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Genetic Analysis of Temperature-Sensitive Mutants Which Define the Genes for the Major Herpes Simplex Virus Type 2 DNA-Binding Protein and a New Late Function

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Eleven temperature-sensitive mutants of herpes simplex virus type 2 (HSV-2) exhibit overlapping patterns of complementation that define four functional groups. Recombination tests confirmed the assignment of mutants to complementation groups 1 through 4 and permitted the four groups to be ordered in an unambiguous linear array. Combined recombination and marker rescue tests (A. E. Spang, P. J. Godowski, and D. M. Knipe, J. Virol. 45:332–342, 1983) indicate that the mutations lie in a tight cluster near the center of U_L to the left of the gene for DNA polymerase in the order 4-3-2-1-polymerase. The seven mutants that make up groups 1 and 2 fail to complement each other and mutants in HSV-1 complementation group 1-1, the group thought to define the structural gene for the major HSV-1 DNA-binding protein with a molecular weight of 130,000. At 38°C, mutants in groups 1 and 2 synthesize little or no viral DNA, and unlike cells infected with the wild-type virus, mutant-infected cells exhibit no detectable nuclear antigen reactive with monoclonal or polypeptide-specific antibody to the major HSV-2 DNA-binding protein. The four mutants that make up groups 3 and 4 do not complement each other, nor do they complement mutants in group 2. They do, however, complement mutants in group 1 as well as representative mutants of HSV-1 complementation group 1-1. At 38°C, mutants in groups 3 and 4 are phenotypically DNA^+, and nuclei of mutant-infected cells contain the HSV-2 DNA-binding protein. Thus, the four functional groups appear to define two closely linked genes, one encoding an early viral function affecting both viral DNA synthesis and expression of the DNA-binding protein with a molecular weight of 130,000 (groups 1 and 2), and the other encoding a previously unidentified late viral function (groups 3 and 4). The former gene presumably represents the structural gene for the major HSV-2 DNA-binding protein.

Functional homology between the genes of herpes simplex virus types 1 and 2 (HSV-1, HSV-2) was first demonstrated by the failure of phenotypically similar temperature-sensitive (*ts*) mutants of the two virus types to complement (8, 29). That the order of genes on the chromosomes of the two viruses is at least roughly similar was demonstrated by the viability of intertypic recombinants and by the colinearity of HSV-1 and HSV-2 DNA sequences specifying polypeptides of approximately the same size and kinetic class (12, 15–17). Although studies of intertypic recombinants suggest extensive functional homology and genetic colinearity between HSV-1 and HSV-2 DNAs, comparative complementation and mapping studies of phenotypically similar mutants of each virus type provide more definitive proof of such homology. To date, comparison of the functional defects of *ts* and other mutants of HSV-1 and HSV-2 by intertypic complementation, combined with efforts to locate the mutations by physical mapping procedures, have demonstrated functional homology and genetic colinearity for three viral genes: thymidine kinase (11), DNA polymerase (3), and alkaline DNase (18, 21). We report herein the results of a study defining a third homologous gene: the gene for the major viral DNA-binding protein.

The following observations prompted this investigation. A collaborative study of 29 *ts* mutants of HSV-2 identified 20 complementation groups (25). A total of 5 of the 29 mutants, as well as 6 mutants not included in the original

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study, exhibit overlapping patterns of complementation among themselves, complement mutants in other groups efficiently, and differ markedly in their viral DNA phenotypes. Importantly, several of the 11 HSV-2 mutants fail to complement mutants in HSV-1 complementation group 1-1, which defines the gene for the major HSV-1 DNA-binding protein with a molecular weight of 130,000 (130K) (5, 29a).

Herein we describe complementation tests, recombination analysis, and selected phenotypic properties of the series of HSV-2 *ts* mutants. In related papers, Spang et al. (27) and Weller et al. (29a) describe fine-structure physical mapping studies of the HSV-2 mutants and the series of analogous HSV-1 mutants, respectively. Taken together, these three studies and the earlier study of Conley et al. (5) define a fourth HSV gene which is both functionally homologous and colinear on the genomes of HSV-1 and HSV-2: the gene for the major HSV DNA-binding protein. In addition, we describe a previously unidentified late viral gene which maps to the left of the HSV-2 DNA-binding protein near position 0.36 on the viral genome.

MATERIALS AND METHODS

Cells. Human embryonic lung (HEL) cells and African green monkey kidney (Vero) cells were used for virus growth and for complementation and recombination tests. HEL cells were used for determining viral DNA phenotypes, virus assays were conducted in Vero cells, and immunofluorescence tests were conducted in primary Syrian hamster embryo cells (Lakeview Syrian hamsters, Lakeview Hamster Colony, Newfield, N.J.). Cells were propagated in Dulbecco modified 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 and virus assays. The *ts* mutants of HSV-2, strains 186, IPB2, and UW268, were isolated after mutagenesis with 5-bromodeoxyuridine, UV light, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and nitrous acid. The properties of these mutants and of the HSV-1 mutant used in intertypic complementation tests, *ts*J12, are presented in Table 1.

Virus stocks were prepared in HEL or Vero cells, and plaque assays were performed in Vero cells as previously described (25). Permissive and nonpermissive temperatures were 34 and 38°C, respectively. The plating efficiencies of mutants [(PFU/ml at 38°C)/(PFU/ml at 34°C)] ranged from 10^{-6} to 10^{-4}.

Genetic methods. Complementation and recombination tests were conducted as described previously (24, 26). Complementation groups of mutants, where previously determined, are shown in Table 1.

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

Immunofluorescence tests. Indirect immunofluorescence tests were conducted by the method of Porter (19) as modified by Flannery et al. (9).

Monoclonal antibody to the 130K HSV-2 DNA-binding protein was kindly provided as mouse ascitic fluids by Anthony Minson (University of Cambridge, England). This antibody is immunoglobulin G antibody; purified 130K protein competes for the antibody in radioimmunoassays; and the antibody precipitates the purified protein and produces the same distribution of fluorescence in HSV-2-infected cells as polypeptide-specific antiserum directed against the entire 130K protein (A. Minson, personal communication).

Polypeptide-specific antiserum to the 130K protein

TABLE 1. Properties of HSV-1 and HSV-2 *ts* mutants used in this study

Virus type	Wild-type strain	Mutant	Mutagen[a]	Complementation group[b]	Reference or investigator
HSV-2	186	*ts*A1	BUdR	2-7	(8)
		*ts*A8	BUdR	2-7	(8)
		*ts*B5[c]	BUdR	2-3	(8)
		*ts*H9 (*ts*314)	UV	2-2	(4)
		*ts*39	UV		P. Schaffer
		*ts*178	NTG		D. Purifoy
		*ts*201	NTG		D. Purifoy
		*ts*208	NTG		D. Purifoy
	IPB2	*ts*1	NA	2-2	(30)
		*ts*42082	NA	2-2, 2-7,2-14[d]	(30)
	UW268	*ts*4	BUdR		(28)
		*ts*19	BUdR	2-2	(28)
HSV-1	KOS	*ts*J12[c]	NTG	1-9	(24)

[a] Abbreviations: BUdR, 5-bromodeoxyuridine; UV, UV light; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NA, nitrous acid.

[b] Determined previously where shown (8, 25).

[c] Mutant *ts*B5 specifies a thermolabile DNA polymerase (22); mutant *ts*J12 of HSV-1 strain KOS is defective in the expression of glycoprotein gAgB (14).

[d] Mutant *ts*42082 failed to complement mutants in three groups (25).

was prepared as described by Courtney and Benyesh-Melnick (6). The patterns of 130K protein-specific fluorescence produced by this antiserum in cells infected with and transformed by HSV-2 have been described previously (9).

Fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) was used to detect monoclonal and polypeptide-specific antibody on fixed monolayers.

RESULTS

Complementation. The results of previous complementation tests placed HSV-2 mutants *ts*H9, *ts*1, and *ts*19 into one complementation group (2-2) and mutant *ts*A8 into another group (2-7) (25). Mutant *ts*42082 failed to complement members of both groups, and because of this overlap it could not be assigned unequivocally to either. The unusual overlapping patterns of complementation exhibited not only by *ts*42082 but also by six other HSV-2 *ts* mutants not included in the original study (*ts*4, *ts*39, *ts*178, *ts*201, *ts*208, and *ts*A1) prompted a reexamination of the functional relatedness of these mutants by complementation analysis.

The results of quantitative complementation tests among the 11 mutants are shown in Table 2. The HSV-2 mutant *ts*B5, which specifies thermolabile DNA polymerase activity and is a member of complementation group 2-3 (22, 25), and *ts*J12, a mutant defective in glycoprotein gAgB and a member of group 1-9 (14, 25), were included as controls. By using an index of 10 rather than 2 (24) to signify positive complementation, the 11 mutants were classified into four groups numbered 1, 2, 3, and 4 (Table 2). Members of a given group failed to complement each other; however, members of different groups exhibited overlapping patterns of complementation. Thus, mutants in group 1 failed to complement mutants in group 2. Moreover, except for pairs *ts*H9 + *ts*178, *ts*19 + *ts*178, and *ts*19 + *ts*201, the five mutants in group 1 complemented mutants in groups 3 and 4 efficiently. In contrast, the group 2 mutants failed to complement mutants in groups 3 and 4. Likewise, mutants in group 3 failed to complement the group 4 mutant. Notably, all mutants in groups 1 through 4 (except for the pairs *ts*H9 + *ts*J12 and *ts*19 + *ts*J12, which were not tested, and *ts*178 + *ts*J12, which yielded an index of 2.1) complemented control mutants *ts*B5 of HSV-2 and *ts*J12 of HSV-1 efficiently. The fact that mutants complemented *ts*B5 and *ts*J12 indicates that they are very likely defective in genes other than polymerase (*ts*B5) or glycoprotein gAgB (*ts*J12).

Recombination. The observation that the 11

TABLE 2. Complementation among *ts* mutants of HSV-2

Mutant	Complementation index[a]												
	Group 1					Group 2		Group 3			Group 4	Controls	
	*ts*1	*ts*42082	*ts*4	*ts*H9	*ts*19	*ts*A1	*ts*A8	*ts*178	*ts*201	*ts*208	*ts*39	*ts*B5	*ts*J12
Group 1													
*ts*1		*0.7*	*0.5*	*1.6*	*1.2*	*2.8*	*1.8*	29.3	65.2	221	344	259	27.6
*ts*42082			*0.6*	*1.8*	*0.9*	*0.8*	*0.7*	49.1	71.2	227	360	224	25.1
*ts*4				*1.2*	*1.7*	*0.6*	*1.2*	40.0	44.1	137	454	237	15.8
*ts*H9					*1.7*	*0.6*	*1.0*	*0.0*	13.5	345	245	260	ND[b]
*ts*19						*1.4*	*0.7*	*1.6*	*2.4*	14.4	114	12.7	ND
Group 2													
*ts*A1							*1.2*	*1.9*	*1.1*	*1.7*	*4.0*	1,438	10.9
*ts*A8								*1.1*	*0.4*	*0.8*	*3.3*	595	12.9
Group 3													
*ts*178									*0.4*	*1.1*	*1.8*	1,924	*2.1*
*ts*201										*0.7*	*1.5*	2,105	42.9
*ts*208											*1.9*	1,310	32.4
Group 4 *ts*39												2,353	57.4
Controls													
*ts*B5													58.2
*ts*J12													

[a] Complementation tests were conducted as previously described (24). Complementation indices <10 are italicized.

[b] ND, Not done.

TABLE 3. Recombination among 11 *ts* mutants of HSV-2

Mutant	Test no.	Recombination frequency[a]										
		Group 1					Group 2		Group 3			Group 4	Control
		*ts*1	*ts*42082	*ts*4	*ts*H9	*ts*19	*ts*A1	*ts*A8	*ts*178	*ts*201	*ts*208	*ts*39	*ts*B5
Group 1													
*ts*1	1		0.002	1.0			0.1	1.1	0.7	9.1	2.5	6.8	6.0
	2		0.1	0.8			0.8	1.2	1.8	4.8	4.3	9.6	5.0
	3				0.1	0.03							
*ts*42082	1			0.1			0.4	2.4	0.6	2.0	1.3	5.3	5.0
	2			0.4			0.6	1.0	1.6	5.8	4.8	9.5	5.9
	3				0.001	0.01							
*ts*4	1						0.3	0.5	0.6	3.7	3.6	7.4	11.0
	2						0.7	0.7	1.7	4.5	4.5	9.0	8.0
	3				0.04	0.04	2.0			4.0		8.0	
*ts*H9	3					0.001	1.3	1.5	2.6	2.5	2.8	7.9	1.4
*ts*19	3						2.2	2.1	0.8	5.1	4.0	8.0	1.3
Group 2													
*ts*A1	1							0.001	0.7	1.1	0.3	2.2	19.2
	2							0.0	0.7	2.3	2.0	6.5	15.2
	3									1.9			
*ts*A8	1								0.9	0.5	0.6	4.0	17.5
	2								0.8	2.1	1.7	7.3	17.1
Group 3													
*ts*178	1									0.0	1.3	2.0	14.5
	2									0.0	0.8	4.8	23.0
*ts*201	1										1.4	0.6	25.8
	2										0.0	4.5	24.9
*ts*208	1											2.0	24.8
	2											4.5	25.9
Group 4													
*ts*39	1												47.6
	2												44.1

[a] Recombination tests were conducted as described previously (26).

mutants could be classified into four functional groups based on overlapping patterns of complementation raised the question whether the mutations in each group could be ordered by recombination. To test this possibility, recombination analysis was conducted by two-factor crosses. The polymerase mutant, *ts*B5, was again included as an outside marker since the physical map location of its mutation (0.40 to 0.42) has been determined (27). The HSV-1 mutant *ts*J12 was not included in these crosses because intertypic recombination frequencies are usually low and may not reflect the order of markers faithfully (7). Unfortunately, no equivalent mutant (i.e., no mutant defective in glycoprotein gAgB) has yet been identified in the HSV-2 system. Three separate recombination tests were performed. Tests 1 and 2 were conducted in HEL cells, and test 3 was conducted in Vero cells. The results of these tests are presented in Table 3, and the

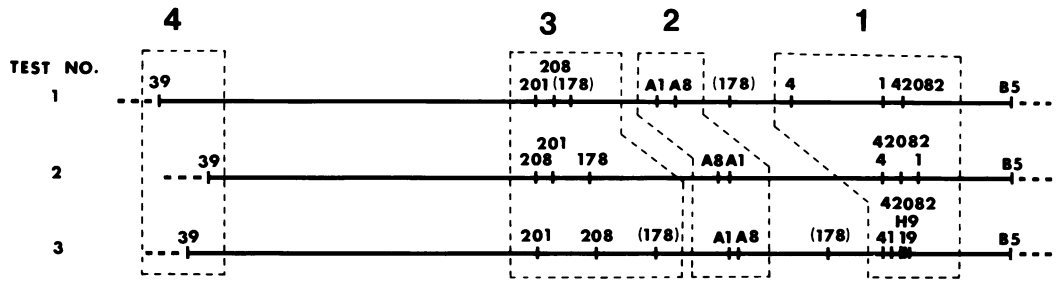

FIG. 1. Linkage maps generated by two-factor crosses among 12 *ts* mutants of HSV-2. Mutations in members of the four functional groups are enclosed by dashed lines and numbered at top.

linkage maps derived from these data are shown in Fig. 1.

Although recombination frequencies between markers varied among the three tests (Table 3), both the order of markers and the relative distances between markers were the same when the three maps were compared on the same scale (Fig. 1).

The classification of mutants into four groups by complementation analysis was confirmed by recombination analysis (Table 3, Fig. 1). Thus, with one exception, mutations of mutants in the same group were more closely linked to each other than to the mutations of mutants in other groups, and the mutations of members of the four groups were ordered unambiguously in all three tests with respect to the outside marker, the mutation of *ts*B5. The exception to this pattern was *ts*178, for which the mutation mapped either to the right of *ts*201 and *ts*208 in group 3 or between groups 1 and 2 in tests 1 and 3 (Fig. 1). It will be recalled that mutant *ts*178 also produced ambiguous results in complementation tests (Table 2).

Phenotypic properties of mutants. (i) Viral DNA phenotypes. The results of complementation and recombination analysis indicated that the four groups of mutants may represent four distinct and separable functions. Comparison of the viral DNA phenotypes of the 11 mutants further supports this hypothesis (Table 4). Thus, viral DNA synthesis at 38°C ranged from <1 to

TABLE 4. Phenotypic properties of 13 HSV-2 *ts* mutants

Virus	Viral DNA synthesis at 38°C (% wild-type)[a]	Fluorescent staining[b]							
		34°C				38°C			
		Nucleus		Cytoplasm		Nucleus		Cytoplasm	
		MC	PS	MC	PS	MC	PS	MC	PS
Wild-type	100	+	+	+	+	+	+	−	+
*ts*B5	5								
Group 1									
*ts*19	<1								
*ts*H9	<1	+	+	±	+	−	−	±	±
*ts*4	<1								
*ts*42082	<1								
*ts*1	5								
Group 2									
*ts*A8	10	+	+	±	+	−	−	−	±
*ts*A1	9	+	+	+	+	−	−	+	+
Group 3									
*ts*208	80								
*ts*201	52	+		±		+		−	
*ts*178	50								
Group 4									
ts 39	119	+		±		+		−	

[a] Measured as described by Aron et al. (1).

[b] Staining with monoclonal antibody (MC) and polypeptide-specific antibody (PS) to the 130K protein. +, Bright fluorescence; ±, moderate to faint fluorescence; −, no specific staining.

5% of wild-type levels in mutants of group 1, from 9 to 10% in mutants of group 2, and from 50 to 80% in mutants of group 3. The group 4 mutant synthesized wild-type levels of viral DNA. All mutants were equally effective in shutting off host cell DNA synthesis; each mutant reduced levels of cellular DNA synthesis to 10 to 30% of uninfected cell levels (data not shown).

(ii) Immunofluorescence tests. Intertypic complementation tests have shown that one member of group 1 (*ts*H9) and both members of group 2 (*ts*A1 and *ts*A8) do not complement a series of six *ts* mutants in HSV-1 complementation group 1-1 (29a). Mutants in group 1-1 are defective in viral DNA synthesis, and their mutations map between coordinates 0.385 and 0.413; furthermore, mutant-infected cells fail to express the 130K DNA-binding protein at 39°C in immunofluorescence tests with monoclonal antibody to the HSV-1 DNA-binding protein. Because our data indicate that mutants *ts*H9, *ts*A1, and *ts*A8, as well as other members of groups 1 and 2, are defective in the equivalent HSV-2 gene, we tested mutants representing all four groups for their ability to express the 130K DNA-binding protein at 38°C. In these tests we used monoclonal antibody to the HSV-2 130K protein. The results of these tests are summarized in Table 4, and photomicrographs of typical fluorescence reactions are shown in Fig. 2. As in cells infected with wild-type virus (Fig. 2A), the protein was detected in the nuclei and to a lesser extent in the cytoplasm of mutant-infected cells at 34°C (Fig. 2C, E, and G). At 38°C, the antigen was detected in nuclei but not in the cytoplasm of cells infected with the wild-type virus (Fig. 2B) or with mutants in groups 3 and 4 (e.g., *ts*201; Fig. 2H). In contrast, the antigen was not detected in the nuclei of cells infected with one mutant in group 1 (*ts*H9) or with both mutants in group 2 (*ts*A1 and *ts*A8). Cytoplasmic fluorescence was, however, detected in cells infected with *ts*H9 (Fig. 2F) and *ts*A1, but not in cells infected with *ts*A8 (Fig. 2D), at 38°C.

Because the monoclonal antibody could, by definition, detect only a single epitope of the 130K protein and because mutant forms of the 130K protein may be sufficiently altered structurally to render the protein undetectable with this monoclonal antibody, we also tested cells infected with selected mutants and with the wild-type virus, using antiserum prepared against the purified 130K protein (Table 4). Such polypeptide-specific antiserum should be reactive with the spectrum of antigenic determinants on the 130K protein. Like the monoclonal antibody, the polypeptide-specific antiserum was reactive with both the nuclei and the cytoplasm of cells infected with wild-type and with mutant viruses at 34°C. Moreover, unlike the monoclonal antibody, the polypeptide-specific antiserum produced positive fluorescence in the cytoplasm of cells infected with wild-type virus at 38°C.

Importantly, no nuclear fluorescence was observed in cells infected with *ts*H9, *ts*A1, or *ts*A8 at 38°C. The failure to detect nuclear fluorescence with polypeptide-specific antiserum strongly suggests that the protein is, in fact, not present in the nucleus and not simply that it may be present in an altered antigenic state, as one might conclude from tests with monoclonal antibody.

Thus, with one exception, the patterns of fluorescence observed in mutant-infected cells were identical with both antibody preparations. The exception was that no cytoplasmic fluorescence was detectable in *ts*A8-infected cells at 38°C with monoclonal antibody, but moderate cytoplasmic fluorescence was observed with the polypeptide-specific antiserum.

Taken together, the results of immunofluorescence tests indicate that mutants in groups 1 and 2 are defective in a function affecting the transport of the 130K protein to the nuclei of infected cells at 38°C, whereas mutants in groups 3 and 4 exhibit no such defect. These observations thus provide additional support for the functional grouping suggested by the other genetic and phenotypic parameters described above.

DISCUSSION

Eleven *ts* mutants define four functional groups. The combined results of complementation, recombination, and phenotypic analysis indicate that the 11 HSV-2 *ts* mutants can be assigned to four functional groups (Fig. 3).

The unusual overlapping patterns of complementation exhibited by these mutants (Table 1) provided the initial evidence for the existence of four groups. One possible explanation for the overlap is that the mutants possess multiple *ts* mutations. This explanation appears unlikely, however, because recombination frequencies were (i) higher than one would expect in crosses between double mutants and (ii) sufficiently additive to construct linkage maps. Moreover, all mutants reverted with low but consistent frequency (10^{-6} to 10^{-4}).

Importantly, the grouping by complementation was supported by recombinational and phenotypic data. Generally speaking, recombination frequencies between mutants in groups that did not complement were low, demonstrating close linkage, whereas recombination frequencies were higher between mutants in groups that did complement.

An interesting finding from recombination studies concerns the unexpectedly high frequen-

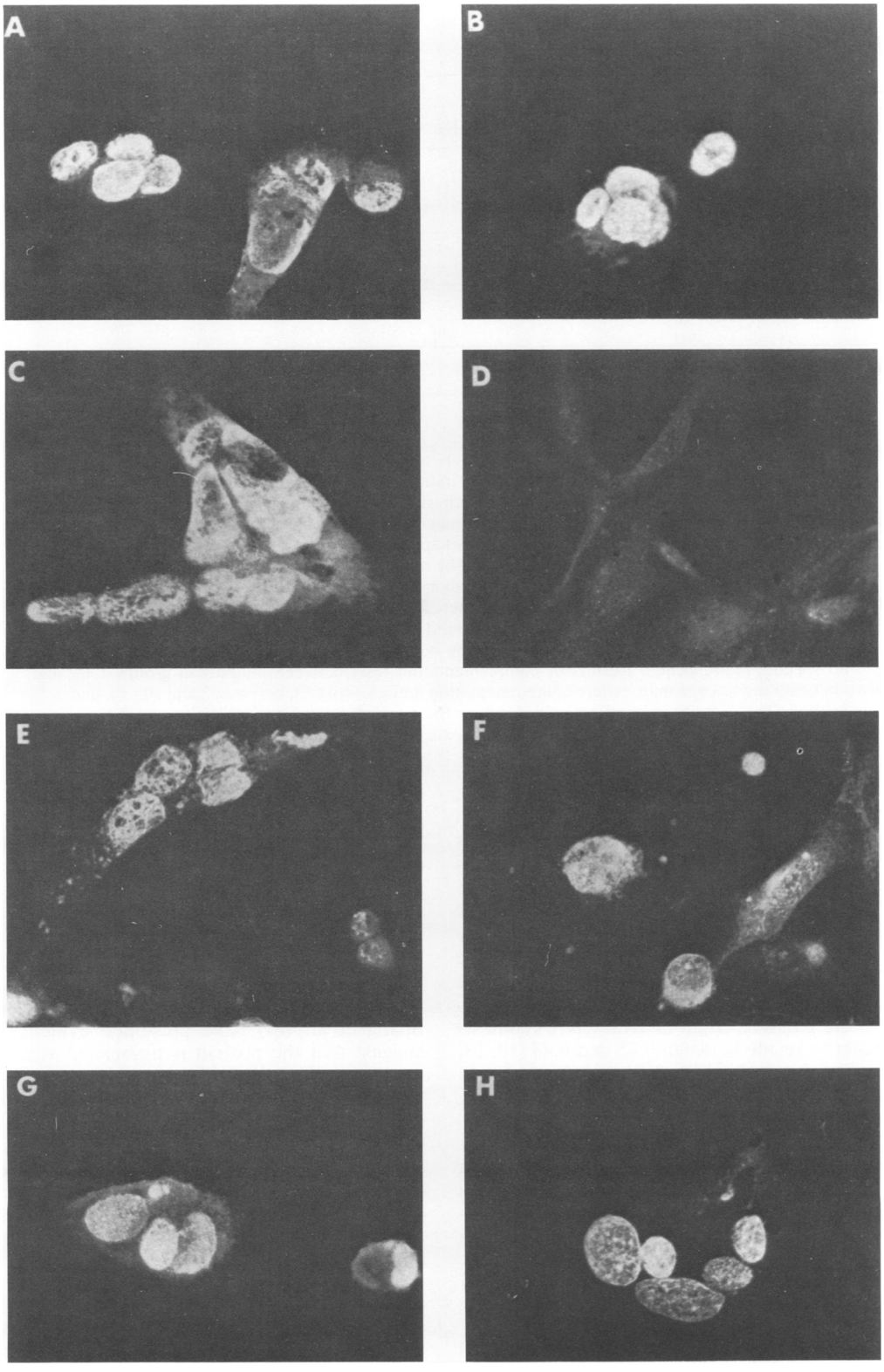

FIG. 2. Photomicrographs of cells infected with wild-type and *ts* mutant viruses, stained with monoclonal antibody to the major HSV-2 DNA-binding protein of 130K. Shown are cells infected with wild-type virus at 34°C (A) and 38°C (B); with *ts*A8, group 2, at 34°C (C) and 38°C (D); with *ts*H9, group 1, at 34°C (E) and 38°C (F); and with *ts*201, group 3, at 34°C (G) and 38°C (H).

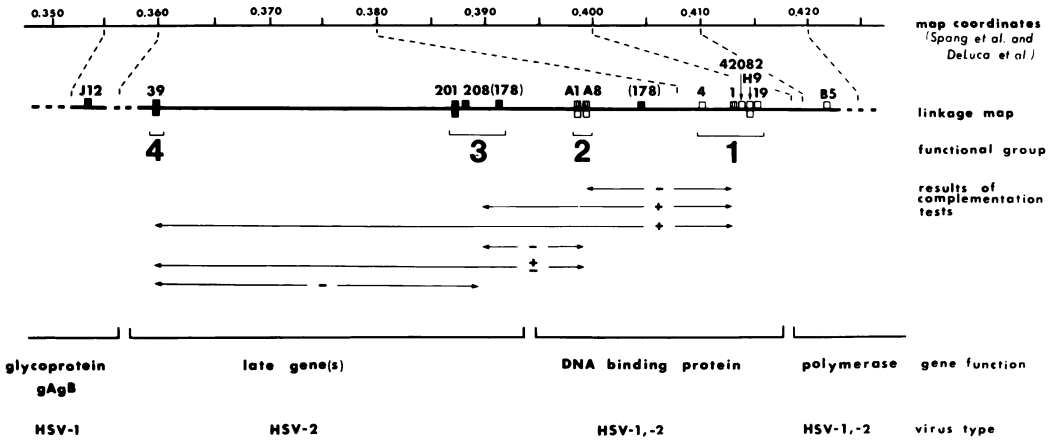

FIG. 3. Genetic and phenotypic properties of 11 *ts* mutants of HSV-2. The heavy line is the linkage map generated by two-factor crosses (Fig. 1). The top line indicates the physical map coordinates of mutations shown on the linkage map. The physical map location of the HSV-1 mutation of *ts*J12 was determined by DeLuca (personal communication). The mutant *ts*J12 is a member of complementation group 1-9 (25), which defines the gene for HSV-1 glycoprotein gAgB (14). The physical map locations of mutations in members of groups 1 through 4 and of *ts*B5 were determined by Spang et al. (27). The mutant *ts*B5 is a member of complementation group 2-3 (25), which defines the gene for HSV-2 viral DNA polymerase (2, 22). The viral DNA phenotypes of mutants at 38°C are indicated by boxes above the linkage map line: ▬, >20% of wild-type levels of viral DNA; ▥, ≤20% of wild-type levels of viral DNA; ⊔, no detectable viral DNA. Expression of the 130K DNA-binding protein in nuclei of mutant-infected cells at 38°C is indicated by boxes below the line: ▛, bright nuclear fluorescence; ⊓, no specific fluorescence. Results of complementation tests between mutants in groups 1 through 4 are shown beneath the linkage map, where complementation indices >10 (+), between 2 and 10 (±), and <2 (−) are distinguished. The identity of viral genes defined by the *ts* mutants studied and the virus type in which these genes have been identified (HSV-1, HSV-2, or both) are illustrated at the bottom.

cy of recombination between *ts*B5 and *ts*39, whose mutations represent the opposite termini of the linkage map. Indeed, recombination between *ts*B5 and *ts*39 approached 50%, whereas the actual physical distance between the two mutations is less than 10% of the viral genome (Fig. 3 and accompanying text). A frequency approaching 50% suggests the presence of one or more recombinational hot spots between the two markers. Whether such a hot spot constitutes the putative origin of viral DNA synthesis thought to reside between 0.35 and 0.45 (10, 13, 23)—the physical map location of the 11 mutations involved in this study—is an intriguing possibility.

Although the results of recombination tests were sufficiently additive to order the four groups of mutations unambiguously with respect to one another and to *ts*B5, the order of mutations within each group is less certain. The order shown in Fig. 3 represents the average of the three positions for each mutation shown in Fig. 1 and should be considered suggestive rather than definitive.

The fact that mutants in the same functional group have similar phenotypic properties with regard to both the level of viral DNA synthesis and the ability to detect the 130K protein in nuclei of mutant-infected cells provides additional support for the classification of the 11 mutants into four functional groups. The failure to detect the 130K protein in the nuclei of cells infected with mutants *ts*H9, *ts*A1, and *ts*A8 is of special interest. Results obtained with polypeptide-specific antiserum suggest that the protein is not, in fact, present in nuclei. It is possible, on the other hand, that the antibody preparations used in these tests were not sensitive enough to detect small quantities of 130K protein. Indeed, Spang et al. (27) have presented evidence to suggest that the protein is associated with the nuclear fraction of cells infected with selected mutants in groups 1 and 2. Clearly, additional experimentation will be necessary to resolve this problem. In any event, it is clear that mutants in groups 1 and 2 possess a defect in nuclear expression of the 130K protein not characteristic of mutants in groups 3 and 4 when expression is assessed with polypeptide-specific antisera in immunofluorescence tests.

The results of the studies described in this report also address a more fundamental problem with the genetic analysis of HSV. Based upon the criterion that complementation indices ≥2 reflect the ability of two mutants to complement (24), 29 *ts* mutants of HSV-2 were shown to define 20 nonoverlapping complementation groups (25). The recent studies of S. K. Weller,

W. R. Sacks, D. M. Coen, and P. A. Schaffer (submitted for publication) now indicate that complementation indices between 2 and 10 are ambiguous and may represent either intracistronic or intercistronic complementation. These findings are supported by the results of our studies with mutants in groups 1 and 2. These results have, therefore, prompted us to reclassify mutants in complementation group 2-2 and 2-7 to a common group, group 2-2. For the same reasons, the results of complementation tests reported herein, as well as the results of all future tests, will utilize a value of 10 to signify positive complementation. It is anticipated that the problem of intracistronic versus intercistronic complementation will ultimately be resolved by combined studies involving physical mapping, transcriptional mapping, and in vitro translation of carefully mapped transcripts.

Order, orientation, and physical map location of mutations in the four groups. The order, orientation, and physical map location of the mutations presented in Fig. 3 are based upon the following observations.

(i) Mutant *ts*B5 synthesizes a thermolabile DNA polymerase (22) and does not complement the HSV-2 DNA polymerase mutant of strain HSG52, *ts*6, a member of complementation group 2-3 (2). Both the *ts*B5 and *ts*6 mutations have been mapped to a 3.2-kilobase region between coordinates 0.40 and 0.42 on the physical map of HSV-2 (3, 27).

(ii) HSV-2 mutations of members of complementation group 2-2 as well as their HSV-1 counterparts in group 1-1 (i.e., those in the major HSV-1 DNA-binding protein) map immediately to the left of mutations in the polymerase gene (27, 29a).

(iii) In the HSV-1 system, the nearest recognized marker to the left of mutations in members of group 1-1 is the gene for glycoprotein gAgB. The HSV-1 *ts*J12 mutant, a late mutant defective in gAgB expression, maps between coordinates 0.357 and 0.360 (N. DeLuca, personal communication); analogous HSV-2 mutations in glycoprotein gAgB have not yet been identified. Because three of four mutants in groups 3 and 4 complement mutant *ts*J12 efficiently (Table 2) and because *ts*39 (group 4), *ts*201 (group 3), and *ts*178 (group 3) do not exhibit altered expression of gAgB (B. Pancake, personal communication) as *ts*J12 does (14), it is unlikely that any of these mutants is defective in the gene for gAgB. If this is indeed the case, then the DNA-positive mutants in groups 3 and 4 must represent a new late gene (or genes) lying between the genes for the 130K protein and glycoprotein gAgB (Fig. 3).

(iv) Spang et al. (27) have recently demonstrated that mutations representing groups 2, 3, and 4 map within a region with map coordinates 0.360 to 0.380. This region lies immediately to the right of the sequence in which the *ts*J12 mutation lies (0.357 to 0.360) and to the left of a sequence from 0.380 to 0.400 containing the mutation in *ts*1 (group 1). The physical mapping data of Spang et al. thus support the order (right to left) *ts*B5 (polymerase), groups 1 (2, 3, 4), *ts*J12 (glycoprotein gAgB) (Fig. 3).

(v) Fine mapping of viral transcripts found in abundance before viral DNA synthesis has shown that two species correspond to the genes for glycoprotein gAgB and the 130K protein (L. Holland, personal communication). Moreover, a protein of 128K was translated in vitro by Conley et al. (5) from transcripts mapping in or near the region encoding the 130K protein.

Four functional groups: How many genes? The most reasonable interpretation of the genetic and phenotypic data summarized in Fig. 3 is that the 11 mutants represent two (or perhaps three) viral genes.

One gene encodes the 130K protein. This gene is represented by mutants in groups 1 (*ts*4, *ts*1, *ts*42082, *ts*H9, and *ts*19) and 2 (*ts*A1 and *ts*A8). This interpretation is based upon (i) the failure of mutants in both groups to complement each other; (ii) the common defect in viral DNA synthesis and the failure to express the 130K polypeptide in the nucleus characteristic of these mutants at 38°C; (iii) the observation that the mutations of HSV-1 *ts* mutants with similar phenotypic properties map to a corresponding region of the HSV-1 genome (29a); (iv) the fact that at least three mutants in group 1 (*ts*1, *ts*19, and tsH9) exhibit altered distribution of the 130K protein in nuclei and cytoplasm as determined by cellular fractionation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27); and (v) the observation that the purified 130K protein from *ts*H9-infected cells exhibits reduced DNA-unwinding activity compared with purified 130K protein from cells infected with wild-type virus (20). The hypothesis that mutants in groups 1 and 2 define a single gene implies that the 130K protein has at least two functions; mutants in groups 1 and 2 are distinguishable by complementation with mutants in other genes (i.e., mutants in group 1 complement mutants in groups 3 and 4 efficiently, whereas mutants in group 2 do not). What specifically these two functions are is currently not known.

Based upon physical mapping studies (27), the *ts* mutations of members of all four groups map within a 6.4-kilobase region between coordinates 0.36 and 0.4. This amount of DNA is sufficient to encode a protein of approximately 210K. Subtracting the coding sequences for the 130K protein from this sum (the coding sequences for the 130K protein map to the right

hand end of the region), the remaining DNA is sufficient to encode a protein(s) of approximately 80K. The template for this protein(s) would lie in the left-hand end of the region, to the left of the gene for the 130K protein and to the right of the gene for glycoprotein gAgB. Unfortunately, insufficient phenotypic and physical mapping data are available to determine whether mutants in groups 3 and 4 define one or two genes. In any event, these mutations define one or more previously undescribed late HSV-2 functions.

As illustrated in Fig. 3, the results of this investigation together with the findings of Spang et al. (27) serve to define the gene for the major HSV-2 DNA-binding protein—a gene that is both functionally homologous and structurally colinear with the gene for the HSV-1 DNA binding protein (29a). Additionally, these studies have identified a previously undescribed HSV-2 late gene(s) for which a counterpart has recently been identified in the HSV-1 system (B. Pancake, personal communication).

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