Anatomy of Herpes Simplex Virus (HSV) DNA

X. Mapping of Viral Genes by Analysis of Polypeptides and Functions Specified by HSV-1 × HSV-2 Recombinants

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Received for publication 13 October 1977

In an earlier paper (Morse et al., *J. Virol.* **24**:231-248, 1977) we reported on the provenance of the DNA sequences in 26 herpes simplex virus type 1 (HSV-1) × HSV-2 recombinants as determined from analyses of their DNAs with at least five restriction endonucleases. This report deals with the polypeptides specified by the recombinants and by their HSV-1 and HSV-2 parents. We have identified (i) the corresponding HSV-1 and HSV-2 polypeptides with molecular weights ranging from 20,000 to more than 200,000, (ii) the polypeptides that undergo rapid post-translational processing, and (iii) polypeptides that vary intratypically in apparent molecular weight. By comparing the segregation patterns of the polypeptides with those of the DNA sequence of the recombinants, we have mapped the templates specifying 26 polypeptides and several viral functions on the physical map of HSV DNA. The data show the following: (i) α polypeptides map at the termini of the L and S components of the HSV DNA. Although α ICP 27 maps entirely within the reiterated region of the L component, the template for α ICP 4 may lie only in part within the reiterated sequences of the S component. Of note is the finding that cells infected with a recombinant that contains both HSV-1 and HSV-2 DNA sequences in the S component produced α ICP 4 of both HSV-1 and HSV-2. (ii) Templates specifying β and γ polypeptides map in the L component and appear to be randomly distributed. (iii) Thymidine kinase and resistance to phosphonoacetic acid mapped in the L component. In addition, we have taken advantage of the rapid inhibition of host protein synthesis characteristic of HSV-2 infections and syncytial plaque morphology to also map the template(s) responsible for these functions in the L component. The implications of the template arrangement in HSV DNA are discussed.

In this paper we report the location, in the DNAs of herpes simplex virus types 1 and 2 (human herpesviruses 1 and 2; HSV-1 and HSV-2), of the templates specifying 26 polypeptides and several viral functions. The experimental design used in these studies was similar to that used in mapping adenovirus templates (9, 25) and consisted of comparing the segregation patterns of polypeptides specified by HSV-1 × HSV-2 intertypic recombinants with their DNA sequence arrangements. The DNA sequence arrangements of the intertypic recombinants used in these studies were reported in a previous paper in this series (26). We now report on the polypeptides produced in cells infected with the intertypic recombinants and on the physical location in HSV DNA of the templates specified by them.

Pertinent to the results presented in this paper

are the following data: (i) Honess and Roizman (13) reported that HSV-1 specifies in infected cells at least 48 polypeptides ranging from 20,000 to greater than 200,000 in molecular weight. Several additional polypeptides less than 20,000 in molecular weight were reported by Marsden et al. (24). A similar list of HSV-2 polypeptides was reported by Powell and Courtney (30). Studies by Courtney and Powell (3), Pereira et al. (29), Gibson and Roizman (8), and Cassai et al. (2) showed that many HSV-1 and HSV-2 virion polypeptides and infected-cell polypeptides (ICP) differ in electrophoretic mobility, but only a few of these polypeptides were identified as functionally identical.

(ii) The synthesis of both HSV-1 and HSV-2 polypeptides is regulated. On the basis of the temporal pattern and requirements for their synthesis, HSV-1 ICPs were shown to form at least

three groups, designated α , β , and γ , whose synthesis is coordinately regulated and sequentially ordered in a cascade fashion (14, 15, 32–34).

(iii) HSV-1 and HSV-2 DNAs are linear double-stranded molecules approximately 97×10^6 to 99×10^6 in molecular weight (21, 39). HSV-1 DNA consists of two covalently linked components, L and S, containing 82 and 18% of DNA, respectively. The terminal reiterated sequence bracketing the L component, designated as *ab*, and its inverted repeat *b'a'* each contain 6.0% of the DNA, whereas the sequences bracketing the S component, designated as *a'c'* and *ca*, each contain 4.3% of the viral DNA (32, 33, 39). DNA extracted from virions or from infected cells consists of four equimolar populations differing in the orientation of L and S components (10, 33, 39). Studies of HSV-2 DNA with restriction endonucleases indicate that it has a similar structure (G. S. Hayward, T. G. Buchman, and B. Roizman, manuscript in preparation). Analyses of HSV-1 $\times$ HSV-2 intertypic recombinants reported in the preceding paper (26) suggested that only a limited number of HSV DNA arrangements participated in the formation of recombinants and by extension were capable of replication. The HSV DNA arrangement shown to be able to participate in the formation of all recombinants analyzed in the preceding study was designated as prototype (P) (26). The three other arrangements were designated as *I_S*, inversion of S component; *I_L*, inversion of L component; and *I_{SL}*, inversion of both L and S components. For the sake of simplicity, only the P arrangements of HSV-1 and HSV-2 are shown in this paper.

(iv) The location of templates specifying known viral functions in HSV DNA are largely unknown. Previous studies have shown that viral RNA sequences accumulating in the cytoplasm in the presence of cycloheximide (α RNA) hybridize predominantly with terminal fragments of L and S components and to a lesser extent with internal fragments in the L component (19).

(v) Recently we reported on the usefulness of intertypic (HSV-1 $\times$ HSV-2) recombinants for mapping viral markers on the DNA (26). The recombinants used in these studies were produced by crossing *ts* HSV-1 $\times$ *ts* HSV-2 or by crossing *ts* PAA' HSV-1 $\times$ HSV-2. The provenance of the DNA sequences in the cloned recombinants were determined by mapping with *Hpa* I, *Bgl* II, *Xba* I, *Eco*RI, *Hsu* I, and, in some instances, *Kpn* I restriction endonucleases. The nomenclature of the recombinants, the mapping procedure, and detailed restriction endonuclease maps were reported in the preceding paper (26).

MATERIALS AND METHODS

Radiochemicals. L-[U-¹⁴C]isoleucine, L-[U-¹⁴C]leucine, and L-[U-¹⁴C]valine (all with specific activities of approximately 300 mCi/mmol) were purchased from New England Nuclear Corp., Cambridge, Mass.

Cells. HEP-2 cells were grown in Eagle minimal essential medium supplemented with 10% calf serum, 0.001% ferric nitrate, and 1% sodium pyruvate.

Viruses. The virus strains used in this study were (i) HSV-1 (KOS *tsE6*), a DNA⁺ *ts* mutant, and HSV-2 (186 *tsB5*), a DNA⁺ mutant described elsewhere (35, 36); (ii) HSV-1 (17 *tsJ*), a DNA⁺ *ts* mutant (24, 38) kindly provided by J. Subak-Sharpe; (iii) HSV-1 (HFEM *tsN102*), a DNA⁺ mutant kindly provided by A. Buchan; (iv) HSV-1 (B2006 *ts*⁺), kindly provided by S. Kit (20); (v) HSV-2 (186), the parent strain of HSV-2 (186 *tsB5*); (vi) HSV-2 (GP6), a *syn*⁺ mutant (1) of HSV-2 (G); (vii) HSV-2 (GP6 PAA'), a mutant of GP6 resistant to phosphonoacetic acid (PAA), selected by procedures described in reference 26; (viii) HSV-1 (F) and HSV-2 (G) (32–34); and (ix) 24 intertypic recombinants derived by crossing HSV-1 $\times$ HSV-2 parental strains (26). Virus stocks were prepared from plaque-purified seed as previously described (26).

Labeling of proteins synthesized by infected cells. Confluent HEP-2 cell monolayers in 25-cm² tissue culture flasks were exposed to 20 PFU of virus per cell in 1.0 ml of maintenance medium. After 1 h of incubation of 33.5°C with constant agitation, the inoculum was replaced with 5.0 ml of maintenance medium, and incubation was continued at 33.5°C. For labeling polypeptides, the cultures were replenished with labeling medium containing one-tenth the normal amount of leucine, isoleucine, and valine, but supplemented with [¹⁴C]leucine, -isoleucine, and -valine (2.0 μ Ci of each amino acid per ml of medium). For labeling early viral polypeptides, HEP-2 cells were exposed to virus at 37°C for 1 h and then labeled for 2 h at 39°C.

At the end of the labeling period, the cells were rinsed with ice-cold phosphate-buffered saline (3 $\times$ 5.0 ml/flask) to terminate incorporation, either harvested immediately (pulse) or washed, and then reincubated in the absence of labeled amino acids (chase).

Preparation of samples for electrophoresis. The labeled cells were stripped from the dish, denatured, and solubilized by heating for 2 to 3 min at 80°C in the presence of 2% sodium dodecyl sulfate (SDS), 5' β -mercaptoethanol, and 0.05 M Tris-hydrochloride (pH 7.0).

Polyacrylamide gel electrophoresis. The electrophoretic, staining, and autoradiographic techniques were as described previously (11, 37). The polyacrylamide gel electrophoresis was done in a discontinuous buffer system containing 0.1% SDS. The stacker and separation gel contained 3 and 9% acrylamide, respectively, cross-linked with *N,N*-diallyltartardiamide (Aldrich Chemical Co., Milwaukee, Wis.) in an amount corresponding to 2.6% of the weight of acrylamide. The separation gel was 20 cm in length. The proteins used for molecular-weight calibration were β' , β , and α subunits of *Escherichia coli* RNA polymerase, bovine serum albumin, and soybean trypsin inhibitor (T_i) (Boehringer Mannheim, Indianapolis, Ind.) with

molecular weights of 165,000, 155,000, 39,000, 69,000, and 21,000, respectively.

RESULTS

Comparison of HSV-1 and HSV-2 ICP.

Table 1 summarizes the data derived in the course of these studies on the apparent molecular weights and other properties of HSV-1 and HSV-2 polypeptides. The polypeptides were designated according to Honess and Roizman (13) as extended by Pereira et al. (29). Thus, the polypeptides are numbered numerically in order of decreasing molecular weight (13). Polypeptides undergoing rapid post-translational processing are identified by number and letters *a*, *b*, *c*, etc., denoting the precursor (*a*) and products (*b*, *c*, etc.) (29). Table 1 (i) enumerates HSV-1 and HSV-2 specific polypeptides in infected-cell lysates, (ii) identifies the polypeptides that undergo rapid post-translational processing, resulting in an appreciable change in the apparent molecular weight, (iii) lists the polypeptides whose apparent molecular weight was found to

vary intratypically among HSV-1 and HSV-2 strains, (iv) provisionally matches the corresponding HSV-1 and HSV-2 polypeptides, and (v) describes the kinetic class to which the polypeptides belong.

The experimental data relevant to the construction of this table are as follows:

(i) The criteria for identifying virus-specific ICP was that of Honess and Roizman (13) as applied by them for identifying HSV-1 polypeptides and by Powell and Courtney (30) for identifying those of HSV-2. We have largely retained the numerical designation of Honess and Roizman (13) for HSV-1 ICPs and, for simplicity, assigned to HSV-2 ICP numbers that correspond to those of HSV-1 ICP.

(ii) Identification of polypeptides whose molecular weights were altered as a consequence of rapid post-translational processing was based on experiments involving pulse-labeling followed by an appropriate chase in the absence of radioactive precursors. An example of the results of such an experiment is shown in Fig. 1, where we

TABLE 1. *Electrophoretic properties of HSV polypeptides^a*

Poly-peptide no.	Form	Mol wt ($\times 10^{-3}$)		Group	Poly-peptide no.	Form	Mol wt ($\times 10^{-3}$)		Group
		HSV-1 (F)	HSV-2 (G)				HSV-1 (F)	HSV-2 (G)	
1	<i>b</i>	>221	221	γ	20		77*	78*	γ
	<i>a</i>				21		72.5	73.5*	
2	<i>b</i>	205	>205	γ	23		71	72	γ
	<i>a</i>	196	198		24	<i>b</i>	68	68	β/γ
3		194	191	γ		<i>a</i>	67.5	67	
4	<i>c</i>	170*	180*	α	25	<i>b</i>	64	63	γ
	<i>b</i>	165	177			<i>a</i>	63	62.5	
	<i>a</i>	163	172		26	<i>b</i>	61.5*	62	β
5	<i>b</i>	151	153	γ		<i>a</i>	60	60	
	<i>a</i>	149	151		27	<i>b</i>	58	59	α
6	<i>b</i>	146	146*	β		<i>a</i>	56.5	58	
	<i>a</i>	143	143		28		56	57	
7		139*	138*		29	<i>b</i>	55	54.5	β
7.5	<i>b</i>	132*	135			<i>a</i>	54.5	53	
	<i>a</i>	130	131		31		52	52	
8		128	127	β	32		51.5	50.5	γ
9	<i>b</i>	122	121	γ	33		50	49	γ
	<i>a</i>	119	119		34		49.5	48	
10		117*	122*	β/γ	35	<i>b</i>	46.5	45*	γ
11		114	114*	γ		<i>a</i>	45		
12		111	113		36		42.5	42.5	β
13		109	112		37	<i>b</i>	39	39.5*	γ
14		106	107			<i>a</i>		38.5	
15		103*	101*	γ	38		37	35.5	
16		101	100		39	<i>b</i>	36	35	β
17		92	91*	β		<i>a</i>	35		
18	<i>b</i>	88*	85*	γ	40		33.7	34	β
	<i>a</i>	85			41		32	33.5	β
19		78	79	γ	43		26.5	26	γ
					44		24.5	25	γ

^a The polypeptides are numbered numerically in order of decreasing molecular weight. The table also identifies the polypeptides that undergo changes in their electrophoretic mobility, which are designated as *a*, *b*, or *c* forms. Polypeptides showing intratypic variability are marked with an asterisk.

show the polypeptides undergoing rapid post-translational cleavages relatively early in infection. Some of these (e.g., ICP 4 and 6) have been previously shown to be processed after synthesis. Table 1 lists 16 polypeptides that were found to be significantly altered in electrophoretic mobility after synthesis. This is a minimal estimate because post-translational modifications that do not result in a perceptible change in electrophoretic mobility would not be detected. Two points are noteworthy concerning the processing of polypeptides. First, with only one exception, all processed forms of the polypeptides showed a decrease in electrophoretic mobility. Secondly, we resolved in these experiments two bands for the major capsid protein, ICP 5. Pulse-chase experiments of the kind described in the legend to Fig. 1 failed to show a product-precursor relationship. One possible interpretation of our observations is that of a fraction of ICP 5 is altered during assembly of the HSV capsids. The designations 5a and 5b are arbitrary.

(iii) Intratypic variability of structural polypeptides among HSV-1 strains has been reported previously (28). Comparison of the parental strains used to construct the recombinants revealed numerous ICPs that varied among HSV-1 and among HSV-2 strains. The data are based on electrophoretic mobility of the labeled ICPs on the same gels. Some of the data are shown in Fig. 2 through 10 and summarized in Table 1. An interesting example of a nonstructural polypeptide that appeared to be variable was ICP 4; as shown in Fig. 2, the fully processed form (ICP 4c) of HSV-2 (G) migrated more slowly than the corresponding polypeptide of HSV-2 (186).

(iv) The identification of an ICP as α , β , or γ was based on the following operational definition and on the basis of the kinetics of their synthesis (14, 15). By definition, α polypeptides are made immediately after withdrawal of cycloheximide present in the medium during infection and for several hours thereafter (14, 15). An example of a cycloheximide withdrawal experiment is shown in Fig. 1. In that experiment, only ICP 4 and 27 were made in HSV-1-infected cells after withdrawal of the drug. α Polypeptides reached peak rates of synthesis between 2 and 4 h post-infection. In contrast, β polypeptides reached

peak rates of synthesis between 5 and 7 h post-infection and were identified on that basis. Identification of γ polypeptides was based on the observation that they are made at increasing rates until 12 to 15 h postinfection.

(v) Identification of corresponding HSV-1 and HSV-2 ICPs was based on the following rationale. Comparison of the two sets of ICPs showed that they fall into two classes. The first comprised HSV-1 and HSV-2 ICPs that fell into identical kinetic classes and did not differ with respect to electrophoretic mobility. Although they are not central to the objectives of this study, for the purpose of identification we assumed that they have identical functions and numbered them accordingly. The second set comprises HSV-1 and HSV-2 ICPs that could not be matched with respect to electrophoretic mobility. Examples of such are ICP 4 and 27, which fall into the α group, and ICP 5, member of the γ group (14). These polypeptides are central to the objectives of this study because identification of the HSV-1 and HSV-2 polypeptides specified by the recombinants was based in part on their electrophoretic mobilities. The pairing of the corresponding polypeptides was based on analyses of ICPs specified by the recombinants and rested on two criteria. First, with one exception identified later in the text, the presence in the cell lysate of a polypeptide specified by one serotype excluded the presence of the other. Second, each ICP specified by HSV-1 or HSV-2 could be mapped independently as either present or absent, and in each instance the paired ICPs mapped in the same location.

Mapping of HSV polypeptides. In principle, by correlating the segregation patterns of the polypeptides specified by the recombinant viruses with the DNA sequence arrangement of the recombinant virus, it should be possible to map the physical location of the templates specifying viral polypeptides on the HSV genome. The experimental procedure was as follows. Cells infected with parental strains or with recombinants were pulse-labeled at three time intervals after infection as described in Materials and Methods and in the legends to Fig. 2 through 10. Table 2 shows the segregation pattern of the paired HSV-1-HSV-2 polypeptides whose templates were mapped. Figure 11 shows the DNA

FIG. 1. Autoradiographic images of electrophoretically separated polypeptides from HSV-1- and HSV-2-infected cells. HEp-2 cells were infected with either HSV-1 (F) or HSV-2 (G) strains and then labeled for 15 min with ^{14}C -amino acids at the times indicated. At the end of the pulse-labeling period, the inoculated cultures were either harvested immediately (P) or chased by incubation in unlabeled maintenance medium for an additional hour (C). The cycloheximide withdrawal experiment was performed by incubating HEp-2 cells infected with HSV-1 (F) in the presence of 50 μg of cycloheximide per ml of medium. After 7 h, the cycloheximide was washed out and replaced with ^{14}C -amino acid labeling medium. The cells were harvested after 15 min of incubation.

Series A and B

FIG. 2. Autoradiographic images of electrophoretically separated polypeptides labeled with ^{14}C -amino acid from 1 to 3 h postinfection. Parental strains for series A intertypic recombinants were HSV-1 (*tsJ*) and HSV-2 (GP6). The parental strains for series B were HSV-1 (*tsJ*) and HSV-2 (186).

sequence arrangement of the recombinants determined previously (26). Figure 12 shows the physical location of the templates mapped in this study. The mapping of the template speci-

fying ICP 5 illustrates the mapping procedure. Thus, examination of the ICP 5 specified by recombinants C4D and A6D (Table 2; Fig. 4, 7, and 10) indicates that the template (Fig. 11) is

Series A and B

FIG. 3. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series A intertypic recombinants from 4.5 to 6 h postinfection.

Series A and B

GP6 A1E1 A1G3 tsJ A7D A2D A4D GP6 A5C A6D tsJ B1E 186 A1E2

FIG. 4. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series A intertypic recombinants from 9 to 10 h postinfection.

Series C

G N102 C2D 186 C3D1 C4D N102 C5D C6D C7D 186 mock

FIG. 5. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series C intertypic recombinants from 1 to 3 h postinfection as described in Materials and Methods. Parental strains for series C intertypic recombinants were HSV-1 (tsN102) and HSV-2 (186).

Series C

FIG. 6. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series C intertypic recombinants from 4.5 to 6 h postinfection.

Series C

FIG. 7. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series C intertypic recombinants from 9 to 10 h postinfection.

Series D

G tsE6 D1E1 A8E D4E3 D1E4 D1E5 tsE6 D4E1 D5E1 D5E2 mock

FIG. 8. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series D intertypic recombinants from 1 to 3 h postinfection. Parental strains for series D intertypic recombinants were HSV-1 (KOS tsE6) and HSV-2 (186 tsB5).

Series D

D3E2 †sE6 A8E D4E3 D1E4 186 D1E5 D4E1 D4E2 †sE6 D5E1 D5E2

FIG. 9. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series D intertypic recombinants from 4.5 to 6 h postinfection.

Series D

tsB5 D1E1 D3E2 tsE6 A8E D4E3 D1E4 tsB5 D1E5 D4E1 D4E2 tsE6 D5E1 D5E2

FIG. 10. Autoradiographic images of electrophoretically separated polypeptides labeled in cells infected with series D intertypic recombinants from 9 to 10 h postinfection.

TABLE 2. *HSV-1- and HSV-2-infected cell polypeptides^a*

Intertypic re-combinant	Polypeptide no.																											
	2	4	5	7	8	9	10	11	15	18	19	20	23	24	25	27	29	32	33	35	36	39	40	41	43	44		
D1E1	2	1.2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2		
A1E1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1		
A1G3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1		
A7D	1	2	1	1	1	1	1	2*	1	1	1	1	1	1	1	1	2	1	1	A	1	1	1	1	1	1		
A1E2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1		
A2D	1	2	1	1	1	2*	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	2*	1	2*	1	1		
A4D	1	2	1	1	1	1	1	1	1	1	2	A	1	1	1	2	2	1	1	1	1	1	1	1	1	1		
A5C	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1		
D3E2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2		
C1D	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1		
C2D	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	1	1		
C3D1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	1	1		
A8E	1	2	2	1	2	2	2	2	2A	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	1		
C4D	1	2	2	1	1	2A	1	1	2	2	2	2	2	2	2	1	2	2	A	2	1	1	2	2	1	1		
A6D	1	2	1	1	1	2	1	1	1	2	2	2	2	1	1	2	2	1	2	1	1	1A	1	2	1	1		
B1E	1	2	1	1	1	2	1	1	1	2	1	1	1	1	1	2	2	1	2	1	1	1	1	2	1	1		
D3E1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1		
D4E3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1		
D1E4	1	2	A	1	2	2	1	2	2A	2	2	2	2	2	1	2	2	1	2	2	2	A	1	2	2	1		
D1E5	1	2	A	1	2	2	1	2	2A	2	2	2	2	2	1	2	2	1	2	2	2	A	1	2	2	1		
D4E1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2A	1	1	1	1	1	1	1		
D4E2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2A	1	1	1	1	1	1	1		
C5D	1	2	1	2	1	2	2	1	2	2	2	2	2	1	2	2	2	2	2	1	1	2	2	2	2	2		
C6D	1	2	1	2	1	2	1	1	1	1	2	2	2	1	2	2	2	1	1	1	1	1	1	1	1	1		
D5E1	1	2	1	1	2	2	1	2	1	2	1	1	1	1	1	1	2	1	2	2	1	1	1	1*	2	2		
D5E1	1	2	1	1	2	2	1	2	1	2	2	2	1	1	1	2	2	1	2	2	1	1	1A	2	1	1		

^a Polypeptides identified as HSV-1 are labeled as 1; those of HSV-2 are labeled as 2. The asterisk identifies the few instances in which the polypeptides produced by the cells infected with the recombinants did not cosegregate with the DNA sequences predicted by other recombinants. The letter A denotes polypeptides with an aberrant electrophoretic mobility.

to the left of the left crossover site of C4D (0.30 map unit) but to the right of the left crossover site of A6D (0.22 map unit). The map position of the ICP 5 template is more precisely defined by the crucial recombinants D1E4 and D1E5, each of which specifies an ICP 5 with an electrophoretic mobility intermediate between those of HSV-1 and HSV-2. This suggests the possibility of an intragenic recombination event. Comparison with the ICP 5 specified by D4E1, D4E3, and C5D indicates that the left boundary of the template for ICP 5 does not extend to the left beyond 0.23 map unit, which indicates that it is the right crossover site of D1E4 and D1E5 that is most likely responsible for the aberrant electrophoretic properties of ICP 5 produced by these recombinants. The map position of the template for ICP 5 was thus determined to be between 0.23 and 0.30 map units. The DNA sequence arrangements and the ICP 5 specified by all other recombinants are consistent with this conclusion.

The segregation patterns of the polypeptides mapped in this study are summarized in Table 2, which identifies two sets of inconsistencies. The first, identified with an asterisk, comprises

a few instances in which the polypeptides produced by the cells infected with the recombinants did not cosegregate with the DNA sequences predicted by other recombinants. Possible explanations for these inconsistencies are: (i) intragenic recombination events resulting in a recombinant gene specifying a polypeptide with an electrophoretic mobility characteristic of one of the parental types, as illustrated diagrammatically in Fig. 13A; (ii) DNA heteroduplex mismatch repair; and (iii) undetected double-crossover events in regions of the DNA lacking restriction enzyme sites. We noted previously (26) that double-crossover events encompassing small regions of the DNA would be detected only if they bracketed a restriction enzyme cleavage site. The second set, identified with the letter A, denotes polypeptides with an aberrant electrophoretic mobility. In some instances (e.g., ICP 5 of D1E4 and D1E5), the electrophoretic mobility was intermediate between that of the parental types. In other instances, the polypeptide migrated faster than the corresponding parental polypeptides. Possible explanations for the emergence of polypeptides with aberrant electrophoretic mobility are: (i) a recombination

FIG. 11. Provenance of the DNA sequences in the intertypic recombinants used in this study. The upper and lower lines of each doublet represent HSV-1 and HSV-2 DNA, respectively. The heavy line identifies the DNA sequences present in the recombinant virus. The diagonal line spans the boundaries of the crossover sites as defined by the five restriction endonucleases. Hatch marks at or near the diagonal line identify critical endonuclease cleavage sites that define the boundaries of the crossover event. The hatch marks with a knob identify the restriction sites retained in recombinant DNA, whereas those without knobs represent parental cleavage sites that are absent in the recombinant DNA. The boxes on the map unit line represent the reiterated sequence of L and S components of HSV DNA.

event may have occurred within a gene, resulting in premature termination of transcription or translation (Fig. 13B); (ii) a recombination event may have occurred within a gene only partially aligned, resulting in a recombinant gene specifying a polypeptide with an apparent molecular weight intermediate between that of the parental types (Fig. 13C); or (iii) the product of a recombinant gene such as illustrated diagram-

matically in Fig. 13A and C may not have been processed properly.

From Fig. 11 and 12, it becomes apparent that some polypeptides are located in cluster groups with overlapping boundaries. These polypeptides can, however, be ordered by analysis of their segregation pattern among the recombinants. For example, ICP 10 must lie to the left of the ICP 7 and 25, since recombinant C6D specifies HSV-1 ICP 10 but HSV-2 ICP 7 and 25. Accordingly, a correlation of this segregation pattern with the DNA map of C6D places ICP 10 to the left of ICP 7 and 25. The linear order of the polypeptides deduced in this fashion is, from left to right, 9, 41, 18, 33 (15, 39), 40, 5, 24, 36 (35, 11), 8, 43, 44, 32, 2, 10 (7, 25), 23, 20, 27, 29, 4.

The map positions of several polypeptides are of special interest and are dealt with below.

ICP 4. The segregation patterns of ICP 4 (Fig. 2, 3, 5, 6, 8, 9, 11, and 12) indicate that the gene for that polypeptide is located to the right of 0.787 map unit as defined by recombinants A1E1, A1G3, A7D, and D1E1. Several observations made in this and other studies define the location of this template more precisely. Specifically:

(i) Recombinant D1E1 specifies in infected cells (Fig. 8 and 9) the fully processed form of HSV-2 ICP 4 (ICP 4e) and the underprocessed forms of HSV-1 ICP 4 (ICP 4a). The only detected HSV-1 sequences in D1E1 are located at map positions 0.93 to 1.00, indicating that the template for HSV-1 ICP 4 is in the S component and, by extension, that the template for HSV-2 ICP 4 must also be in the S component. It would follow, therefore, that in D1E1 HSV-2 ICP 4 maps between 0.83 and 0.93, whereas HSV-1 ICP 4 maps between 0.93 and 1.0. In Fig. 12, the map position of HSV-1 ICP 4 is shown as determined from analyses of recombinants D1E1. There is evidence, however, that HSV-1 DNA is diploid for the ICP 4 gene and that D1E1 is one example of an ICP 4 heterodiploid recombinant; this conclusion emerges from the observation that recombinants formed between intact HSV-1 DNA and *Xba*-1 fragment I of HSV-2 DNA also specify both HSV-1 and HSV-2 ICP 4. In these recombinants the HSV-2 sequences are in map positions 0.945 to 1.000; i.e., they are reciprocal to those of the D1E1 recombinant (D. Knipe, W. Ruyechen, B. Roizman, and I. Halliburton, submitted for publication).

(ii) A possible complication that might affect the map positions of the genes specifying ICP 4 stems from the observation that although viral RNA extracted from polyribosomes primed for synthesis of α polypeptides hybridizes to restriction endonuclease fragments of the S component, the amount of the reiterated sequences

FIG. 12. Location of templates specifying HSV markers, functions, and polypeptides determined from an analysis of HSV-1 $\times$ HSV-2 recombinants.

represented in this RNA was too small, by a factor of 2, to contain the entire ICP 4 template (19). However, the sum of the sequences that are reiterated and unique sequences that are presented in the α RNA could contain the ICP 4 template. Additional evidence derives from studies (5, 6) on defective virus generated by serial passage of HSV-1 (Justin). Cells infected with certain populations containing defective particles overproduce ICP 4. The defective DNA was shown to consist of a reiterated segment arising from the *ac* and *U_S* sequences of *S* located between map positions 0.939 and 1.000 in the arrangement of HSV DNA shown in Fig. 11 (6). Analyses of the sequences of the defective DNA that hybridize with α RNA (H. Locker and N. Frenkel, in *Proceedings of the Symposium on Herpesvirus and Oncogenesis*, in press) also show that the amounts of reiterated *ac* sequences of the *S* component represented in α RNA are not sufficient to specify ICP 4. Thus, although the evidence clearly indicates that HSV is at least partially diploid for ICP 4 gene, we cannot at this time determine whether the putative remainder of ICP 4 gene extends into the unique sequences of *S* or into the *L* component.

ICP 27. Because A7D, A5C, D5E1, D4E1, and D4E2 all specified ICP 27 of HSV-1 and because the right crossover site in A7D and A5C near the L-S junction had to be to the left of 0.84 map unit as determined by *Kpn* I digestion (26), the template specifying this polypeptide must map within the *L* component of the DNA. By similar reasoning, analyses of recombinants A1E1, A1G3, and A2D suggest that ICP 27 maps within

FIG. 13. Hypothetical models of recombinational events giving rise to inconsistent or aberrant polypeptides specified by some of the HSV-1 $\times$ HSV-2 recombinants. (A) Intragenic recombinational event between genes in which only the 5' ends are aligned. (B) Intragenic recombinational event resulting in a premature termination of transcription or translation. (C) Intragenic recombinational event between genes only partially aligned resulting in a recombinant gene specifying polypeptides with an apparent molecular weight intermediate between those specified by HSV-1 and HSV-2 parents. The heavy line represents the corresponding polypeptide template. The dashed line represents the DNA sequences in the recombinant.

the right-hand end of the *L* component between 0.787 and 0.83 map unit. In support of this location for the template specifying ICP 27 is the observation that the parental strains used to construct the recombinants in series A were a

PAA^r mutant of 17 *tsJ* (HSV-1) and wild-type HSV-2 (GP6) and that the selection was for PAA^r *ts*⁺ progeny. From those experiments, we previously concluded that *tsJ* maps to the right of map position 0.78 (26). Marker rescue experiments (Knipe, Ruyechan, and Roizman, unpublished data) showed that the temperature-sensitive lesion of *tsJ* is located between map positions 0.70 and 0.83 and therefore that the selection is consistent with a requirement for replacement of HSV-1 sequences with HSV-2 sequences.

Three comments concerning ICP 27 should be made. First, ICP 27 is an α polypeptide. It is therefore of interest to note that the DNA sequences arising from the region containing the template for ICP 27 are represented in α RNA (Fig. 12). Second, our data place ICP 27 within the regions of the reiterated sequence *ab* of the L component. However, recombinants containing heterologous *ab* sequences (e.g., A4D, A1G3, A2D, A1E2, and D5E2) do not produce both HSV-1 and HSV-2 ICP 27. Third, ICP 27 can be used to better define the position of crossover events in the DNA of several recombinants. Thus, because of the absence of known restriction endonuclease cleavage sites between map positions 0.78 and 0.83 for HSV-1 and 0.76 and 0.83 for HSV-2, the crossover events near the junction of the L and S components in the DNA of recombinants D5E1, A5C, A1G3, A1E1, and A7D are shown as occurring within a broad region spanning the junction. Comparisons of the DNA sequence arrangements, polypeptides, and biological properties of recombinants shown in Fig. 14 suggest that in A1E1 the crossover event is nearer the map position 0.78 than in A5C and in A7D. The conclusion is based on the observation that A1E1 specifies the ICP 27 of HSV-2, whereas the others specify the ICP 27 of HSV-1 and are partially temperature sensitive.

ICP 6. We suspect that the broad band designated as ICP 6 and ICP 7 contains at least two polypeptides. In earlier studies (29), it was shown that a single band appears in that region during a pulse and that only a portion of that band is then chased to a higher molecular weight. Current data indicate that early in infection, the pulse-labeled band simply broadens to a higher apparent molecular weight during the chase (Fig. 1). We have tentatively defined ICP 6 as that polypeptide which shifts to a higher apparent molecular weight and ICP 7 as that polypeptide which does not become appreciably altered during the chase. We have not been able to map ICP 6 because the electrophoretic mobility of HSV-1 ICP 6 is not different enough from that of HSV-2 ICP 6 to obtain a reliable segregation pattern.

FIG. 14. Possible location of crossover sites near the junctions of L and S components in the DNAs of recombinants A1E1, A5C, and A7D. (A) The boundaries of the crossover sites based on restriction endonuclease maps of the recombinant DNAs. As indicated in the text, and previously reported, the recombinational event occurred between the *Hpa* I cleavage site in HSV-1 and the *Kpn* I site in HSV-2. (B) The probable location of the crossover site in recombinant A1E1 defined by the electrophoretic mobility of ICP 27 and efficiency of plating. (C) The probable location of the crossover site in recombinants A5C and A7D based on the electrophoretic mobility of ICP 27 and partial temperature sensitivity of the recombinants. In (A) and (B), the heavy line represents the template for ICP 27. The dashed line represents the DNA sequences in the recombinant.

Mapping of specific viral functions. (i) HSV-1 (HFEM) *syn*⁻ locus. The biological properties of the intertypic recombinants were reported previously (26). In that report it was observed that intertypic recombinants (C3D₂, C4D, C5D, C6D, and C7D) expressed the *syn*⁻ plaque morphology phenotype characterized by fusion of cells into polykaryocytes. Inasmuch as series C recombinants were derived from a cross of HSV-1 (HFEM *tsN10*₂ *syn*⁻) × HSV-2 (186 *syn*⁺), those recombinants expressing the *syn*⁻ phenotype most likely contain HFEM DNA sequences specifying this function. Analysis of recombinant DNAs shows that the template responsible for the *syn*⁻ phenotype must map to the right of the left crossover site of C4D (0.30) and to the left of the right crossover site of C5D (0.42).

PAA^r. PAA has been shown to selectively inhibit the HSV DNA polymerase (23, 27). Resistance to PAA was used for selection of recombinant progeny in series A, B, and C. Previously we reported that the PAA resistance marker

maps between 0.43 and 0.52 map unit based on DNA sequence arrangements of C1D, C2D, and C3D1.

Shutoff of host protein synthesis. HSV-1 and HSV-2 shut off host protein synthesis within a few hours after infection. Pereira et al. (29) and Powell and Courtney (30) noted that HSV-2 shuts off host protein synthesis much more rapidly and efficiently than HSV-1. We have used this differential property to map the function responsible for the accelerated inhibition of host protein synthesis.

Analysis of HSV-infected HEp-2 cells labeled from 1 to 3 h postinfection (Fig. 2, 5, and 8) clearly demonstrated that five recombinants (C2D, C3D1, D1E1, C5D, and C6D) rapidly and efficiently shut off host protein synthesis in a manner characteristic of HSV-2. Correlation of this phenotype with the physical DNA map of the recombinants suggests that function involved in the accelerated inhibition of host protein synthesis must map between 0.52 and 0.59 map unit. It may be significant that this phenotype correlated with the presence of HSV-2 ICP 7 and 27. Contrary to expectations, C1D did not exhibit this phenotype, possibly because the gene function was inactivated by the recombination event in that region. Since we were mapping accelerated inhibition of host protein synthesis, these observations should not be construed to indicate that the number and locations of all genes responsible for inhibition of host functions are known.

Thymidine kinase. Several lines of evidence suggest that the gene specifying the thymidine kinase maps between 0.27 and 0.35 map unit.

(i) HSV-1 $\times$ HSV-2 intertypic recombinants were constructed from a cross between HSV-1 (B2006 *tk*⁻) $\times$ HSV-2 (GP6 PAA⁺). Progeny were selected that were PAA⁺ *tk*⁻ by plating in the presence of 100 μ g of PAA (17, 23) and 100 μ g of thymidine arabinoside (ara-T) (7) per ml of medium. The only recombinant obtained in this cross contained HSV-2 sequences between map positions 0.38 and 0.44, suggesting that the *tk* gene maps to the left of 0.38 map unit or to the right of 0.44 map unit.

(ii) The parental HSV-1 mutant (*tsE6*) used in the construction of the D series of recombinants is *tk*⁻ (4). We have analyzed the ability of recombinants D1E4, D1E5, D5E1, and D5E2 to form plaques in the presence of 20 μ g of ara-T per ml of medium. Only D5E1 and D5E2 were able to plaque in the presence of ara-T and were therefore *tk*⁻. This suggests that the *tk*⁻ gene maps between 0.27 and 0.35 map unit.

(iii) The molecular weight of the HSV-1-induced thymidine kinase polypeptide in SDS-gels was determined by Honess and Watson (16) to

be 44,000 and by Courtney et al. (4) to be 43,000. ICP 35 (molecular weight 42,000) mapped between 0.27 and 0.35 map unit, whereas ICP 36, another candidate with a molecular weight of 47,000, mapped between 0.31 and 0.38 map unit. These results are also consistent with a recent report that the *Hpa* I DNA fragment contained the genetic information necessary to biochemically transform *tk*⁻ cells to the *tk*⁺ phenotype (41; S. Silverstein, personal communication).

DISCUSSION

In this paper we have (i) enumerated and provisionally matched HSV-1 and HSV-2 polypeptides with molecular weights of 20,000 to more than 200,000, (ii) identified polypeptides that vary in apparent molecular weight from strain to strain and those that undergo rapid post-translational processing, resulting in an appreciable change in the apparent molecular weight, and (iii) mapped the approximate location of the templates specifying 26 polypeptides and several viral functions. Several aspects of the present data merit discussion.

Limitations of the mapping procedures. The HSV-1 and HSV-2 genes that were amenable to mapping by the procedure used in this paper are those that differ in the apparent molecular weight of their product. The genes whose products do not differ in apparent molecular weight or in immunological specificity will have to be mapped by other procedures.

The precision of the mapping procedure used in this study is governed by two factors: the number and distribution of crossover sites and the number of restriction enzyme cleavage sites available to pinpoint the exact location of crossover sites. There were no crossover sites between map positions 0 and 10, and only one recombinant contained a crossover site in the S component suitable for mapping purposes. In addition, the exact position of crossover sites was determined (26) by a total of 56 restriction enzyme cleavage sites in HSV-1 DNA and 40 sites in HSV-2 DNA. In consequence, no polypeptides were mapped in map positions 0 to 10, only one polypeptide was unambiguously shown to arise from a template in the S component, and, as shown in Fig. 11, the boundaries for some of the polypeptide locations are quite broad and may have to be redefined by other techniques.

Considering the complexity of the genome, the correspondence between the segregation patterns of the DNA sequences and that of the polypeptides specified by the recombinants was quite good. The number of inconsistencies was small (Table 2) and could be ascribed to one or more of several possibilities as described earlier in the text.

Apparent gene arrangement in HSV DNA. Several aspects of the arrangement of HSV gene templates are of interest. First, there appears to be a clear segregation of templates specifying α polypeptides. Thus both α templates map at the termini of L and S components at least partly within the reiterated regions. The position of α templates coincides with the map position of some α RNA sequences as reported previously. The proximity of α templates to the termini of the L and S components suggests that the promoter for transcription of these functions may be at the termini. Second, although no β or γ polypeptides were mapped in the S component, those in the L component appeared to be intermixed. It is also interesting to note that viral functions most likely involving β polypeptides (e.g., *tk*, PAA', and accelerated shutoff of host macromolecular synthesis) were intermixed with γ functions (syncytial plaque morphology). These observations have several implications.

The apparent random distribution of templates of β and γ polypeptides is of special interest in view of the evidence indicating that the sequential switch-on of the coordinately regulated β and γ polypeptide syntheses is determined at the transcriptional and post-transcriptional levels (14, 15, 32, 34). One possible explanation for the data is that β and γ templates are segregated on opposite strands. To illustrate this hypothesis further, it could be that (i) the promoter for α template transcription is in the reiterated sequences of the S component and allows transcription from right to left across the function, (ii) the promoter for β polypeptides in the L component is to the left of map position 0.78 and allows transcription to the left, and (iii) at least one promoter for γ templates is to the left of map position 0.12 and promotes transcription to the right. In support of this hypothesis are the following data. (i) Trace amounts of α RNA hybridize to sequences occupied by β and γ templates (19). Consistent with this finding, in many experiments in which attempts were made to restrict viral protein synthesis to α polypeptides, trace amounts of a subset of β polypeptides designated as β_1 (ICP 6, 8, 17, 29, 39, and 41) were also made (29; Fig. 2, 5, and 8). Normal synthesis of β_1 polypeptides, however, required transcription in the presence of functional β polypeptides (29). One possible explanation is that the trace amounts of β_1 polypeptides and corresponding RNA represent read-through from right to left through the putative β_1 promoter. (ii) The hypothesis predicts nuclear transcripts considerably greater in length than the polyribosomal mRNA as well as the accumulation of large amounts of RNA transcribed from both strands and capable of annealing to form

double-stranded RNA. We have in fact reported that nuclear transcripts are many times larger than polyribosomal viral RNA (18, 40) and that a very large fraction of the DNA is transcribed symmetrically (17, 22).

Presence of gene templates in reiterated sequences of viral DNA. As indicated in Results, ICP 27 maps entirely within the *ab* region of the DNA, whereas ICP 4 maps at least in part in the reiterated *ac* sequences of the S component (Fig. 12). These observations would indicate that HSV is diploid for ICP 27 and at least partially diploid for ICP 4. However, whereas ICP 4 heterodiploidy was readily demonstrated in recombinants with heterotypic *ac* sequences (e.g., DIE1), it was not demonstrable for ICP 27 in recombinants with heterotypic *ab* sequences (A1G3, A1E2, A2D, A4D, and D5E2). Two possible explanations may account for this observation. First, it is conceivable that an ICP 27 template is located in the left end of the L component, but that it lacks a promoter site and is not transcribed. It should be noted that if the promoter for transcription of ICP 27 were in S, as considered in the preceding section, then both the right and left templates of ICP 27 would be transcribed off the P and I_L arrangements of HSV DNA, respectively, and both HSV-1 and HSV-2 ICP 27 would be made. The second hypothesis is that at least portions of the terminal inverted sequences must be identical but inverted and that the ICP 27 gene lies entirely within such sequences, whereas the ICP 4 gene does not. This hypothesis arises from the notion, to be detailed elsewhere (manuscript in preparation), that the inversion of L and S components relative to each other arises as a consequence of an obligatory post-synthesis event in which the terminal *ab* and *ac* sequences are repaired or regenerated, using the reiterated *baac* junction as a template. To evaluate this hypothesis, we reexamined the data indicating that the recombinants A1G3, A1E2, A2D, A4D, and D5E2 have heterotypic *ab* regions. The conclusions are based on analyses of the DNAs of recombinant viruses with the restriction endonuclease *Hpa* I which cleaves HSV-1 DNA within the *ab* sequences at map positions 0.037 and 0.787. The ICP 27 template was mapped between map positions 0.787 and 0.83. Since we have no restriction enzyme cleavage sites in the *ab* sequences between 0.000 and 0.037 and between 0.787 and 0.83, we cannot exclude the possibility that these regions are in fact homotypic, and consequently only one ICP 27 would be expected.

Contribution of the recombinants to the study of the evolutionary divergence of HSV-1 and HSV-2. Numerous studies have

shown the close genetic relationship between HSV-1 and HSV-2, and it has been suggested that their current evolutionary divergence reflects acquisition of affinity for different ecological niches as a consequence of different modes of transmission (31). Implicit in this hypothesis is the heuristic concept that the virus carries two sets of functions: those that pertain to the interaction of viral macromolecules among themselves and those that involve interaction of viral and host macromolecules (31). Analyses of the DNAs of the recombinant viruses reported previously (26) led to the conclusion that HSV-1 and HSV-2 DNAs are at least grossly colinear, i.e., that the arrangement of the genes was not altered drastically in the course of the evolutionary divergence. This study contributed an additional conclusion based on the finding that at least some of the recombinants specify a nearly equal number of HSV-1 and HSV-2 polypeptides, implying that HSV-1 and HSV-2 polypeptides are capable of interacting. Although not all permutations have been observed, and therefore we do not know whether prohibited, i.e., lethal, gene combinations exist, the data are adequate to conclude that the sites on viral polypeptides specifically defining the interaction of viral macromolecules among themselves have been conserved. The conclusions are consistent with those of Hilliker and Botstein (12) from studies on bacteriophage lambda that evolution proceeded such that sequence divergence occurred between recombinable segments.

ACKNOWLEDGMENTS

We gratefully acknowledge the help of Ian Halliburton in setting up the new conditions for polyacrylamide gel electrophoresis.

The studies conducted at the University of Chicago were aided by grants from the National Cancer Institute, Public Health Service (CA 08494 and CA 19264), the American Cancer Society (VC 103L), and the National Science Foundation (PCM 76-06254). The studies at Sidney Farber Cancer Center, Harvard Medical School, were aided by grants from the National Cancer Institute, Public Health Service (CA 10893 and CA 20260). L.S.M. is a predoctoral trainee supported by Public Health Service training grant 5-T32 GM07183 from the National Institute of General Medical Sciences. L.P. is a postdoctoral trainee supported by Public Health Service training grant AI-00184 from the National Institute of Allergy and Infectious Diseases.

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