Deletion Mutants Which Affect the Nuclease-Sensitive Site in Simian Virus 40 Chromatin

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A short segment of simian virus 40 (SV40) chromatin on the late side of the origin of replication is hypersensitive to nuclease cleavage. The role of DNA sequence information in this nuclease-sensitive feature was examined by constructing deletion mutations in this region. Deletions were introduced into the inserted segment of *in(Or)*-1411 (a viable, partially duplicated variant of SV40), and nuclease sensitivity of the inserted segment was compared with that of the unaltered sequences in their normal location in the viral genome. Extended deletions (118 to 161 base pairs) essentially abolished nuclease sensitivity within the inserted segment. Shorter deletions (21 to 52 base pairs) at separate locations retained the nuclease-sensitive feature. In some short-deletion mutants nuclease susceptibility was substantially reduced. We conclude that more than one genetic element in this region contributes to the organization of the nuclease-sensitive feature and that the SV40 72-base repeat is not, in itself, sufficient signal for this feature.

Viral chromatin extracted from nuclei of simian virus 40 (SV40)-infected cells is structurally similar to cellular chromatin (1, 4, 7, 15). Most of the viral genome is packaged into nucleosomes; however, in a substantial subfraction of chromatin molecules, a short region of the viral DNA, including the origin of replication and adjacent sequences, is preferentially exposed to endonuclease attack (17, 24-26). Although a role in viral gene transcription has yet to be demonstrated, this structure may be analogous to DNase I-hypersensitive sites which have been reported near the 5' end of many transcriptionally active genes (5).

Since the nuclease-sensitive feature has a specific location on the viral genome, we expected that *cis*-acting genetic elements could be identified which are responsible for the occurrence of this structure. We have shown (29) that such elements must be located near the origin of DNA replication (*ori* site) but that replication from that *ori* site is not required for expression of the nuclease-sensitive feature. DNA sequences some distance from the *ori* site also contribute to the nuclease susceptibility. We have sought to delineate *cis*-acting elements by the use of deletion mutants. Introduction of deletions into a partially duplicated SV40 variant allowed us to make a quantitative comparison between the nuclease sensitivity of the segment carrying the deletion and the unaltered copy elsewhere on the viral genome. Some deletions essentially removed the nuclease-sensitive feature. Other

mutants retained this feature, but the degree of nuclease susceptibility was reduced. Although the results did not allow us to exclude the possibility that a single short DNA segment was essential for expression of this feature, deletions of sequences at well-separated locations strongly influenced the degree of nuclease sensitivity, suggesting that multiple genetic elements contribute to this chromatin structure.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (BSC-1) were propagated in minimal essential medium (Earle salts) supplemented with 8% newborn calf serum (Biocell Laboratories, Inc.), 2% fetal calf serum (K C Biological), and 40 μ g of gentamicin (Schering Corp.) per ml at 37°C in an atmosphere of 5% CO₂. The nondefective constructed duplication mutant, *in(Or)*-1411 (18), was grown from a stock provided by T. Shenk.

Isolation of deletion mutations in *in(Or)*-1411. Nuclear extract (17) from *in(Or)*-1411-infected BSC-1 cells was incubated for 30 min at 37°C in the presence of 3 mM MnCl₂. Enzymatic cleavage was stopped with sodium dodecyl sulfate (0.5%) and EDTA (10 mM). DNA was isolated by phenol extraction and concentrated against 1-butanol. Full-length linear DNA molecules were separated by electrophoresis in 1.4% agarose, detected by ethidium bromide stain, and recovered by dissolution of the gel in 6 M KI-0.01 M sodium phosphate (pH 7.0), followed by chromatography on hydroxylapatite (17).

BSC-1 cell monolayers were transfected with this DNA by the DEAE-dextran technique (14). Resulting

viral plaques [designated *in(Or)*-1411-*dl1*, -*dl2*, etc.] were isolated and purified by replaques.

Enzymatic digestion of viral nucleoproteins and analysis of resulting DNA fragments. Confluent monolayer cultures of BSC-1 cells were infected with virus (multiplicity of infection = 0.4) in minimal essential medium (Earle salts) containing 2% fetal calf serum and gentamicin. At 22 h postinfection, $^{32}\text{P}_i$ (carrier-free; ICN Chemical and Radioisotope Div.) was added (20 $\mu\text{Ci}/\text{ml}$ in 15 ml of phosphate-free medium per 150-mm culture dish). At 42 h postinfection, nuclear extracts were prepared by a Triton X-100-EDTA procedure as previously described (17).

Cleavage of viral chromatin by endogenous endonuclease and isolation of full-length linear viral DNA were carried out as described above except that incubation with MnCl_2 was for 20 min and electrophoretic bands were detected by autoradiography. After dialysis against 15 mM NaCl-1.5 mM sodium citrate (pH 7.0), the initial cleavage site was mapped by redigestion with either *EcoRI* or *KpnI*. Autoradiography was performed on dried gels with Kodak XRP-1 film. Films were scanned with a Zeineh scanning densitometer (LKB Instruments), and film densities over segments corresponding to specific DNA fragment size ranges were quantitated by integration. Specific cutting frequencies for segments I and II (F_I and F_{II}) were calculated as previously described (29).

Restriction enzymes were obtained from New England Biolabs except for *EcoRI* which was purchased from Miles Laboratories, Inc. Digestion conditions were those suggested by the supplier.

DNA sequence analysis. Mutant viral DNA was digested with a mixture of *BamHI* and *BclI* or *BamHI* and *HinfI*. The appropriate double-digest fragment was isolated by preparative electrophoresis on a polyacrylamide slab gel and subjected to further digestion with *HaeIII*, *AluI*, *PvuII*, *BstNI*, *DdeI*, or *SphI*. The 5'-terminal phosphate residues were removed with bacterial alkaline phosphatase (BAPC; Worthington Biochemical Corp.) before end labeling with [γ - ^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp.) in the presence of T4 polynucleotide kinase (P-L Biochemicals, Inc.). The fragment containing the deletion was purified by polyacrylamide gel electrophoresis, and one 5'-labeled terminus was removed by digestion with *BstNI*, *DdeI*, *PvuII*, *HaeIII*, *BglI*, or *BclI* before subjecting the isolated fragment to the degradative reactions described by Maxam and Gilbert (13).

RESULTS

Construction and mapping of deletion mutants.

To direct deletions to the nuclease-sensitive regions, nuclear extracts from *in(Or)*-1411-infected cells were incubated with MnCl_2 , allowing endogenous nuclease to cleave within the nuclease-sensitive regions. Full-length linear DNA was isolated and used to transfect BSC-1 cell monolayers, and viral plaques were recovered. We anticipated that this approach would favor recovery of deletions within the inserted segment since sequences in that segment are nonessential for virus production (18). Sequences adjacent to the initial cleavage site are

deleted, presumably due to cellular processes during recircularization of the viral DNA molecules (12).

Viral plaques were screened by sizing the smaller fragment produced by double digestion with *BamHI* and *BclI*. Of 234 plaque isolates, 39 were altered in this segment. Seven of these were selected for further characterization. Mutant DNAs were mapped with *BglI* and *BstNI* and by the S1 nuclease heteroduplex mapping procedure (19). In all cases, restriction enzyme analysis and heteroduplex mapping gave patterns identical to that of *in(Or)*-1411 DNA except for deletion of sequences within the inserted segment. The results are shown graphically in Fig. 1. Figure 2 shows DNA sequence analysis of these mutants.

Distribution of nuclease-sensitive sites in mutant chromatin. Endonuclease which is endogenous to BSC-1 cell nuclei was used to cleave ^{32}P -labeled chromatin to a limited extent. Full-length linear DNA was isolated and redigested with a single-cut restriction enzyme (illustrated in Fig. 3). Results of experiments with *in(Or)*-1411 and deletion mutant chromatin are shown in Fig. 4.

Consistent with previous data on double-origin mutants (29), variant *in(Or)*-1411 is preferentially cut within each of the duplicated segments. In each case, deletion mutant chromatin displays nuclease sensitivity in segment I which is comparable to the parent.

in(Or)-1411-*dl12*, -*dl14*, and -*dl46* show little

FIG. 1. Map of *in(Or)*-1411 and deletion mutants derived from *in(Or)*-1411. The physical map of *in(Or)*-1411 is from Shenk (18). Stippled regions correspond to duplicated segments (designated I and II for later reference). Wedge-shaped extensions from the circle indicate deleted sequences in *in(Or)*-1411 by comparison with wild-type SV40. Numbering on the expanded section of the map is in base pairs from the center of the 27-bp palindrome, with positive values in the late direction with respect to the inserted *ori* site. The limits of the inserted segment (-58 to +214) are indicated by (-). Deletion mutants are shown at the right by open bars.

or no preferential cleavage in region II. A faint band visible in each channel may represent a remnant of the nuclease-sensitive feature, but it is clear that these mutants are highly defective in their ability to organize this chromatin structure. Mutants *dl9*, *dl10*, *dl16*, and *dl42* retain the characteristic nuclease sensitivity in region II.

The frequency of cleavage in this region is substantially reduced in *dl9* and *dl10* by comparison with the parent. Locations of the preferentially nuclease-sensitive regions were confirmed by restriction enzyme mapping of the linear DNA with *KpnI* (gels not shown).

The specific cutting frequency within each

FIG. 2. Nucleotide sequence of deletion mutant DNAs. (A) Four representative gels showing sequence determination. Nucleotide residues at the boundaries of deletions are noted by numbers that correspond to numbering used in (B). (B) Nucleotide sequence of the inserted segment in *in(Or)-1411* is shown together with sequences immediately adjacent to the insert. The positions of joints formed by the insertion are indicated by arrowheads. Numbering is from the center of the 27-bp palindrome as in Fig. 1. "Early" and "late" directions are shown with respect to the inserted segment. "Site 1," "site 2," and "site 3" refer to binding sites for SV40 T-antigen (23). The 21-bp repeated sequences and the 72-bp segment, which is repeated in wild-type SV40 but not in either segment I or segment II of *in(Or)-1411*, are shown by boxes. The Goldberg-Hogness sequence is indicated by a darker box. DNA structures for all of the mutants are shown diagrammatically with boundaries and sizes of the deletions included. The insertion of 1 bp is also indicated in *dl9*.

FIG. 3. Expected regions of nuclease sensitivity in *in(Or)*-1411 and the size distribution of DNA fragment clusters that would be produced by secondary cleavage with *Eco*RI. Duplicated segments are indicated by the stippled regions. Predicted regions of nuclease sensitivity correspond to segments extending 300 bp in the late direction from each *Bgl*II site (indicated by cross-hatched sectors extending from the circle). kb, Kilobase.

segment of the genome was quantitated by scanning the autoradiogram with a densitometer. Table 1 gives the specific cutting frequencies (corrected for a background assumed to be due to random cleavage) obtained for segments I and II in the mutants. Calculations were based on scans of the autoradiograms shown in Fig. 4 and are typical of results obtained.

DISCUSSION

The 273-base pair (bp) segment inserted in *in(Or)*-1411 contains *cis*-acting genetic information sufficient to form a nuclease-sensitive region in viral chromatin. This nondefective variant provides an opportunity to determine which DNA sequences are necessary and sufficient for the occurrence of the unique nucleoprotein structure, since the insert (segment II) can be altered, leaving segment I to perform functions vital to viral infection.

We have obtained deletion mutants which show little or no nuclease sensitivity in segment II while nuclease sensitivity in segment I remains unchanged. These mutants identify sequences critical to the nuclease-sensitive feature which cannot act at a distance of 2.5 kilobases [the distance between segments I and II in *in(Or)*-1411]. The most extensive deletions we

FIG. 4. *Eco*RI digests of linear DNA produced by the action of endogenous endonuclease on chromatin from *in(Or)*-1411 and deletion mutants. 32 P-labeled linear DNA was isolated after cleavage of viral chromatin with endogenous endonuclease as described in the text and redigested with *Eco*RI before electrophoresis on 1.4% agarose. Size markers (M) consist of a mixture of double-digestion products of circular *dl*14 DNA (A) or circular *dl*46 DNA (B) by *Bgl*II and *Eco*RI, *Bam*HI and *Eco*RI, and *Bcl*II and *Eco*RI. I and II refer to predicted nuclease-sensitive segments indicated in Fig. 3. kb, Kilobase.

TABLE 1. Comparison of specific cleavage frequencies within regions I and II for *in(Or)*-1411 and deletion mutant chromatin^a

Mutant	F _I	F _{II}	F _I /F _{II} ratio
1411	0.22	0.18	1.2
<i>dl9</i>	0.22	0.07	3.1
<i>dl10</i>	0.31	0.05	6.2
<i>dl12</i>	0.30	0.03	10
<i>dl14</i>	0.32	0.02	16
1411	0.21	0.16	1.3
<i>dl16</i>	0.24	0.14	1.7
<i>dl42</i>	0.22	0.11	2.0
<i>dl46</i>	0.31	<0.02	>16

^a The fraction of the total chromatin population specifically cleaved in segment I (F_I) or in segment II (F_{II}) was determined by scanning autoradiograms with a densitometer (see the text). Data in the first five lines correspond to the experiment shown in Fig. 4A; data in the last four lines correspond to the experiment shown in Fig. 4B.

have studied (*dl12*, *dl14*, *dl46*) are in this category, and their effect on chromatin structure may be related to their size. The shortest deletion of this type (*dl12*) lacks sequences associated with the *ori* site (9, 20, 22), tumor antigen (T-antigen) binding sites 2 and 3 (23), the Goldberg-Hogness sequence (6), and all copies of the 21-bp repeated sequence (21).

Several other mutants we have studied retain well-defined nuclease-sensitive sites in segment II (as well as in segment I), indicating that sequences deleted in these mutants are not necessary for the nuclease-sensitive feature. Genetic information in this category includes much of the *ori* site and T-antigen binding site 2 (*dl42*) and at least one copy of the 21-bp repeat (*dl16*). Mutants *dl9* and *dl10* define sequences which are also included in this category. Taken together, these latter two mutations delete all of the 72-bp segment although neither alone removes that entire sequence. Sequences on the early side of nucleotide -58 and on the late side of nucleotide +214 (Fig. 2) are likewise not required for the nuclease-sensitive feature. From our previous work (29) we know that a segment lacking sequences on the late side of nucleotide +179 (Fig. 2) retains the nuclease-sensitive feature.

If there is a single DNA segment which accounts for the strong effect of *dl12*, we can narrow down its location by excluding those sequences deleted in mutants which overlap *dl12* but which do not have such a large effect. Such a segment must lie between nucleotides +8 and +82 (sequences deleted in *dl12*, but not in *dl10*, *dl16*, or *dl42*). DNA sequences which have not yet been eliminated in this manner include the Goldberg-Hogness box, T-antigen binding site 3, and one copy of the 21-bp repeat. Additional

short-deletion mutants must be characterized to determine if such a genetic element can be identified. The 72-bp segment (+107 to +178), which is present as a tandem repeat in wild-type SV40 but is not duplicated in either segment of *in(Or)*-1411, is not affected in *dl12*. Therefore the presence of this information is not sufficient for the occurrence of the nuclease-sensitive feature.

It is possible that no single sequence will be found which is essential. Individual genetic elements, each capable of organizing a nuclease-sensitive site, could occur at separate locations, and the preferential cleavage seen in this region may be a summation of their individual contributions. All major elements of this type must be disrupted in *dl12*. Shorter-deletion mutants which reduce nuclease sensitivity to intermediate levels may affect only a subset of such elements. Since the number of mutants analyzed so far is small, general conclusions regarding sequences required for nuclease sensitivity are limited. The F_I/F_{II} ratio for all of the mutants studied is higher than it is for parental *in(Or)*-1411 chromatin. This could mean that all of the sequences play a greater or lesser role in determining nuclease sensitivity (however, the significance of this effect in *dl16* and *dl42* is open to question). Also, there is a general correlation between the size of a deletion and its effect upon nuclease sensitivity. This would be expected if many sequence elements played independent roles. Of the mutants described in this report, only *dl9* has effects which cannot be explained in this manner since this deletion lies wholly outside the DNA segment deleted in *dl12*.

Within the nuclease-sensitive region, discrete subregions of nuclease sensitivity can be distinguished (16, 17). This pattern of cleavage sites may reflect independent genetic organizers for nucleoprotein structure of the type described above, or it may be due to the overall nucleoprotein configuration. High-resolution mapping of cleavage sites in segment II in our mutants should provide information about these possibilities.

Contributions of sequences in the neighborhood of the 72-bp segment to the nuclease-sensitive chromatin structure are of particular interest. The recent finding (2, 8) that the 72-bp segment is required for early gene transcription *in vivo* has led to speculation that this sequence may be necessary for formation of a chromatin configuration required for early gene transcription. All but 10 bp of the 72-bp segment are deleted in *dl10*. Segment II of chromatin formed by this mutant retains a well-defined nuclease-sensitive site which is cleaved at reduced frequency. The result suggests that the 72-bp segment plays a role in determining the degree of nuclease sensitivity but does not provide pri-

mary determinants for this nucleoprotein structure. We have previously shown (29) that cleavage of viral chromatin by *Bgl*I is influenced by sequences greater than 230 bp in the late direction from the *Bgl*I site. Preliminary results with *dI9* (Gerard and Scott, unpublished data) indicate that reduced nuclease sensitivity in segment II of this mutant is also detected with *Bgl*I, suggesting that the effect of this mutation extends throughout the nuclease-sensitive region.

The possibility should be considered that deletions may have effects on chromatin structure because they change the spatial arrangement of DNA sequence elements rather than affecting the elements themselves. For example, accessibility of DNA segments to nuclease or to binding proteins may depend on the position of these sequences in a nucleoprotein structure, and this, in turn, may depend on the length of DNA separating genetic elements which organize the nucleoprotein structure. These ideas have been developed in more detail by Weintraub (28). Evaluation of this possibility will require construction of additional deletion and insertion mutants.

The nuclease-sensitive chromatin structure correlates with a gap in the nucleosome pattern seen with the electron microscope (10, 16). Nucleosomes which are allowed to assemble in vitro show some selectivity for the DNA sequences where they form (3, 27). Wasylyk et al. (27) have shown that a region in SV40 DNA extending about 400 bp in the late direction from the *ori* site is unfavorable for in vitro nucleosome formation. This is the same region which shows a gap in the nucleosome pattern in viral chromatin isolated from infected cells. Genetic elements which we have postulated above to explain the effects of deletion mutants may be the same DNA segments which prevent in vitro nucleosome formation. Kornberg (11) has suggested that binding sites for nonhistone proteins spaced at 166-bp intervals could prevent formation of nucleosomes along the entire DNA segment between such sites. To eliminate nucleosome formation over the 300-bp nuclease-sensitive region in SV40, three such sites would be required.

The role of the nuclease-sensitive feature of SV40 chromatin in viral infection is not yet understood. We currently favor the idea that this structure is relevant to viral gene expression. The region of nuclease sensitivity spans both the early and late promoters, and the 75S subfraction of intracellular viral nucleoprotein which contains species active in replication and transcription contains most of the molecules which display the nuclease-sensitive feature (W. A. Scott and J. P. Hartmann, manuscript in preparation). Elucidation of the role of specific DNA

sequences in the formation of this structure should provide insight into its biological role.

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