmic and nuclear
fractions, in sucrose gradients to minimize examination of a
preselected nuclear fraction (refs. 12 and 14 and Materials
and Methods). In a typical experiment, the light- and heavy-
sedimenting chromatin of the 75S peak were pooled and digested separately at 33°C with saturating amounts of various restriction enzymes. After digestion, the DNA was freed from proteins and analyzed by electrophoresis and fluorography. The results of such experiments showed that >95% of the tsC219 chromatin assembled at 40°C was cut by
Bgl I in the Ori region (Fig. 2A). In contrast only 50% of the
minichromosomes isolated from cells labeled at 33°C were
accessible to Bgl I (Fig. 2B and Table 1). These results show that the region spanning the replication origin and the early

transcription initiation sites of tsC219 chromatin are highly
exposed when the initiation of virion assembly is blocked.
The enhancer-specific restriction endonuclease Sph I cleaved
98% of the tsC219 chromatin assembled at 40°C and 83% of
the chromatin produced at 35°C (Fig. 2). In contrast, within
experimental error, comparable amounts of digestion were
observed when tsC219 chromatin prepared from cells labeled
at different temperatures was probed with enzymes that
cleave the coding sequences. For example, BamHI digested
only 8–20% of tsC219 chromosomes assembled at 33°C, 37°C,

Table 1. Restriction enzyme digestion analysis of tsC219 chromatin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>At 33°C</th>
<th>At 37°C</th>
<th>At 40°C</th>
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<tbody>
<tr>
<td></td>
<td>Heavy</td>
<td>Light</td>
<td>Heavy</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BamHI</td>
<td>8</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>BglI</td>
<td>50</td>
<td>40</td>
<td>64</td>
</tr>
<tr>
<td>MspI</td>
<td>35</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>SphI</td>
<td>78</td>
<td>87</td>
<td>89</td>
</tr>
<tr>
<td>AccI</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>EcoRI</td>
<td>ND</td>
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Relative amounts of linear DNA with respect to total DNA were
estimated from densitometric tracings of underexposed fluorograms
of gels obtained in experiments conducted as in Fig. 2. Also shown
are the results of digestion experiments conducted with tsC219
chromatin isolated from cells labeled at the semipermissive tempera-
ture (37°C). The results shown are means of results obtained from
several independent experiments. The range of error was ±7%. ND,
not determined.

Fig. 1. Map of SV40 DNA. The locations of the SV40 coding and
regulatory sequences and of the cleavage sites of the restriction
enzymes used for experiments reported in this work are indicated.

Fig. 2. Restriction enzyme digestion analysis of tsC219 chroma-
tin assembled in cells at the nonpermissive (A) and permissive (B)
temperatures. The chromatin was prepared using a hypotonic buffer.
The sucrose gradient fractions spanning the light (L) and heavy (H)
sides of the 75S chromatin peak were pooled and digested separately
in buffer containing an unlabeled plasmid, included to monitor reaction
completeness. The digestion products were analyzed by agarose gel
electrophoresis. The DNA bands corresponding to
chromatin were detected by fluorography. Similar results were obtained in reactions carried out in the absence of unlabeled control
DNA (data not shown). N, L, and SC, mobilities of nicked circular,
linear, and supercoiled SV40 DNAs, respectively.
or 40°C (Fig. 2 and Table 1). Surprisingly, all minichromosomes were highly resistant to cleavage by Acc I (Table 1).

Restriction Endonuclease Digestion Pattern of wt776 Chromatin. Published reports indicate a wide range of SV40 chromatin accessibility (25–90%) to Bgl I. A range of 25–75% cleavage has been reported for wt776 chromatin (16–18). Depending on the isolation procedure, 30–50% cleavage has been observed for the chromatin from the wild-type strain wt800 (9, 10), and minichromosomes of the 777 strain are almost completely cleaved by Bgl I (4). With these apparently conflicting results in mind, we examined the restriction enzyme cleavage patterns of chromatin from cells infected with wt776, the parental strain of tsC219 (19).

For the experiment presented in Fig. 3, three sets of cell cultures were infected with wt776. The first set was incubated at 40°C and the other two were incubated at 37°C. All three sets were labeled at the respective temperatures and harvested 48 hr after infection as above. We used a hypotonic buffer, buffer B, to extract the chromatin from cells labeled at 40°C and from one set of cells labeled at 37°C. An isotonic buffer was used to extract the chromatin from the other set of cells labeled at 37°C. The chromatin fractions were purified by sucrose gradient centrifugation, pooled, and digested as above. Qualitatively, our results agree with the observations of Tack et al. (10); we found that hypotonic and isotonic extraction procedures yield minichromosomes having different degrees of sensitivity to Bgl I (Fig. 3). For example, only 16–30% of the chromatin was cleaved if the isolation buffer contained NaCl (Fig. 3C). In contrast, an unexpectedly high fraction of the chromatin extracted in the hypotonic buffer was cleaved by Bgl I (Fig. 3A and B). Close examination of the sucrose gradient profiles of these chromatin preparations showed that inclusion of NaCl in the extraction buffer resulted in the selective dissociation of a large fraction of the 220S virions, yielding virion-derived chromatin that also sedimented at 75S (data not shown).

From several independent experiments, we have observed that there is a correlation between the dissociation of virions, or intermediates in virus assembly, and accessibility of the isolated 75S chromatin to Bgl I. We have observed that the wild-type minichromosomes, which show a very high accessibility to Bgl I, are obtained from preparations in which virion/chromatin ratios are >4:1 (Fig. 4). We have pursued this unexpected finding by examining the correlation between virion/chromatin ratios and the extent of wt776 chromatin cleavage by other restriction enzymes. Qualitatively, as observed for Bgl I, the sensitivity of wt776 chromatin to Sph I depends on the virion/chromatin ratio (Fig. 4). In contrast, within the limitations of experimental error, a lower sensitivity to differences in the virion/chromatin ratio was observed for Acc I, EcoRI, and BamHI, enzymes that recognize sequences within the coding region (Fig. 4).

**DISCUSSION**

Electron microscopic and micrococcal nuclease digestion analyses have shown that, in eukaryotic cells, almost all genes transcribed by RNA polymerase II are packaged into nucleosomes (2). However, appropriate restriction endonucleases and DNase I cleave preferentially at the 5' termini of genes that are or have been transcriptionally active (2, 8).

Numerous hypotheses have been proposed to explain the molecular basis of these so-called hypersensitive sites. For example, these sites could arise from (i) the preferential interaction of transcription factors with selected DNA sequences, resulting in the exclusion of histones from these sites via a competition mechanism; (ii) possible selectivity of the core histones for the DNA sequences that flank the

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**Fig. 3.** Effects of temperature and isolation buffer on the restriction enzyme digestion pattern of wt776 chromatin. (A) Cells infected with wt776 were labeled at 40°C, and the minichromosomes were isolated using a hypotonic buffer. (B) The minichromosomes were prepared using a hypotonic buffer from wt776-infected cells labeled at 37°C. (C) The minichromosomes were prepared from cells labeled at 37°C using an isotonic buffer. H and L, sucrose gradient fractions spanning the heavy and light sides of the 75S chromatin peak, respectively. The digestions were carried out as described in the legend of Fig. 2.

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**Fig. 4.** Correlation between restriction enzyme accessibility of wild-type 75S chromatin and virion/chromatin ratios. This figure summarizes the results of several digestion experiments with wt776 chromatin as described in Fig. 3. The virion/chromatin ratios were determined from sucrose gradient sedimentation profiles for each preparation and are plotted vs. the percentage of linear DNA observed after digestion of the chromatin with the respective restriction enzymes. The presentation of the data was simplified by taking an average of cleavage extents observed for heavy and light chromatin.
regulatory region (nucleosome positioning); and/or (iii) the regular arrangement of nucleosomes with respect to a reference point, such as a replication origin.

The possible function of the SV40 replication origin (Ori) in establishing the open region in minichromosomes has been a controversial issue and the subject of numerous studies (see refs. 4, 9, and 10). This function was first proposed by Weintraub (8) based on the observation that 90% of the minichromosomes, isolated from cells infected with the 777 strain, are accessible to cleavage by Bgl I (4). Based on studies conducted on wt800 chromatin, DePamphilis and coworkers (9, 10) challenged this result. They found that the sensitivity of wt800 minichromosomes to Bgl I cleavage depended on the buffer used for chromatin isolation: 50% cleavage for minichromosomes extracted in a hypotonic buffer and only 25% for those isolated in an isotonic buffer (9, 10). These observations, along with their previous results on micrococcal nuclease digestion analysis (20), led DePamphilis and coworkers to conclude that nucleosomes are not phased with respect to the Ori region and are deposited randomly on SV40 DNA during replication (9, 10).

Our studies on wt776 strain have shown that the inclusion of salt in the extraction buffer results in disappearance of the 220S virion peak with a concomitant increase of the 75S chromatin peak. Such virion instability has been observed previously to occur at salt concentrations higher than 0.2 M and was attributed to mediation by unknown nuclear factors (21). Nevertheless, our salt-instability result indicates that the increased fraction of wt776 chromatin resistant to cleavage by Bgl I is probably derived from the dissociated virion and, thus, does not correspond to transcriptionally active or newly replicated chromatin. Consistent with this, we have found that there is a direct correlation between the accessibility of wt776 chromatin to Bgl I vs. the virion/chromatin ratio observed for a given preparation (Fig. 4). Such an unexpected correlation was also observed to a lesser extent for SpH I but it was not apparent for enzymes whose recognition sequences lie within the coding regions. Even though Varshavsky et al. (4) used an isotonic buffer in their 777 chromatin isolation, they observed high accessibility to Bgl I (4). This could be explained by the previous finding that 75S SV40 is a linear virus. It contains an in-phase deletion in the gene encoding VP1 near the EcoRI site (22) and may thus be defective in the initiation or propagation of virion assembly.

Our studies on SV40 assembly have shown that at 40°C, tsC219 chromatin is not packaged by the temperature-sensitive VP1 and accumulates as a stable 75S nucleoprotein complex (12, 13). This 75S chromatin contains a full complement of the core histones but only a trace amount, if any, of the capsid proteins (12, 13). In contrast, appreciable amounts of the capsid proteins co sediment with wt776 chromatin (23) and with C219 chromatin assembled at the permissive temperature (33°C) (unpublished observations). Our restriction endonuclease digestion analyses indicate that >95% of the tsC219 chromatin assembled at 40°C is accessible to cleavage by Bgl I (Fig. 2A). This accessibility is reduced in wild-type chromatin (Fig. 4) and to ≈50% for tsC219 chromatin isolated from cells incubated at 33°C (Fig. 2B).

The results of our comparative restriction enzyme digestion analysis allow us to address the following two issues: (i) the nonrandom placement of nucleosomes with respect to the Ori region and (ii) the effect of virus assembly on the structure of SV40 chromatin. The finding that >95% of tsC219 chromatin formed at 40°C is cleaved by Bgl I indicates that nucleosomes are assembled nonrandomly with respect to the replication origin when virion assembly initiation is blocked. Nonrandom arrangement of nucleosomes has been observed to occur in cellular chromatin: around the centromeres of yeast chromosomes (24), near the telomers of Oxytricha macronuclei (25), and between the origins of replication of the DNA encoding rRNA (rDNA) in Tetrahymena (26). In each of these examples, the binding of specific proteins to the regulatory sequences of DNA seems to provide a boundary condition for nucleosome assembly (25). In the case of SV40, the large tumor (T) antigen, other replication factors, and/or transcription factors are obvious candidates for directing nucleosome assembly with respect to the Ori region. Such a mechanism is further supported by the finding that a DNase I-hypersensitive site is created when the 72-base-pair enhancer segment is translocated to other sites in the SV40 genome (27). It appears, however, that DNA replication must also be involved since the DNase I cleavage pattern of a transfected SV40 DNA differs between nonreplicating and postreplicated chromatin (28).

Our data do not exclude the possibility that nonrandom distribution of nucleosomes with respect to the Ori region could be mediated by selectivity of histones for certain sequences in DNA. The various possibilities presented here are not mutually exclusive and may, in fact, cooperate to create a chromatin structure required for transcription and replication.

The viral chromatin that is used in encapsidation is drawn from the same pool of molecules available for replication and transcription. Therefore, it is plausible that the capsid proteins interact with the minichromosomes to channel them along the virion assembly pathway (13). Coca-Prados et al. (29) have observed that the intermediates in SV40 (180S) assembly (the previrions) possess a longer nucleosome repeat length than the 75S chromatin (211 ± 14 bp as opposed to 196 ± 8) and suggested that the addition of capsid proteins could alter the spacing of nucleosomes in minichromosomes. Consistent with this idea, we have recently discovered that the tsC minichromosomes, blocked in the initiation of virus assembly, possess a considerably shorter nucleosome repeat length (177 ± 4 bp) as compared to that of the wt776 chromatin or to the tsC219 chromatin assembled at the permissive temperature (30). It appears that the major capsid protein VP1, directly or through specific interactions with other viral proteins, is involved in changing the arrangement of nucleosomes on SV40 DNA, possibly via a "nucleosome sliding" mechanism. The sliding of nucleosomes toward the regulatory region may function in repressing the transcription of the viral genes to initiate virion assembly. It is plausible that a similar nucleosome sliding mechanism, possibly mediated by histone H1, could operate in cellular chromatin to repress gene activity.

We thank Arnold Stein for helpful discussions and Christine Gobble for typing this manuscript. This work was supported by Research Grant DCB-830-1944 from the National Science Foundation.