Volume 5 Number 10 October 1978 Nucleic Acids Research

SV40 viral minichromosome: preferential exposure of the origin of replication as probed by restriction endonucleases

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Received 10 August 1978

ABSTRACT

Isolated SV40 minichromosomes [1-3] were treated with different single-cut restriction endonucleases to probe the arrangement of nucleosomes in relation to the SV40 DNA sequence. While Eco RI and Bam HI each cut 22-27% of the SV40 minichromosomes under limit-digest conditions, Bgl I, which cuts SV40 DNA at or very near the origin of replication [4,5], cleaves 90-95% of the minichromosomes in a preparation. Similar results were obtained with minichromosomes which had been fixed with formaldehyde before endonuclease treatment. One possible interpretation of these findings is that the arrangement of nucleosomes in the compact SV40 minichromosomes is nonrandom at least with regard to sequences near the origin of DNA replication.

INTRODUCTION

Virions of papovaviruses, in particular those of polyoma and SV40, have been shown to contain histones as internal core proteins [6-9]. In nuclei of lytically infected cells viral DNA is associated with cellular histones in a chromatin-like structure called a minichromosome which is replicated and specifically transcribed via cellular enzymatic machinery [1-3, 10-12]. A subunit (nucleosomal) organization of the cellular chromatin which is based on specific repeating patterns of histone-histone and histone-DNA interactions (see refs. 13, 14 for reviews) is also characteristic of viral minichromosomes [15-18]. The question as to whether nucleosomes are deposited on DNA at random or whether there is a specific relation(s) between repeating units (nucleosomes) formed by histones and base sequences in DNA has been approached previously in a number of ways [17, 19-23]. Although the results of some of these studies suggested that nucleosomal arrangement in the minichromosome is not completely random [22,23], the data did not permit one to arrive at any specific interpretation. In the present work we show that the origin of SV40 DNA replication in the isolated compact minichromosomes is highly exposed in comparison with other sites elsewhere in the SV40 genome as probed by single-cut restriction endonucleases.

MATERIALS AND METHODS

Isolation of SV40 minichromosomes. Plaque-purified SV40 virus (strain 777, a gift from Dr. P. Sharp) was grown on monolayers of green monkey kidney cells (CV-1 line) as described previously [1,16]. The cells were labeled with [Me-^{3}H]thymidine (20-56 Ci/mmole; New England Nuclear) 30 hr after infection (30 μCi per ml of the medium). At 40 hr after infection the cells were washed twice with 0.14 M NaCl, 1 mM Tris-HCl, pH 6.8 (pH of all buffers was measured at 20-22°C). To each 350-cm^2 roller bottle (from 2 to 4 in different experiments) 8 ml of 0.25% Triton X-100, 10 mM Na-EDTA, 10 mM triethanolamine (TEA)-HCl, pH 6.8, were added. Phenylmethylsulphonyl fluoride (PMSF; Sigma; 0.5 M solution in absolute ethanol) was then added to a final concentration of 1 mM followed by incubation for 10 min at ∿4°C. Thereafter 1 M NaCl was added to a final concentration of 0.12 M. The lysate obtained was gently scraped with a rubber policeman and centrifuged at 5,000 g for 5 min at 3°C. The nuclear pellet obtained from 5-10x10^7 cells was briefly resuspended in 30 ml of 0.25% Triton X-100, 0.12 M NaCl, 10 mM Na-EDTA, 1 mM PMSF (freshly added), 10 mM TEA-HCl, pH 6.8, and then centrifuged at 5,000 g for 5 min. The pellet was resuspended for extraction of the SV40 minichromosomes in 3-4 ml of 0.25% Triton X-100, 0.12 M NaCl, 10 mM Na-EDTA, 0.1 mM PMSF, 10 mM TEA-HCl, pH 8.0, and the suspension subjected to continuous gentle stirring for 3 hr at ∿4°C. The suspension was then centrifuged at 5,000 g for 15 min and the supernatant layered onto three 36 ml linear sucrose gradients (5-25%) in 0.10 M NaCl, 1 mM EDTA, 10 mM TEA-HCl, pH 7.5. The samples were centrifuged in the SW27 rotor (Beckman) at 15,000 rpm for 17 hr at 3°C. Appropriate fractions (see Fig. 1) were pooled and immediately used for the next experimental stage.

Digestion of unfixed minichromosomes with restriction endonucleases. Digestions were carried out directly in fractions from preparative sucrose gradients (Fig. 1). The final composition of the digestion buffer (buffer A, 15-20% sucrose, 0.10 M NaCl, 1 mM Na-EDTA, 8 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF, 10 mM TEA-HCl, pH 7.5) was produced by addition of appropriate volumes of 50 mM $MgCl_2$, 0.2 M DTT and 0.5 M PMSF to fractions from sucrose gradients (Fig. 1) shortly before digestion. Restriction endonucleases used in this work (Eco RI, Bam HI and Bgl I) were purchased from New England Biolabs. The amount of restriction endonuclease used for digestion of minichromosome samples was approximately 20-fold in excess over that required to completely digest an equal quantity of purified SV40 DNA under identical digestion conditions. Digestions were carried out by addition of a restriction endonuclease to the minichromosomes in buffer A followed by incubation at 37°C

for 20 min. The reaction was stopped by addition of 100 mM Na-EDTA, pH 7.5, to a final EDTA concentration of 15 mM. Purified total yeast tRNA (Boehringer) was then added to a final tRNA concentration of 10 µg/ml followed by addition of 2.9 volumes of 95% ethanol and incubation overnight at -20°C. The samples were centrifuged at 15,000 g for 15 min, the pellets were air-dried, dissolved in an SDS-containing sample buffer and subjected to gel electrophoresis in the presence of SDS (see below). Purified SV40 DNA (specific radioactivity 1-4x10^5 cpm/µg) was obtained from isolated SV40 minichromosomes by gel chromatography on Sepharose 2B in the presence of SDS. The void volume-fractions were pooled, precipitated with ethanol and the precipitate was washed with 70% ethanol, 60 mM NaCl. Treatment of SV40 DNA with restriction endonucleases was carried out in buffer A with a 5-fold excess of an endonuclease over the minimum amount required for a complete digestion under conditions used (37°C, 20 min). An admixture of the unlabeled 28S rRNA (present in 60S ribosomal subunits partially cosedimenting with compact SV40 minichromosomes in a sucrose gradient) did not interfere with either endonuclease treatments of DNA and minichromosomes or gel electrophoresis of DNA.

Fixation of SV40 minichromosomes with formaldehyde. Fractions from a sucrose gradient (Fig. 1) were made 1% in HCHO by addition of 11% HCHO, 0.10 M NaCl, 1 mM Na-EDTA, 10 mM TEA-HCl, pH 7.5 (pH of the initial HCHO solution was adjusted with NaOH). The samples were incubated at ∿4°C for 30-60 hr followed by dialysis against ∿500 volumes of 0.10 M NaCl, 1 mM Na-EDTA, 10 mM TEA-HCl, pH 7.5, for 30-40 hr at ∿4°C with two changes of the buffer.

Digestion of fixed SV40 minichromosomes with restriction endonucleases. Digestions were carried out in buffer A under conditions described above for unfixed minichromosomes. After termination of the digestion with Na-EDTA, 20% SDS was added to a final SDS concentration of 0.2% followed by incubation of the samples at ambient temperature for two days to allow inactivation of PMSF to occur [24]. Thereafter nuclease-free pronase (Calbiochem; 10 mg/ml in 10 mM TEA-HCl, pH 7.5) was added to a final pronase concentration of 30 µg/ml followed by incubation at 37°C for 2 hr. Then total yeast tRNA was added to a final tRNA concentration of 10 µg/ml followed by ethanol precipitation, low-speed centrifugation and addition of an SDS-containing electrophoretic buffer to the pellet as described for the unfixed minichromosomes. In control experiments the purified SV40 DNA was treated with HCHO followed by removal of HCHO and treatment with restriction endonucleases and pronase as described above.

Gel electrophoresis of DNA. Nuclease-digested, ethanol-precipitated minichromosomes (see above) were dissolved in 20% sucrose, 1% SDS, 0.005%

Bromphenol Blue, 1 mM Na-EDTA, 1 mM TEA-HCl, pH 7.5, heated at 55°C for 15 min and then subjected to agarose gel electrophoresis in the presence of SDS. Free proteins in SDS-containing samples did not interfere with either electrophoresis of DNA or fluorographic detection of ^{3}H-DNA bands. Electrophoresis was carried out at 1-2 V/Cm in 15 cm long, 2.5 mm thick slab gels containing 2% agarose (BioRad, ultrapure), 0.1% SDS, 1 mM Na-EDTA, 5 mM CH_3COONa, 40 mM Tris-HCl, pH 7.5. The contents of both upper and lower compartments were stirred during electrophoresis and the buffer was recirculated between the compartments. After electrophoresis the gel was soaked in a few successive changes of CH_3OH, then in 4% PPO in CH_3OH for 2 hr followed by removal of CH_3OH by immersion in H_2O for 1 hr followed by immersion in 4% glycerol in H_2O for 1 hr and drying of the gel under reduced pressure. Dried gels were exposed at -70°C to presensitized Kodak X-Omat R films [25]. Quantitation of the DNA electrophoretic patterns was carried out by scintillation counting of ethidium-stained DNA bands excised under UV illumination (for staining with ethidium bromide the gels were washed in a few successive changes of 1 mM Na-EDTA, pH 7.5, for 1.5 hr to remove SDS). Agarose slices were dissolved directly in scintillation vials by heating at 90°C in 1 ml of 1 N HCl followed by addition of 11 ml of Aquasol-2 (NEN) and counting. Measurements of percentages of linear SV40 DNA III in various digests by densitometry of ethidium-stained DNA electrophoretic patterns gave results indistinguishable from those obtained by scintillation counting of excised ^{3}H-DNA bands.

RESULTS AND DISCUSSION

Figure 1 shows a sedimentation profile of the [^{3}H]thymidine-labeled compact SV40 minichromosomes (see also refs. 1-3). Peak fractions were pooled, then digested with single-cut restriction endonucleases Eco RI, Bam HI or Bgl I as described in Methods; the DNA products of digestion were fractionated by agarose gel electrophoresis (Fig. 2). Measurements of the percentage of linear SV40 DNA III in the digests were carried out by scintillation counting of excised ethidium-stained ^{3}H-DNA bands (see Methods). The measurements show that while Eco RI and Bam HI each cut 22-27% of the SV40 minichromosomes under conditions of a limit-digest, the corresponding figure for Bgl I is 90-95% for the same preparation of minichromosomes (Figs. 2 and 3). To verify whether these values correspond to limit-digest conditions, we varied the quantity of enzyme (Fig. 2q; cf. Fig. 2r) and measured the kinetics of digestion for all three enzymes (Fig. 2 t-w shows the results for Eco RI). These controls show that the chosen conditions of digestion (see Methods) indeed correspond to

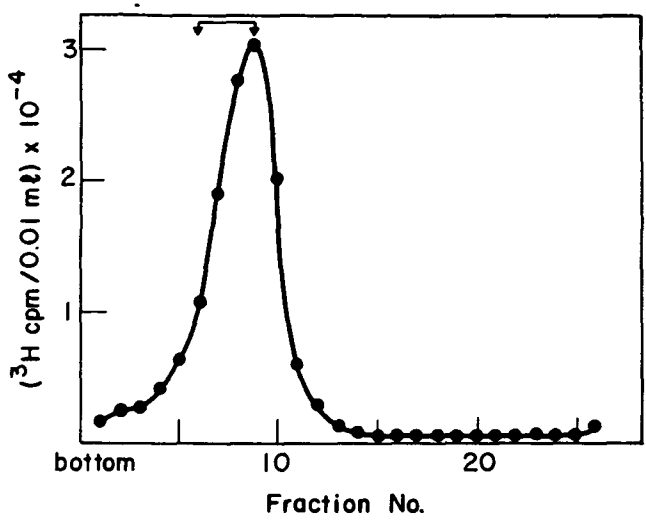

Fig. 1. Preparative sucrose gradient centrifugation of SV40 minichromosomes. See Methods for details. Fractions indicated by arrows were used for endonuclease treatments.

limit-digest conditions for all three enzymes.

Minichromosomes aggregate upon addition of $MgCl_2$ to EDTA-containing sucrose gradient fractions (data not shown). Therefore it was possible that in such aggregates some potential sites of cleavage were shielded from a restriction endonuclease added to the digestion mixture after $MgCl_2$ (see Methods). However, this seems unlikely since it can be shown that the addition of an endonuclease to the minichromosome preparation either before or after the addition of $MgCl_2$ does not result in any change in the percentage of conversion of the circular SV40 DNAs I and II into the linear DNA III (Fig. 2 m,n, p,q). Furthermore, it seems difficult to explain how a non-specific aggregation sterically hinders the action of Eco RI and Bam HI but not Bgl I (Fig. 2 f,g; cf. Fig. 2 b,d,h,i). Finally, the same preferential digestion of the minichromosomes by Bgl I was obtained with formaldehyde-fixed minichromosomes (see below) which do not aggregate upon addition of $MgCl_2$.

In another series of experiments we fixed isolated minichromosomes with formaldehyde before endonuclease treatments to see whether the cross-linked minichromosomes would display the same enhanced sensitivity towards Bgl I. The results (shown only for Eco RI in Fig. 2c and for the control in Fig. 2j) were quite similar to those obtained with unfixed minichromosomes, i.e., Eco RI and Bam HI each cut 24-29% of the minichromosomes, whereas more than 95% of the minichromosomes were converted to linears by Bgl I.

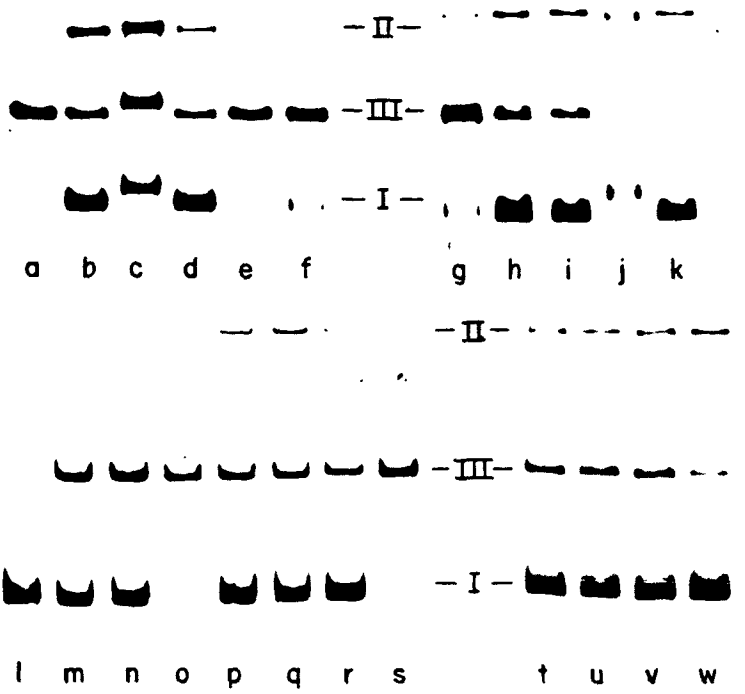

Fig. 2. Digestion of SV40 minichromosomes with single-cut restriction endonucleases.

Minichromosomes were digested with Eco RI, Bam HI or Bgl I. DNA products of digestion were fractionated by agarose gel electrophoresis as described in Methods.

(a) Eco RI limit-digest of the purified SV40 DNA I; (b) the same but of the unfixed SV40 minichromosomes; (c) the same but of the HCHO-fixed minichromosomes (see Methods); (d) Bam HI limit-digest of the unfixed minichromosomes; (e) the same but of the purified SV40 DNA I; (f) Bgl I limit-digest of the unfixed SV40 minichromosomes; (g) the same but from another experiment; (h) the same as (b) but from another experiment; (i) the same as (d) but from another experiment; (j) DNA from the HCHO-fixed, pronase-treated minichromosomes (control; see Methods); (k) DNA from unfixed, undigested minichromosomes; (l) the same but the minichromosomes were incubated in buffer A without endonucleases at 37° for 20 min (control; see Methods); (m) Eco RI limit-digest of the unfixed minichromosomes ($MgCl_2$ was added before Eco RI; the same as (b); see Methods); (n) the same but $MgCl_2$ was added after Eco RI; (o) Bgl I limit-digest of the purified SV40 DNA I; (p) Bam HI limit-digest of the unfixed minichromosomes ($MgCl_2$ was added before Bam HI; the same as (d)); (q) the same but $MgCl_2$ was added after Bam HI; (r) the same but a 3-fold lower amount of the enzyme; (s) Bgl I limit-digest of the purified SV40 DNA I which was pretreated with 1% HCHO at 4°C for 70 hr (see Methods); (t-w) kinetics of digestion of the unfixed minichromosomes with Eco RI; the points correspond to 60, 20, 5 and 1 min of digestion, respectively.

Notice reproducibly lower mobilities of DNA bands from HCHO-fixed, pronase-treated minichromosomes in comparison with DNA from unfixed minichromosomes.

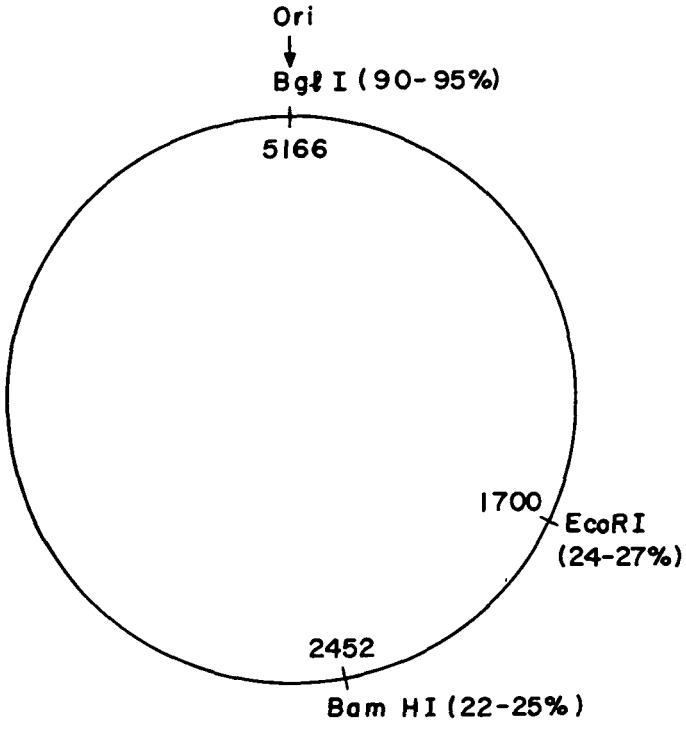

Fig. 3. Summary of the results.
A circular restriction map of the SV40 DNA with Bgl I, Eco RI and Bam HI cleavage sites shown. The numbering of nucleotide residues is according to Reddy et al. [4]. Values in parentheses are the percentages of the linear SV40 DNA III produced by Eco RI, Bam HI or Bgl I from the isolated SV40 minichromosomes under limit-digest conditions.

It should be noted that DNAs isolated from formaldehyde-fixed, pronase-treated minichromosomes migrate in the gel significantly more slowly than their unfixed counterparts (Fig. 2 c,j; cf. Fig. 2 b,k). This effect is apparently not due to a formaldehyde-induced change in the structure of DNA itself since control digestion with Eco RI of the purified SV40 DNA I which had been pretreated with formaldehyde does not reveal any anomaly in either cleavage pattern or electrophoretic mobility (Fig. 2 s). Recent data suggest that the observed shift in mobility (Fig. 2 c,j; cf. Fig. 2 b,k) is due to pronase resistance of a significant portion of the cross-linked protein in the minichromosome. Further analysis of this effect and its implications in studies on minichromosome structure is in progress.

Thus, the origin of replication in the isolated SV40 minichromosomes is

apparently highly exposed in comparison with the rest of the SV40 genome as probed by single-cut restriction endonucleases (Fig. 3). At least three interpretations of these findings are formally compatible with the data. The first interpretation is that the arrangement of nucleosomes (i.e., the arrangement of octameric histone cores) in the isolated compact minichromosomes is nonrandom at least with regard to sequences near the origin of replication. The second interpretation is that nucleosomes are arranged randomly but there are sequence-specific DNA-binding proteins (e.g., T antigen or T antigen-related proteins [28]) which, by binding at or near the origin of replication modify their microenvironment in such a way that the Bgl I recognition sequence becomes accessible to the enzyme. Finally, it is not formally excluded by the present data that the enzymatic properties of Bgl I restriction endonuclease differ from those of other type II restriction endonucleases used in this work (Eco RI and Bam HI) in such a way as to permit Bgl I to cleave DNA in a chemical microenvironment which would make other restriction endonucleases inactive. However, this latter interpretation was made extremely unlikely by our most recent results (manuscript in preparation), according to which an almost entire stretch of "late" SV40 DNA 400-500 base pairs long which includes the origin of replication is highly exposed in comparison with the rest of the SV40 genome as probed by both multiple-cut restriction endonucleases and staphylococcal nuclease.

ACKNOWLEDGEMENTS

We thank Bruce Yazejian and Frederic Boyce for their excellent technical assistance. One of us (A.J.V.) is deeply indebted to many colleagues both at and outside M.I.T. for their encouragement, support and generous sharing of equipment and facilities during the course of this work.

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