Fine-Structure Mapping and Functional Analysis of Temperature-Sensitive Mutants in the Gene Encoding the Herpes Simplex Virus Type 1 Immediate Early Protein VP175

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Herpes simplex virus (HSV)-specific proteins fall into at least three kinetic classes whose synthesis is sequentially and coordinately regulated. Temperature-sensitive (*ts*) mutants of one complementation group (1-2) are defective in the transition from immediate early to early and late protein synthesis. To elucidate the function of the 1-2 gene product in the HSV type 1 replicative cycle, nine *ts* mutants in this group were mapped by fine-structure analysis and characterized biochemically. Physical mapping by homotypic marker rescue has shown that all members of the group lie within the terminally repeated sequences of the S region of the genome. Fine-structure genetic and physical mapping permitted the mutations to be ordered within these sequences. Because it has been shown that the message for VP175 and the DNA template specifying this protein extend beyond the limits of the physical map of the mutations, it follows that the mutations must lie within the structural gene for VP175. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that most members of the group overproduced the immediate early proteins VP175, -136, -110, and -63 and markedly underproduced early and late proteins at the nonpermissive temperature. In temperature shiftup experiments, it was found that the synthesis of early and late proteins ceased, whereas the synthesis of immediate early proteins began again. Thus, it is postulated that VP175 is (i) involved in the transition from immediate early to early protein synthesis, (ii) required continuously to maintain early protein synthesis, and (iii) autoregulated, acting to inhibit immediate early protein synthesis.

The genome of herpes simplex virus type 1 (HSV-1) is large (molecular weight, 96×10^6) and structurally complex (1, 10, 11, 18, 38, 44). Despite rapid progress in the genetic analysis of HSV, little is known about the regulation of viral gene expression. The observation that the synthesis of three classes of viral polypeptides is sequentially and coordinately regulated was followed by the demonstration that coordinate regulation is at least partly controlled at the level of transcription (7, 8, 13, 14, 17, 22, 24, 25, 32, 43, 47). More recently, a viral gene has been identified which acts very early in the HSV replicative cycle and controls the transition from immediate early to early and late protein synthesis (8, 24, 25, 32). This gene is located in the terminal repeats of S (20, 29, 33, 42) and is thought to encode a viral polypeptide of molecular weight 175,000, designated ICP4 (13) or VP175 (8). A number of temperature-sensitive (*ts*) mutants in this gene have been isolated (5, 28, 36). Studies with these mutants have revealed that VP175 controls a transcriptional event(s) which is essential for the transition from immediate early

functions to early and late functions (28, 32, 47). Additionally, the gene is diploid (20, 27, 33), presenting an unique situation for genetic analysis.

It is currently believed that VP175 is the product of gene 1-2; however, most of the evidence to support this contention is circumstantial. Thus, mutants defective in this gene overproduce VP175, the mutant protein exhibits altered intracellular compartmentalization and migration in sodium dodecyl sulfate (SDS)-gels, and the template specifying VP175 maps in the same region of the genome as do mutants in group 1-2 (27, 29, 41, 42). To determine whether the lesions in mutants in group 1-2 are in the coding sequence for VP175, we have mapped nine *ts* mutants in group 1-2 by physical and genetic methods and correlated their order and map locations with the known location of DNA sequences which specify VP175 (27, 33) and the mRNA which encodes this protein (48).

Other investigators have demonstrated that one function of the product of gene 1-2 involves transcriptional regulation (32, 47). To further

define the role of this protein in regulation, we have examined viral polypeptide synthesis by one mutant in the group, *tsB21u*, in temperature shiftup experiments. Our results demonstrate that VP175 is involved in mediating the transition from immediate early to early protein synthesis, that it is required continuously for the synthesis of some early and late proteins, and that it acts to inhibit its own synthesis.

MATERIALS AND METHODS

Cells and cell culture. Serially propagated human embryonic lung fibroblasts (HEL), primary rabbit kidney cells (RK), and African green monkey kidney cells (Vero) were used. Cells were grown in Eagle minimal essential medium (Autopow; Flow Laboratories, Rockville, Md.) containing 10% fetal bovine serum, 0.03% glutamine, and 0.025% NaHCO_3 . Cells were maintained in the same medium containing 5% fetal bovine serum. Virus stocks were grown, and complementation and recombination tests were performed in HEL cells; RK cells were used for the isolation of viral DNA and for marker rescue studies. Virus infectivity assays were performed in Vero cells.

Viruses and virus assays. The KOS strain of HSV-1 was used as the wild-type virus (40). The isolation of mutants *tsB2b*, *tsB21u*, *tsB27h*, *tsB28h*, *tsB32h*, *tsJ12g*, *ts901*, *tsLB2*, *tsD*, and *tsc75* has been reported previously (4, 5, 12, 15, 20, 34). The last four mutants were obtained from M. Levine, I. Halliburton, J. Subak-Sharpe, and R. Honess, respectively. Virus stocks were prepared and infectivities were determined as previously described (36). In all tests, the permissive temperature was 34°C and the nonpermissive temperature was 39°C.

Complementation tests and mapping procedures. Mutants *tsB2b*, *ts901*, *tsD*, and *tsLB2* were shown in a previous study to define complementation group 1-2 (36). Complementation analysis with the other mutants used in this study is described herein. Complementation tests and recombination tests using two-factor crosses were carried out as described previously (34, 37). Marker rescue experiments were performed as reported by Chu et al. (5). The restriction maps of strain KOS used to determine the physical locations of mutations in marker rescue studies were taken from Skare and Summers (*EcoRI*) (39) and Wagner and Summers (*HincII*) (45) or were generated in this laboratory (*BamHI*).

Isolation of viral DNA, digestion with restriction enzymes, and agarose gel electrophoresis. DNA was isolated from partially purified cytoplasmic and extracellular virions as described by Chu et al. (5). Restriction enzymes were purchased from New England Biolabs (Boston, Mass.), and DNA was digested with 2 U of enzyme per μg of DNA. Fragments were separated by electrophoresis on horizontal slab gels and isolated as previously described (5).

Analysis of infected cell polypeptides. HEL cell monolayers were infected at a multiplicity of 100 PFU/cell and incubated in water baths at either 34 or 39°C under the conditions described in the text. Polypeptides were labeled with 5 μCi of ^{14}C -amino acids per ml (Schwarz/Mann, Orangeburg, N.Y.; amino acid

mixture, 50 to 60 mCi/matom) in maintenance medium containing 10% of the normal amounts of all amino acids except arginine and glutamine, which were used in normal amounts. Immediate early proteins (31) were induced in infected cultures treated with 50 μg of cycloheximide (Calbiochem, La Jolla, Calif.) per ml from 0 to 7 h postinfection. The drug was removed after 7 h, monolayers were washed five times with Tris-saline, and medium containing 5 μCi of ^{14}C -amino acids and 15 μg of actinomycin D (Calbiochem) per ml was added. Cells were harvested at 8 h postinfection by scraping into the medium. They were washed three times in Tris-buffered saline and suspended in water, and the resulting suspensions were frozen. Samples were later thawed, sonicated for 2 min, adjusted to 0.05 M Tris-hydrochloride (pH 6.7)–1% SDS–1% 2-mercaptoethanol–0.5 M urea, and boiled for 2 min. Electrophoresis was carried out on 7 to 15% linear gradient SDS-polyacrylamide slab gels (23). The gels were fixed in 7% acetic acid and 50% methanol, impregnated with Enhance (New England Nuclear Corp., Boston, Mass.), and dried. The dried gels were then fluorographed at -70°C on Kodak SB-5 film.

RESULTS

Complementation studies with *ts* mutants. In two separate tests, eight of the nine *ts* mutants used in this study were shown to belong to complementation group 1-2 (5, 36). To confirm the assignment of these mutants and of *tsc75* (20) to group 1-2, a quantitative yield-of-progeny complementation test was performed (Table 1); *tsJ12g*, a member of group 1-9, was used as the positive control (36). None of the nine mutants tested complemented each other, whereas all complemented *tsJ12g* (Table 1). Thus, all nine mutants belonged to group 1-2. It should be noted that the nine mutants included in this study behave as if they possess point mutations in a single gene; i.e., they revert with low to moderate frequency (data not shown) and exhibit nonoverlapping patterns of complementation in tests with members of other groups (5, 36).

Physical mapping experiments. Marker rescue studies were undertaken to determine the physical map locations of the nine *ts* mutants. We have reported previously that *tsB21u* lies within the "c" sequences in the terminal repeats of the S region of the genome (29). This location is in agreement with the findings of Stow and Wilkie (42) and Knipe et al. (20, 21), who placed *tsLB2*, *tsD*, and *tsc75* within *EcoRI* fragment K, which contains S-terminal sequences "c" and "a."

Table 2 shows the results of marker rescue experiments using purified *EcoRI* fragments shown in Fig. 1. *tsLB2*, *tsD*, and *tsc75* had been shown previously to be in fragment K (20, 21, 42). Efficient rescue with the remaining six mutants was observed with fragment K (0.966 to

TABLE 1. *Complementation between members of group 1-2*

Virus	B2	B21	B27	B28	B32	901	LB2	<i>ts</i> D	<i>tsc</i> 75	<i>ts</i> J12g
<i>ts</i> B2b		1.5 ^a	0.70	0.81	0.37	0.40	0.55	0.48	0.70	170
<i>ts</i> B21u			0.31	0.27	0.86	0.76	0.88	0.33	0.32	4.9
<i>ts</i> B27h				0.28	0.18	1.9	1.5	0.61	0.68	19
<i>ts</i> B28h					0.20	0.42	0.68	0.13	0.50	72
<i>ts</i> B32h						1.4	0.84	0.28	0.38	580
<i>ts</i> 901							0.45	0.10	0.20	570
<i>ts</i> LB2								0.44	0.91	22
<i>ts</i> D									0.35	444
<i>tsc</i> 75										30

^a Complementation index = [yield (A + B)]/[yield (A) + (B)]; infected cultures were incubated at 39°C, and yields were assayed at 34°C; values greater than 2.0 (in italics) were considered positive (34).

TABLE 2. *Marker rescue tests with mutants in group 1-2: rescue with EcoRI fragments*

Virus	Fragment ^a						None
	A, B, C	D, E, F, G	H	I, J	K		
<i>ts</i> B2b	4.0	0.0	0.0	0.0	0.7	0.0	
<i>ts</i> B21u	10.0	0.0	0.0	0.0	9.6	0.0	
<i>ts</i> B27h	1.5	0.0	0.0	0.0	1.0	0.0	
<i>ts</i> B28h	0.7	0.0	0.0	0.0	0.6	0.0	
<i>ts</i> B32h	7.0	0.0	0.0	0.0	41.0	0.0	
<i>ts</i> 901	22.0	0.0	0.0	0.2	1.0	0.0	

^a Values represent the ratio of the yield of progeny virus from transfected cells when assayed at 39°C as compared with the yield at 34°C (×100). Virus titers at 34°C were always greater than 10⁵ PFU/ml.

1.000) and with a mixture of fragments A, B, and C; fragments B and C are joint-spanning fragments, both of which contain sequences in K. The *Eco*RI K fragment lies entirely within the terminally repeated sequences of the S region. Although we have not determined whether each mutation is present within both repeats, it is likely that this is the case, since fragments representing both ends of S have been shown to rescue *ts*B21u and the presence of the lesion in only one end would permit self-rescue of the mutant (29). Moreover, like *ts*B21u, the terminal fragments of the L region (J and E) failed to rescue, indicating that none of the mutations lies within "a" sequences present at the joint and at the termini of the molecule. Rather, the mutations lie within the "c" sequences adjacent to U_S (20, 29). In the case of *ts*901, the low level of rescue observed with combined fragments I and J was probably due to contamination of the I,J mixture with fragment K, because although fragments J and K share some common sequences, rescue was not observed with fragment E.

To further refine the map positions of the mutants, *Eco*RI fragment K was isolated and redigested with *Bam*HI and *Hinc*II; each of these enzymes makes a single cut within the K fragment, resulting in three fragments (2.0, 1.85, and 1.25 kilobases [kb]) which are easily re-

solved (Fig. 1). When these fragments were used in marker rescue tests, the results shown in Table 3 were obtained. With *ts*B2b, *ts*B21u, *ts*B27h, *ts*B28h, and *ts*B32h, rescue occurred maximally with the terminal *Hinc*II fragment K2 (0.988 to 1.000), whereas with *ts*901, rescue occurred only with the middle fragment, K3 (0.979 to 0.988). Fine-structure mapping of *ts*LB2 and *tsc*75 demonstrated that with the former mutant rescue occurred only with fragment K1, whereas with the latter mutant rescue occurred with both K2 and K3.

A summary of the physical map locations of all nine mutants is presented in Fig. 2. In this figure, the limits of fragments which exhibited rescue are shown as bars. The white portion of each bar indicates sequences in which mutations are thought not to lie by the process of elimination; e.g., failure of the termini of L to rescue *ts*B32h, *ts*B28h, *ts*B27h, *ts*B2b, and *ts*B21u excludes their lying in the "a" sequences, whereas rescue of *tsc*75 by all four terminal fragments excludes its lying in the "c" sequences. Because direct tests of the sequences excluded by the process of elimination have not been conducted, the outermost limits of each bar should be considered definitive. The location of *ts*D (0.966 to 0.900) was taken from Stow et al. (41, 42). It should be mentioned that Wilkie and his colleagues have recently refined the limits of *ts*D and of *ts*K, another member of group 1-2, to coordinates, 0.979 to 0.990 and 0.966 to 0.978, respectively (personal communication). The sequence of mutations on the physical map is therefore *ts*K-*ts*LB2, *ts*D-*ts*901, *ts*B32h-*ts*B28h-*ts*B27h-*ts*B2b-*ts*B21u, and *tsc*75.

The location of the sequence specifying VP175 as determined by heterotypic recombination and marker rescue (27, 33) and the 4.7-kb mRNA which codes for VP175 (48) are also shown in Fig. 2. Because the map coordinates of the mutants lie within the limits set for the mRNA and VP175 structural polypeptide and because no evidence for splicing of the gene for VP175 has been reported, it is assumed that each of the

FIG. 1. *EcoRI* restriction map of HSV-1, strain KOS. (1) *L* and *S* components of the viral genome; (2) sequence organization; (3) fractional distance; (4) *EcoRI* restriction map in the *P* orientation of the genome (27) (fragments *B* and *C* are joint-spanning fragments containing *E* plus *K* and *J* plus *K*, respectively); (5) *Bam*HI/*Hinc*II restriction map of *EcoRI* fragment *K*. The termini of *K*₁ are determined by *EcoRI* and *Bam*HI sites, the termini of *K*₃ are determined by *Bam*HI and *Hinc*II, and the termini of *K*₂ are determined by the *Hinc*II site and the physical end of the *S* region. The gel profile on the left is a preparative gel of *EcoRI*-cleaved HSV-1 DNA fragments. The *K* fragment was excised and digested with *Bam*HI and *Hinc*II. The resulting fragments are shown in the gel profile on the right.

mutants is defective in the structural gene for VP175.

Genetic mapping experiments. Because marker rescue tests did not succeed in separating all mutations physically within the repeat, a linkage map was constructed by two-factor crosses in an effort to order the mutants with respect to one another (Table 4, Fig. 2). The following observations concerning the linkage map are notable. First, the mutants clustered

into four groups, each separated by 1.4 to 1.9 recombination units. The first group contained mutants *tsB2b*, *tsB21u*, and *tsB27h*; the second contained mutants *tsB28h*, *tsB32h*, and *tsLB2*; the third contained *tsD*; and the fourth contained *ts901*. Second, although the mutants clustered into groups, no mutational hot-spotting was evident (i.e., all mutations induced with one mutagen were not located in one place). Both thymidine-specific (bromodeoxyuridine and

UV) and cytosine-specific (hydroxylamine) mutagens were used for mutant induction, and mutations derived with either type of mutagen were randomly arranged on the map. Third, although the physical map is not sufficiently detailed to establish the order of mutants within each of the groups, the genetic and physical maps are colinear with respect to the order of seven of the nine mutants tested: *tsD-ts901* and *tsB32h-tsB28h-tsB27h-tsB2b-tsB21u*. Only *tsLB2* is out of order on the genetic map. Fourth, the recom-

bination frequencies observed were larger than one might expect given the total physical distance involved (3.1 kb). In previous studies, the greatest distance between mutations in a single gene in unique sequences in L was 3.3 recombination units (37) (unpublished data). The high frequency of recombination between mutants of group 1-2 could reflect (i) a unique property of the repeated "c" sequences in that they may constitute a region of very active recombination, (ii) the diploid nature of the gene, or (iii) the large size of the structural gene for VP175. In

TABLE 3. Marker rescue tests with mutants in group 1-2: rescue with *Bam*HI, *Hinc*II-cleaved *Eco*RI-K

Virus	Fragment ^a			
	K1	K2	K3	None
<i>tsB2b</i>	0.0	2.1	0.0	0.0
<i>tsB21u</i>	0.0	4.5	0.3	0.0
<i>tsB27h</i>	0.0	0.3	0.0	0.0
<i>tsB28h</i>	0.0	0.1	0.0	0.0
<i>tsB32h</i>	0.0	1.4	0.0	0.0
<i>ts901</i>	0.0	0.0	5.4	0.0
<i>tsLB2</i>	7.3	0.0	0.0	0.0
<i>tsc75</i>	0.0	14.0	7.6	0.0

^a See Table 2.

TABLE 4. Recombination between mutants in group 1-2

Virus	B2	B21	B27	B28	B32	901	LB2	D	c75
<i>tsB2b</i>		0.36 ^a	0.51	3.2	4.1	4.0	1.4	2.2	0.87
<i>tsB21u</i>			0.91	4.2	2.8	7.7	2.3	2.1	1.6
<i>tsB27h</i>				4.9	2.7	6.1	1.4	1.3	1.8
<i>tsB28h</i>					0.11	3.8	0.73	4.5	0.31
<i>tsB32h</i>						3.4	0.71	1.45	0.55
<i>ts901</i>							2.0	1.9	0.89
<i>tsLB2</i>								3.5	0.40
<i>tsD</i>									0.0
<i>tsc75</i>									

^a Recombination frequency = [yield (A + B) at 39°C]/yield (A + B) at 34°C] × 2 × 100.

FIG. 2. Physical and genetic maps of mutants in complementation group 1-2. The VP175 coding sequence was taken from Preston et al. (33) and Morse et al. (27). The limits of the 4.7-kb mRNA and direction of transcription are taken from Watson et al. (48) and Clements et al. (6). The fractional distance bar indicates the positions of cleavage sites of restriction enzymes used in this study: 0.966 = *Eco*RI, 0.979 = *Bam*HI, and 0.988 = *Hinc*II. The maximum limits of the sequences in which the mutations map are presented as solid bars (indicating sequences in which the mutation is thought to lie) and open bars (indicating sequences in which the mutation probably does not lie as deduced by the process of elimination; see text for explanation). The genetic map was constructed from the data presented in Table 3.

contrast to most mutants, low recombination frequencies were consistently observed between *tsc75* and other mutants (20) (Table 4). Moreover, *tsc75* appears to be closely linked to *tsD*, yet it is located in the "a" sequences at the end of the physical map. It may be that mutant *tsc75* contains multiple mutations, the possible origins of which will be discussed later.

Polypeptide synthesis in *ts* mutant-infected cells. We have shown previously that *tsB2b* and *tsB21u* overproduce immediate early polypeptides VP175, -136, -110, and -63 (8, 9). Similar findings have been reported by Marsden et al. (26) and Knipe et al. (21) with *tsD*, *tsK*, *tsT*, and *tsLB2*, all members of complementation

group 1-2. To determine whether all members of the group examined in this study exhibit this property, cells were infected with each mutant, incubated at 39°C, and labeled from 5 to 24 h postinfection. All of the mutants exhibited a similar but not identical polypeptide phenotype at 39°C (Fig. 3). Thus, for all mutants, immediate early polypeptides VP175, -68, and -63 were overproduced. These polypeptides correspond to ICP4, -22, and -27, respectively, of Honess and Roizman (13). Although VP136 (ICP6) was overproduced by all mutants except *tsB28h* and *tsB32h*, VP110 (ICP0) was overproduced only by mutants not derived from strain KOS, i.e., *tsLB2*, *tsc75*, and *tsD*. Additionally, some early

FIG. 3. SDS-PAGE analysis of viral proteins synthesized in cells infected with *ts* mutants in complementation group 1-2. HEL cells were infected with 100 PFU/cell at 39°C, labeled with ^{14}C -amino acids from 5 to 24 h postinfection, and processed for PAGE as described in Materials and Methods. Abbreviations: M, mock-infected cells; IE, immediate early polypeptides; KOS, wild-type virus; std, molecular weight standards in decreasing order (220,000, 93,000, 69,000, 44,000, 30,000, and 12,000). The molecular weights of viral proteins presented are those reported here and by other investigators: A, this study; B, Marsden et al. (26) and Preston (31); C, Courtney et al. (9); D, Honess and Roizman (13) and Pereira et al. (30). Values indicate molecular weights $\times 10^3$. Numbers in parentheses are ICP designations of Honess and Roizman (13).

and late viral polypeptides (e.g., VP64 and -155, respectively) were synthesized by all mutants. That the variations may reflect strain-specific differences in gene expression cannot be excluded. However, an alternative explanation for the observed variation concerns the fact that some mutants exhibit greater leak at 39°C than others derived from the same strain. Thus, for example, *ts*B28h and *ts*B32h synthesized greater amounts of late proteins at 39°C than did the other KOS mutants (data not shown). Figure 3 also includes the various molecular weights and nomenclatures used in the literature to describe these polypeptides. In this report, we have used the nomenclature of Preston (31) (Fig. 3, column B).

Temperature shift experiments. To determine whether functional VP175 is required only transiently or throughout the replicative cycle, temperature shiftup experiments were performed with *ts*B21u. In the first experiment, *ts*B21u-infected cells were incubated for various lengths of time at 34°C before shiftup to 39°C; all cultures were labeled at the time of shift and harvested at 24 h (Fig. 4).

When cultures were incubated continuously at 39°C (Fig. 4, lane b), overproduction of immediate early polypeptides VP175, -136, -110, and -63 was evident. Late polypeptides VP154 and VP100 and early polypeptide VP64 were minor species in these cells. In contrast, early and late polypeptides predominated in cultures incubated at 34°C from 1, 2, 4, or 8 through 24 h (Fig. 4, lanes c, e, g, and i).

In cultures shifted up at 1, 2, and 4 h (Fig. 4, lanes d, f, and h), immediate early polypeptides VP175, -136, and -63 were predominant, considerably more being synthesized than in the 34°C controls. Thus, immediate early protein synthesis was continuous (i.e., not shut off) after shiftup. VP110 was also present, but to a lesser extent than the other three polypeptides. In cultures shifted up at 4 h (lane h), VP175, -136, -110, and -63 were present in greater amounts than in the 34°C controls (lane g), but increased amounts of VP154 and -64 were also observed. The synthesis of VP110 is difficult to evaluate in these experiments. Although little was seen in the shift at 2 h and more was seen in the shifts at 4 and 8 h, this increase may be due to the fact that an early polypeptide comigrates with VP110 (30). Thus, the protein seen at later times may be the immediate early polypeptide, VP110, the early polypeptide, or both. Clearly, however, the synthesis of VP175, -136, and -VP63 was not shut off after shiftup even though many early and late proteins were being synthesized simultaneously. Thus, despite the current hypothesis that early viral proteins are required to turn off

immediate early protein synthesis, these proteins continued to be synthesized in the presence of early proteins. Because the only essential lesion in *ts*B21u is in the gene for VP175, these observations suggest the possibility that VP175 is involved in the inhibition of immediate early protein synthesis or, alternatively, that VP175 is required continuously to produce a factor required to shut off the synthesis of this class of polypeptides. Even when the shift occurred as late as 8 h (Fig. 4, lane j), the expression of VP175 was still greater than in the 34°C control (lane i). Additional differences were noted when proteins were synthesized after shift to 39°C (lane j) compared with unshifted cultures (lane i; see dots between lanes i and j). Thus, it appears that functional VP175 is required even after 8 h for the expression of these proteins, but not for the expression of all early and late proteins.

An additional observation made from this gel concerns variation in the electrophoretic mobility of VP175. In all cases, the VP175 polypeptide made at 34°C migrated more slowly than that made at 39°C (most clearly seen in lanes e, f, g, and h of Fig. 4). This observation is in agreement with the observations of Preston, who showed that in cells infected with *ts*K, different forms of VP175 were synthesized at permissive and non-permissive temperature (31), and of Pereira et al. (30) and Wilcox et al. (49), who found three forms of wild-type VP175 in pulse-chase experiments.

Functional VP175 has been shown to be required only transiently for viral DNA synthesis, the requirement being bypassed by a 2-h preincubation at 34°C (35). The results of the present study demonstrate that continued synthesis of VP175 is required for the expression of certain late viral gene products (see above). The requirement for functional VP175 in the synthesis of infectious virus, however, has not been examined. Therefore, the yield of infectious virus in cultures infected as for shiftup tests (Fig. 4) was measured (Table 5).

In unshifted cultures, *ts*B21u was temperature sensitive for growth when compared with the wild-type virus, as expected. When mutant-infected cultures were shifted up at 1, 2, 4, and 8 h and yields were assayed after 24 h, they were only 10-fold higher than the yield from the culture incubated at 39°C. Thus, unlike viral DNA synthesis, functional VP175 is apparently required after 8 h for the production of infectious virus. Moreover, VP175 appeared to be required throughout the 18-h growth cycle, since the yield of infectious virus was reduced 10-fold compared with unshifted cultures when cultures were shifted up at 18 h (data not shown). Alternatively, given the greatly diminished amount of

FIG. 4. SDS-PAGE analysis of viral proteins synthesized in cells infected with *tsB21u* after temperature shiftup. (a) Mock-infected HEL cells labeled from 4 to 24 h at 39°C; (b) *tsB21u*-infected cells incubated at 39°C and labeled from 4 to 24 h; (c-l) cells infected at 34°C and held continuously at this temperature or shifted up to 39°C at designated times; (c, e, g, and i) cells incubated at 34°C and labeled at 1, 2, 4, and 8 h, respectively; (d, f, h, and j) cells shifted up and labeled at 1, 2, 4, and 8 h, respectively; (k and l) wild-type virus-infected cells labeled from 4 to 24 h at 34 and 39°C, respectively. All samples were harvested at 24 h and prepared for PAGE and virus assay (Table 5) as described in Materials and Methods.

VP175 synthesized after shiftup at 8 h (Fig. 4, lane j), it is possible that the same sequences which specify VP175 also specify a later function required for infectivity, and that a mutation in

the sequences specifying VP175 could also inactivate this putative later function.

The experiment illustrated in Fig. 4 demonstrated that VP175 synthesis was not shut off

TABLE 5. Yield of *tsB21u* and wild-type virus after temperature shiftup

Virus	Initial temp of incubation (°C)	Time of shiftup (h)	Virus yield
<i>tsB21u</i>	34	— ^a	6×10^7
	39	—	8×10^3
	34	1	3×10^4
	34	2	3×10^4
	34	4	4×10^4
	34	8	8×10^4
Wild type	34	—	1×10^8
	39	—	1×10^8

^a —, Not shifted.

when cultures were shifted up at 1 to 4 h (lanes f and b, respectively; not clear from this gel, but seen in other experiments), indicating that maximum synthesis of VP136 occurs later than other immediate early polypeptides, an observation that led Pereira et al. to classify this polypeptide in the group of early or β polypeptides (30). When cells were shifted up at 4 h and labeled from 4 to 8 h (lane d), immediate early (VP175, -136, -110, and -63), early (e.g., VP64), and late (e.g., VP154) polypeptides were synthesized. Because little synthesis of VP175 was observed at 34°C during this period (lane f), this finding suggests that continued (or possibly renewed) expression of immediate early polypeptides occurred in conjunction with the expression of late polypeptides. Pulse-labeling for periods as short as 30 min produced the same results (data not shown). Shifting up at later times (i.e., 8 to 12 and 12 to 24 h; lanes g and j) did not result in the accumulation of VP175 or VP136; however, the synthesis of VP63 (and, to a lesser extent, VP110) was enhanced relative to its synthesis in 34 and 39°C control cultures (lanes h and i and lanes k and l). As in the case of infected cells shifted up from 4 to 8 h, the synthesis of immediate early polypeptides (VP63 and -110) occurred simultaneously with the synthesis of late (VP100 and -154) polypeptides.

To test the possibility that the accumulation of immediate early polypeptides in cultures shifted to 39°C was due to their increased stability at this temperature, mutant-infected cells were pulse-labeled at 39°C from 4 to 4.5 h (Fig. 5, lane m) and chased from 4.5 to 8 h at 39°C (lane n) or after shiftdown to 34°C (lane o). The labeled, chased polypeptides were equally stable at both temperatures (lanes n and o), supporting the concept that the accumulation of immediate early proteins at 39°C after shiftup was due to continued or increased synthesis rather than to decreased turnover. Although it appears that

some synthesis continued during the chase period (Fig. 5), such synthesis was not observed in other experiments. The relative stability of the protein at the two temperatures was identical, however.

Additional evidence for the reexpression of immediate early polypeptides after shiftup comes from the observation that when *tsB21u*-infected cells were shifted at 4 or 8 h and either pulse-labeled immediately for 1 h or pulse-labeled 2 h after the shiftup, the same level of synthesis of VP175 was observed (data not shown). Because the half-life of the early mRNA's is only 1 h (46), it would be necessary to synthesize new mRNA in order to maintain the same rate of protein synthesis. In this experiment, expression of VP175 in cultures shifted up at 8 h was observed, whereas no expression of VP175 was observed in cultures labeled at 34°C during the same period, again indicating that VP175 is reexpressed and therefore involved in inhibiting its own synthesis.

An additional observation made from the gels shown in Fig. 5 concerns the active form of VP175. It is apparent from Fig. 4 and 5 that the form of VP175 which accumulated at 34°C was of higher apparent molecular weight than the form which accumulated at 39°C. After pulse-labeling, a fast-migrating form was synthesized, and this form could be chased to a slower-migrating form which accumulated at 39°C (Fig. 5, lanes m and n). After shift from 39 to 34°C and chase (lane o), a slight change in apparent molecular weight was evident. As mentioned above, the finding of three electrophoretic variants of VP175 is consistent with the reports of Pereira et al. (30), Preston (31), and Wilcox et al. (49), who have described three forms of the protein (VP175a, -b, and -c or ICP4a, -b, and -c) which share a precursor-product relationship. Preston noted that upon shift from 34 to 39°C, VP175c was converted to VP175b, with loss of activity (31). Consistent with this observation, we found that VP175b was converted to VP175c upon shiftdown. Of additional interest is Preston's finding that upon shiftdown in the presence of cycloheximide, no new protein synthesis was required for early transcription (32). From these observations, it appears that the "c" form is the active form of VP175. Although the nature of the modification in VP175 is not known, Wilcox et al. (49) have presented evidence which implicates phosphorylation in the modification process. Whatever the mechanism of modification, it is essential for functional activity.

DISCUSSION

The results presented here support and complement previous findings concerning the loca-

FIG. 5. SDS-PAGE analysis of viral proteins synthesized in cells infected with *tsB21u* after temperature shiftup and pulse-labeling. HEL cells were infected with 100 PFU/cell and labeled as follows: (a) mock-infected cells labeled from 4 to 8 h at 34°C; (b and c) *tsB21u*-infected cells labeled from 1 to 4 h at 34 and 39°C, respectively; (d) *tsB21u*-infected cells shifted from 34 to 39°C at 4 h and labeled from 4 to 8 h; (e and f) *tsB21u*-infected cells labeled from 4 to 8 h at 39 and 34°C, respectively; (g) *tsB21u*-infected cells shifted up from 34 to 39°C at 8 h and labeled from 8 to 12 h; (h and i) *tsB21u*-infected cells labeled from 8 to 12 h at 39 and 34°C, respectively; (j) *tsB21u*-infected cells shifted from 34 to 39°C at 12 h and labeled from 12 to 24 h; (k and l) *tsB21u*-infected cells labeled from 12 to 24 h at 39 and 34°C, respectively; (m-o) *tsB21u*-infected cells labeled at 39°C from 4 to 4.5 h and harvested at 4.5 h (m) or chased with cold amino acid-containing media from 2.4 to 8 h at 39°C (n) or 34°C (o).

tion and function of the gene encoding VP175. The original description of cascade regulation in the HSV-1 replicative cycle (13) led to the identification of a group of proteins (immediate early, or α , proteins) which are synthesized immediately after infection and whose synthesis is

required for the initiation of synthesis of a second group of proteins, the early, or β , proteins. Early proteins were shown to play a role in the shutoff of immediate early protein synthesis (13, 14).

Among the immediate early proteins first de-

scribed were VP175, -110, and -63 (13). The existence of a mutant, *ts*B2b, which overproduced VP175 and was defective in the transition from immediate early to late protein synthesis was reported simultaneously (8). The latter study also demonstrated that in *ts*B2b-infected cells, VP175 was not transported to the nucleus as efficiently as in wild-type virus-infected cells at the nonpermissive temperature. Both the transition from immediate early to late protein synthesis and transport to the nucleus could be reversed upon shiftdown and bypassed by preincubation at the permissive temperature, indicating that the defective function in this mutant was both early and reversible. Subsequent experiments demonstrated that functional VP175 was essential for viral DNA synthesis and that the requirement could be bypassed by preincubation for 2 h at the permissive temperature (35). More recently, it was shown that upon shiftdown, new transcription was required for viral DNA synthesis in cells infected with *ts*B2b (28). Other investigators have isolated additional *ts* mutants of complementation group 1-2 (D, T, K, and LB2) (36) which possess similar phenotypic properties. Preston (32) has reported that at least six immediate early polypeptides (VP175, -136, -110, -68, -63, and -12) are synthesized *in vivo* after release of a cycloheximide block and that *in vitro* translation of immediate early mRNA yields these same products. Preston has also compared the synthesis of immediate early polypeptides in *ts*K and wild-type virus-infected cells and has obtained evidence which demonstrates that (i) immediate early proteins are directly involved in the transition from immediate early to early protein synthesis, (ii) three forms of VP175 exist, and (iii) upon temperature shiftdown, the multiple effects of the mutations are reversible. In contrast, Pereira et al. (30) placed VP136 in the group of early proteins despite the fact that it is synthesized in the presence of cycloheximide or canavanine. This classification was based upon the observation that VP136 was synthesized slightly later than the other immediate early proteins.

To confirm that the product of gene 1-2 is indeed VP175 and to further define the function of this protein in the regulation of viral transcription, studies were undertaken to map physically and genetically a series of mutants in group 1-2 and to characterize the residual activity of mutant gene products in infected cells at the nonpermissive temperature.

Mapping studies. Previously, studies have shown that *ts*B21u (29), *ts*B2b (3), *ts*D (41, 42), *ts*LB2 (21), and *ts*c75 (22) lie in the repeated sequences bounding *Us*. In this study, we have attempted to refine the map positions of several

mutants in complementation group 1-2 and to separate them physically within these sequences.

It is our contention that the data presented here together with the findings of other investigators place the mutants within the structural gene for VP175. The pertinent arguments for this contention are as follows: the nine mutants (i) fail to complement each other, (ii) exhibit similar viral DNA and protein phenotypes, (iii) produce an altered form of VP175, (iv) are separable by physical and genetic mapping procedures, and (v) map within the DNA sequences which encode VP175. Moreover, VP175 is the only protein which has been shown to be encoded within these sequences, and (vi) a 4.7-kb immediate early mRNA coding for a protein of molecular weight 175,000 also maps in these sequences (6). Therefore, unless extensive splicing occurs in production of the message for VP175, and no evidence for such splicing has yet been reported, the nine mutants must lie in the structural gene for VP175. Mapping studies therefore exclude the possibility that the phenotypic properties of mutants of group 1-2 are due to mutations in a gene which regulates VP175 synthesis.

As described above (Results), most mutants map nearer the terminal "a" sequences than the unique region of S. Two possible explanations for this observation exist: (i) this segment of the VP175 gene, if mutated, results in significant, observable phenotypic changes (e.g., this segment encodes an active site), or (ii) some property of the DNA makes it highly mutable. We cannot test the former possibility at this time; however, the latter lends itself to speculation. Knipe et al. (21) have demonstrated regions of identity between the terminal "ca" sequences in S and have proposed a copying mechanism whereby, during DNA replication, one terminus serves as the template for the other. They further postulated that "c" sequences further from the terminal "a" sequences can tolerate sequence heterogeneity more readily than the "c" sequences nearer "a." Thus, the former sequences have been designated variable "c" sequences, and those nearer terminal "a" sequences have been designated invariable "c" sequences. These investigators also demonstrated that *ts* lesions in VP175 are recessive. Thus, sequences at only one terminus need be wild type to generate the wild-type phenotype, whereas sequences at both termini must be mutant to generate the *ts* phenotype (21, 31, 33). Our data are consistent with this hypothesis in that a mutation induced in invariable "c" sequences would be more likely to be copied into the opposite terminus, yielding a mutant phe-

notype, than would a mutation in variable "c" sequences, which could produce a "silent" mutant phenotype. Thus, perhaps, the copying mechanism of DNA synthesis constitutes the underlying reason for the isolation of a predominance of *ts* mutations in invariable, terminal "c" sequences. Further analysis of the remaining members of group 1-2 and efforts to introduce mutations into the variable and invariable sequences in one terminus but not the other will be necessary to prove this hypothesis.

The genetic map of the nine mutants exhibits striking linearity and additivity, much more so than linkage maps of markers in unique sequences in L (37; Dixon and Schaffer, manuscript in preparation). Moreover, the recombination frequencies observed among mutants in group 1-2 were much higher than those observed between markers within a single gene in the unique region (Dixon and Schaffer, manuscript in preparation). The diploid nature of the gene may account for the high frequency of recombination since only one terminus need recombine to yield a *ts*⁺ recombinant: crossovers at either end would produce twice the effective recombination frequency.

Another interesting observation which arose from recombination studies concerns the behavior of *ts*c75. This mutant recombines inefficiently with other members of group 1-2. In addition, it behaves as if it were closely linked to *ts*D, yet it has been mapped physically in the terminal "a" sequences (20). A possible explanation for these findings is that the "a" sequence may, in fact, be repeated within "c" sequences. This suggestion was first made by Wadsworth et al. (44), who demonstrated small lariat structures at the ends of molecules which had been digested with exonuclease III, and proposed that the sequence arrangement of the S region may be ...a'x'a c'---U_S---ca'xa. We will assume for the sake of argument that this arrangement is correct. If the "a" sequence is diploid in each repeat and if *ts*c75 does lie within the "a" sequence, at least two copies of the mutant "a" sequence would be present in order to prevent self-rescue. Although Knipe et al. (20) have proposed that the coding sequences for VP175 include the terminal "a" sequence, this need not be the case. In fact, it may be a mutation in the internal "a" sequence which produces the mutant phenotype, or perhaps mutations in both "a" sequences. Our genetic and physical mapping data support this contention. If the mutation in the internal "a" sequence were essential for the expression of the *ts* phenotype, and if this sequence were adjacent to *ts*D, then the position of *ts*c75 found by recombination analysis would be predicted. Moreover, if "a" sequences were

also present in "c" sequences, one would predict that *ts*c75 would be rescued by fragment K2 (containing the terminal "a" sequence) and by fragment K3 (containing the putative "a" sequence in "c"). Indeed, this is precisely the result we obtained.

Alternatively, the low frequency of recombination observed between *ts*c75 and other members of group 1-2 may reflect the fact that recombination occurs with decreased frequency at the ends of molecules. Thus, low recombination frequencies with markers in the terminal "a" sequence could result in mismapping. This explanation is inconsistent, however, with rescue of *ts*c75 by both K2 and K3.

Function of VP175. Having demonstrated that all nine mutants in complementation group 1-2 are located in the structural gene for VP175, further functional analysis of this polypeptide was undertaken.

We first demonstrated that all nine mutants exhibit similar phenotypes with regard to polypeptide synthesis at the nonpermissive temperature. Minor variations in the amounts of certain polypeptides were observed, owing perhaps to strain variation or leak, but in most cases VP175, -136, -110, -68, and -63 were seen to be overproduced. In *ts*c75-infected cells, however, increased amounts of VP110 accumulated relative to the other mutants. Although this may simply reflect a strain-specific difference, it may also reflect some unusual properties of VP110. Clements et al. (6) and Watson et al. (48) have shown that VP110 is encoded by the terminal repeats of the L region. Because the "a" sequence is common to the repeats of both L and S and because the mRNA coding for VP110 may contain a terminal "a" sequence, it is possible that *ts*c75 is mutant for both VP175 and VP110 and is, therefore, a nonresolvable double mutant. If *ts*c75 contains a mutation in the gene for VP110, then the accumulation of VP110 in mutant-infected cells may reflect a role for VP110 in its own regulation or, alternatively, it may reflect a failure to process the mutant form of VP110 analogous to mutants in VP175. Because no single mutant in VP110 is available, we cannot distinguish between the two possibilities.

Several groups have reported that functional VP175 is required to switch from immediate early to early protein synthesis and that the requirement is probably at the level of transcription (7, 8, 13, 14, 22, 24, 25, 32, 43, 47). What is not clear is the role of early proteins in regulating immediate early protein synthesis and the role of VP175 in regulating its own synthesis. Temperature shiftup experiments have shown that VP175 as well as other immediate early proteins can be synthesized in the presence of both early

and late functional proteins, indicating that VP175 is involved either directly or indirectly in turning off its own synthesis and perhaps that of other immediate early proteins. This conclusion was particularly apparent in the case of shifts late in infection which resulted in the reexpression or increased synthesis of immediate early proteins (Fig. 4). If this is indeed the case, then the model of cascade regulation must be modified to include autoregulation of immediate early proteins in addition to feedback inhibition by early proteins.

Another point to be made concerns the existence of multiple control groups in each polypeptide class. Pereira et al. (30) first suggested that subgroups of immediate early and early proteins existed, a concept supported by Preston (32). Our experiments have defined a group of proteins which are active late in infection (after 8 h) and yet require the continued expression of VP175. This is apparent from the differences seen in polypeptide synthesis after shiftup late in infection and from the observation that no virus was produced even though most proteins were synthesized (35). In addition, VP136, first classified as an immediate early protein by Marsden et al. (26) and an early protein by Pereira et al. (30), does not appear to be under VP175 control, yet it accumulates after VP175, -110, and -63 reach maximum synthesis. Thus, as pointed out by others, VP136 synthesis exhibits the kinetics of an early protein and the control requirements of an immediate early protein.

A model consistent with all available data would require that all promoters on the HSV genome be active at all times but that promoters differ in the frequency with which transcription is initiated. Promoters for VP175, -110, and -63 would have the highest initiation frequencies, whereas those for VP136 and other early and late proteins would have a lower frequency, but still be recognized by the transcriptional apparatus. One function of VP175 would be to increase the frequency of transcription from some early or even late promoters and decrease the frequency from its own promoters. Consistent with this model are the following observations: (i) certain late proteins (e.g., VP154) can be expressed in the absence of functional VP175 (this study), (ii) VP175 is required for certain late viral functions (this study), (iii) the resident HSV TK gene in biochemically transformed cells is expressed in the absence of VP175 (19, 24, 25), (iv) functional VP175 is able to enhance HSV TK expression in HSV TK-transformed cells (19, 24, 25), (v) VP175 is reexpressed in group 1-2 mutant-infected cells after shiftup (i.e., after inactivation of the autoregulatory function of VP175) (this study), and (vi) the active form

of VP175 has DNA binding properties (49). If this model were correct, one might expect VP175 to bind directly to promoter sequences or perhaps to form complexes with the RNA polymerase to alter its specificity. This transcriptional model is similar in many ways to the regulatory mechanism operative during adenovirus replication in which pre-early proteins are required to initiate the synthesis of early proteins, which in turn initiate later protein synthesis (2, 16). However, during transcription *in vitro*, the major late promoter(s) is recognized in preference to the early promoter(s) (J. Manley, personal communication). This indicates that although early functions are needed to enhance late promoter activity, they can be bypassed by the cellular transcriptional complex.

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