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Genetic Analysis of Temperature-Sensitive Mutants Which Define the Gene for the Major Herpes Simplex Virus Type 1 DNA-Binding Protein

SANDRA K. WELLER, KATHRYN J. LEE, DENNIS J. SABOURIN, AND PRISCILLA A. SCHAFFER*

Laboratory of Tumor Virus Genetics, Sidney Farber Cancer Institute, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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We have assigned eight temperature-sensitive mutants of herpes simplex virus type 1 to complementation group 1-1. Members of this group fail to complement mutants in herpes simplex virus type 2 complementation group 2-2. The mutation of one member of group 1-1, *ts*HA1 of strain mP, has been shown to map in or near the sequence which encodes the major herpes simplex virus type 1 DNA-binding protein (Conley et al., J. Virol. **37**:191–206, 1981). The mutations of five other members of group 1-1 map in or near the sequence in which the *ts*HA1 mutation maps, a sequence which lies near the center of U_L between the genes for the viral DNA polymerase and viral glycoprotein gAgB. These mutants can be divided into two groups; the mutations of one group map between coordinates 0.385 and 0.398, and the mutations of the other group map between coordinates 0.398 and 0.413. At the nonpermissive temperature mutants in group 1-1 are viral DNA negative, and mutant-infected cells fail to react with monoclonal antibody to the 130,000-dalton DNA-binding protein. Taken together, these data indicate that mutants in complementation groups 1-1 and 2-2 define the gene for the major herpes simplex virus DNA-binding protein, an early gene product required for viral DNA synthesis.

At least 50 virus-specific polypeptides are synthesized in cells infected with herpes simplex virus type 1 (HSV-1) (19). Although considerable progress has been made in mapping viral transcripts and polypeptides on the HSV-1 genome, specific functions have been assigned to fewer than 10 viral polypeptides. The most direct approach to elucidating the roles of viral proteins in the replicative process has been to isolate mutants which exhibit alterations in the synthesis or activity of these proteins.

The availability of HSV-1 mutants has facilitated both the identification of the functional roles of the following HSV-1 proteins and the map locations of the genes which specify them: HSV-1 thymidine kinase (2, 48); HSV-1 DNA polymerase (1, 6); the immediate-early regulatory protein (molecular weight, 175; ICP4 or VP175) (12, 34); HSV-1 glycoprotein gAgB (24, 39, 40; N. DeLuca, personal communication); and the major DNA-binding protein (11, 33). Mutants which exhibit altered distribution of the latter protein are the subject of this study.

The major DNA-binding protein, a polypeptide of approximately 130,000 daltons (130K) belonging to the early kinetic class of viral gene products, was described first for HSV-1 by Bayliss et al. (3) and subsequently for HSV-2 by Purifoy and Powell (36). The major HSV-1 DNA-binding protein, ICP8 (19) or VP143 (15), corresponds to HSV-2-infected cell protein ICSP 11/12 (33). To date, we know that the 130K protein binds preferentially to single-stranded DNA, is probably required for HSV DNA synthesis (34), and may act to stabilize DNA polymerase activity (32). Interestingly, antisera prepared against HSV-1- or HSV-2-infected cell proteins cross-react with DNA-binding proteins having approximate molecular weights of 130,000 in cells infected with bovine mammilitis virus, pseudorabies virus, and equine abortion virus, indicating that the gene for the 130K protein has been conserved evolutionarily (25, 49).

The map location of the 130K protein was first suggested by the studies of Conley et al., who isolated a DNA-negative, temperature-sensitive mutant, *ts*HA1, which maps between coordinates 0.385 and 0.402 (11). The smallest DNA fragment able to rescue *ts*HA1 selected an mRNA species from HSV-1-infected cells which, when translated in vitro, encoded a polypeptide having a molecular weight of 128,000. The simplest explanation for these results is that

the mutation in *ts*HA1 lies in or near the gene coding for the 130K DNA-binding protein. It is interesting that the HSV-1 DNA fragment which rescued *ts*HA1 (coordinates 0.385 to 0.402) (11) lies within the region of the HSV-1 genome which has been shown to induce morphological transformation of mouse and hamster cells in vitro (5, 38), suggesting a potential role for this protein in morphological transformation.

The availability of *ts*HA1, whose mutation maps near the coding sequences for the 130K protein, prompted the genetic analysis described below. These studies demonstrated that *ts*HA1 is a member of a larger group of mutants which constitute complementation group 1-1. In addition, mutants in group 1-1 fail to complement mutants in HSV-2 complementation group 2-2, whose members are characterized by altered cellular distribution of the HSV-2 130K DNA-binding protein (11a).

In this paper we describe the physical mapping of the mutations of five members of complementation group 1-1 and demonstrate that these mutants, like their HSV-2 counterparts, exhibit altered distribution of the major DNA-binding protein. Our findings confirm the relationship between mutants in group 1-1 and the HSV-1 DNA-binding protein and provide a more precise definition of the limits of the gene encoding this protein.

MATERIALS AND METHODS

Cells and cell culture. Human epidermoid carcinoma no. 2 (HEp-2), primary rabbit kidney (RK), and African green monkey kidney (Vero) cells were propagated in the Dulbecco modification of Eagle minimal essential medium containing 10% newborn calf serum, 0.03% glutamine, and 0.25% $NaHCO_3$. Cells were maintained in the same medium containing 5% newborn calf serum. Virus stocks were grown and assayed, and complementation tests were performed in Vero cells. RK cells were used for marker rescue studies, and viral DNA was isolated from infected HEp-2 cells.

Virus and virus assays. The origins of the 11 HSV-1 mutants and four HSV-2 mutants used in this study are shown in Table 1. The wild-type virus strains and mutagens used for the induction of these mutants are also shown in Table 1. The results of previous complementation tests placed HSV-1 mutants *ts*A1, *ts*A15, *ts*A16, *ts*656, and *ts*N103 into one complementation group, group 1-1 (41, 42). Mutants *ts*C7 and *ts*D9 are members of complementation groups 1-3 and 1-4, respectively, and exhibit thermolabile DNA polymerase activity (1, 35, 37, 42). The HSV-1 mutant *ts*J12 is a member of complementation group 1-9 and exhibits altered synthesis of glycoprotein gAgB at the nonpermissive temperature (24, 42). The HSV-2 mutants *ts*H9, *ts*A1, and *ts*A8 were previously assigned to two complementation groups (groups 2-2 and 2-7); however, a recent study has demonstrated that in all likelihood these mutants are members of a common group designated group 2-2 (11a). The HSV-2 mutant *ts*D6, which is able to synthesize viral DNA at the nonpermissive temperature, is in complementation group 2-15 (14, 42).

Virus stocks were prepared and assayed as previously described (42). The permissive and nonpermissive temperatures used were 34 and 39°C, respectively, unless otherwise specified. All virus stocks used in this study exhibited efficiencies of plating less than or equal to 10^{-4}. In general, mutants exhibited low levels of leak and reversion ($<10^{-5}$); however, one mutant, *ts*A15, consistently exhibited a slightly higher reversion frequency (10^{-4} to 10^{-3}) than the other mutants.

Complementation. Complementation tests were conducted in Vero cells as described previously (41).

Viral DNA phenotype. Viral DNA phenotypes of mutants were determined as described by Aron et al. (1).

Immunofluorescence tests. Indirect immunofluorescence tests were conducted in Vero cells by the method of Porter et al. (30). Monoclonal antibody to the HSV-1 130K DNA-binding protein was kindly provided by Martin Zweig (National Cancer Institute, Frederick, Md.) (44). Fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin G was used to detect monoclonal antibody on fixed monolayers.

Recombinant DNA plasmids containing HSV-1 DNA. Recombinant DNA plasmids containing fragments of the genome of HSV-1 strain KOS were generously provided by Myron Levine and Rozanne Sandri-Goldin (University of Michigan, Ann Arbor), David Knipe (Harvard Medical School, Boston, Mass.), Wai-Choi Leung (McMaster University, Hamilton, Ontario, Canada), Neal DeLuca (Pennsylvania State University, University Park), and Richard Dixon and Donald Coen (Sidney Farber Cancer Institute, Boston, Mass.). The recombinant plasmids used all contained HSV-DNA sequences located between map coordinates 0.312 and 0.422 on the physical map of the genome (see Fig. 2). A brief description of these plasmid clones follows.

pSG18 was constructed by inserting *Eco*RI fragment F (map coordinates 0.315 to 0.422) into the *Eco*RI site of pBR325 (17). *Sal*I subfragments of pSG18 were inserted into the *Sal*I site of pBR325, generating clones pSG18-SA, pSG18-SD, and pSG18-SE (D. Knipe, personal communication). P1BI1 contains *Bgl*II fragment I (map coordinates 0.312 to 0.415) inserted into the *Bgl*II site of pKC7 (23). pKEF-P1, pKEF-P2, and pKEF-P3 contain strain KOS DNA extending from the *Pst*I sites at map coordinates 0.398, 0.388, and 0.370, respectively, to the *Eco*RI site at map coordinate 0.422 (N. DeLuca, D. J. Bzik, V. C. Bond, S. Person, and W. Snipes, Virology, in press). pKOS10 and pKOS15 contain *Bam*HI fragment G (map coordinates 0.345 to 0.398) inserted into the *Bam*HI site of pBR322 (10). In addition to *Bam*HI fragment G, pKOS15 contains strain KOS *Bam*HI fragment W (Weller and Lee, unpublished data).

DNA isolation. HSV-1 strain KOS was propagated in HEp-2 cells, and viral DNA was obtained from partially purified virions as described by Parris et al. (29).

Plasmid DNA was prepared as follows. Bacteria containing plasmids were grown in Frazier medium, and plasmid DNA was extracted by the method of

Clewell and Helinski (9). Plasmid DNA was purified by equilibrium centrifugation in a solution containing 50% (wt/wt) CsCl and 1 mg of ethidium bromide per ml for 48 h at 45,000 rpm in a Beckman 60Ti rotor. After centrifugation the plasmid band was extracted with *n*-butanol, dialyzed against 0.1× SSC (0.015 M NaCl plus 0.0015 M sodium citrate, pH 7.0), ethanol precipitated, and stored in 0.1× SSC or 1× TE (0.01 M Tris plus 0.001 M EDTA [trisodium salt], pH 7.4).

Mapping of restriction endonuclease cleavage sites in viral and plasmid DNAs. Viral and plasmid DNAs were digested to completion with restriction enzymes *Eco*RI, *Sal*I, *Hin*dIII, *Kpn*I, *Pst*I, and *Hpa*I (New England Biolabs, Beverly, Mass.) and with *Bam*HI and *Bgl*II (Biotec, Madison, Wis.) by using the prescribed buffers. Electrophoresis was carried out in 0.5 to 1.5% agarose horizontal slab gels (5 mm thick) containing 0.5 μg of ethidium bromide per ml. A DNA marker mixture containing bacteriophage lambda DNA digested with *Hin*dIII and pBR322 DNA digested with *Bst*NI was used to make accurate size determinations of the DNA fragments generated after restriction enzyme digestion. The sizes of the standards ranged from 100 base pairs to 27 kilobases (kb). DNA was transferred to nitrocellulose filters by the method of Southern (46) and was hybridized with HSV-1 *Bgl*II fragment I labeled by nick translation with ^{32}P (see below). Autoradiography was carried out at −80°C with Kodak X-Omat X-ray film, using Cronex enhancing screens.

Isolation of DNA fragments. Restriction enzyme-generated DNA fragments were isolated from agarose gels in one of two ways. The first method was a modification of the glass powder elution procedure described by Vogelstein and Gillespie (47; B. Paterson, personal communication). Briefly, agarose gel slices containing restriction fragments were dissolved at 37°C in 6.1 M sodium iodide saturated with sodium sulfite. The DNA was absorbed to fine particles of 325-mesh silica glass for 2 h at 0°C. The glass was recovered by centrifugation and washed once with sodium iodide solution and twice with 50% ethanol–10 mM Tris (pH 7.5)–0.1 M NaCl–1 mM EDTA at 0 to 4°C. DNA was eluted with 1× TE at 37°C for 30 min.

Alternatively, electrophoresis was carried out in low-melting-point agarose as described by Parker and Seed (28). The DNA fragment was obtained free of agarose by chromatography on a benzoyl-naphthoyl-DEAE-cellulose column, using a modification of the procedure of Sedat et al. (43).

Preparation of nick-translated viral DNA hybridization probe. DNA from plasmid p1BI 1 was purified as described above, except that the DNA was extracted twice with redistilled phenol containing 1.6 mg of hydroxyquinoline per ml and saturated with 10 mM Tris-hydrochloride (pH 7.3) and once with chloroform-isoamyl alcohol (24:1) before dialysis. The HSV-1 *Bgl*II fragment I insertion from the p1BI 1 clone (map coordinates 0.314 to 0.415 on the HSV-1 genome) was purified after digestion with *Bgl*II by preparative low-melting-point agarose electrophoresis as described above. The purified *Bgl*II fragment I was nick translated by the procedure of Maniatis et al. (27). [^{32}P]dCTP and [^{32}P]dGTP were obtained from Amersham Corp., Arlington Heights, Ill.

DNA infectivity assays. DNA samples were assayed for infectivity by the calcium phosphate co-precipitation method of Graham and Van der Eb (18), with the following modification. RK cells were transfected in suspension and plated as infectious centers by using uninfected RK cells as indicators, as described by Parris et al. (29).

Marker rescue. Marker rescue experiments were performed by cotransfection of RK cells with intact infectious HSV-1 DNA from a temperature-sensitive (*ts*) mutant and cloned wild-type DNA fragments as plasmids. Before transfection, plasmid DNA was digested with a restriction enzyme which either linear-

TABLE 1. Mutants of HSV-1 and HSV-2 used in this study

Virus type	Wild-type strain	Mutant[a]	Mutagen[b]	Group	Reference or investigator
HSV-1	KOS	*ts*A1	BUdR	1-1	41
		*ts*A15	NTG	1-1	41
		*ts*A16	NTG	1-1	41
		*ts*A24	UV light	—[c]	P. A. Schaffer
		*ts*A42	HA	—	S. P. Little
		*ts*C7	BUdR	1-3	41
		*ts*D9	BUdR	1-4	41
		*ts*J12	NTG	1-9	41
	KOS 1.1	*ts*656	BUdR	1-1	20
	HFEM	*ts*N103	NA	1-1	A. Buchan
	mP	*ts*HA1	HA	—	11
HSV-2	186	*ts*H9	UV light	2-2	8
		*ts*A1	BUdR	2-2[d]	14
		*ts*A8	BUdR	2-2[d]	14
		*ts*D6	BUdR	2-15	14

[a] Nomenclature from Schaffer et al. (42).
[b] BUdR, 5-Bromodeoxyuridine; NTG, nitrosoguanidine; NA, nitrous acid; HA, hydroxylamine.
[c] —, Not previously reported.
[d] HSV-2 mutants *ts*A1 and *ts*A8 were previously assigned to complementation group 2-7; however, recent evidence (11a) indicates that these mutants belong to complementation group 2-2 (see text).

ized the plasmid or excised the inserted HSV-1 DNA; the restriction enzyme was inactivated either by incubation at 60°C for 10 min or by phenol extraction and ethanol precipitation. Each transfection mixture contained 1 μg of digested plasmid DNA, 200 to 1,000 PFU of infectious HSV-1 DNA, and 12 μg of RK DNA in 0.6 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline. Transfections were performed as described previously (29). When generalized cytopathic effects were observed, virus was harvested, and infectious progeny virus was assayed in Vero cells at 34 and 39°C. The efficiency of plating was calculated as follows: plaque-forming units per milliliter at 39°C/plaque-forming units per milliliter at 34°C.

RESULTS

Properties of *ts* mutants. (i) Viral DNA phenotypes and viral DNA polymerase activities. Mutants *ts*A1, *ts*A15, *ts*A16, *ts*C4, and *ts*D9 have been described previously as to their viral DNA phenotypes and viral DNA polymerase activities (1). The following two new mutants of strain KOS are described below: *ts*A24 and *ts*A42. Mutant *ts*A42 was isolated after hydroxylamine mutagenesis of a mixture of *Xba*I fragments (S. P. Little, personal communication), and *ts*A24 was isolated after UV mutagenesis (Schaffer, unpublished data). No viral DNA synthesis was detected in lysates of cultures infected with any of the mutants in group A, C, or D at 39°C (Table 2) (1).

When mutants *ts*C4 and *ts*D9 were tested for induction of DNA polymerase activity at 39°C, they exhibited a marked reduction in polymerase activity (1). Moreover, Purifoy et al. (35, 37) have shown that the purified polymerases synthesized by *ts*C7 (another member of complementation group 1-3) and *ts*D9 are temperature sensitive. In contrast, mutants *ts*A1, *ts*A15, and *ts*A16 induce wild-type levels of polymerase at 39°C (1). Moreover, the mutations of *ts*C4 and *ts*D9 map to a region of DNA which defines the polymerase locus (6), whereas the mutations of *ts*A1, *ts*A15, and *ts*A16 do not (see below).

(ii) Immunofluorescence tests: the 130K polypeptide. Because physical mapping studies placed members of complementation group 1-1 within the region of the HSV genome spanning coordinates 0.312 to 0.422, the region thought to contain the structural gene for the 130K protein (11; S. K. Weller, W. R. Sacks, D. M. Coen, and P. A. Schaffer, submitted for publication), we were interested in knowing whether mutants with mutations mapping in this region were affected in the expression of this protein. For this purpose cells infected with wild-type virus and mutants in complementation group 1-1, as well as *ts*J12 (group 1-9) and *ts*D9 (group 1-4), were tested for reactivity with a monoclonal antibody to the HSV-1 130K protein by immunofluorescence as described above. Photomicrographs of typical fluorescence reactions for strain KOS and *ts*A1 at 34 and 39°C are shown in Fig. 1. Whereas cells infected with strain KOS exhibited bright nuclear fluorescence at both 34 and 39°C (Fig. 1A and 1B), cells infected with *ts*A1 exhibited nuclear fluorescence at 34°C (Fig. 1C) but no specific fluorescence in the cytoplasm or nucleus at 39°C (Fig. 1D). Other members of complementation group 1-1 (*ts*A15 and *ts*A16) resembled *ts*A1 in that no specific nuclear or cytoplasmic fluorescence was observed at 39°C (Table 2). Importantly, cells infected with mutants *ts*A24, *ts*A42, and *ts*HA1 resembled cells infected with established group 1-1 mutants. In contrast, *ts*J12- and *ts*D9-infected cells exhibited nuclear fluorescence at both 34 and 39°C (Table 2). The presence of nuclear fluorescence in *ts*D9-infected cells at 39°C demonstrated that the absence of nuclear fluorescence in cells infected with group 1-1 mutants was not simply due to the absence of viral DNA

TABLE 2. Viral DNA synthesis and fluorescent staining with anti-130K monoclonal antibody of wild-type virus and *ts* mutants of HSV-1 at 34 and 39°C

Virus	Viral DNA synthesis[a]		Nuclear fluorescence with anti-130K monoclonal antibody	
	34°C	39°C	34°C	39°C
KOS	100	100	+	+
*ts*A1[b]	44	0	+	−
*ts*A15[b]	19	0	+	−
*ts*A16[b]	33	0	+	−
*ts*A24	93	0	+	−
*ts*A42[c]	93	0	+	−
*ts*J12	95	92	+	+
*ts*C7[b]	27	0	+	+
*ts*D9[b]	57	0	+	+

[a] Results are expressed as percentages of viral DNA in wild-type virus-infected cultures.
[b] Data on viral DNA synthesis and DNA polymerase activity were taken from Aron et al. (1).
[c] Assay of viral DNA synthesis was performed by S. P. Little (personal communication).

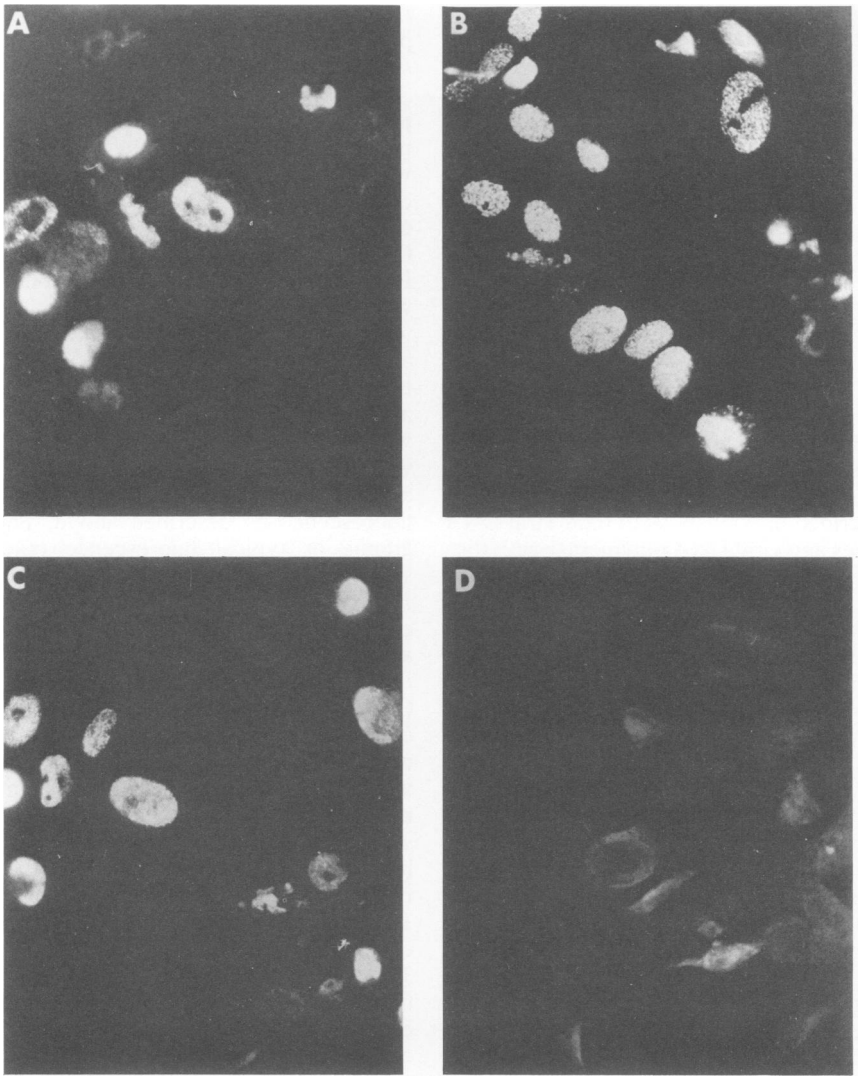

FIG. 1. Immunofluorescent staining of HSV-1 strain KOS- and *ts*A1-infected Vero cells with monoclonal antibody to the 130K DNA-binding protein: photomicrographs of cells infected with HSV-1 strain KOS at 34°C (A) and 39°C (B) and with mutant *ts*A1 at 34°C (C) and 39°C (D) and treated successively with monoclonal antibody to the HSV-1 130K protein and fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin G.

synthesis at this temperature, as *ts*D9 is DNA negative. It should be emphasized that the failure of mutants in group 1-1 to exhibit specific nuclear fluorescence when they were tested with monoclonal antibody to the 130K protein indicates either that the protein was not synthesized at 39°C or that the protein was synthesized in a configuration not detectable by the monoclonal reagent. In either case, we conclude that members of complementation group 1-1 are mutated in a function affecting the cellular distribution of the 130K DNA-binding protein.

Complementation. Because members of complementation group 1-1, as well as mutants *ts*A24, *ts*A42, and *ts*HA1, exhibited altered expression of the 130K protein and because the *ts*HA1 mutation has been shown to map in or near sequences specifying this protein (11), we were interested in determining the relationship among *ts*HA1, *ts*A24, *ts*A42, and members of complementation group 1-1. Therefore, we conducted complementation tests with these six mutants. Mutants *ts*J12, *ts*C4, and *ts*D9 were included as controls. Table 3 shows the results of quantitative complementation tests with these mutants.

TABLE 3. Complementation among nine *ts* mutants of HSV-1

Mutant	Complementation indices[a]								
	*ts*A1	*ts*A15	*ts*A16	*ts*A24	*ts*A42	*ts*HA1	*ts*J12	*ts*C7	*ts*D9
*ts*A1		**1.2**[b]	**0.6**	**0.9**	**0.9**	**0.3**	11	2,250[c]	170
*ts*A15			**1.9**	6.0	7.3	**1.3**	20	190[c]	35
*ts*A16				**0.3**	**0.7**	**0.7**	68	19.5[c]	2,424
*ts*A24					**0.7**	**0.5**	583	488	554
*ts*A42						**0.7**	2,684	ND[d]	831
*ts*HA1							186	68.1	80
*ts*J12								61[c]	23
*ts*C7									76
*ts*D9									

[a] Complementation tests were conducted as described previously (42).
[b] Boldface values considered to be negative for complementation.
[c] The complementation test was performed with *ts*C4, another member of complementation group 1-3.
[d] ND, Not done.

In previous studies, a complementation index of 2 was used to signify positive complementation (41, 42). However, the levels of complementation which represent inter- and intragenic complementation were not known at the time that this arbitrary value was set. We now feel that complementation indices between 2 and 10 should be regarded as ambiguous in that they may represent either intra- or intergenic complementation, whereas values of 10 or greater are more likely to reflect intergenic complementation (S. K. Weller, W. R. Sacks, D. M. Coen, and P. A. Schaffer, submitted for publication).

*ts*HA1 and the new mutants *ts*A24 and *ts*A42 failed to complement *ts*A1, *ts*A15, and *ts*A16, indicating that all six of these mutants are members of complementation group 1-1 (Table 3). Mutants *ts*A24 and *ts*A42 gave ambiguous complementation indices of 6.0 and 7.3, respectively, when they were paired with mutant *ts*A15; however, these mutants yielded complementation indices of less than 2.0 when they were tested with other members of the group, such as *ts*A1 and *ts*A16. In addition, mutants *ts*A24 and *ts*A42 failed to complement two other members of complementation group 1-1, *ts*656 and *ts*N103 (data not shown). All members of complementation group 1-1, including *ts*656 and *ts*N103, complemented the control mutants *ts*C4, *ts*D9, and *ts*J12 efficiently (i.e., indices were 10 or greater). Thus, we conclude that the five strain KOS mutants *ts*A1, *ts*A15, *ts*A16, *ts*A24, and *ts*A42, as well as the *ts*HA1 mutant of strain mP, the *ts*656 mutant of strain HFEM, and the *ts*N103 mutant of strain KOS 1-1, belong to complementation group 1-1.

Intertypic complementation. Preliminary intertypic complementation tests (R. Dixon, unpublished data) indicated that mutants in HSV-1 group 1-1 and HSV-2 group 2-2 failed to complement each other. To confirm this finding, we performed intertypic complementation tests with six HSV-1 mutants from group 1-1 and three HSV-2 mutants from group 2-2 at 38°C, the nonpermissive temperature for HSV-2 *ts* mutants. HSV-1 mutants *ts*J12 and *ts*C4 and HSV-2 mutant *ts*D6 were included as controls. The results of these tests are shown in Table 4. This

TABLE 4. Intertypic complementation among eight mutants of HSV-1 and four mutants of HSV-2

HSV-1 mutant	Complementation indices with the following HSV-2 mutants[a]:			
	*tsH*9	*tsA*1	*tsA*8	*tsD*6
*ts*A1	0.1[b]	0.5[b]	0.2[b]	8.9
*ts*A15	0.03[b]	0.2[b]	0.06[b]	16.0
*ts*A16	0.3[b]	3.9	0.2[b]	27.0
*ts*A24	0.5[b]	0.5[b]	0.7[b]	8.6
*ts*A42	0.5[b]	1.9[b]	1.9[b]	6.0
*ts*HA1	0.5[b]	0.2[b]	0.2[b]	9.8
*ts*C4	2.4	ND[c]	1,053	2,333
*ts*J12	ND	10.9	12.9	ND

[a] Complementation tests were conducted as described previously (42).
[b] Values considered to be negative for complementation.
[c] ND, Not done.

table shows that the five HSV-1 mutants in group 1-1 failed to complement the three HSV-2 mutants in group 2-2. However, ambiguous results were obtained with the pair *ts*A16 and *ts*A1. In contrast, the HSV-1 mutants in group 1-1 complemented HSV-2 mutant *ts*D6 efficiently. It should be noted that lower complementation indices are routinely obtained in intertypic complementation tests due in part to excessive leak of HSV-1 *ts* mutants at 38°C. Therefore, the complementation indices between 6 and 10 observed in tests with *ts*D6 were thought to reflect positive complementation. With one exception, HSV-2 mutants *ts*H9, *ts*A1, and *ts*A8 complemented HSV-1 mutants *ts*C4 and *ts*J12 efficiently. The complementation index generated by the pair *ts*H9 and *ts*C4 was 2.4, which is low and thus ambiguous. We conclude from these studies that the mutations of members of groups 1-1 and 2-2 very likely represent a function common to both HSV-1 and HSV-2.

Restriction enzyme mapping of *Eco*RI fragment F. As mentioned above, preliminary marker rescue tests with mutants in complementation group 1-1 indicated that the mutations of these mutants lie in *Eco*RI fragment F of strain KOS (Weller et al., submitted for publication). To map the mutations of these mutants more finely, it was first necessary to construct a more detailed restriction map of this region of the genome. The region of strain KOS DNA contained in *Eco*RI fragment F (map coordinates 0.315 to 0.422) was mapped with respect to the cleavage sites of the following restriction enzymes: *Bgl*II, *Kpn*I, *Sal*I, *Pst*I, *Hpa*I, and *Bam*HI. Strain KOS DNA and plasmid subclones containing portions of *Eco*RI fragment F were digested, subjected to agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to nick-translated *Bgl*II fragment I DNA (map coordinates 0.312 to 0.415). The plasmid subclones used for restriction enzyme mapping are shown in Fig. 2. Single, double, and triple digestions permitted relative ordering of cleavage sites of each restriction enzyme within this region. The physical map summarizing the results of these studies is shown in Fig. 3. Our restriction map of this region of strain KOS DNA is identical to the map constructed by N. DeLuca et al. for the same region of strain KOS DNA (DeLuca et al., in press). However, there are minor differences between the maps of strain KOS *Eco*RI fragment F and the equivalent fragments of HSV-1 strains Justin, F (26), and 17 (7). With one exception (the *Bgl*II and *Eco*RI sites at the far left end of *Eco*RI fragment F are reversed on our map of strain KOS and the previously published map [7] of strain 17), the *Eco*RI, *Bgl*II, *Hpa*I, and *Bam*HI sites which lie between coordinates 0.312 and 0.422 appear to be conserved among these four strains of HSV-1. It should be noted that several of the strain KOS *Bam*HI and *Hpa*I fragments have designations which differ from those of strains F and Justin; however, the positions of the sites are conserved. On the other hand, the *Kpn*I and *Sal*I sites within strain KOS *Eco*RI fragment F are similar (but not identical) to the *Kpn*I and *Sal*I sites in the equivalent regions of strains 17 and Justin (7, 26); more significant differences exist between strains KOS and F (26). The *Pst*I sites for other strains are not available for comparison.

Deleted sequences in cloned HSV DNA. Several investigators have observed that HSV-1 DNA fragments consisting of sequences which lie between coordinates 0.407 and 0.413 contain deletions when they are cloned into *Escherichia coli* (31; N. DeLuca, personal communication; N. Frenkel, personal communication; C. Gray, personal communication; D. Knipe, personal communication).

We made similar observations in the course of mapping the *Kpn*I and *Bam*HI cleavage sites within subclones of *Eco*RI fragment F. Thus, we found substantial differences between the electrophoretic mobilities of uncloned fragments of strain KOS DNA and their cloned counterparts. One such comparison is shown in Fig. 4. The DNAs of strain KOS, pSG18, pKEF-P1, and pKEF-P2 were digested with *Kpn*I and analyzed as described in the legend to Fig. 3. Figure 4A shows the ethidium bromide-stained gel, and Fig. 4B shows an autoradiogram of the same gel after hybridization. *Kpn*I fragment d (map coor-

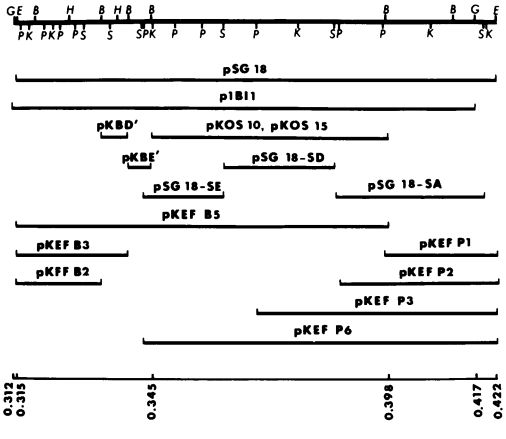

FIG. 2. Recombinant DNA plasmids containing HSV-1 DNA. The line at the top is a composite map of the restriction endonuclease sites taken from Fig. 3. The HSV-1 DNA insertions in each recombinant plasmid described in the text are shown below this map. The numbers at the bottom are selected map coordinates. *B*, *Bam*HI; *E*, *Eco*RI; *G*, *Bgl*II; *H*, *Hpa*I; *K*, *Kpn*I; *P*, *Pst*I; *S*, *Sal*I.

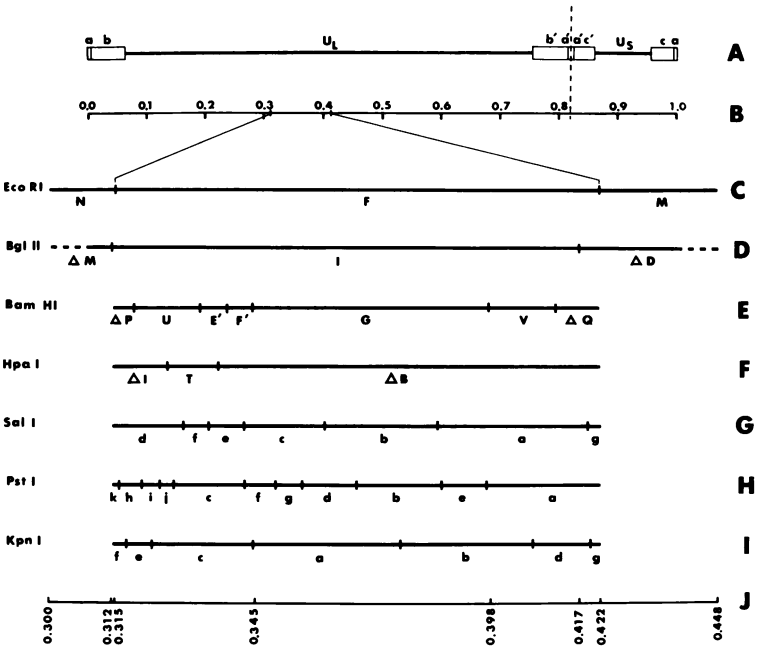

FIG. 3. Restriction map of HSV-1 strain KOS *Eco*RI fragment F. Line A represents the sequence arrangement of HSV-1 DNA. The numbers on line B are the physical map coordinates of the genome. In lines C through I, *Eco*RI fragment F (map coordinates 0.315 to 0.422) is expanded to show the *Eco*RI, *Bgl*II, *Bam*HI, *Hpa*I, *Sal*I, *Pst*I, and *Kpn*I cleavage sites within coordinates 0.315 to 0.422. In the cases of enzymes for which the entire strain KOS map is known (*Eco*RI, *Bgl*II, *Bam*HI, and *Hpa*I), fragments are designated by capital letters corresponding to the fragments generated after cleavage of total strain KOS DNA (45; J. Skare, unpublished data; Weller and Lee, unpublished data). The open triangles indicate that only a portion of the designated fragment is contained within *Eco*RI fragment F. In the cases of enzymes *Sal*I, *Pst*I, and *Kpn*I, fragments are designated with lower-case letters corresponding to the sizes of the fragments generated after cleavage of *Eco*RI fragment F. Line J shows selected map coordinates within *Eco*RI fragment F.

dinates 0.407 to 0.420) of each DNA is indicated with an arrow. Plasmids pSG18, pKEF-P1, and pKEF-P2 each produced a *Kpn*I fragment d which was smaller than strain KOS *Kpn*I fragment d. The size of the deletion in these plasmids varied from approximately 80 base pairs in the 1.82-kb species in pSG18 to 660 base pairs in pKEF-P2. Substantial heterogeneity was also observed in *Bam*HI fragment V (map coordinates 0.398 to 0.413), which shares sequences with *Kpn*I fragment d (Fig. 3). Hence, we conclude that the deleted sequences are located between the *Kpn*I site at the b-d junction and the *Bam*HI site at the V-Q junction (map coordinates 0.407 to 0.413). The presence of these deletions had no apparent effect on the ability of the subclones to rescue *ts* mutations in physical mapping studies (see below). It should be noted that the region of HSV-1 DNA represented by deletion-prone *Bam*HI fragment V is contained within the DNA sequences represented by a class of defective DNAs which are thought to include an origin of viral DNA synthesis (16, 21, 38). The difficulties experienced by us and other workers in cloning these sequences in an undeleted form may be related to the presence of this putative origin.

Marker rescue. The fine-structure physical map locations of the mutations of five strain KOS mutants in HSV-1 complementation group 1-1 within *Eco*RI fragment F were determined by marker rescue. A series of plasmid subclones of *Eco*RI fragment F (Fig. 2) were used in marker rescue experiments with infectious DNAs from *ts*A1, *ts*A15, *ts*A16, *ts*A24, and *ts*A42. In addition, pKEF-P1 was digested with *Bam*HI, and *Bam*HI fragment V was isolated after gel electrophoresis.

The results of marker rescue experiments with *ts*A1, *ts*A15, *ts*A16, *ts*A24, and *ts*A42 are shown in Table 5. The map coordinates of each of the subcloned fragments are also shown in Table 5. In these experiments rescue efficiencies greater than 1.0 were considered positive. Mutants *ts*A1, *ts*A15, and *ts*A42 were rescued efficiently with digested DNAs from pSG18, pKOS10, pKOS15, pKEF-B5, and pSG18-SA. Fragments which rescued these mutants shared sequences

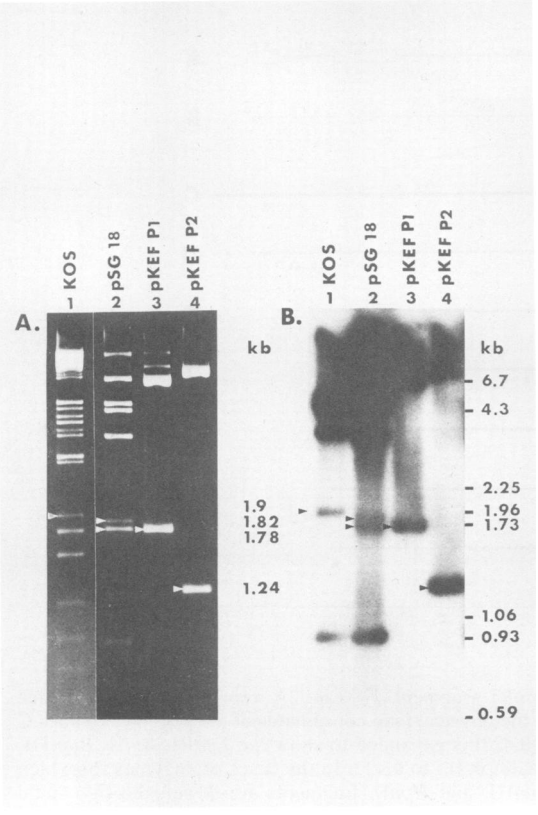

FIG. 4. Analysis of *Kpn*I-digested strain KOS and plasmid DNAs. Strain KOS and plasmid pSG18, pKEF-P1, and pKEF-P2 DNAs (lanes 1 through 4, respectively) were digested with *Kpn*I and subjected to electrophoresis on a 0.7% agarose gel. DNAs were transferred to nitrocellulose and hybridized to ^{32}P-labeled *Bgl*II fragment I DNA as described in the text. (A) Ethidium bromide-stained gel. (B) Autoradiograph of the same gel. A DNA marker mixture containing bacteriophage λ DNA digested with *Hin*dIII and pBR322 DNA digested with *Bst*NI was subjected to electrophoresis on the same gel; the sizes of fragments are shown on the right. The fragments corresponding to *Kpn*I fragment d for each DNA preparation are marked with arrows. The strain KOS fragment (lane 1) migrated at a position corresponding to 1.9 kb. Digestion of pSG18 with *Kpn*I generated two *Kpn*I fragment d bands which migrated slightly faster than the *Kpn*I fragment d from strain KOS (1.82 and 1.78 kb) (lane 2). The *Kpn*I-digested plasmids pKEF-P1 and pKEF-P2 (lanes 3 and 4) each contained a single band corresponding to *Kpn*I fragment d which also migrated faster than the fragment from strain KOS (1.78 and 1.24 kb, respectively).

that mapped between the *Sal*I site at the b-a junction and the *Bam*HI site at the G-V junction (map coordinates 0.385 to 0.398) (Fig. 3). All other tests yielded plating efficiencies less than 1.0. In experiments with *ts*A15, unusually large quantities of ts^+ virus were observed with plasmids pKEF-P1, pSG18-SD, and pSG18-SE, *Bam*HI fragment V, and a "no fragment" control, resulting in plating efficiencies that were approximately 10-fold lower than the plating efficiencies observed with pKOS10 and pKOS15, which rescued most efficiently. Because the reversion frequency of *ts*A15 is higher (10^{-4} to 10^{-3}) than that of other mutants (10^{-5} to 10^{-4}), we conclude that the high background observed in tests with *ts*A15 DNA was probably due to a high frequency of reversion of the *ts* lesion.

Mutants *ts*A16 and *ts*A24 were rescued efficiently by pSG18, pSG18-SA, and pKEF-P1 (Table 5). In addition, *Bam*HI fragment V rescued both *ts*A16 and *ts*A24 efficiently. The fragments which rescued *ts*A16 and *ts*A24 shared sequences which lay within *Bam*HI fragment V between coordinates 0.398 and 0.413.

Selected progeny of marker rescue experiments with *ts*A1, *ts*A24, and *ts*A42 were tested for plating efficiency at 34 and 39°C to confirm their ts^+ phenotypes (i.e., the *ts* lesion was rescued). All plaque isolates produced large plaques at 39°C. Thus, these plaques were ts^+ and resembled wild-type plaques at both 34 and 39°C, suggesting that mutants *ts*A1, *ts*A24, and *ts*A42 each contain a single *ts* mutation which can be rescued efficiently to generate infectious wild-type progeny.

The progeny of marker rescue tests with *ts*A16 and *ts*A15 (Table 5) produced small plaques at 39°C. When these plaques were picked, grown, and assayed at 34 and 39°C, 15 of 15 *ts*A16 and 21 of 21 *ts*A15 plaque isolates were ts^+ and produced very small plaques at 39°C (data not shown). We conclude, therefore, that *ts*A15 and *ts*A16 are double mutants containing a *ts* lesion and an additional small plaque mutation present elsewhere in the genome. It is notable that both *ts*A15 and *ts*A16 were isolated after mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a mutagen noted for its ability to induce multiple mutations (4).

DISCUSSION

Complementation analysis. We have described the results of intra- and intertypic complementation tests with a series of DNA-negative *ts* mutants of HSV-1 and HSV-2. HSV-1 mutants *ts*A1, *ts*A15, *ts*A16, *ts*A24, *ts*A42, *ts*HA1, *ts*656, and *ts*N103 are members of complementation group 1-1. In a similar study, Dixon et al. (11a) have shown that HSV-2 mutants *ts*H9, *ts*A1, and *ts*A8, which were used in this study, as well as four other HSV-2 mutants, are members of a single HSV-2 complementation group, group 2-2. Because HSV-1 mutants in group 1-1 failed to complement mutants in group 2-2, we conclude that the mutants in the two groups very likely

TABLE 5. Marker rescue of *ts*A1, *ts*A15, *ts*A16, *ts*A24, and *ts*A42[a]

Plasmid or fragment[b]	Map coordinates	Marker rescue efficiencies ($\times 10^3$) with the following mutant DNAs:				
		*ts*A1	*ts*A15	*ts*A16	*ts*A24	*ts*A42
pSG18	0.315–0.422	25.1	35.0	10.0	12.1	20.8
pSG18-SA	0.385–0.419	4.0	2.2	10.2	7.9	3.7
pSG18-SD	0.360–0.385	ND[c]	0.2[d]	<0.005[d]	ND	<0.01[d]
pSG18-SE	0.342–0.360	ND	0.4[d]	<0.005[d]	ND	<0.003[d]
pKOS10	0.345–0.398	32.0	13.4	<0.005[d]	<0.002[d]	1.8
pKOS15	0.345–0.398	11.0	17.6	<0.005[d]	<0.01[d]	ND
pKEF-B5	0.315–0.398	4.0	2.1	<0.005[d]	<0.01[d]	3.8
pKEF-P1	0.397–0.422	<0.03[d]	0.5[d]	7.8	1.0	<0.02[d]
*Bam*HI-V	0.398–0.413	0.56[d]	0.8[d]	4.1	11.0	ND
None		<0.01[d]	<0.005[d]	<0.005[d]	<0.05[d]	<0.02[d]

[a] Results are expressed as plating efficiencies, which were determined as follows: PFU per milliliter at 39°C/PFU per milliliter at 34°C.

[b] Plasmids pSG18, pKEF-B5, and pKEF-P1 were digested with *Eco*RI, and the enzyme was inactivated by incubation at 60°C for 10 min before transfection. Plasmids pSG18-SA, pSG18-SD, and pSG18-SE were digested with *Sal*I, and pKOS10 and pKOS15 were digested with *Bam*HI. The plasmids digested with *Sal*I or *Bam*HI were phenol extracted and ethanol precipitated before transfection. *Bam*HI fragment V was eluted from an agarose gel by glass powder elution, as described in the text.

[c] ND, Not done.

[d] Values considered to be negative for marker rescue.

represent a function common to both HSV-1 and HSV-2.

Mapping studies. In preliminary tests we demonstrated that the mutations of mutants in complementation group 1-1 mapped to *Eco*RI fragment F (map coordinates 0.315 to 0.422) (Weller et al., submitted for publication). The fine-structure mapping of the mutations of five members of complementation group 1-1 reported here shows that the DNA fragments which rescue *ts*A1, *ts*A15, and *ts*A42 share a sequence which maps between the *Sal*I site at the b-a junction and the *Bam*HI site at the G-V junction (map coordinates 0.385 to 0.398) (Fig. 5). Conley et al. have reported that mutant *ts*HA1 was rescued by a *Sal*I subfragment obtained from a plasmid (pRB102) containing *Bam*HI fragment G of HSV-1 strain F (11). Although there are discrepancies between the locations of the *Kpn*I and *Sal*I sites in pRB102 and the *Kpn*I and *Sal*I sites reported for strain F DNA by Locker and Frenkel (26) and for strain KOS DNA by us (Fig. 3), it appears that the locations of the *ts* mutations in *ts*HA1, *ts*A1, *ts*A15, and *ts*A42 lie within the same 1.7-kb DNA sequence between the *Sal*I site at the b-a junction and the *Bam*HI site at the G-V junction (Fig. 3 and 5). Mutants *ts*A16 and *ts*A24 were rescued by DNA fragments which map within *Bam*HI fragment V (map coordinates 0.398 to 0.413) (Fig. 5). Taken together, the fragments which rescue mutants in group 1-1 span approximately 4 kb on the map of the HSV-1 genome (map coordinates 0.385 to 0.413).

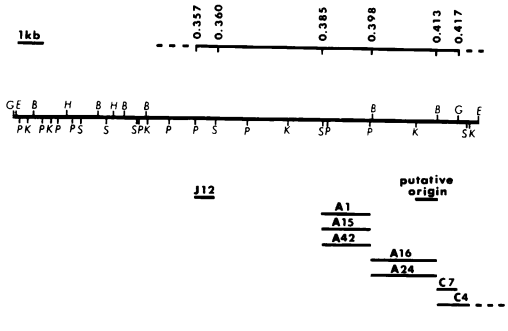

FIG. 5. Physical map of eight HSV-1 strain KOS *ts* mutants and a putative origin of viral DNA synthesis. The line at the top shows the physical map coordinates of the region. The map locations of the *ts*J12, *ts*A1, *ts*A15, *ts*A16, *ts*A24, *ts*C4, and *ts*C7 mutations are shown. The mutation of *ts*J12 (complementation group 1-9) was mapped by marker rescue by Neal DeLuca (personal communication), and the mutations of *ts*C4 and *ts*C7 were mapped by analysis of intertypic recombinants (6). *B*, *Bam*HI; *E*, *Eco*RI; *G*, *Bgl*II; *H*, *Hpa*I; *K*, *Kpn*I; *P*, *Pst*I; *S*, *Sal*I.

Limits of the gene for the 130K DNA-binding protein. The nearest marker in strain KOS to the left of the mutations in the DNA-binding protein (i.e., mutants in group 1-1) is the *ts*J12 marker (group 1-9), which maps between coordinates 0.357 and 0.360 (N. DeLuca, personal communication) and probably lies in the structural gene for viral glycoprotein gAgB (Fig. 5). This marker lies approximately 4 kb to the left of the location of the mutations *ts*A1, *ts*A15, and *ts*A42 in group 1-1. Although we do not know the precise physical limits of the genes for gAgB and the 130K protein, there is sufficient genetic information between the *ts*J12 (gAgB) mutation and the

mutations of members of complementation group 1-1 for at least one additional gene. Indeed, in the HSV-2 system a gene coding for a late function maps in this region (11a). The nearest markers to the right of the mutations in group 1-1 are in the DNA polymerase locus represented by mutants *ts*C4 and *ts*C7 (Fig. 5). The mutations in the polymerase gene map between coordinates 0.398 and 0.438 (6; D. Coen, personal communication).

Relationship of mutants in complementation group 1-1 to the 130K DNA-binding protein. Several pieces of evidence indicate that the mutations of members of groups 1-1 and 2-2 define the gene for the 130K DNA-binding protein (11, 11a; this paper). First, members of group 1-1 fail to complement *ts*HA1, a mutant which was isolated by Conley et al. (11) and whose mutation maps in or near sequences specifying a 130K DNA-binding protein (i.e., ICP8). The complementation and mapping results described in this study are consistent with this interpretation.

Second, the purified 130K protein from HSV-2 *ts*H9-infected cells exhibits a reduced ability to denature polydeoxyadenylic acid-polydeoxythymidylic acid helices at 40°C compared with purified 130K protein from wild-type virus-infected cells (32).

Third, immunofluorescence tests have shown that, whereas cells infected with strain KOS exhibit bright nuclear fluorescence at both 34 and 39°C when they are reacted with anti-130K monoclonal antibody, cells infected with *ts*HA1, *ts*A1, *ts*A15, *ts*A16, and *ts*A42 exhibit nuclear fluorescence at 34 but not 39°C. In addition, cells infected with *ts* mutants in HSV-2 complementation group 2-2 also failed to exhibit detectable nuclear fluorescence at 38°C in tests in which the wild-type virus (strain 186) exhibited brilliant nuclear fluorescence (11a). Importantly, immunofluorescence tests with polypeptide-specific antiserum to the 130K protein confirmed the absence of this protein in nuclei of cells infected with selected members of complementation groups 1-1 and 2-2 (R. Courtney, personal communication). These observations indicate that all members of groups 1-1 and 2-2 bear mutations which affect the distribution of the 130K DNA-binding protein at the nonpermissive temperature. Taken together, these data strongly suggest that mutants in HSV-1 and HSV-2 complementation groups 1-1 and 2-2 define analogous gene(s) for the 130K DNA-binding protein.

Significance of the DNA-binding protein in replication and transformation. To date, we know that the 130K protein is required for HSV DNA synthesis and that it may act to stabilize DNA polymerase activity (K. Powell, personal communication). Antiserum to the HSV-2 DNA-binding protein (ICSP11/12) has been shown to react with biopsy material from patients with severe dysplasia and carcinoma of the cervix, squamous cell carcinoma in situ, and severe dysplasia of the vulva (13, 22). Moreover, the expression of the antigen in hamster cells transformed in vitro by UV-inactivated HSV (15) has been correlated with the tumorigenicity of these cells when they are injected into newborn hamsters (unpublished data).

The possibility that the DNA-binding protein may play a role in morphological transformation and tumorigenicity is currently under investigation. Mutants with mutations affecting the expression of the DNA-binding protein should prove useful in elucidating the functions of this protein both in the viral replicative process and in transformation. The region of the genome between map coordinates 0.385 and 0.460 contains a variety of essential replicative functions, including the gene for the DNA-binding protein, a putative origin of DNA replication, and the HSV DNA polymerase gene. The possible analogy between HSV and the papovaviruses, in which the essential transforming gene (a gene encoding an early protein required for viral DNA synthesis) maps adjacent to the origin of DNA synthesis, deserves careful consideration.

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