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Structure and Function of Herpesvirus Genomes

I. Comparison of Five HSV-1 and Two HSV-2 Strains by Cleavage of Their DNA with *Eco R* I Restriction Endonuclease

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The restriction endonuclease *Eco R* I cleaves HSV-1 and HSV-2 DNA into specific fragments that can be resolved by agarose gel electrophoresis. Comparison of HSV-1 strains KOS, 14-012, MP, F, and CI 101, and HSV-2 strains 333 and 186, suggests that the DNAs from type 1 strains are similar but not identical, and that the type 2 strains differ greatly from type 1 strains. The molecular lengths of the fragments have been determined by electron microscopy and can be used to calibrate gel electrophoretic analyses of DNA fragments.

Herpes simplex virus (HSV) has a genome of double-stranded DNA which has been reported to be about 84 to 100 Mdaltons (10, 29). This genome is replicated and transcribed in the nucleus of susceptible mammalian cells. Thus, it is a useful probe to study the mechanisms of gene duplication and expression in eucaryotic cells. In addition, recent evidence (4, 8) demonstrates that under several types of nonpermissive conditions, HSV can apparently initiate oncogenic transformation in vitro. A clear understanding of the molecular biology of HSV may illuminate details of oncogenic transformation that are common to all tumor viruses.

Several groups have begun to subject HSV to genetic analysis (20, 28). To date, these studies have largely been confined to thermosensitive mutant phenotypes. We have initiated studies directed toward a physical analysis of the genetic map of HSV. One method for such analysis of a DNA genome is to study the location of sites of cleavage by sequence-specific endonucleases (the so-called restriction nucleases). A number of such enzymes have been described (23) that have different specificities. The fragments of HSV DNA produced by these enzymes can be separated by gel electrophoresis and the resultant pattern is highly characteristic of an individual HSV strain. In this paper, we describe a comparison of such cleavage patterns obtained with several different strains of HSV (types 1 and 2), using the restriction nuclease *Eco R* I (R. N. Yoshimori, Ph.D. thesis, Univ. of California, San Francisco, 1971).

MATERIALS AND METHODS

Cell culture. The African green monkey kidney cell line, Vero, was obtained from the ATCC (pass 128) and has been maintained by weekly passage for about 2 years. Medium 199, supplemented with 5% fetal calf serum (FCS), was used in all experiments except where noted. All media components were obtained from the Grand Island Biological Co., Grand Island, N.Y.

Virus stocks. The various virus isolates used are indicated in Table 1. A single plaque of each strain was used to infect 2×10^5 cells and the progeny were harvested 6 days later. These viruses were used to prepare master stocks by infecting 10^7 cells. Working stocks were made as follows: confluent cultures of Vero cells in roller bottles with 650 cm^2 surface area (5×10^7 cells) were infected with 0.1 PFU per cell in 5 ml of M199 with 2% FCS. After a 2-h adsorption period, 70 ml of M199 with 2% FCS was added. In 36 h, the cells showed maximum cytopathic changes and were harvested. The infected cell pellet from one roller bottle was resuspended in 2 ml of M199 with 10% FCS and frozen at −80 C. The suspension was thawed and then sonicated briefly. After low-speed centrifugation, the clarified supernatant was diluted to 4 ml in M199 with 10% FCS and stored in aliquots at −80 C. The titers of these preparations were between 2×10^7 and 2×10^8 PFU/ml. The infectious bovine rhinotracheitis virus was grown in a similar manner on primary bovine embryo cells obtained from Flow Laboratories.

Viral DNA preparation. To prepare highly labeled DNA, 1 mCi of $P^{32}O_4$ (as the free acid) was added to a culture of 10^7 cells in 30 ml of Dulbecco medium which lacked non-radioactive phosphate and serum. After 4 h of incubation, 10^6 PFU of virus was added and the culture was incubated for about 30 h at which time the cytopathic effect was maximum in all cells. The cells were collected and washed once in phosphate-buffered saline and frozen at −70 C. The cells were thawed and resuspended in 3 ml of phosphate-buffered saline and sonicated in a Cole-Parmer Ultrasonic cleaner. Large debris was removed by centrifugation for 10 min at $1,000 \times g$. The supernatant was applied to a discontinuous sucrose gradient containing 10 ml of 50% sucrose in 0.15 M NaCl, 0.01

TABLE 1. *Strains of herpesviruses used in this study*

Virus strain (type)	Origin	Source	Reference
HSV-1-KOS	Lip lesion	P. A. Schaffer	24
HSV-1-F	Facial lesion	B. Roizman	9
HSV-1-MP	Facial lesion	B. Goz	15
HSV-1-14-012	Bonchitis	R. Duff	8
HSV-1-CI 101	Facial lesion	S. Kit	6
HSV-2-333	Penile lesion	F. Rapp	7
HSV-2-186	Penile lesion	P. A. Schaffer	19
IBR[a]-K22	Genital lesion	F. Michalski	16

[a] IBR, Infectious bovine rhinotracheitis.

M Tris-chloride, pH 7.5, and 15 ml of 25% sucrose in the same buffered salt solution. After 2 h of centrifugation in a Spinco SW27 rotor at 24,000 rpm at 5 C, the turbid material at the sucrose interface was collected, diluted threefold with phosphate-buffered saline, and pelleted by a 2-h centrifugation under the same conditions. The virus pellet was resuspended in 0.3 ml of 0.050 M Tris-chloride, pH 8, and 0.010 M EDTA, and then the nucleic acid was extracted with 1 volume of water-saturated phenol. The aqueous phase was applied to an 11-ml gradient of sucrose (5 to 20%) in 1 M NaCl, 0.01 M Tris-chloride (pH 8), and 0.001 M EDTA and centrifuged for 4 h at 40,000 rpm in a SW41 rotor at 4 C. Intact viral DNA sedimented ⅔ down the gradient. The peak of intact DNA was pooled and precipitated by addition of 2.5 volumes of ethanol, and redissolved in 20 μl of 0.002 M Tris-chloride, pH 7.5, and 0.0002 M EDTA.

Endonuclease digestions. *Eco R* I was prepared by the method of Greene et al. (13). For routine assay of this enzyme, the superhelical replicative form of phage G4 DNA (11) was used. This DNA, kindly supplied by G. N. Godson, has one site at which *Eco R* I cleaves (12). HSV DNA was treated as follows: the digestion mixture consisted of 2 μl of 1.0 M Tris-chloride, pH 7.5, 0.10 M $MgSO_4$, and 1 μl of the enzyme preparation; the remaining 20 μl consisted of distilled water and the DNA solution. The digestion proceeded at 37 C for 1 h and then 4 μl of 0.075 M EDTA, 25% glycerol, and 0.2% bromphenol blue was added. If the samples were not subjected to electrophoresis immediately, they were frozen at −20 C. All samples were heated at 55 C for 15 min just prior to electrophoresis to disrupt any aggregation resulting from the complementary termini generated by the *Eco R* I cleavage.

Gel electrophoresis. A modification of previously reported methods (1, 14, 21, 26) was used for separating the DNA fragments. Gels of 0.5% agarose (Sigma) were employed. Agarose (2.0% wt/vol) in distilled water was autoclaved for 25 min and stored at 55 C. Twenty-five milliliters of this was added to a warmed solution containing 65 ml of distilled water and 10 ml of 0.4 M Tris base, 0.3 M NaH_2PO_4 (pH 8.5), 0.01 M EDTA, and 5 mg of ethidium bromide (Sigma) per liter. The solution was mixed and poured into an acid-cleaned slab gel mold (38 by 15 by 0.159 cm) (25) which had a 5-ml plug of polyacrylamide (10% acrylamide, 0.3% bisacrylamide) at the bottom. The well-forming comb was inserted into the agarose and removed after the gel had stood upright for an hour. The bottom spacer was removed and the gel plates were clamped to an electrophoresis apparatus that supported the gel at an angle of 20°. The reservoir buffer was one-half the concentration of that in the gel and electrophoresis of the samples was carried out at 4 C with rectified line voltage (average voltage of 106 C) for about 24 h (until the tracking dye had migrated 20 to 30 cm). DNA containing 25,000 counts/min was applied in a 0.5-cm-wide sample well in the gel. This amount gave sufficient radioactivity so that fragments of less than 1% of the genome could be seen after a 1-week autoradiographic exposure.

The gel was removed from the apparatus and dried onto filter paper as previously described (18). The dehydrated gel was autoradiographed using Kodak RP/R54 medical X-ray film (6 by 17 inch).

Electron microscopy. DNA-containing regions of the gel were located by autoradiography of the wet gel and by their fluorescence in UV light. These regions were cut out and the DNA was electrophoretically eluted (2). The DNA was precipitated with 2 volumes of ethanol at −20 C followed by centrifugation at 10,000 × *g* for 20 min. The precipitate was taken to dryness and resuspended in 50 μl of solution containing 0.1 M Tris-chloride (pH 8.2), 0.01 M EDTA, 40% formamide, 0.01 μg of φX174 phage DNA (RF II), and 50 μg of cytochrome *c* per ml. This solution was spread as a protein film on a hypophase of 0.01 M Tris-chloride (pH 8.2), 0.001 M EDTA, and 10% formamide. Samples were picked up from the protein film, stained, shadowed, and analyzed as described by Davis et al. (5). Length measurements of HSV *Eco R* I fragments were made using the φX174 RF as a length standard (3.7×10^6 daltons). Molecular weight estimates were calculated from these data.

RESULTS AND DISCUSSION

Endonuclease *Eco R* I has proven to be useful in determining the homogeneity and strain of various herpesvirus preparations. It cleaved each herpesvirus DNA we tested into a relatively small number of fragments, and each clinical isolate generated a different set of fragments (Fig. 1).

The molecular weights of the fragments were measured by quantitative electron microscopy. Strain MP virus, which had not been plaque purified, was the source of the fragments that were observed. The results are shown in Table 2. (The 5-Mdalton fragments were lost during subsequent plaque purification of MP.) After electrophoresis on 0.5% agarose gels, migration distance was found to be inversely proportional to the logarithm of the molecular weight of the fragment for fragment masses less than 13 Mdaltons (Fig. 2).

As an independent check on fragment size determinations, the fraction of label in each fragment can be used to estimate its fraction of the total genome, assuming it is equimolar with

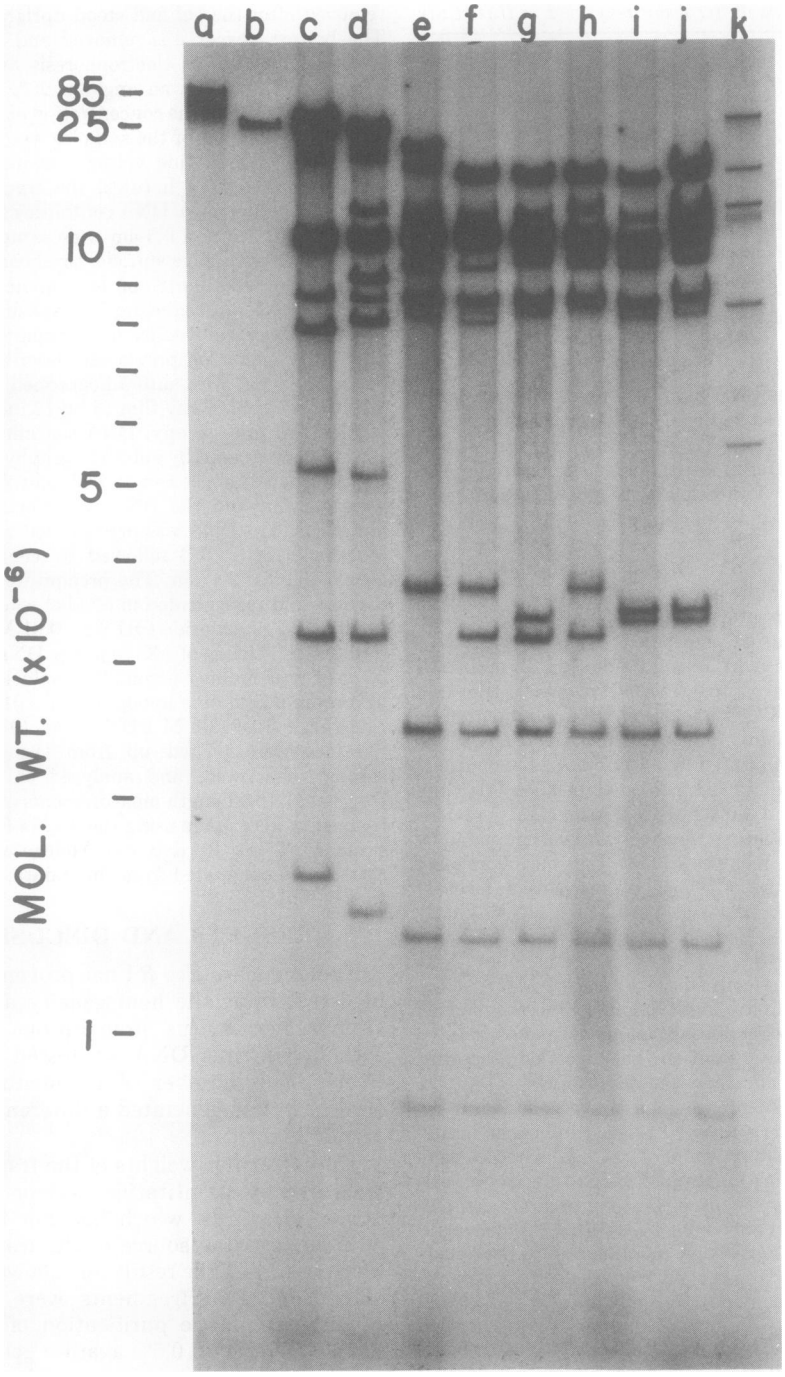


FIG. 1. *Autoradiograph of Eco R I digestion products generated from five HSV type I strains (e to j), two HSV type II strains (c, d), and infectious bovine rhinotracheitis. (a) Strain F DNA uncleaved by Eco R I; (b) T7 DNA (generously supplied by Robert A. Ludwig); (c) strain 333; (d) strain 186; (e) strain F; (f) strain CL-101; (g) strain MP; (h) strain 14-012; (i) strain KOS; (j) a large mass of strain KOS with a much lower specific activity; (k) infectious bovine rhinotracheitis strain K22. Electrophoresis was performed on a 0.5% agarose gel as described. Molecular weight estimates are plotted from Fig. 2.*

TABLE 2. *Molecular weights of RI fragments of HSV MP-DNA as determined by electron microscopy*

Fragment[a]	No. of molecules measured	Molecular weight ± standard deviation
1	14	12.7 ± 1.0
2–5 (unresolved)	21	9.6 ± 1.1
6	9	8.4 ± 0.5
6′	10	5.4 ± 1.7
8	10	3.3 ± 0.3
8′	13	3.0 ± 0.5
9	19	2.6 ± 0.2

[a] Fragments are designated in terms of HSV-KOS fragments. A primed number indicates that the MP fragment has a mobility greater than the corresponding KOS fragment, but less than the subsequently numbered KOS fragment.

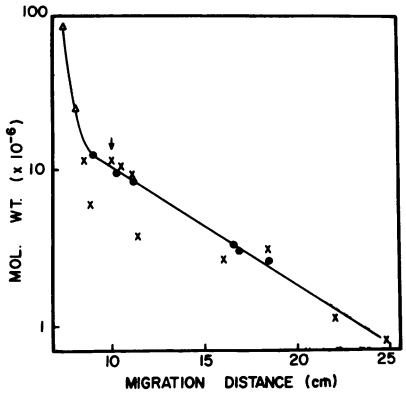


FIG. 2. *Plot of log molecular weights of HSV-MP DNA fragments (●) (Table 2) and HSV-F DNA fragments (×) (calculated from ^{32}P counts) versus electrophoretic migration in 0.5% agarose gels. Intact HSV DNA (Δ) and phage T7 DNA (Δ) are indicated at 85 × 10⁶ and 25 × 10⁶ daltons, respectively. The F fragment denoted with an arrow is one-third of the total calculated because we believe it contains three fragments.*

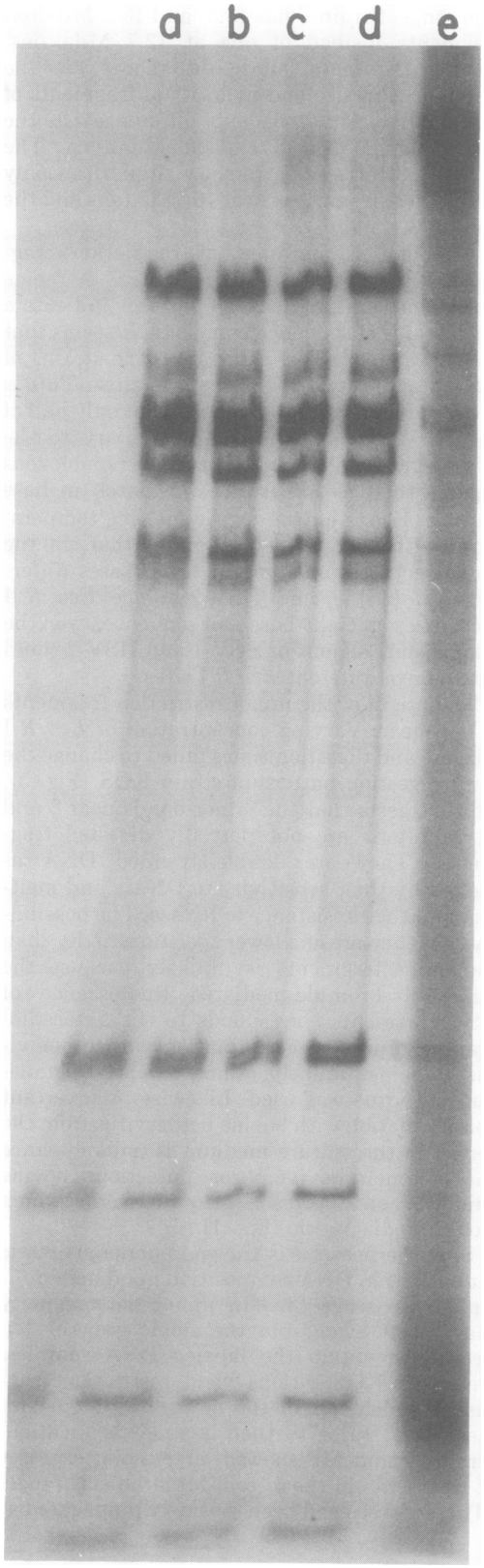
all the other fragments. Figure 2 shows results of such an analysis. A dehydrated gel containing fragments from HSV-1 strain F was sliced and the radioactivity in each slice was measured. To obtain the size of a fragment, the ratio of counts in it to the total counts in all fragments is multiplied by the size of the F genome (85×10^6 daltons) (29). The results show that the fragments are not all equimolar and that those which are equimolar have sizes close to those predicted from electron microscopic measurements.

There are obvious and subtle differences in the restriction patterns of the different virus strains. All of the HSV-1 viruses except 14-012 and CI 101 are different in the 3 to 4 Mdalton size range. Strain F has 15.5- and 13.2-Mdalton fragments instead of one at 12.7 Mdaltons. Among the more subtle differences are the differences in size and molarity of fragments of 9.5 and 9.6 Mdaltons and differences in the minor bands near 8 and 11 Mdaltons. The HSV-2 patterns seem to show similarities only in the three fragments near 10 Mdaltons and the ones of 3.1 and 0.4.

Although comigration of restriction fragments does not prove their identity, it seems likely that similar cleavage patterns indicate a high degree of sequence homology. It seems that the strains of HSV-1 differ from the strains of HSV-2 at most of their cleavage sites. This is not surprising in light of reports that only half of the HSV-1 genome is complementary to the HSV-2 genome (17) and that in the homologous regions there is about 15% mismatch in base pairing (17), as judged by the melting temperature of the hybrid. This implies that, in the homologous regions, one in seven bases differs between HSV-1 and HSV-2. Since *Eco R* I recognizes an eight-base pair sequence, even the homologous regions of HSV-1 and HSV-2 could easily have different *Eco R* I sites.

The origin of the minor restriction fragments is not clear. Varying concentration of *Eco R* I enzyme and digestion times failed to change the limit digestion pattern of strain KOS (Fig. 3). This suggests that the minor bands near 8 and 11 Mdaltons are not partially digested fragments. They are double-stranded DNA as judged by their sensitivity to DNase and melting, and their resistance to RNase. The possibility that they are of a lower specific activity than the other fragments is unlikely because the ethidium bromide-mediated fluorescence of HSV fragments corresponds to the autoradiographic results. Contamination with other viruses seems unlikely, since recently plaque-purified virus was used. Likewise, inadvertant contamination with bovine herpesvirus from the serum in the culture medium is unlikely since the common bovine virus, infectious bovine rhinotracheitis, gives rise to fragments which would be diagnostic (Fig. 1).

Since herpesvirus is known to undergo drastic alterations in DNA composition upon high multiplicity passage (3, 30), minor heterogeneity may result even from the single growth step needed to obtain the labeled DNA samples. Defective KOS virions yield restriction fragments which fall in the 4- to 7-Mdalton size class (30). Prior to their plaque purification, strains F and MP showed large molar excesses of fragments in the 5- to 6-Mdalton size range. (These viruses had been routinely propagated at



multiplicities of infection near one.) The observation of Sheldrick and Berthelot (22), which shows that HSV DNA contains an internal, inverted, tandem duplication of the terminally repetitious region, suggests a molecular basis for the facile generation of variant genomes. Such genetic duplications could serve as frequent sites of unequal recombination to produce DNA molecules which have large segments in common, but which are heterogeneous in other regions of the DNA. It may be, of course, that the minor bands are intermediates in the evolution of high passaged DNA fragments, but it may also be the case that only infectious particles have DNA giving rise to the minor bands. They may be minor only because of the larger mass of defective genomes present in the DNA preparation. Mapping each of the restriction fragments may help to elucidate the role of genomes giving rise to the minor band fragments.

In a sense it is misleading to speak of minor bands because analysis of Fig. 1 reveals that the fragments of many of the major bands are probably present in nonequimolar amounts. Table 3 shows the results of densitometer analysis of the autoradiograph in Fig. 1. (Figure 4 shows a densitometer tracing of an autoradiograph of HSV-1 strain KOS as an example.) We have assumed that there are 3 mol of fragment in the 10.4 to 10.0 size range for the computation. (The KOS doublet at 10.1 Mdaltons is unresolved even after a migration of three times the distance it migrated in Fig. 1.) Since the molar ratios are not easily interpretable, assignment of molecular weights to the different strains by adding the molecular weights of the fragments is not possible.

For the present, we can only guess at the biological significance, if any, contained in the relationships shown by *Eco R* I cleavage patterns. One would not guess from studying the fragment patterns that strains 14-012 and KOS are functionally similar to one another and yet significantly different from the other HSV-1 strains. Duff and Rapp (8) were able to obtain

FIG. 3. *Autoradiograph of gel in which various Eco R digests of HSV-KOS were electrophoresed. (a) 3 μl of enzyme digested the DNA for 2 h, and then another 3 μl was added. A third dose of 3 μl was added at 4.5 h and the digestion was terminated at 6 h. (b) 3 μl of enzyme was incubated with the DNA for 2 h. (c) 3 μl of enzyme was incubated with the DNA for 15 min. (d) 0.3 μl of enzyme was incubated with the DNA for 15 min. (e) 0.03 μl of enzyme was incubated with the DNA for 15 min. All digestions were in about 15 μl except (a) which had a final volume of 20 μl.*

TABLE 3. *Molecular weights of fragments and their approximate molar ratios*[a]

KOS	14-012	MP	CL-101	F	186	333
12.7-1.0	12.7-1.2	12.7-1.1	12.7-0.9	15.5-0.7	22 > 1.5	32
11.0 > 0.5	11.4 > 0.5	11.1-0.5	11.1-0.2	13.2-0.4	19	25 > 1.9
10.9	11.2	10.4-1.0	10.9-0.3	11.2	14.5-0.4	19
10.4-1.0	10.4-1.0	10.1-2.0	10.4-1.0	11.0 > 0.9	13.7-0.4	13.5-0.4
10.1-2.0	10.1-2.0	9.6-0.9	10.1-2.0	10.7	11.2-0.6	10.3-2.0
9.6-0.9	9.6-0.8	8.5-1.1	9.5-0.4	10.4-1.0	10.3-2.0	10.0-1.0
8.5-1.1	8.5-1.2	8.3-0.3	8.5-1.0	10.1-2.0	10.0-1.0	8.6-0.6
8.4 > 0.3	3.6-0.8	3.3-1.1	8.3-0.2	9.5-1.0	9.2-1.2	7.8-1.0
8.2	3.1-0.9	3.1-1.2	8.0-0.3	8.5-1.2	8.5-0.6	5.2-0.5
3.4 > 2.4	2.4-1.0	2.4-1.2	3.6-1.0	8.2-0.2	8.1-1.1	3.1-1.4
3.3	1.3-1.3	1.3-0.9	3.1-1.0	3.6-1.2	5.1-0.5	1.6-1.4
2.4-1.2	0.8-1.1	0.8-0.6	2.4-1.1	2.4-1.7	3.1-1.2	0.4-1.1
1.3-0.5			1.3-0.9	1.3-1.2	1.4-1.3	
0.8-0.5			0.8-0.7	0.8-1.1	0.4-0.9	

[a] Molecular weights in megadaltons and approximate molar equivalents of HSV fragments. The confidence level for the molecular weight estimates is about 5% for fragments from 2 to 13 Mdaltons. Linear extrapolation below 2 Mdaltons and nonlinear interpolation above 13 Mdaltons result in less precise estimations in those regions. Molar ratio computations assumed the presence of 3 mol of fragment in the 10.4- to 10.0-Mdalton range. The gross deviations from integral values suggest that the viral DNA is inhomogeneous.

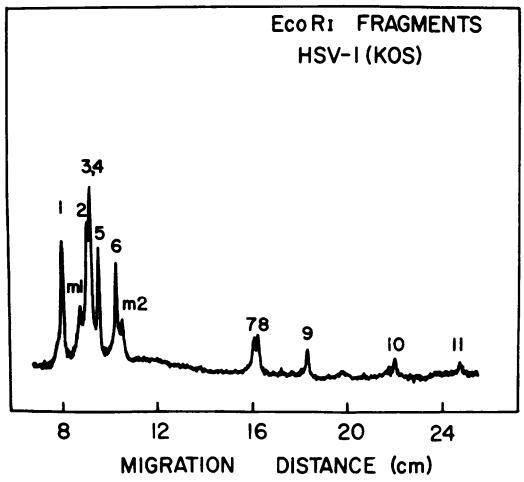


FIG. 4. *Microdensitometer analysis of an autoradiograph of a gel in which HSV-KOS fragments had been electrophoresed. Fragments are numbered in order of increasing mobility. The minor fragments are designated m1 and m2.*

in vitro transformation of hamster embryo fibroblasts with KOS and 14-012 but were unsuccessful with a number of other HSV-1 strains. Although it is premature to speculate on the differences between the oncogenic and nononcogenic strains, the availability of well-defined fragments of HSV-DNA has permitted a more detailed examination of the question (27).

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