Origin of DNA replication in papovavirus chromatin is recognized by endogenous endonuclease

(simian virus 40 and polyoma virus nucleoprotein complexes/gel electrophoresis/restriction endonuclease digestion/physical maps)

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ABSTRACT Isolated simian virus 40 (SV40) and polyoma nucleoprotein complexes contain endonuclease that, under in vitro conditions, converts part (up to 30%) of the covalently closed superhelical DNA to full-length linear rods. The positions of the cleavage sites within the genomes of SV40 and polyoma were determined by digestion with various single-cut restriction endonucleases and subsequent agarose gel electrophoresis of the cleavage products. Both SV40 and polyoma covalently closed superhelical DNA were cleaved open at their respective origins of DNA replication (±75 base pairs). The full-length linear DNA rods whose ends map adjacent to the origin of DNA replication could also be isolated by sodium dodecyl sulfate/phenol extraction both from SV40-infected permissive cells and from purified SV40 virions. These data reveal the presence of a unique structure of the papovavirus chromatin close to the initiation site of DNA replication.

Isolated papovavirus chromatin has been used as a simple model for the study of the fine structure of chromosomes (1–5). A close similarity between the structural organizations of viral and eukaryotic chromatins was revealed with the main feature being that beaded globular structures termed “nucleosomes” (6), which consist of cellular histones (5, 7) are aligned along the DNA filaments on a small number of distinct alternative positions (4).

Viral chromatin also has been successfully used to determine the in vitro factors and conditions that are required for DNA replication. The availability of soluble nucleoprotein complexes derived from the nuclei of simian virus 40 (SV40)-infected cells (8) has fostered such studies (8, 9). A number of enzymes such as α and γ DNA polymerases (10, 11) and RNA polymerase (12) are associated with papovavirus nucleoprotein complexes. As will be shown in this report, there is, in addition, an endonuclease activity present that introduces one double-strand break into the viral DNA. In the case of both SV40 and polyoma DNA, the position that is susceptible to the endonuclease activity within the chromatin was shown to be located at the origin of replication. This finding has revealed a specific structure of the papovavirus chromatin in a biologically important region of the genome.

MATERIALS AND METHODS

Virus and Cells. SV40 strain Rh 911 was grown on CV-1 cells as described (13). Propagation of polyoma virus and infection of 3T6 cells were carried out as described (14). For isolation of nucleoprotein complexes the cells were infected at a multiplicity of 10 plaque-forming units per cell.

Preparation of Nucleoprotein Complexes. Nuclei were isolated from SV40-infected CV-1 cells (10^6 cells in a 15-cm petri dish, 38 hr after infection) and from polyoma-infected 3T6 cells (2×10^7 cells in a 15-cm petri dish, 26 hr after infection) as described (14). For preparation of nuclear extracts as initially described by Su and De Pamphilis (8) the nuclei were suspended in 2 ml of modified hypotonic extraction buffer (10 mM Hepes, pH 8.0/1 mM MgCl₂/0.5 mM CaCl₂/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride). Nucleoprotein complexes were extracted by incubating the mixture for 1 hr at 0°C (in ice) with occasional agitation. The nuclei were removed from the supernate, which was stored at 0°C, by centrifugation for 5 min at 8000 × g, resuspended in 1 ml of extraction buffer, and eluted again for 1 hr, as described above. After removal of the nuclei by centrifugation for 5 min at 8000 × g, the supernates containing the nucleoprotein complexes were pooled and stored at −70°C.

Isolation of DNA from Nucleoprotein Complexes. The nucleoprotein complex preparations were mixed with an equal volume of 1.2% sodium dodecyl sulfate containing 10 mM EDTA, and the DNA was extracted by addition of buffer-saturated phenol/chloroform, 1:1 (vol/vol). The aqueous phase was adjusted to 1 M NaCl and the DNA was precipitated by addition of 2 vol of unadenated ethanol. After pelleting, the DNA was suspended in 20 mM Tris, pH 7.5, and incubated at 37°C for 30 min with RNase (10 μg/ml) that previously had been heated for 15 min at 85°C.

Preparation of Radiolabeled Viral DNA. ³H-Labeled DNA from infected cells was prepared as described (13).

Purification of SV40 Virion DNA. CV-1 cells in roller vessels were infected with SV40 strain Rh 911 at a multiplicity of 0.1 plaque-forming unit per cell. One week after infection the culture medium was adjusted to pH 9.0 with NaHCO₃ and then the cells were frozen and thawed three times. The cell debris was pelleted by centrifugation in a Christ–Heraeus Minifuge at 2000 rpm for 15 min. The supernate was adjusted to 0.5 M NaCl and polyethylene glycol was added to 10% (wt/vol). The suspension was incubated at 4°C overnight, after which the polyethylene glycol was pelleted by centrifugation at 3000 rpm for 15 min in a Christ–Heraeus IV KS centrifuge. One-half milliliter of the pellet was layered on top of a CsCl gradient (1.34 g/cm³) in 10 mM Tris, pH 7.5) for band equilibrium centrifugation in a Beckman SW 65 rotor at 4°C and 40,000 rpm for 48 hr. The virions banding at a density of 1.34 g/cm³ were harvested and dialyzed against 2000 vol of 20 mM Tris, pH 7.5. After a 15-min treatment at 37°C with 1% sodium dodecyl sulfate/20 mM Tris/10 mM EDTA, the viral DNA was extracted with buffered phenol.

Abbreviations: SV40, simian virus 40; nick, single-strand break; FOI DNA, covalently closed superhelical DNA; FOII DNA, double-stranded circular DNA containing a nick in one of the strands; FOIII DNA, double-stranded linear rods of unit length.

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Purification of Intracellular SV40 Double-Stranded Linear DNA Rods of Unit Length (FOIII DNA). Isolation of intracellular SV40 FOIII DNA was performed as detailed (18). Briefly, SV40 DNA was selectively extracted and purified in a CsCl/ethidium bromide gradient. The DNA contained in the light band of the gradient (80 µg) was electrophoresed in a 1.4% agarose gel (1.0 X 9 X 0.3 cm) for 90 min at 100 V. After staining of the DNA within the gel with ethidium bromide a photograph was taken and the section containing the FOIII DNA was excised. The DNA was recovered from the agarose as described (18).

Agarose Gel Electrophoresis. The DNA was analyzed by electrophoresis in vertical 1% or 1.4% agarose (SeaKem, MCI Biomedical, Rockland, ME) gels as described (15). After electrophoresis the gels were stained in electrophoresis buffer containing ethidium bromide at 0.5 µg/ml for 10 min and photographed under UV light using Polaroid film type 667.

Blotting. Denaturation and transfer of DNA from the slab gels to nitrocellulose sheets (Schleicher and Schuell, BA 85) were performed essentially as described by Southern (16).

Hybridization with 32P-labeled nick-translated DNA (17) was carried out for 24 hr at 65°C. After hybridization, the filters were washed extensively in 75 mM NaCl/7.5 mM sodium citrate, pH 7.0, at 65°C and dried overnight at 37°C. Filters were overlaid with Kodak Superspeed film for 2 days.

RESULTS

FOIII DNA Is Generated In Vitro in Nucleoprotein Complexes. Isolated polyoma and SV40 nucleoprotein complexes contained covalently closed superhelical DNA (FOI DNA) and some double-stranded circular DNA containing a nick in one of the strands (FOII DNA) but no detectable FOIII DNA (Fig. 1, lanes a and d). However, as early as 2 min after incubation in extraction buffer, linear FOIII DNA could be detected (Fig. 1, lanes b and c). As indicated in Fig. 2, the relative amount of SV40 FOIII DNA increased with time and appeared to reach a plateau by 30 min of incubation. These kinetics indicate that FOIII is generated rather rapidly and that only a fraction of the viral DNA molecules, not exceeding 30%, can be rendered linear by introduction of a double-strand break. The simultaneous presence of FOI, FOII, and FOIII DNA in nucleoprotein complexes that had been incubated at 37°C in vitro is shown in Fig. 1, which also indicates that the major product of the reaction is FOII DNA.

The appearance of FOIII DNA in the presence of FOI DNA is incompatible with the assumption that the conversion of FOI DNA to FOIII DNA might occur by introduction of random nicks into both strands of the DNA, leading eventually to a double-strand break. If so, one would expect first an increasing amount of FOII DNA concomitant with a decrease of FOI DNA. Only after complete conversion of FOI DNA to FOII DNA should linear FOIII DNA appear if random nicking by nuclease were driving the reaction.

Essentially the same kinetics of conversion of FOI DNA to FOIII DNA were noticed when polyoma nucleoprotein complexes were incubated at 37°C (data not shown).

Determination of Cleavage Site. The above data suggest a mechanism that cleaves open FOI DNA by a double-strand break. To locate the position of opening on the viral genome, FOIII DNA was generated by incubation of the nucleoprotein complexes for 30 min at 37°C followed by DNA extraction with phenol. Then it was digested with single-cut restriction endonucleases to assess whether two discrete cleavage products were formed. The isolated SV40 DNA (FOI, FOII, and FOIII) which served as a substrate for single-cut restriction endonucleases is shown in Fig. 3, lane a. Digestion of an aliquot of this DNA with BamHI generated FOIII DNA by introduction of one double-strand break into circular FOI and FOII DNA at map position 0.17 and, most interestingly, two fragments (see arrows) which must have arisen from specific cleavage of the FOIII DNA. As estimated from a comparison with the size markers (HindIII SV40 DNA fragments shown in lane d), the size of these fragments is 55.5 and 44.5% of the length of SV40 DNA. Another single-cut enzyme that cleaves SV40 DNA at map position 0.67 coincident with the origin of DNA replication (18), Bgl I, generated only FOIII DNA. Based upon these data, the site of opening of SV40 DNA in chromatin can be assigned to the origin of DNA replication at map position 0.67.

Investigation of FOII DNA isolated from polyoma chromatin with single-cut restriction endonucleases revealed essentially the same results as those seen with SV40. When digested with BglI, which cleaves the polyoma DNA at map position 0.58 (19), two fragments were formed whose molecular weights were 88 and 12% of the length of polyoma DNA (Fig. 3, lane f). The larger fragment is indicated by the arrow (both in the stained gel and in the autoradiograph in the lower part of the figure); the smaller fragment cannot be visualized, probably because of its small size. Furthermore, EcoRI, which cleaves polyoma DNA at map position 0 (20) generates, besides FOIII DNA, two fragments (arrows in Fig. 3, lane g). They were 71 and 29% of the length of polyoma DNA.

FIG. 1. Agarose slab gel electrophoresis of viral DNA isolated from nucleoprotein complexes. The nucleoprotein complexes were isolated from polyoma-infected 3T6 cells and incubated in the extraction buffer for 10 min either at 0°C (lane a) or at 37°C (lane b). SV40 nucleoprotein complexes isolated from CV-1 cells were incubated in the extraction buffer for 10 min either at 37°C (lane c) or at 0°C (lane d). Electrophoresis of the purified DNA was carried out for either 2 hr (polyoma DNA) or 5 hr (SV40 DNA). Direction of electrophoresis was from top to bottom.

FIG. 2. Kinetics of appearance of SV40 FOIII DNA in nucleoprotein complexes at 37°C in vitro. Nucleoprotein complexes were incubated at 37°C in extraction buffer. At the indicated periods of time, 20-µl aliquots were withdrawn and, after phenol-extraction, the DNA was analyzed in ethidium bromide-stained agarose slab gels. From photographs (under UV light), densitometer tracings were obtained (Joyce–Loebl densitometer) from which the relative amount of FOIII DNA was calculated.

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These data indicate that the position at which FOIII DNA was cleaved by endonuclease is the origin of polyoma DNA replication at map position 0.71 (20, 21).

Specifically Cleaved SV40 FOIII DNA Is Present in Productively Infected Cells. We have previously shown the presence of FOIII DNA in SV40-infected cells (13). To test whether this population contains molecules whose ends map at or adjacent to the origin of SV40 DNA replication, we selectively isolated [as detailed recently (13)] the FOIII DNA from productively infected cells by dye/buoyant density gradient centrifugation and by preparative agarose gel electrophoresis. The purified FOIII DNA was subjected to digestion with various single-cut restriction enzymes and the resulting cleavage products were electrophoresed into agarose and then detected by blot hybridization with nick-translated SV40 [32P]DNA. When treated with Bgl I, the FOIII DNA substrate remained unaltered and no cleavage products could be discerned (Fig. 4). Two cleavage products were obtained after EcoRII treatment, with molecular weights of 2.5 × 10^6 and 1.1 × 10^6. The larger product formed a diffuse band, suggesting that the opening had occurred in a short region rather than within a specific site. This notion is corroborated by the data obtained after Hpa II cleavage which also generated a diffuse band slightly smaller than unit length. The resulting small product is not revealed, probably because it migrated out of the gel. Finally, BamHI digestion produced two fragments, 2 × 10^6 and 1.6 × 10^6 daltons.

These results show the presence of SV40 FOIII DNA molecules in infected cells whose ends terminate either at or in close proximity to the origin of DNA replication.

Specifically Cleaved SV40 FOIII DNA Isolated from Virion Particles. The presence of FOIII DNA in productively infected cells renders it likely that such molecules might become encapsidated during the process of SV40 virus maturation. When analyzed by agarose gel electrophoresis, there indeed was some FOIII DNA revealed, usually not exceeding 20% of the total virion DNA (Fig. 5). Digestion of the purified virion DNA with single-cut restriction endonucleases according to the protocols described in Figs. 3 and 4 showed that the virion FOIII DNA consisted of molecules whose ends mapped close to the position of the origin of DNA replication at map position 0.69–0.70 (Fig. 6).

DISCUSSION

The covalently closed circular DNA of SV40 and polyoma viruses is cleaved open in vitro to full-length linear rods by an enzymatic reaction that is carried out by an endonuclease endogenous to isolated viral chromatin. The precise nature of the endonuclease is unclear and awaits further characterization. It is not only bound to chromatin but appears, in addition, in free form in nuclear extracts. Protein-free viral DNA is cleaved open by both the soluble and the chromatin-associated endonuclease in a random fashion (data not shown here). In contrast, we have shown in this report that the cleavage takes place in viral chromatin either at or closely adjacent to the respective origins of DNA replication. The assignment of the region where the viral DNA is cleaved to a specific map position was ac-

FIG. 3. Agarose slab gel electrophoresis of single-cut restriction endonuclease digestion products of viral DNA isolated from nucleoprotein complexes. SV40 (lanes a–d) and polyoma (lanes e–h) nucleoprotein complex preparations were incubated for 30 min at 37°C in extraction buffer. Then the DNA was purified, digested to completion with restriction endonucleases that introduce one double-strand break and analyzed by agarose slab gel electrophoresis. Lanes: a, undigested aliquot of the SV40 FOI, FOII, and FOIII DNA; b, after digestion with Bam HI; c, after digestion with Bgl I; d, Hind III-generated SV40 DNA fragments obtained after cleavage of FOI DNA, coelectrophoresed as size markers; e, polyoma FOI and FOII DNA from nucleoprotein complexes incubated at 0°C; f, Bam HI-digested polyoma DNA; g, EcoRII-cleaved polyoma DNA; h, an undigested aliquot of g preparation. (Lower Right) Autoradiograph of the polyoma DNA in the gel which had been transferred after denaturation to a nitrocellulose filter and hybridized with nick-translated [32P]DNA. The fragments resulting from cleavage of FOIII DNA with single-cut endonucleases are pointed out by the arrows. Direction of electrophoresis was from top to bottom.

FIG. 4. Autoradiograph of single-cut restriction endonuclease digestion products of intracellular SV40 FOIII DNA. From productively infected CV-1 cells, the SV40 FOIII DNA was selectively isolated as described (13) and subjected to agarose slab gel electrophoresis after complete digestion with Bgl I (lanes a), EcoRII (lane b), Hpa II (lane c), and BamHI (lane d). After electrophoresis the DNA was denatured, transferred to a nitrocellulose filter, and hybridized with nick-translated SV40 [32P]DNA. The molecular weights of the fragments were estimated by comparison of their relative mobilities with the distances migrated by authentic SV40 DNA restriction fragments of known molecular weights in the same gel (not shown here). Direction of electrophoresis was from top to bottom.
FIG. 5. Agarose slab gel electrophoresis pattern of SV40 virion DNA. CV-1 cells were infected with SV40 (10 plaque-forming units per cell) and labeled with [3H]thymidine (5 μCi/ml) in roller production vessels (100 ml of medium) for 5 days, starting 1 day after infection. Then the virions were purified and the virion DNA was subjected to agarose slab gel electrophoresis. After electrophoresis the gel was sliced into 1-mm fractions and the radioactivity was determined. The positions of FOI, FOII, and FOIII DNA coincided with the positions of partial EcoRI digestion products of SV40 DNA in the same gel (not shown here). Direction of electrophoresis was from left to right.

The nucleotide sequences of SV40 and polyoma share limited similarities at the origin of DNA replication (ref. 22; B. E. Griffin, personal communication). One might invoke, therefore, hitherto unknown cellular restriction endonuclease-like enzymes that cleave open the viral DNA at this position by recognizing the same sequences. That protein-free viral DNA is randomly cleaved by the endonuclease does not, in our opinion, rigorously preclude the existence of such enzymes, because specific cleavage might be masked by the preponderance of randomly attacking endonucleases. Nevertheless, structural particularities of the viral chromatin must play a role, because FOIII DNA is generated in not more than 30% of the nucleoprotein complexes. Our observation extends the previously reported findings that a protein is linked to SV40 DNA at the origin of DNA replication in molecules bearing a single-strand nick at this position (23).

The presence in virions of FOIII DNA molecules whose ends map in the same region raises the question of whether an endonuclease might be encapsidated and become activated during the processes of virus purification and DNA isolation. Because we have also found such FOIII DNA in productively infected cells, it is equally possible that they might be encapsidated already as linear rods of unit length.

Both SV40 and polyoma viruses replicate according to the same mechanism along covalently closed parental DNA template strands (20, 24–27) where replication proceeds bidirectionally at an equal pace. It is conceivable that the chromatin structure at or closely adjacent to the origins of DNA replication might have unique properties such that the DNA is rendered accessible to endonuclease in replicating complexes. However, this is not compelling because the relative amount of molecules undergoing replication late in infection is thought not to exceed about 3% of the superhelical SV40 DNA (28). In contrast, we estimate that not more than 30% of the nucleoprotein complexes are capable of yielding FOIII DNA upon in vitro incubation. One might conceive, therefore, that other important biological properties of this region of the genome may account for the structural particularities of the chromatin such as transcriptional activities that are likely to be initiated here close to the leader sequences (29, 31).

Note Added in Proof. While this paper was in press we learned that similar evidence regarding the structure of the SV40 chromatin at the origin of DNA replication was obtained by Scott and Wigmore (32).

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