PROTEIN-SV40 DNA COMPLEX STABLE IN HIGH SALT AND SODIUM DODECYL SULFATE

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SUMMARY: A protein-DNA complex which is stable in concentrated salt solutions and in the presence of sodium dodecyl sulfate has been extracted from purified viruses and is found in the nicked circular DNA fraction. The protein is visualized as a "dot" on the DNA molecule by electron microscopy using a modified version of the ethidium bromide mounting technique. The position of the dot is at 0.67 genome units clockwise from the *eco*RI restriction site on the SV40 DNA map.

INTRODUCTION

Griffith et al. (1) have observed that several gentle lysis procedures for extracting SV40 DNA from viruses and infected cells yield a fraction of the covalently closed circular DNA with a high molecular weight protein moiety attached to the DNA at a specific site. We now wish to report that when viruses are subjected to more stringent lysis, using SDS, a considerable amount of the DNA is recovered as a nicked circular form. Electron microscope and biochemical studies show that a smaller protein or protein complex is attached to a fraction of this DNA at the same specific site.

MATERIALS AND METHODS

Semi-confluent monolayers of TC7 cells (2) grown either in 10 mm petri dishes or in a half gallon bottle were infected with SV40 (a small plaque forming strain, sp12, plaque purified) at a multiplicity of 5 to 10 plaque forming units per cell. We wish to thank Dr. J. Robb for sending us the TC7 cell line and a stock of virus. Viruses were purified by a combination of previously described methods as follows. The cell-virus suspension was frozen and thawed 3 times in a dry-ice ethanol bath and in a 37°C water bath, then centrifuged at about 1000g for 10 min. Viruses in the supernatant were precipitated with polyethyleneglycol (3). Viruses in the cell pellet were collected by the method described in Tai et al. (4). Viruses from both the supernatant and pellet fractions were further purified by centrifugation to equilibrium several times in CsCl (ρ = 1.34 g/cc).

SV40 DNA (16 μg) was cleaved with *eco*RI endonuclease in 60 μl of 0.1M Tris buffer (pH 7.6), 5mM $MgCl_2$, 50mM NaCl, at 37°C for 6 hours.

Abbreviations: Tricine, N-Tris (hydroxymethyl) methylglycine; EM, electron microscopy; EtdBr, ethidium bromide; SDS, sodium dodecyl sulfate.

The reaction was terminated by dialysis against 0.1M Tris, 0.01M EDTA, pH 7.6. Further cleavage of *eco*RI linear SV40 DNA with *hpa*II endonuclease was performed in 10mM Tris buffer (pH 7.6), 10mM $MgCl_2$, 1mM DTT, at 37°C for 4 hours. The reaction was terminated by dialysis against 0.1M Tris, 0.01M EDTA, pH 8.5. EM observation showed that more than 80% of the molecules were properly cleaved by the two enzymes. The restriction endonucleases, *eco*RI and *hpa*II, were generous gifts from Drs. H. Boyer and P. Sharp, respectively.

Pronase (Calbiochem.) was used at a concentration of 0.2 to 0.4 mg/ml in 10mM Tricine, 1mM Na_3 EDTA buffer (pH 8.0) or in 1 x SSC (pH 7.0) at 37°C for 1 hour.

DNA was prepared for electron microscopy (EM) by a modified version of the ethidium bromide (EtdBr) mounting technique (5). Purified DNA was dissolved at a concentration of 0.05 μg/ml in 10mM Tricine (pH 9.0), 1mM Na_3 EDTA. Immediately before use, EtdBr dissolved in water (1 mg/ml) was added to give a final concentration of 100 μg/ml. Droplets of 10 μl of this solution were placed on a clean sheet of parafilm and covered to prevent contamination. After 10 min. at room temperature, a thin carbon coated grid was touched to the surface of the DNA droplet. The grid was then washed with redistilled water, dehydrated in ethanol, stained with 1 x 10^{-4}M uranyl acetate in 90% ethanol for 30 sec., rinsed in isopentane and rotary shadowed with platinum at a 1 : 8 angle. Electron micrographs were usually taken at a magnification of 18,000 with a Philip 300 electron microscope. With this technique, duplex DNA is about 3 nm wide.

RESULTS

1) Visualization of a "dot" on nicked circular SV40 DNA

Two DNA peaks were observed when the sodium dodecyl sulfate (SDS) lysate of the viruses was sedimented through a linear sucrose gradient. A representative absorbance profile of such a preparation is shown in Fig. 1a. Approximately 65% of the total DNA sedimented rapidly, peak I; and 35% slowly, peak II. The DNAs in the pooled fast and slow fractions were concentrated by ethanol precipitation and separately sedimented to equilibrium in EtdBr-CsCl gradients (Fig. 1b and Fig. 1c, respectively). The majority of the DNA in peak I banded at the same density as covalently closed circular DNA (fractions 4 through 6 in Fig. 1b). These pooled fractions are referred to as lower band DNA. There was a minor peak at the same density as linear- or nicked-circular DNA. The DNA in peak II formed only one broad band at a density near that of linear- or nicked-circular DNA (fractions 4 through 9 in Fig. 1c). The DNA in these fractions is referred to as upper band DNA.

The lower band and upper band DNAs were examined by EM as described in Materials and Methods. The majority of the lower band DNA appeared as rather short linear or branched structures (Fig. 2a). This is characteristic

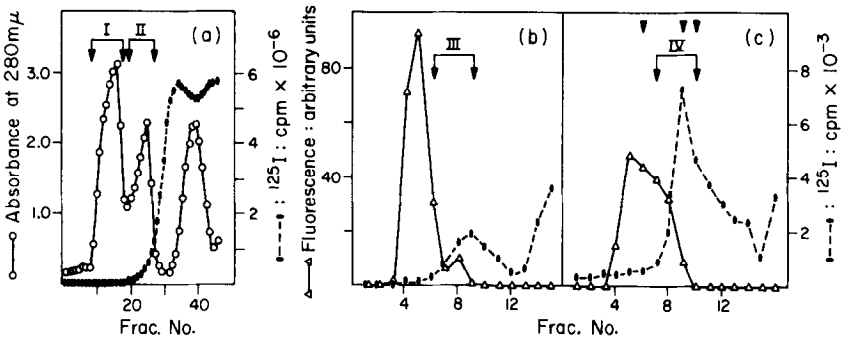

Figure 1. Fractionation of SV40 DNA after extraction.
(a) 200 μg of virus in 20 μl of 0.1% SDS, 0.1M borate buffer (pH 8.5) was labeled with ^{125}I as described by Bolton and Hunter (7). The virus was mixed with 13 mg of unlabeled virus, incubated for 10 min. at 50°C and then 2 hours at 37°C in 0.1M borate buffer (pH 8.5) containing 5% SDS, 5mM EDTA and 5mM DTT. The sample (3 ml) was layered onto 28 ml of a 15% to 30% linear sucrose gradient resting on a 7 ml layer of 60% sucrose in an SW27 centrifuge tube. It was then centrifuged at 23K rpm for 25 hours at 20°C. 0.8 ml fractions were collected from the bottom of the gradient and examined for absorbance at 280 nm and for ^{125}I counts. The viral polypeptides sediment near the top of the gradient (fraction 33 through 43). The fractions in peak I or in peak II were pooled, the DNAs precipitated with ethanol, and analyzed in EtdBr-CsCl gradients as shown in (b) and (c). The ^{125}I counts were measured in a Nuclear Chicago γ-radiation counter.

(b) and (c) EtdBr-CsCl gradient profiles of isolated peak I DNA (b) and peak II DNA (c). Isolated DNAs were sedimented to equilibrium in CsCl (ρ = 1.55 g/cc) containing 300 μg/ml of EtdBr. 0.15 ml fractions were collected from the bottom of the tube, diluted to 3 ml with 10mM Tris, 1mM EDTA (pH 7.6), and examined for ^{125}I counts and for fluorescent intensity. The fluorescence measurements were performed with a Hitachi Perkin-Elmer MPF2A fluorescence spectrophotometer at the excitation and emission wavelengths of 365 nm and 584 nm respectively. The concentration of DNA in the undiluted fraction 5, Fig. 1b, was about 700 μg/ml as determined by the diphenylamine method (18). The fractions, indicated as III and IV, were used in the experiments shown in Fig. 2. The triangle marks at the top of Fig. 1 (c) indicate the fractions taken for analysis by electron microscopy (Table 1). The fraction numbers in the two samples begin at an arbitrary point and not at the bottom of the tube. The position of the DNA bands in experiments (b) and (c) corresponded to the usual positions for closed circular and nicked circular or linear DNAs respectively.

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of closed circular DNA molecules under this spreading condition. After X-ray nicking (6), the molecule had an open circular structure and the contour length increased about 4-fold to that expected for SV40 DNA. Two kinds of DNA structures were observed in upper band DNA: clean open circles and open circles with one or more dots, as shown in Fig. 2b.

Data on the frequency of dots in various fractions and preparations is given in Table 1. About half of the circular molecules in the upper band

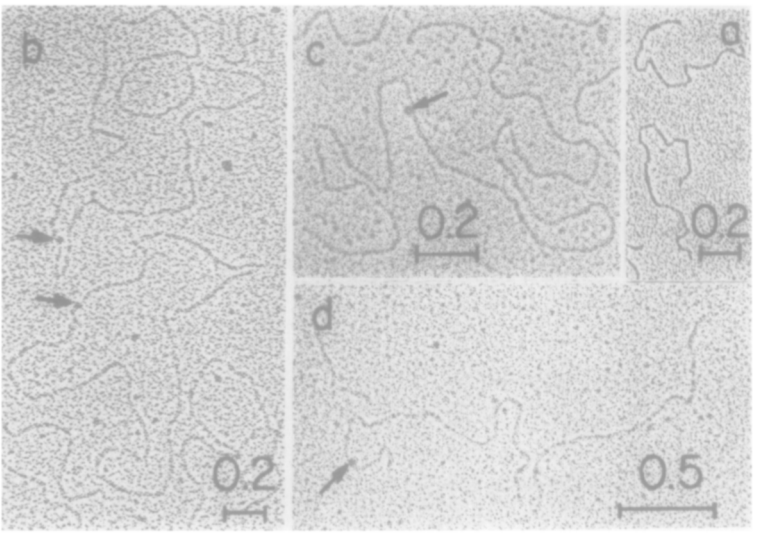

Figure 2. (a) Electron micrograph of closed circular DNA molecules from lower band. (b) Upper band DNA with one dot on each molecule as indicated by arrows. (c) A dot attached to a linear DNA molecule from the *eco*RI digested upper band DNA preparation. (d) One large fragment from an upper band DNA sample which was treated with *eco*RI and *hpa*II restriction enzymes. The arrow points to a dot close to one terminus. All calibration markers are in kilobase units.

preparation possessed one or more than one dot. In one case, of 100 circular molecules which possessed dot(s), there were 62, 34, and 4 with 1, 2, and 3 dots respectively. The nicked DNA sample obtained after X-ray treatment of closed circular DNA contained very few dotted molecules (Table 1). The diameter of a dot on a DNA molecule was measured as ca 7 nm whereas the width of the DNA is about 3 nm.

2) Some dots are proteins

A protein-DNA complex should have a lower buoyant density than that of DNA. Upon removal of the DNA by DNase digestion, the protein should band at a much lower density. Also, if the visualized dots on DNA molecules were proteins, the frequency of appearance of dotted-molecule should be higher in the fractions with lighter buoyant densities.

The results of the examination of fractions of upper band DNA by EM are presented in Table 1. The frequency of dotted molecules in the lighter

Table 1. Frequencies of Dots on Different Kinds of Molecules

	Number of Molecules				Fraction of Nicked Circle or Linear		
	Total	Closed Circle	Nicked Circle	Linear	No Dot	Dot(s)	Dubious[1]
Upper Band DNA							
Preparation 1	105	-	104	1	0.41	0.55	0.04
Preparation 2	107	-	107	0	0.32	0.66	0.03
Preparation 1+pronase	157	-	135	22	0.72	0.18	0.10
Fraction[2] 6	130	-	108	22	0.64	0.31	0.05
Fraction[2] 9	139	-	138	1	0.38	0.57	0.04
Fraction[2] 10	133	-	124	9	0.37	0.54	0.09
*eco*RI linear, Prep. 1	100	-	-	100	0.45	0.55	0
*eco*RI linear, Prep. 1+pronase[3]	105	-	-	105	0.88	0.10	0.03
*eco*RI/*hpa*II/large fragment[4]	67	-	-	67	0.34	0.63	0.03
*eco*RI/*hpa*II/small fragment[4]	111	-	-	111	0.80	0.16	0.04
Lower Band DNA							
Preparation 1	137	97	40	0	-	-	-
Preparation 2	100	100	0	0	-	-	-
Preparation 3	163	141	20	0	-	-	-
Preparation 3, X-ray nicked	109	-	109	-	0.81	0.16	0.04

Table 1. EM specimens were prepared as described in Materials and Methods. DNA molecules were photographed randomly.
1) An amorphous structure attached to the DNA is classified as dubious.
2) Fractions designated in Fig. 1c.
3) The protein was extracted by phenol after pronase treatment.
4) Fragments were purified by velocity sedimentation in a 5% to 20% linear sucrose gradient after the restriction enzyme treatments (figure not shown).

fractions 9 and 10 was higher than in fraction 6. Pronase treated upper band DNA showed a decreased frequency of dotted molecules (Table 1). These data suggest that some of the dots visualized by EM are proteins tightly bound to the DNA.

SDS disrupted viruses were treated with ^{125}I labeled 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester as described in the legend to Fig. 1 in order to label the viral polypeptides (7). An association of ^{125}I counts to a part of the upper band DNA was observed (Fig. 1b and c). The fractions designated III and IV (Fig. 1b and c) were collected and treated with DNase. The DNase treated and non-treated samples were analyzed for their buoyant behavior. Without DNase treatment, most of the ^{125}I counts banded at a density just slightly lower than that of a pure marker DNA with a ^{3}H label. After the treatment, the ^{125}I counts banded at the top of the

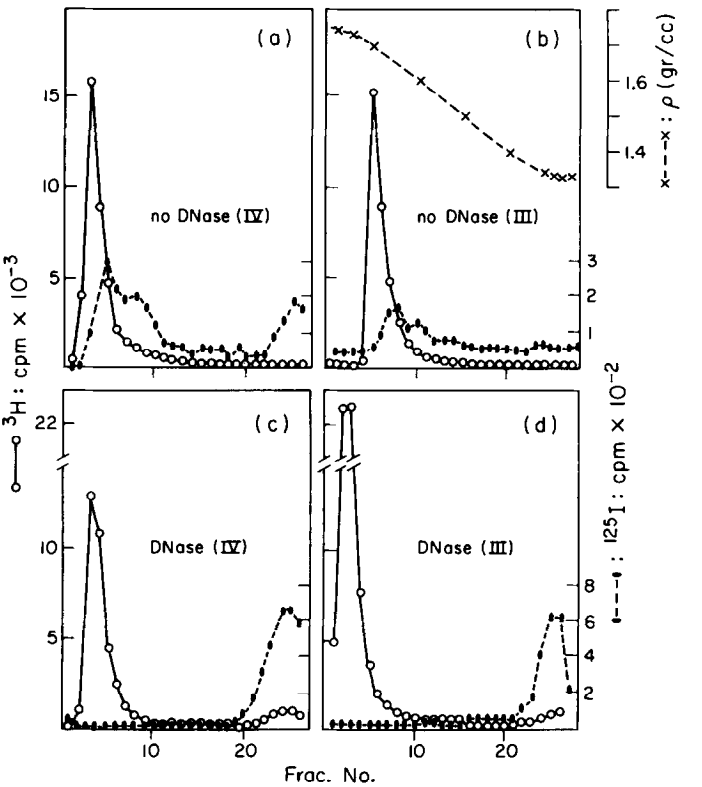

Figure 3. CsCl sedimentation profiles of DNAs at equilibrium before and after DNase treatment. The DNAs in the fractions indicated in Fig. 1 (b) (III) and Fig. 1 (c) (IV) were precipitated with ethanol at -21°C for 8 hours, collected by centrifugation and resuspended in 2 ml of 0.01M Tris (pH 7.8), 0.01M $MgCl_2$, 0.125M NaCl. A portion of each sample was set aside after addition of EDTA to 0.1M ((a), fraction IV; (b), fraction III). The rest of each DNA sample ((c), fraction IV; (d), fraction III) was treated with DNase I (Worthington Biochemical Corporation; RNase free) 100 μg/ml at 37°C for 30 min. The reaction was terminated by addition of EDTA to 0.1M. Samples, treated and non-treated with DNase, were mixed with ^{3}H-labeled PM2 DNA and centrifuged to equilibrium in CsCl gradients ($\rho \cong 1.5$ g/cc, 5 ml, SW50 rotor, 48K rpm, 20°C, 60 hours). 0.18 ml fractions were collected from the bottom of the gradient and examined for ^{125}I and ^{3}H counts.

gradient (ρ = 1.34 g/cc), that is at a density characteristic of proteins.

3) Location of the "dots"

Upper band DNA was digested either with *eco*RI restriction endonuclease or with *eco*RI and *hpa*II restriction endonucleases. Each endonuclease cleaves SV40 DNA once at unique but different locations (8, 9, 10, and 11). A histogram of dot positions on the full length linear DNA molecules resulting from

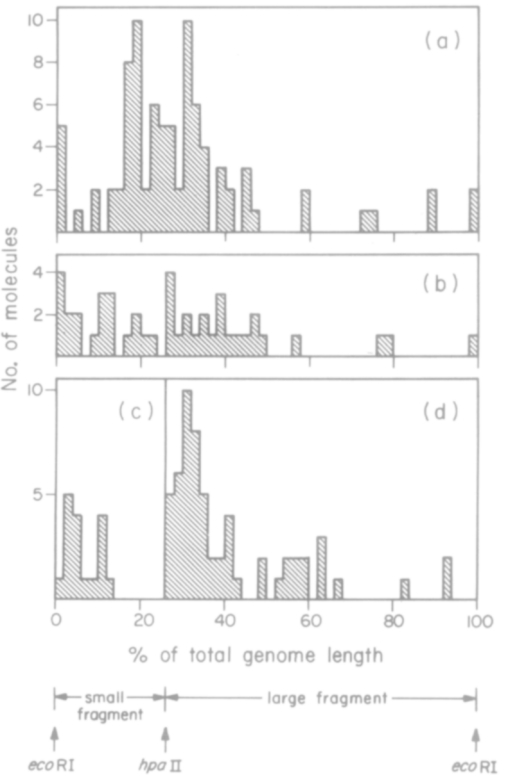

Figure 4. (a) and (b) Positions of protein dots found in association with *eco*RI SV40 linear DNA . (a) The upper band DNA (preparation 1, Table 1) was incubated with *eco*RI and prepared for EM study as described. Fifty five out of 100 randomly scanned intact linear molecules had one or more dots on each. The distribution of 87 dots on 55 molecules is plotted. (b) Control experiment with the lower band DNA (preparation 1, Table 1). Thirty three out of 100 molecules had a total of 44 dots. Length measurement for linear molecules was 5.1 ± 0.3 kilobases, relative to an open circular SV40 DNA standard taken as 5.2 kilobases.

(c) and (d) Positions of protein dots found in association with DNA fragments formed by the action of *eco*RI and *hpa*II. The upper band DNA was first incubated with *eco*RI, then with *hpa*II as described in Materials and Methods. Fragments of 0.74 ± 0.05 (large) and 0.26 ± 0.02 (small) fractional length were distinguishable under EM. Fifteen out of 48 randomly selected small fragments had a total of 17 dots attached. The distribution is shown in (c). Twenty seven out of 51 large fragments had 62 dots as shown in (d).

*eco*RI digestion is shown in Fig. 4. The relative position of a dot on molecules containing one dot is presented as the distance to the closer end. When two dots were present on one DNA molecule, the dot closer to an end was assigned the smaller coordinate. Fig. 4a shows that most of the dots map between 0.15 and 0.35 fractional lengths on the viral DNA from the upper

band. As mentioned above, there is a lower frequency of dots on lower band DNA molecules (Table 1). When cleaved with *eco*RI, these dots mapped more or less uniformly over the DNA molecules (Fig. 4b).

Two fragments of length 0.74 and 0.26 are produced by treatment with *eco*RI and *hpa*II restriction enzymes. As shown in Fig. 4c and d, most of the dots mapped between 0.26 and 0.35 fractional lengths on the larger fragment; a small number of dots were uniformly distributed over the small fragment.

While the dot position is somewhat variable, there is a very clear concentration of dots at a position of about 0.33 map units counterclockwise from the *eco*RI site on SV40 DNA.

In order to obtain well-extended and easily measurable molecules on EM specimens, it was necessary to use DNA solutions at high pH and low ionic strength. This condition might favor the non-specific sticking of some proteins to DNA, thereby accounting for the background of dots. The distribution of dots along the DNA molecules from the upper band is clearly nonrandom, Fig. 4d. The background which we interpret as non-specific binding of some protein was quantitatively different in different preparation. The presence of restriction enzymes or pronase resulted in a higher background. The removal of these proteins by phenol extraction prior to adsorption to the EM grid reduced the background substantially.

DISCUSSION

A salt-stable and SDS-stable protein DNA complex has been observed in a population of nicked SV40 DNA molecules isolated from purified viruses. The protein appears as a dot on electron micrographs and is located at 0.67 map units clockwise from the *eco*RI restriction site. Griffith et al. (1) have observed a salt-stable protein DNA complex in closed circular SV40 DNA extracted from viruses by treatment at pH 10.5. After X-ray nicking and cleavage with restriction endonucleases, their "knob" mapped at the same position, within experimental error, as did our dot. All this suggests that the complex we have observed might originate from the knob under the more stringent condition of SDS lysis (12).

The dot and the Griffith et al. knob maps at or close to the origin of DNA replication (13, 14).

The buoyant density profiles of the complexes as defined by the ^{125}I counts in both Fig. 3a and b show broad bimodal distributions. We can make a rough estimate of the molecular weight of the protein(s) responsible for the smaller density shift relative to the marker. The shift of 40 mg/cc corresponds to a protein molecular weight of 3×10^5. The observed dot diameter of 7 nm corresponds to a molecular weight for a protein of 1.4×10^5. No single protein in this molecular weight range has been identified among the SV40 polypeptides.

The fact that a protein is associated with nicked circular DNA but not with covalently closed circular DNA raises several interesting questions. Is the protein associated with the DNA at the nick(s)? Preliminary data from electron microscope single strand length measurements of the *eco*RI cut upper band DNA suggest that at least one single strand break exists near the location of the protein, supporting the hypothesis that the dot is bound at the nick.

Our present hypothesis is that a complex of proteins is associated with covalently closed viral DNA at the origin of DNA replication, that one of the proteins possesses endonucleolytic activity, and that one of the proteins is tightly associated with the DNA. Evidence for the existence of endonucleolytic activity in both SV40 and polyoma virus has been reported (15, 16, 17). Further studies of the implications of the above findings are in progress.

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