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EVIDENCE FOR A PROTEIN(S) BOUND TO HERPES SIMPLEX VIRUS DNA

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SUMMARY

The Xba I cleavage pattern of highly purified, but not specifically deproteinized, herpes simplex virus DNA does not match published patterns. If the purified herpes simplex virus DNA is first extracted with phenol and then digested with Xba I, the cleavage pattern matches the published patterns. This comparison is taken as supportive of the hypothesis that there is a protein(s) bound to herpes simplex virus DNA.

INTRODUCTION

Herpes simplex virus[1] DNA possesses a terminal repetition (1,2). In an earlier publication, we found that HSV DNA purified from the Hirt supernatant of HSV-infected cells by sequential preparative glycerol gradient sedimentation and CsCl buoyant density centrifugation could not be digested with E. coli exonuclease III sufficiently to produce circular DNA unless the HSV DNA was subjected to additional deproteinization (3). This datum was taken as indirect evidence for the presence of a protein(s) bound at or near the end(s) of HSV DNA. More direct evidence for the presence of the hypothesized HSV DNA-bound protein(s) has been sought. We have chosen to use the agarose gel electrophoresis assay first investigated by Sharp et al. (4) for the Ad5 DNA-protein complex. Those authors found that, when the Ad5 DNA-protein complex was cleaved by Eco R_I (which cleaves Ad5 DNA twice to yield three specific fragments) and subjected to electrophoresis in an agarose gel, only the middle fragment entered the gel (4). The two end fragments, each of which has a protein covalently attached, remained at the origin. For deproteinized Ad5 DNA, all three Eco R_I fragments enter the gel.

HSV DNA is more complex than Ad5 DNA. Firstly, HSV DNA is four to five times larger than Ad5 DNA. Secondly, HSV DNA is composed of a population of four molecules which differ with respect to the orientation of their sequences (5-8). Therefore, the restriction enzyme cleavage patterns of HSV DNA are

[1]ABBREVIATIONS

Ad5, adenovirus type 5; HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; SDS, sodium dodecyl sulfate.

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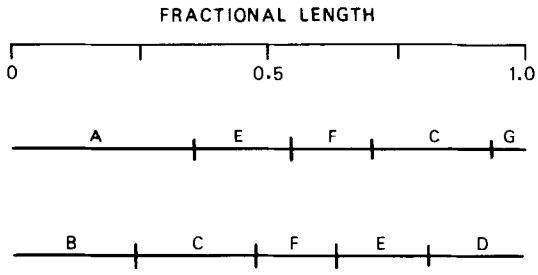

Figure 1. Schematic diagram of Xba I cleavage sites on HSV DNA. Because HSV DNA exists as a population of four molecules (5-8), two line drawings are required to diagram the Xba I cleavage sites on HSV DNA. The data used to produce the diagram were taken from Wilkie (7) and Skare and Summers (8). The four end fragments (A, B, D, and G) are each present in one-half molar amount relative to the internal fragments (C, E, and F).

significantly more complex than the corresponding patterns for Ad5 DNA. Endonuclease Xba I cuts HSV DNA the fewest times (four) of any known restriction enzyme (7,8) and, therefore was chosen for our study. Because of the inversions within the HSV genome, Xba I cleavage yields seven specific HSV DNA fragments; four are end fragments each present in one-half molar amount (7,8). A schematic diagram of the Xba I cleavage sites on HSV DNA is presented in Figure 1. We have compared the Xba I cleavage pattern of highly purified, but not specifically deproteinized, HSV DNA with the pattern of highly purified, deproteinized HSV DNA. Although the data were not as straightforward to interpret as the cleavage pattern of the Ad5 DNA-protein complex, nevertheless, the data support the hypothesis that there is a protein(s) bound to HSV DNA.

MATERIALS AND METHODS

Virus and DNA preparation. HSV-1 (Patton) and HSV-1 (KOS) were plaque-purified three times before use. Standard procedures were used for the large scale growth of HSV DNA (3,9). The HSV DNA was radiolabeled in vivo by the addition of [^{32}P]orthophosphate (75 μCi/ml, New England Nuclear) to phosphate-depleted MEM (10). HSV DNA was purified from the Hirt supernatant of HSV-infected Vero cells by sequential glycerol gradient sedimentation and CsCl buoyant density centrifugation (9). The highly purified HSV DNA was divided into three portions: one was mock-treated and was the "non-deproteinized" sample; the second was extracted with phenol (redistilled and equilibrated with buffer) in the presence of 0.5% SDS (Bio-Rad Labs, electrophoresis grade) and was the "deproteinized" sample; the third was digested with Pronase (Calbiochem, 1 mg/ml, 37°C, 0.5 hr; previously autodigested) in the presence of 0.5% SDS and then extracted with phenol and was the "rigorously deproteinized" sample. A fourth sample was an equimolar mixture of non-deproteinized and rigorously deproteinized HSV DNA.

Restriction enzyme cleavage and gel electrophoresis. Endonuclease Xba I was purchased from New England Biolabs. Restriction endonuclease digestions

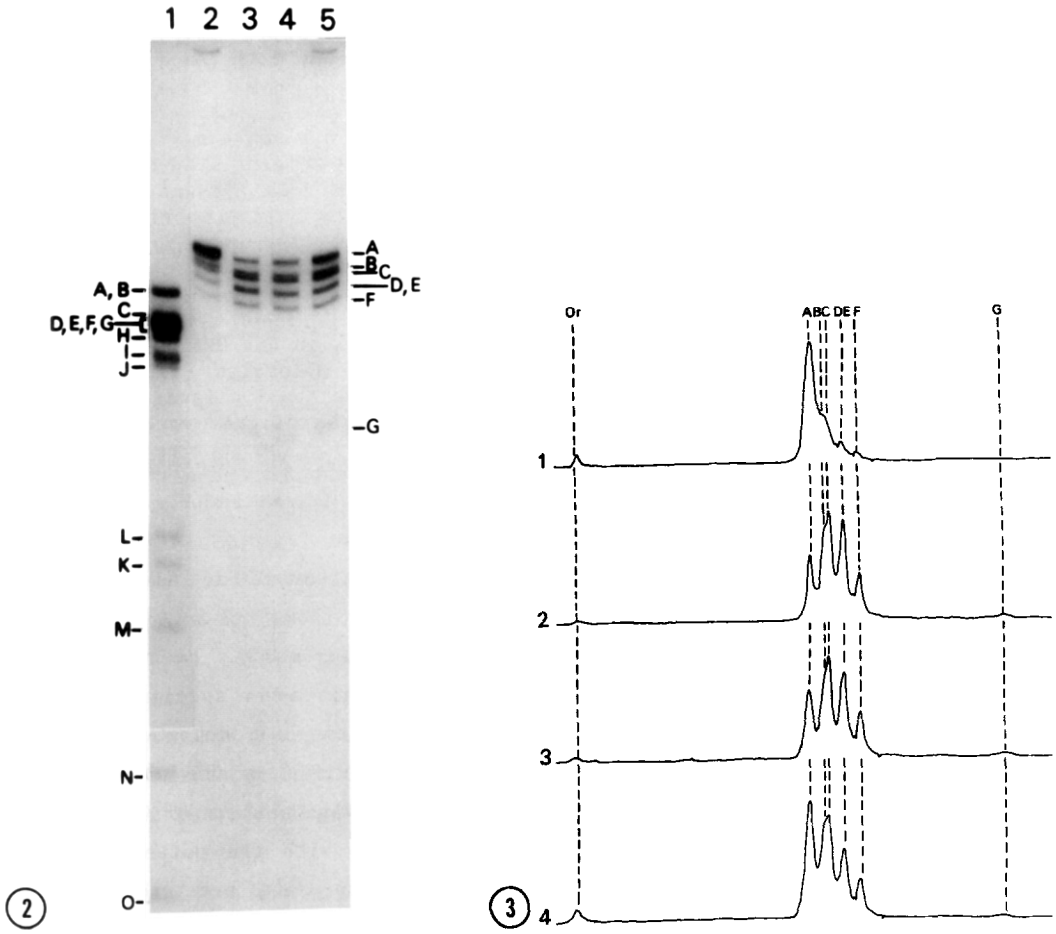

Figure 2. Autoradiograph of an agarose gel of endonuclease-cleaved and electrophoretically separated HSV DNA. The samples of HSV DNA, radiolabeled in vivo with [^{32}P], were digested by restriction endonuclease and subjected to electrophoresis through an agarose gel as described in Materials and Methods. The designations of the bands by letters are as described by Skare and Summers (8). Track 1: The control, Eco R_I-cleaved HSV-1 (KOS) DNA. The print was a combination of two autoradiographs of the same gel so that all the bands can be seen. Track 2: Xba I-cleaved non-deproteinized HSV-1 (Patton) DNA. Track 3: Xba I-cleaved deproteinized HSV-1 (Patton) DNA. Track 4: Xba I-cleaved rigorously deproteinized HSV-1 (Patton) DNA. Track 5: The Xba I-cleaved equimolar mixture of non-deproteinized and rigorously deproteinized HSV-1 (Patton) DNA.

Figure 3. Traces of autoradiographs. The autoradiograph of Figure 2 was traced. The track numbers and band designations correspond to Figure 2. Or represents the origin of the gel.

were performed in a fivefold enzyme excess for 2 hr at 37°C in a buffer consisting of 0.15 M NaCl, 0.006 M Tris (pH 7.9), and 0.006 M $MgCl_2$. The reaction was terminated by the addition of loading buffer: 30% Ficoll, 0.06 M

EDTA, 0.25 M Tris (pH 8.0), 0.5% SDS, and 0.2% bromphenol blue. Samples were heated at 55°C for 5 min, centrifuged at 40 x g for 1 min at room temperature, and then loaded into preformed slots in the agarose gel. Both 0.5% and 0.3% agarose (Bio-Rad Labs, electrophoresis grade) slab gels were used. The gels were horizontal, submerged agarose gels (11,12). Electrophoresis was carried out at 2.7 V/cm for 14 hr at room temperature in running buffer: 0.04 M Tris, 0.005 M sodium acetate, 0.001 M EDTA adjusted to pH 7.9 with glacial acetic acid. Following electrophoresis, the gels were dried on filter paper. For autoradiography, the dried gel was placed on Kodak Royal X-omat film and sandwiched between two Cronex screens (13). At appropriate times, the film was developed. Traces of the autoradiographs were made on a Transidyne 2955 Scanning Densitometer.

RESULTS

Restriction enzyme cleavage patterns for the various HSV DNA samples are presented in Figure 2. Traces of the autoradiographs are presented in Figure 3. Figure 2 is one autoradiograph from one gel. However, the patterns of the bands within the gel were quantitatively the same for different autoradiographs of the same gel, for different gels of the same HSV DNA preparation, and for three independent preparations of HSV DNA. Track 1 in Figure 2 is the standard, Eco R_I-cleaved HSV-1 (KOS) DNA. The pattern in Track 1 matched the pattern previously published by Skare et al. (14). Track 4 in Figures 2 and 3 was the pattern for Xba I-cleaved, rigorously deproteinized HSV-1 (Patton) DNA. This pattern was indistinguishable from the patterns published by Skare and Summers (8) and by Wilkie (7). The pattern of Track 4 was also indistinguishable from the pattern of Track 3, the pattern for Xba I-cleaved deproteinized HSV DNA. Track 2 was the pattern for Xba I-cleaved non-deproteinized HSV DNA. While the pattern was qualitatively the same as the patterns in Tracks 3 and 4, it was quantitatively very different: e.g., band A was severely over-represented. Track 5, the control, was the Xba I-cleavage pattern of an equimolar mixture of non-deproteinized and rigorously deproteinized HSV DNA. The most important point in Figures 2 and 3 was that the Xba I-cleavage pattern for highly purified but non-deproteinized HSV DNA does not match the published patterns. If the HSV DNA was subjected to deproteinization, then the Xba I-cleavage pattern matched published patterns. As an additional control, we attempted to reproduce the Xba I cleavage pattern of non-deproteinized HSV DNA by digestion of rigorously deproteinized HSV DNA with reduced amounts of restriction enzyme. The cleavage pattern of incompletely Xba I-digested rigorously deproteinized HSV DNA did not match the Xba I cleavage pattern of non-deproteinized HSV DNA (data not shown).

Quantitation of the restriction enzyme cleavage patterns (Fig. 3) is given in Table 1. Bands BC and DE were too close together to separate quantitatively. Therefore, the data for these doublet bands are given as the sum of the two components. The numbers are the average values from three traces of

Table 1. Quantitation of the individual bands of Xba I-cleaved HSV DNA.

	Percent Mass					Molar Yield (relative to band F)					
Band	Wilkie (7)	Track 2	Track 3	Track 4	Track 5	Theory (7,8)	Wilkie (7)	Track 2	Track 3	Track 4	Track 5
A	19	62	16	21	32	0.5	0.55	4.5	0.45	0.59	1.2
BC	37	22	38	37	36	1.5	1.7	2.6	1.7	1.7	2.2
DE	27	9	28	25	19	1.5	1.5	1.5	1.7	1.6	1.6
F	13	5.6	15	14	11	1.0	0.85	1	1	1	1
G	3.2	1.5	2.4	2.6	2	0.5	0.48	0.45	0.29	0.32	0.34

Band and Track designations are the same as for Figure 2: Track 2, non-deproteinized HSV DNA; Track 3, deproteinized HSV DNA; Track 4, rigorously deproteinized HSV DNA; Track 5; equimolar mixture of non-deproteinized and rigorously deproteinized HSV DNA. To calculate the percent mass, graphical integrations followed by normalization were performed for traces such as Figure 3. The numbers are the average of three traces of each of three autoradiographs such as Figure 2. For the calculation of molar yield relative to band F, the known molecular weights of the bands of the Eco R_I-cleaved HSV-1 (KOS) DNA (14) were used to construct a graph of log molecular weight versus distance migrated (data not shown). The molecular weights of the bands of Xba I-cleaved HSV-1 (Patton) DNA were then determined. All the bands except band G fall on the non-linear portion of the curve. Therefore, the molecular weights so determined must be taken as approximate. Nevertheless, they were the same, within experimental error, as published values (7,8). The percent mass for each band was divided by its molecular weight to give the molar yield in arbitrary units. As band F is clearly separated from other bands in the gel (Fig. 2), is near the center of the genome (7,8), and is present in one molar yield (7,8), band F was taken as a reference. The molar yield in arbitrary units for each band was divided by the molar yield in arbitrary units for band F to give the molar yield relative to band F.

each of three autoradiographs of the single gel given in Figure 2. Quantitation of other gels and of independent HSV DNA preparations yielded data indistinguishable from the data in Table 1. For comparison, the data of Wilkie (7) are included in Table 1. The values for percent mass published by Wilkie (7) are the same, within experimental error, as we have achieved for deproteinized (Track 3) and rigorously deproteinized (Track 4) HSV DNA. Also given in Table 1 are the results of the calculations of the relative molar yields of each band. For comparison, Table 1 gives the theoretical molar yields (7,8) and the values achieved by Wilkie (7). The relative molar yields of all the bands in the patterns for deproteinized (Track 3) and rigorously deproteinized (Track 4) HSV DNA were in agreement with each other, with the values published by Wilkie (7), and with the theoretical values (7,8). For the pattern of Xba I-cleaved non-deproteinized HSV DNA (Track 2), the data in Table 1 showed that bands A and BC are over-represented, relative to band F, but that bands DE and G were represented in the usual amounts. The control digestion, the Xba

I-cleavage of the equimolar mixture of non-deproteinized and rigorously deproteinized HSV DNA (Track 5), gave molar yields of the bands as the arithmetic mean of the two components, within experimental error.

The quantitation of the bands within the gel, though presented for only one gel (Table 1), was the same, within experimental error, for different gels and for three independent HSV DNA preparations. The percent of the total HSV DNA that remained at the origin of the gel was between 2 and 3% for the deproteinized and rigorously deproteinized HSV DNA (Fig. 3: Tracks 3 and 4). The percent of the total non-deproteinized HSV DNA that remained at the origin of the gel varied among different gels: from a low of 5% (Fig. 3: Track 2) to a high of 80%. We have no explanation for this variation at present. Possible reasons for the variation are under investigation.

DISCUSSION

The Xba I-cleavage pattern of highly purified, but not specifically deproteinized HSV DNA did not quantitatively match published patterns (Track 2; Fig. 2). If the HSV DNA was specifically deproteinized (for example, by extraction with phenol), then the Xba I-cleavage pattern matched the published patterns (Tracks 3 and 4; Fig. 2). The control, Xba I digestion of an equimolar mixture of non-deproteinized and rigorously deproteinized HSV DNA, demonstrated that the results cannot be explained by assuming the presence of an inhibitor of the restriction enzyme in the non-deproteinized HSV DNA preparation (Track 5; Fig. 2). Additional controls demonstrated that the results cannot be explained by assuming only partial digestion of the HSV DNA. Therefore, overall, the results were consistent with the presence of a protein(s) bound to highly purified HSV DNA.

The end fragments of Xba I-cleaved HSV DNA are A and B for one end and D and G for the other, each present in one-half molar amount (7,8). If this experiment on the HSV DNA-protein complex were as straightforward as the Ad5 DNA-protein complex, then one or more of the HSV DNA-protein complex end fragments should be missing from the Xba I-cleavage pattern (Track 2; Fig. 2). This was clearly not the case. Bands D and G were present in the usual molar yield for deproteinized HSV DNA (Track 2; Table 1). Bands A and BC were present in over-abundant molar yield (Track 2; Table 1). The important point is that bands A and BC reverted to the theoretically expected molar yields if the HSV DNA was extracted with phenol before digestion with Xba I. The Xba I cleavage pattern for HSV DNA extracted with phenol in the presence of SDS has consistently been indistinguishable from the Xba I cleavage pattern for HSV DNA digested with Pronase in the presence of SDS followed by extraction with phenol. This datum suggests, but does not prove, that the protein(s) is not covalently bound to HSV DNA.

We conclude that the data presented here give further credence to the hypothesis that there is a protein(s) bound to HSV DNA. Additional experiments to support the hypothesis are in progress.

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