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Isolation and Characterization of Herpes Simplex Virus Type 1 Host Range Mutants Defective in Viral DNA Synthesis

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Cell lines were generated by cotransfection of Vero cells with pSV2neo and a plasmid containing the herpes simplex virus type 1 (HSV-1) *Eco*RI D fragment (coordinates 0.086 to 0.194). One such cell line (S22) contained the genes for alkaline exonuclease and several uncharacterized functions. Three mutant isolates of HSV-1 strain KOS which grew on S22 cells but not on normal Vero cells were isolated and characterized. All three mutants (*hr*27, *hr*48, and *hr*156) were defective in the synthesis of viral DNA and late proteins when grown in nonpermissive Vero cells. Early gene expression in cells infected with these host range mutants appeared to be normal at the nonpermissive condition. The mutations were mapped by marker rescue to a 1.5-kilobase fragment (coordinates 0.145 to 0.155). The mutation of one of these mutants, *hr*27, was more finely mapped to an 800-base-pair region (coordinates 0.145 to 0.151). This position of these mutations is consistent with the map location of a putative 94-kilodalton polypeptide as determined by sequence analysis (D. McGeoch, personal communication). Complementation studies demonstrated that these mutants formed a new complementation group, designated 1-36. The results presented in this report indicate that the 94-kilodalton gene product affected by these mutations may have a direct role in viral DNA synthesis.

The herpes simplex virus (HSV) genome is large (160 kilobases [kb]) and encodes many of the gene products involved in the replication of its double-stranded DNA chromosome. HSV functions involved either directly in DNA synthesis or in nucleotide metabolism include the major DNA-binding protein (10, 28, 54), DNA polymerase (42), alkaline nuclease (18, 38), a 54-kilodalton (kDa) polymerase-associated protein (51), ribonucleotide reductase (15), thymidine kinase (14), and dUTPase (39).

The most direct approach to identifying gene products essential for viral DNA replication has been to isolate mutants which exhibit alterations in DNA synthesis. Of the functions identified, only the major DNA-binding protein (ICP8) (10, 37, 54) and the HSV DNA polymerase (5, 6, 9, 22, 23, 36) have been shown by the analysis of temperature-sensitive (*ts*) and other mutants to be absolutely essential for viral DNA replication. Genetic studies indicate that under certain conditions alkaline nuclease (19, 32), ribonucleotide reductase (15, 40), and thymidine kinase (24) may also be required. To identify additional proteins required for viral DNA synthesis, DNA-negative mutations in genes whose functions are currently unknown have been isolated and studied. These include *ts*K13 and *ts*M19, representing complementation group 1-10, whose mutations map to the far left end of the genome between coordinates 0.095 and 0.108 (53; S. K. Weller, E. P. Carmichael, D. P. Aschman, D. J. Goldstein, and P. A. Schaffer, Virology, in press). In addition, the lesion in *ts*S38, a member of complementation group 1-26, has been mapped to coordinates 0.126 to 0.133 (Weller et al., in press). *ts*S38 produces small to moderate levels of viral DNA at the nonpermissive temperature (8). In this case, it is not clear whether the gene product is essential for viral DNA synthesis since the *ts* mutation may be leaky (i.e., some residual activity of the affected protein may be present at the nonpermissive temperature). To circumvent problems with potentially leaky *ts* mutants, we have used an alternative approach for the isolation of mutations in functions thought to be involved in viral DNA synthesis. This strategy, originally developed by Benjamin (2), involves the isolation of host range mutants which can be propagated on complementing transformed cell lines. We have isolated mutants in a new complementation group whose mutations map to the left of alkaline nuclease within the *Eco*RI D fragment of HSV-1 strain KOS (coordinates 0.086 to 0.194). These host range mutants fail to synthesize detectable levels of viral DNA in nonpermissive cells.

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MATERIALS AND METHODS

Cells and viruses. Procedures for the maintenance and growth of African green monkey kidney cells (Vero) have been described previously (53). S22 cells (see below) were maintained as above, but with the addition of the antibiotic geneticin (G418; 250 μg/ml; GIBCO Laboratories, Grand Island, N.Y.).

The KOS strain of HSV-1 was used as the wild-type (WT) virus. *ts* mutants used in this study are shown in Table 1. HSV-1 strain KOS *ts* mutants were generously provided by P. A. Schaffer (Dana-Farber Cancer Institute, Boston, Mass.). *ts*J12 contains a *ts* mutation in the structural gene for the glycoprotein gB which has been mapped to coordinates 0.357 to 0.360 (44). HSV-1 strain 17 mutants *ts*R, *ts*S, and *ts*X were kindly provided by V. Preston and H. Marsden (MRC Institute of Virology, Glasgow, Scotland) (30). Viruses were propagated and assayed as described previously (45).

Plasmids and bacteria. All recombinant clones containing HSV-1 DNA are shown in Fig. 1. pSG10, generously provided by R. Sandri-Goldin (University of California at Irvine) and M. Levine (University of Michigan, Ann Arbor), contains the *Eco*RI D fragment of HSV-1 strain KOS (coordinates 0.086 to 0.194) cloned into the *Eco*RI site of pBR325 (20). The construction of pSG10-SB, pSG10-B2, and pSG10-BD2 is described elsewhere (Weller et al., in press). pSG10-XII was constructed by ligating an *Xho*II fragment (coordinates 0.145 to 0.155) into the *Bam*HI site of pUC19. pSG10-BD2XH was constructed as follows. pSG10-BD2 was

TABLE 1. HSV-1 *ts* mutants used in this study

Mutant	Mutagen[a]	Complementation group	DNA phenotype[b]	Reference
*ts*K13	NTG	1-10	−	Schaffer et al. (44)
*ts*F18	NTG	1-6	+	Schaffer et al. (44)
*ts*S38	HA	1-26	±	Chu et al. (8)
*ts*J12	NTG	1-9	+	Schaffer et al. (44)
*ts*R	BUdR	nd[c]	−	Matz et al. (30)
*ts*S	BUdR	nd	−	Matz et al. (30)
*ts*X	BUdR	nd	−	Matz et al. (30)

[a] HA, Hydroxylamine; BUdR, 5-bromodeoxyuridine; NTG, nitrosoguanidine.

[b] Phenotypes determined by Aron et al. (1) for *ts*F18 and by Weller et al. (53) for *ts*K13; others determined by authors indicated. Symbols indicate synthesis of viral DNA at the nonpermissive condition relative to that in the wild type: +, >20% of the wild-type level; ±, ≤20%; −, no detectable viral DNA synthesized.

[c] nd, Not previously determined.

digested with *Xho*I and *Hpa*I, and the ends were filled in with the large fragment of DNA polymerase I and religated. pSG10-P5 and pSG10-P6 were constructed by ligating the *Pst*I fragments of *Eco*RI-D (coordinates 0.129 to 0.151 and 0.151 to 0.158, respectively) into the *Pst*I site of pUC19. pSV2neo contains the bacterial gene for neomycin resistance under the control of the simian virus 40 early promoter (47). Recombinant plasmids were propagated in *Escherichia coli* JM83 or DH5 by standard procedures (29).

Viral and cellular DNA isolation. Viral DNA from host range mutants of HSV-1 strain KOS was prepared from partially purified virions as described by Parris et al. (35). Cellular DNA was isolated as described in Weller et al. (55).

Transformation of Vero cells. Plasmids pSV2neo (5 μg) and pSG10 (3 μg) were coprecipitated in the presence of 16 μg of salmon sperm DNA in a total volume of 1.0 ml by the procedure of Graham and van der Eb (21). Freshly trypsinized Vero cells (4×10^6) were added to the precipitate and incubated at 37°C for 30 min with continuous agitation. This cell-DNA mixture was suspended in 10 ml of culture medium and transferred to a 100-mm petri dish. After 4 h at 37°C, the cells were shocked with 15% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline, washed twice with HEPES-buffered saline, and incubated further at 37°C after the addition of medium. G418 selection was carried out as described by DeLuca et al. (12). The cells were grown to confluency (about 2 days), trypsinized, and plated at a 1:10 dilution in medium containing G418 (500 μg/ml). After approximately 2 weeks at 37°C (with periodic change of medium), individual G418-resistant colonies were isolated, amplified, and screened as described in Results. One positive cell line, S22, was chosen for further studies.

Analysis of cellular and viral DNA. Total cellular or viral DNA was digested with restriction endonucleases, fractionated by agarose gel electrophoresis, and transferred to a GeneScreen Plus nylon membrane (New England Nuclear

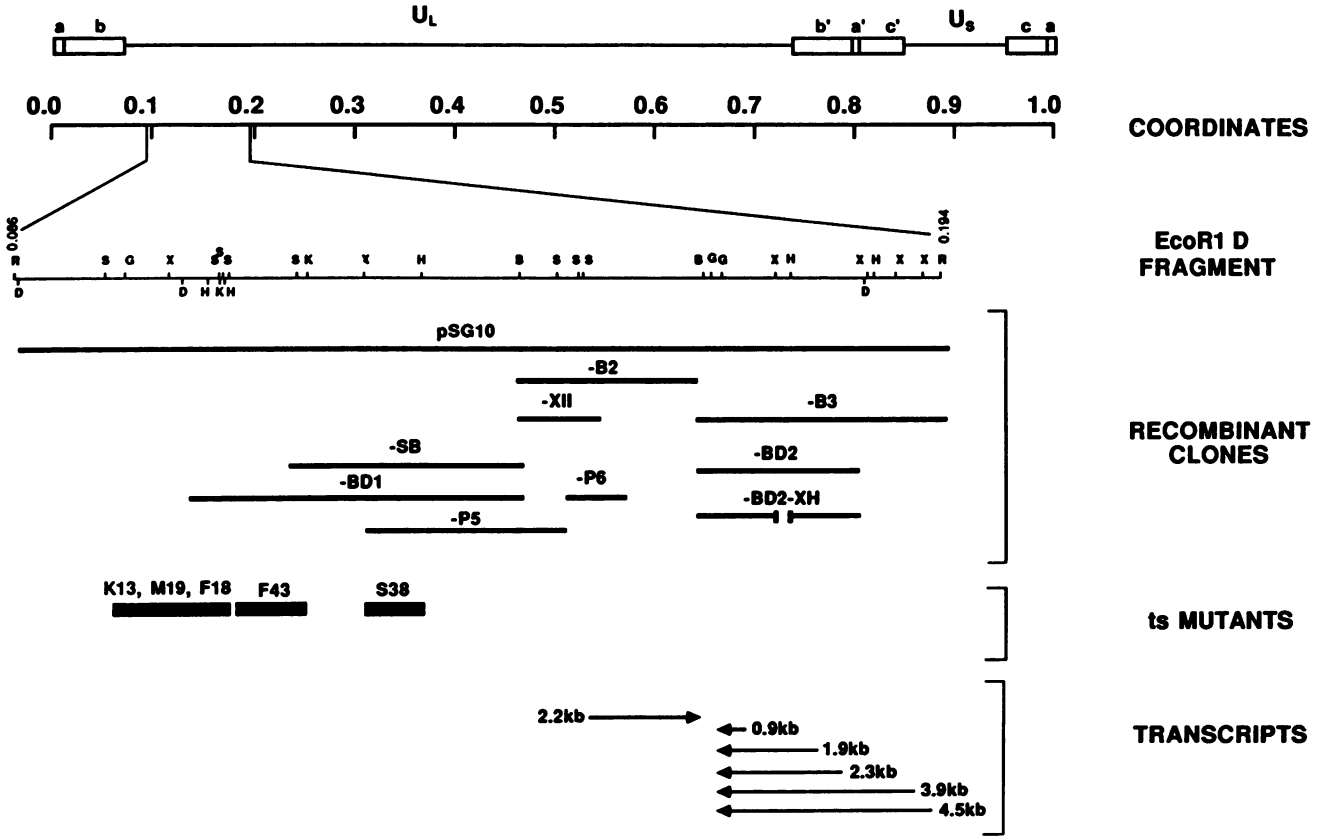

FIG. 1. Recombinant DNA plasmids and physical map locations of mutations in five *ts* mutants within the *Eco*RI D fragment. The *Eco*RI D fragment (map coordinates 0.086 to 0.194) has been expanded to show internal cleavage sites: *Eco*RI (R), *Bgl*II (G), *Hin*dIII (H), *Kpn*I (K), *Hpa*I (H), and *Xho*I (X). The HSV-1 DNA insertions in each recombinant plasmid described in the text are shown below the restriction map. The physical map locations of mutations in five HSV-1 strain KOS *ts* mutants are shown in relation to transcripts which have been finely mapped within this region (11, 13, 52).

TABLE 2. Titers of mutants at 39°C

Strain	Titer (PFU/ml)		
	Vero cells	S22 cells	Ratio, S22/Vero
KOS	7.5×10^8	8.9×10^8	1.2
*ts*S38	8.5×10^4	1.7×10^8	2.1×10^3
*hr*27	$<10^2$	1.5×10^8	$>1.5 \times 10^6$
*hr*48	$<10^2$	2.2×10^8	$>2.2 \times 10^6$
*hr*156	$<10^2$	5.8×10^7	$>5.8 \times 10^5$
*ts*R	$<10^3$	1.5×10^7	$>1.5 \times 10^4$
*ts*S	$<10^3$	1.8×10^5	$>1.8 \times 10^2$
*ts*X	$<10^3$	6.7×10^6	$>6.7 \times 10^3$

Corp.) as suggested by the supplier. Recombinant DNAs used as probes for hybridization were labeled by the method of Feinberg and Vogelstein (17).

Marker rescue analysis. Marker rescue experiments were performed by the procedure of Parris et al. (35) with the modifications described by Chiou et al. (7).

Complementation tests. Complementation tests were conducted as described previously (45). Complementation indices (CIs) were calculated from the formula CI = $(A + B)_{np}/(A_{np} + B_{np})$, where A and B are two mutants. Virus yields from infections carried out in Vero cells at 39.6°C (nonpermissive conditions) were assayed for plaque formation in S22 cells at 34°C (permissive conditions).

Synthesis of viral DNA in infected cells. Analysis of viral DNA synthesis was performed as described by Aron et al. (1) except that proteinase K was used instead of pronase. Growth in Vero cells was used as the nonpermissive condition for host range mutants, and growth in S22 cells was used as the permissive condition.

Viral protein labeling and gel electrophoresis. Labeling of HSV-infected Vero or S22 cells with [^{35}S]methionine was performed as described previously (Weller et al., in press) except that label was added twice, first at 5 h postinfection and again at 10 h postinfection. Addition of isotope at 10 h was found to improve labeling of viral late proteins. Cells were lysed in a solution containing 0.05 M Tris chloride, pH 7.2, 0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100, and the protease inhibitors *N*-α-*p*-tosyl-lysine chloromethyl ketone (Sigma Chemical Co.) and phenylmethylsulfonyl fluoride (Sigma) at a final concentration of 0.1 mM and 0.5 mM, respectively. The extracts were then sonicated for 45 s, and cell debris was removed by centrifugation for 5 min in an Eppendorf microcentrifuge. An equal volume of sample buffer containing 0.37 M Tris chloride, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 10% sodium dodecyl sulfate, and 0.001% bromphenol blue was added to each sample, which was then boiled for 3 min and loaded onto a 9% bisacrylamide-cross-linked polyacrylamide gel (26). Gels were treated with Autofluor (National Diagnostics), dried, and exposed to Kodak XAR-5 film at −70°C.

RESULTS

Isolation of host cells. The HSV-1 *Eco*RI D fragment of strain KOS (16.5 kb; coordinates 0.086 to 0.194) contains a number of viral genes, several of which have been implicated in viral DNA synthesis (Fig. 1). Transcript mapping within the *Eco*RI D fragment revealed six mRNAs between coordinates 0.165 and 0.194, including the gene for alkaline nuclease (11, 13) (Fig. 1). *ts* mutants of KOS whose lesions map in the *Eco*RI D fragment include two DNA-positive mutants in complementation group 1-6 (*ts*F18 and *ts*F43, coordinates 0.095 to 0.108 and 0.112 to 0.118, respectively) and three mutants representing two complementation groups which were defective in viral DNA synthesis. These include *ts*K13 and *ts*M19 (complementation group 1-10), whose mutations mapped to coordinates 0.095 to 0.108, and *ts*S38 (complementation group 1-26), whose mutation mapped to coordinates 0.126 to 0.133 (Fig. 1) (8, 44, 53; Weller et al., in press).

To carry out a functional analysis of the *Eco*RI-D region, we constructed cell lines which contained the *Eco*RI D fragment. We anticipated that these cell lines could be used to complement both conditionally lethal and null mutations in this region since the wild-type protein would be provided by the cells in *trans*. Vero cells were cotransfected with plasmids pSV2neo and pSG10 (Fig. 1). Two weeks after transfection, G418-resistant colonies were isolated and tested at 39.9°C for the ability to complement the growth of the mutant *ts*S38, whose mutation had been mapped within *Eco*RI-D. *ts*S38 formed plaques at the nonpermissive temperature in one of the lines tested, S22 cells, but did not form plaques efficiently in Vero cells (Table 2). This result confirms that S22 cells are capable of expressing at least one of the viral proteins mapping within the *Eco*RI D fragment.

To confirm the presence of the *Eco*RI D fragment and to determine the approximate copy number, total cellular DNA isolated from S22 cells was analyzed by the method of Southern (46) (Fig. 2). After digestion with *Bam*HI and *Hin*dIII, S22 DNA was subjected to electrophoresis, transferred to a nylon membrane, and probed with the ^{32}P-labeled *Bam*HI-*Hin*dIII fragment (coordinates 0.165 to 0.186) obtained from pSG10-BD2 (Fig. 1). This fragment, which lay within *Eco*RI-D, contained a large portion of the coding region of the alkaline nuclease gene. The standards present

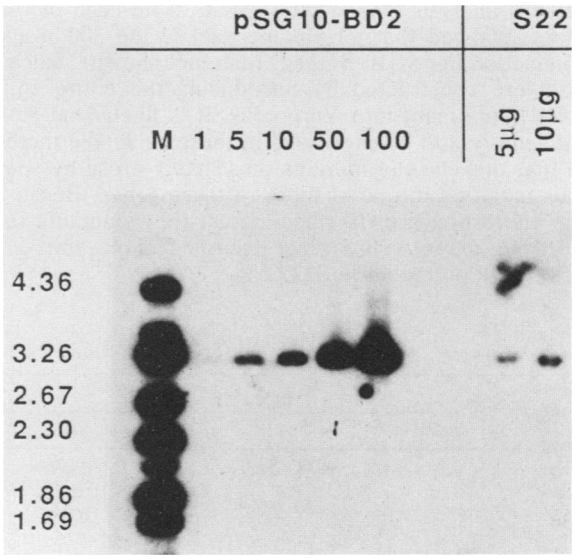

FIG. 2. Analysis of HSV-1 DNA in S22 cells. *Bam*HI-*Hin*dIII digests of S22 DNA (5 and 10 μg) were analyzed by the method of Southern (46). pSG10-BD2 was digested with the same enzymes to visualize 1 (10 pg), 5 (50 pg), 10 (100 pg), 50 (500 pg), and 100 (1 ng) copies of viral DNA per 3×10^9 bp of cellular DNA. The blot was probed with the ^{32}P-labeled *Bam*HI-*Hin*dIII fragment from pSG10-BD2 (Fig. 1). Numbers to the left indicate sizes of molecular weight markers (lane M).

TABLE 3. Marker rescue of *hr*27, *hr*48, and *hr*156

Plasmid[a]	Map coordinates[b]	Marker rescue efficiency[c]		
		*hr*27	*hr*48	*hr*156
pSG10	0.086–0.194	399	316	196
pSG10-BD1	0.103–0.145	<0.05	<0.05	<0.05
pSG10-P5	0.129–0.151	328	nd[d]	nd
pSG10-XII	0.145–0.155	40	1.0	1.0
pSG10-B2	0.145–0.165	2.0	nd	0.20
pSG10-P6	0.151–0.158	<0.05	nd	nd
pSG10-B3	0.165–0.194	<0.05	<0.05	<0.05
None		<0.05	<0.05	<0.05

[a] Plasmids were linearized before transfection.
[b] Coordinates are in the P orientation.
[c] Results are expressed as plating efficiencies, which were determined by the formula $[(PFU/ml_{Vero}/PFU/ml_{S22})] \times 10^3$. A value of <0.05 indicates no marker rescue occurred.
[d] nd, Not done.

on the gel (pSG10-BD2 digested with *Bam*HI and *Hin*dIII) indicated that S22 cells contained approximately five copies of the *Bam*HI-*Hin*dIII fragment per cell. Further Southern analysis of this DNA revealed that the cells contained DNA from approximately coordinates 0.118 to 0.194 (data not shown). In addition, the DNA appeared to be stably maintained in the cells for at least 25 passages (data not shown).

Isolation of host range mutants. Our original intention was to isolate deletion mutations in the gene for alkaline nuclease. To this end, S22 cells were cotransfected with wild-type KOS DNA and pSG10-BD2XH, which contains a deletion of 300 nucleotides in the alkaline nuclease gene (see Materials and Methods and Fig. 1). Three hundred plaques were isolated and tested for their ability to grow on S22 and Vero cells. Sixteen host range mutants were identified which formed plaques on S22 but not on Vero cells. Data from titrations of three of these mutants (*hr*27, *hr*48, and *hr*156) are shown in Table 2. Restriction enzyme digestion and Southern analysis (46) of viral DNAs from each of the 16 mutants showed that all mutants lacked the 300-base-pair (bp) deletion in pSG10-BD2XH (data not shown). Since S22 cells were constructed by introducing the entire 16.5-kb *Eco*RI D fragment into Vero cells, it is likely that several viral genes could be expressed in these cells. We therefore feel that this class of mutants most likely arose by spontaneous mutation in one or more of these genes. Because of their interesting growth phenotypes, three mutants (*hr*27, *hr*48, and *hr*156) were plaque-purified three times, and stocks were prepared on S22 cells.

Genetic analysis. (i) Marker rescue. To determine whether the lesions in *hr*27, *hr*48, and *hr*156 were in the nuclease gene or in another gene contained in S22 cells, marker rescue was performed with cloned DNA fragments from wild-type HSV-1 KOS DNA (Fig. 1 and Table 3). As expected, plasmid pSG10, which contains HSV-1 *Eco*RI-D, efficiently rescued the lesions in all three mutants. To map the mutations more finely, subclones of *Eco*RI-D were used in marker rescue tests. Plasmids pSG10-BD1, pSG10-B3 (which contains the entire nuclease gene), and pSG10-P6 did not rescue the mutations in *hr*27, *hr*48, and *hr*156; however, plasmids pSG10-XII and pSG10-B2 rescued the mutation(s) in all three mutants. Thus, we localized the mutations to a 1.5-kb fragment, at a position 1.5 to 3.0 kb to the left of the 3′ end of the alkaline nuclease gene (coordinates 0.145 to 0.155). In addition, the mutation in *hr*27 was more finely mapped with *Pst*I clones pSG10-P5 and pSG10-P6 (Fig. 1). Since plasmid pSG10-P5 rescued the lesion in *hr*27, its mutation was localized to an 800-bp fragment between coordinates 0.145 and 0.151.

(ii) Complementation studies. To determine the functional relationships between *hr*27, *hr*48, *hr*156 and other mutants whose mutations have been mapped to the *Eco*RI D fragment, complementation tests were conducted. Table 4 shows the results of quantitative complementation tests with the host range mutants and several *ts* mutants. *hr*27, *hr*48, and *hr*156 failed to complement each other for growth in Vero cells, indicating that they are members of the same complementation group. Mutants in complementation groups 1-6 (*ts*F18), 1-10 (*ts*K13), and 1-26 (*ts*S38) all complemented the growth of the host range mutants. Another member of complementation group 1-10 (*ts*M19) also complemented the mutants efficiently (data not shown). Furthermore, mutant *ts*J12, whose mutation mapped between coordinates 0.357 and 0.360, was able to complement the host range mutants. These results demonstrate that *hr*27, *hr*48, and *hr*156 belong to a complementation group distinct from the previously isolated *ts* mutants of strain KOS. We have designated this complementation group 1-36 following the nomenclature established by Schaffer et al. (45).

The lesions in three HSV-1 strain 17 mutants (*ts*R, *ts*S, and *ts*X) have been localized to the far-left-hand end of the long unique region between coordinates 0.145 and 0.167, to a position overlapping the map location of the host range mutations described in this study (30). In addition, these mutants were capable of plaque formation on S22 cells but not on Vero cells at the nonpermissive temperature (Table 2). These data led us to examine the functional relationships

TABLE 4. Complementation among 10 mutants of HSV-1

Mutant	CI[a]									
	*hr*27	*hr*48	*hr*156	*ts*R	*ts*S	*ts*X	*ts*K13	*ts*F18	*ts*S38	*ts*J12
*hr*27		**0.75**	**1.23**	**1.48**	**0.40**	**0.82**	15.8	48	64.1	69.3
*hr*48			**1.08**	nd[b]	nd	nd	nd	nd	nd	nd
*hr*156				**0.28**	**0.50**	**0.83**	16.0	52.3	55.1	24.8
*ts*R					**1.81**	**1.69**	27.6	49.2	82.8	195
*ts*S						**1.48**	2.7	68.3	nd	8.0
*ts*X							1.84	11.3	nd	13.2
*ts*K13								nd	200	94.0
*ts*F18									nd	nd
*ts*S38										132
*ts*J12										

[a] Numbers in boldface are considered negative for complementation.
[b] nd, Not done.

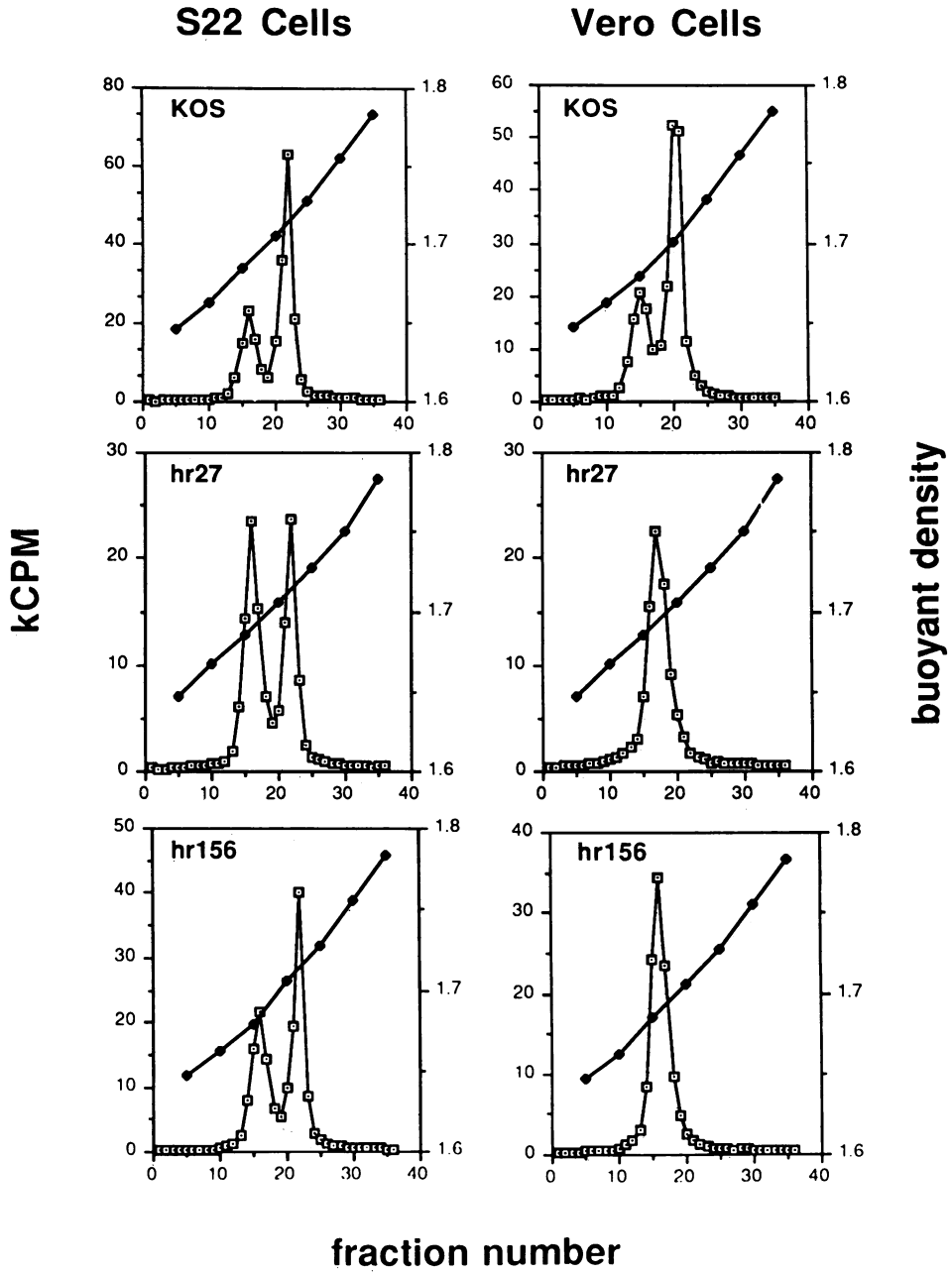

FIG. 3. Separation of viral and cellular DNAs by CsCl equilibrium centrifugation. Mutant- or wild-type-infected Vero or S22 cells incubated at 34°C were exposed to 10 μCi of [^{3}H]thymidine per ml from 6 to 24 h postinfection. Lysates of these cells were then subjected to equilibrium centrifugation in neutral CsCl gradients as described by Aron et al. (1). Cellular DNA corresponds to a peak with a buoyant density of 1.690, and viral DNA gives a peak with a buoyant density of 1.725.

between *ts*R, *ts*S, and *ts*X and the host range mutants. *hr*27, *hr*156, and *hr*48 failed to complement the strain 17 mutants (Table 4). We have observed that CIs obtained with *ts*X are reproducibly low; therefore, we do not feel that the 1.84 value obtained with *ts*X and *ts*K13 is significant. With this one exception, mutants in all other complementation groups did complement *ts*R, *ts*S, and *ts*X efficiently. We conclude from these data that *ts*R, *ts*S, and *ts*X are also members of complementation group 1-36.

Phenotypic analysis. (i) Synthesis of viral and cellular DNA by wild-type and host range mutants. To determine whether the defect in these mutants was at the level of DNA synthesis, the incorporation of [*methyl*-^{3}H]thymidine into viral DNA in mutant-infected Vero or S22 cells was measured. The three host range mutants were analyzed in this way, and data for two are shown in Fig. 3. Wild-type and mutant-infected cells were labeled and total DNA was harvested as described by Aron et al. (1). Cellular and viral DNAs were then resolved by equilibrium centrifugation in cesium chloride. Figure 3 shows the pattern of incorporation of [^{3}H]thymidine into viral and cellular DNA by the wild-type virus (KOS), *hr*27, and *hr*156 grown in Vero cells and in S22 cells. S22 and Vero cells infected with KOS and S22 cells infected with *hr*27 and *hr*156 were capable of inducing

TABLE 5. Viral DNA synthesis of wild-type and host range mutants in Vero and S22 cells[a]

Virus	% of wild-type DNA synthesis	
	Vero cells	S22 cells
*hr*27	0	40
*hr*48	0	32
*hr*156	0	56
KOS	100	100

[a] Values under viral peaks were determined, and results are expressed as the percentage of viral DNA in wild-type virus-infected cultures. Values represent the average of two separate experiments.

significant levels of viral DNA. However, in Vero cells infected with *hr*27 and *hr*156, no detectable viral DNA was synthesized. The average values for viral DNA synthesis with the three mutants and wild-type virus are shown in Table 5. Based on this observation, it appears that the lesion(s) in these mutants may be in a gene whose product is involved in viral DNA synthesis.

(ii) Viral protein synthesis at the permissive and nonpermissive conditions. Mutants with a block in viral DNA synthesis would be expected to be defective in induction of viral late (γ_1 and γ_2) polypeptides (10). Vero and S22 cells were infected with host range mutant or wild-type viruses at a multiplicity of 10 PFU/cell. The pattern of protein synthesis of S22 cells infected with *hr*27, *hr*48, and *hr*156 resembled that of cells infected with wild-type virus (Fig. 4, compare lanes 8, 9, and 10 with lanes 1 and 7). In contrast, *hr*27-, *hr*48-, and *hr*156-infected Vero cells (Fig. 4, lanes 3, 4, and 5) showed no detectable synthesis of true late (γ_2) polypeptides such as ICP15, ICP19/20, ICP33, and ICP43/44, and early-late (γ_1) polypeptides such as ICP5 and ICP25 were synthesized in greatly reduced amounts compared with the wild type. The synthesis of early polypeptides such as ICP8 and ICP36 was not affected in mutant-infected Vero cells, indicating that there was no gross defect in early gene expression. The electrophoretic profile seen in mutant-infected Vero cells was quite similar to that seen in cells infected with wild-type virus in the presence of the viral replication inhibitor phosphonoacetic acid (Fig. 4, lane 2). Thus, we conclude that although early gene expression is not affected in Vero cells infected with host range mutants, γ_1 and γ_2 proteins are decreased, consistent with a defect in DNA synthesis.

DISCUSSION

We report here the isolation and characterization of a new group of HSV-1 host range mutants defective in viral DNA synthesis. The isolation of these mutants was made possible by the availability of a permissive complementing cell line, S22. This cell line was isolated following transformation of Vero cells with a plasmid containing the *Eco*RI D fragment. Sixteen mutants whose growth was supported by S22 cells were isolated, and three mutants (*hr*27, *hr*48, and *hr*156) were selected for further study. These mutants defined a newly designated complementation group, 1-36, as shown by their ability to complement other mutants of strain KOS. The inability of these mutants to complement three DNA-negative mutants of strain 17, *ts*R, *ts*S, and *ts*X, indicates that all six were members of the same group. The lesions in the three host range mutants *hr*27, *hr*48, and *hr*156 were localized by marker rescue to a 1.5-kb fragment (coordinates 0.145 to 0.155) mapping to the left of the alkaline nuclease gene. Further fine mapping has demonstrated that the lesion in *hr*27 lies within an 800-bp fragment between coordinates 0.145 and 0.151. Recent sequencing data reveal the existence of an open reading frame of 94 kDa in this region (D. McGeoch, personal communication). The mutation in mutant *hr*27 mapped to a position entirely within this open reading frame, and the mutations in *hr*48 and *hr*156 were also consistent with their being in this gene (Fig. 5).

Mutants *hr*27, *hr*48, and *hr*156 were defective in viral DNA synthesis when grown in Vero cells. Furthermore, the pattern of viral protein synthesis induced by these mutants in Vero cells was consistent with their DNA-negative phenotype in that they were defective in the synthesis of late polypeptides while synthesis of early proteins appeared to be normal. These results suggest that the putative 94-kDa protein plays a role in viral DNA synthesis.

An alternative approach has recently been used to identify gene products required for amplification of HSV origin-containing plasmids (4). It has been known for some time that HSV origin-containing plasmids can be amplified in Vero cells if HSV functions are supplied in *trans* by either cotransfection with HSV DNA or superinfection with intact

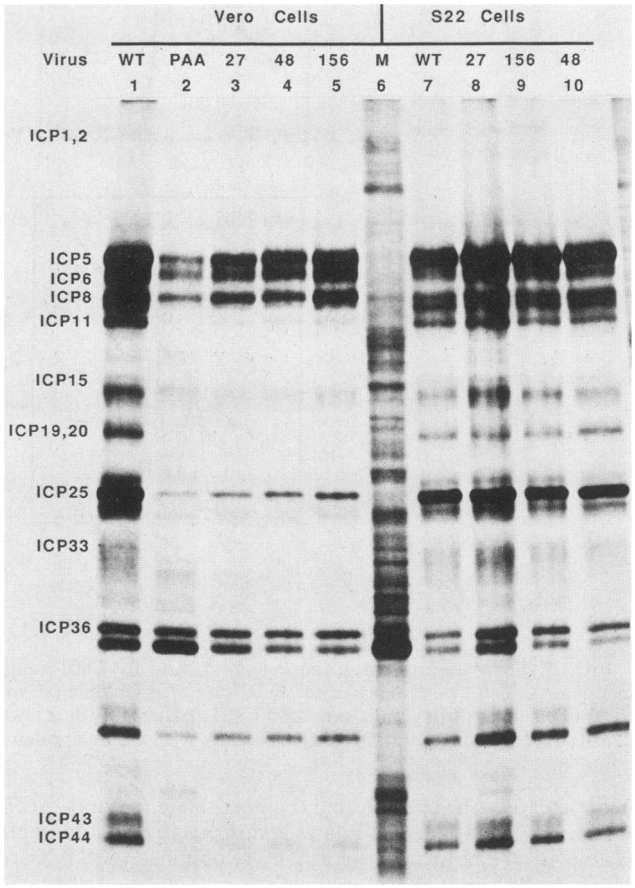

FIG. 4. Synthesis of viral polypeptides in wild-type- and mutant-infected cells. Monolayers of S22 and Vero cells were infected with wild-type (WT) or mutant viruses *hr*27, *hr*48, or *hr*156. [^{35}S]methionine was present during incubation from 5 to 18 h postinfection (see Results). At 18 h postinfection, total cell lysates were prepared for electrophoresis. The polypeptides were separated by electrophoresis through a 9% polyacrylamide gel. Lane 2 represents KOS-infected Vero cells in the presence of 150 μg of phosphonoacetic acid (PAA) per ml. Lane M, Mock-infected cells.

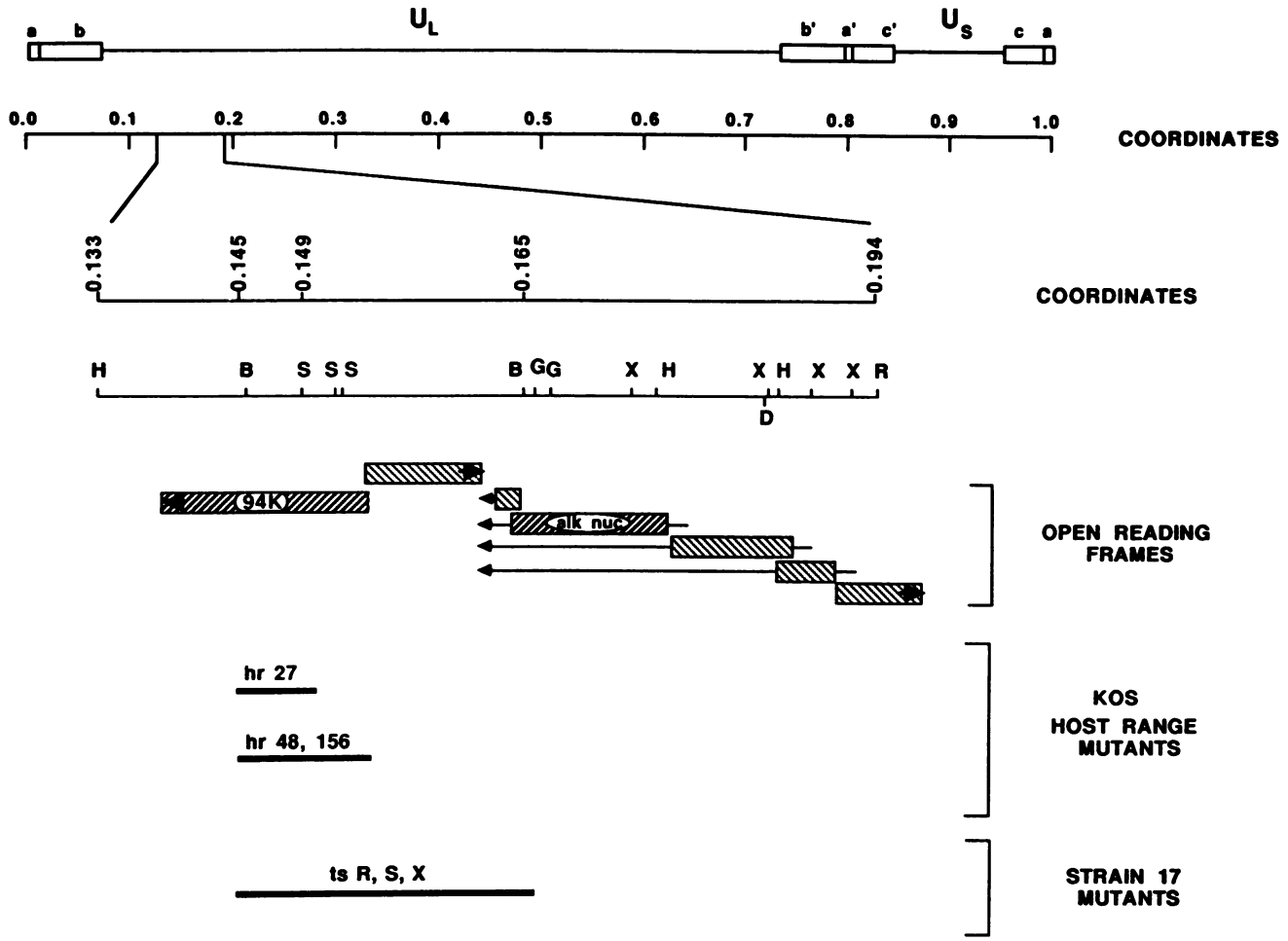

FIG. 5. Putative open reading frames in the right half of *Eco*RI-D. The *Hpa*I-*Eco*RI restriction fragment has been expanded to show restriction sites (see legend to Fig. 1). DNA sequence analysis (31; D. McGeoch, personal communication) revealed the presence of several open reading frames in this region. Shown below are the map locations of KOS host range mutants *hr*27, *hr*48, and *hr*156 (described in this paper) and strain 17 mutants *ts*R, *ts*S, and *ts*X (30). alk nuc, Alkaline nuclease gene; 94K, 94-kilodalton. Arrows indicate direction of transcript or open reading frame.

HSV virus (48, 49, 50, 55). Challberg (4) has found that certain combinations of HSV clones can be used to support origin-containing plasmid amplification. One clone that has been shown to be required in this system contains the gene for the 94-kDa protein (Challberg, personal communication). This observation is consistent with results described herein demonstrating that the 94-kDa protein gene product is essential for viral DNA synthesis in vivo.

Many putative DNA replication functions have been localized to the left end of the viral genome. In addition to alkaline nuclease (32, 33) and the gene for the 94-kDa protein, two other genes in this region have been implicated in DNA synthesis. Mutants *ts*K13 and *ts*M19 (complementation group 1-10), whose mutations map to the far left end of *Eco*RI-D (Fig. 1), failed to synthesize detectable levels of viral DNA at the nonpermissive temperature (Weller et al., in press). In addition, we and others (8; E. P. Carmichael and S. K. Weller, unpublished data) have shown that *ts*S38 (complementation group 1-26) produces small to moderate amounts of viral DNA at the nonpermissive temperature. However, this mutant has been shown to be leaky at 39.9°C. A null mutation in the gene which is defective in *ts*S38 will be required to elucidate whether this gene product is essential for DNA synthesis. It is anticipated that the use of host range mutants will greatly facilitate the analysis of genes whose products are essential for DNA synthesis.

At present, HSV is known to encode at least seven gene products involved in DNA synthesis and nucleotide metabolism. These include DNA polymerase (25, 41, 42), DNA-binding protein (10, 51, 54), thymidine kinase (14), ribonucleotide reductase (15, 40), alkaline nuclease (18, 32, 33, 38), a 54-kDa polymerase-associated protein (51), and a dUTPase (39). Other activities have been reported to be induced after HSV infection which may ultimately prove to be virus encoded. These include a protein which specifically binds an HSV origin of DNA synthesis, ori_S (16), topoisomerase (27, 34), uracyl DNA glycosylase (3), and dCMP deaminase (43). We might speculate that the virus encodes other activities essential for viral DNA synthesis, such as a primase and a DNA helicase. To identify the genes for these putative DNA synthetic functions and to determine their role in viral replication, additional mutants which are defective in viral DNA synthesis will be necessary. We feel that host range mutants such as those described in this paper will be especially powerful in the identification of DNA synthesis functions. To avoid problems resulting from spontaneous

mutations, cell lines can be constructed which contain only one viral gene. This will be facilitated by the availability of DNA sequence information.

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