

Role of Herpes Simplex Virus Type 1 UL46 and UL47 in α TIF-Mediated Transcriptional Induction: Characterization of Three Viral Deletion Mutants

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The transcriptional induction of the α or immediate-early gene class of herpes simplex virus type 1 effected by the α *trans*-induction factor (α TIF, ICP25, VP16, Vmw65) requires an α -specific *cis*-acting site. Increased transcription does not result from the direct, independent binding of α TIF, but rather from an α TIF-dependent formation of a protein-DNA complex containing, in addition to α TIF, at least one host cell factor. One of the host factors is a POU domain protein which recognizes an octamer element in the α -specific consensus. There is evidence that α TIF may drive the formation of multiple protein-DNA complexes containing a POU protein and additional host factors. Previously, the gene products of UL46 and UL47 have been implicated in modulating the α TIF-dependent transcriptional induction of α genes. Our current studies have extended these analyses from a transient-expression system to a series of viral deletion mutants. In these studies we demonstrate that neither UL46- nor UL47-encoded gene product, either separately or in combination, is required for viral growth in cell culture. The absence of UL47 reduces by up to 80% the ability of the virus to induce an α -regulated thymidine kinase reporter gene resident in 143TK⁻ cells. Autoradiograms of [³⁵S]methionine pulse-labeled infected cell proteins, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, show that deleting UL46 and/or UL47 has no discernable effect on the synthesis of α TIF or α TIF-containing proteins. Subsequent Western immunoblot analysis, with rabbit anti- α TIF antibodies made to an α TIF-*Staphylococcus aureus* protein A fusion, demonstrated that the accumulation and steady-state levels of α TIF or α TIF-containing proteins was indistinguishable from that of the thymidine kinase-negative isogenic parental virus, RA305.

The herpes simplex type 1 (HSV-1) genome contains a minimum of 70 open reading frames whose expression during lytic infection can be divided into three major temporal classes, the α (immediate-early), β (early), and γ (late) genes (19, 20; reviewed in reference 45). In addition to their temporal pattern, the α , β , and γ genes can be functionally classified. The five α genes encode regulatory proteins, whereas most of the β and γ genes encode viral DNA replication functions and structural proteins involved in virion assembly, respectively (reviewed in reference 45). The α genes are expressed first, in the absence of *de novo* protein synthesis, and are required for subsequent viral gene expression in productively infected cells (9, 19, 20, 45). The transcriptional induction of the α genes is effected by the α -*trans*-induction factor (α TIF, ICP25, VP16, Vmw65), a 64-kDa virion tegument component (4, 6, 17, 38). To effect transcriptional induction, α TIF requires a specific *cis* element (TAATGARAT) which is present in all α -class genes (22, 28, 29). Increased transcription does not result from the independent binding of α TIF, but rather from an α TIF-dependent formation of a protein-DNA complex containing, in addition to α TIF, at least one host factor (31, 32, 43). Analysis of functional domains of α TIF have demonstrated the presence of a binding domain, necessary for complex formation, which is separable from the COOH-terminal transactivation domain of the protein (8, 13, 52). Several host factors, including α H1 or Oct-1 (also referred to as OTF1 and NFIII) and Oct-2, have been identified which are

able to complex with α TIF (3, 12, 21, 23, 24, 32, 37, 44, 49). Complex formation appears to be mediated through the POU domain present in these proteins, which recognizes an octamer element contained within the α element (gyATGN TAATgaratctygtnggg) (3, 12, 18, 21, 24, 36). A recent report suggests that α TIF may drive the formation of multiple protein-DNA complexes containing a POU protein and additional host factors, one of which may be cell type specific (21).

Previous work has identified a role for at least two additional viral factors, encoded by HSV-1 UL46 and UL47, in α TIF-dependent transcriptional induction (33, 34). In transient-expression assays the gene product of UL46 was able to enhance α TIF-mediated induction of an α TK reporter construct 2- to 3-fold, while the gene product of UL47 acted to reduce the levels of α TIF-mediated induction 2.5- to 3-fold in the presence or absence of UL46. These results were the first report of additional viral factors involved in α -gene induction, and they predicted the presence of a regulatory gene cluster composed of UL46, UL47, and α TIF (UL48) (33, 34).

The goals of our current study were to investigate the roles of UL46 and UL47 in the context of the virus to determine whether they are essential for viral growth in cell culture, whether their gene products acted in a fashion consistent with previous observations with a transient-expression system, and to begin to determine the levels of their interaction with α TIF. To this end, we constructed a series of deletion mutants lacking either UL46, UL47, or both. In this paper we demonstrate that neither UL46 or UL47, either separately or in combination, is required for growth in cell

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culture. We also report on the differences observed between the viral and the transient-expression systems regarding the effect of these genes on α TIF-specific transcriptional induction. Finally, we demonstrate that although UL46 and UL47 do not affect either the synthesis or steady-state levels of α TIF, the UL47 deletion results in the loss of the 64-kDa α TIF and the concomitant expression of a novel protein of approximately 85 kDa, which is recognized by anti- α TIF antibodies and appears to retain α TIF-specific induction activity.

MATERIALS AND METHODS

Mutant viruses. All mutant viruses described in these studies were derived from HSV-1 (F), whose properties and propagation have been described elsewhere (10). *ts502* Δ 305 contains the Δ 305 thymidine kinase (TK) deletion in addition to a temperature-sensitive (*ts*) lesion in the α 4 gene (41). R Δ 305, a TK⁻ mutant, was used as the parental strain for the construction of the mutants depicted in Fig. 1B (42). R3622 and R3787 were constructed by the cotransfection of 1 to 2 μ g of purified cytoplasmic R Δ 305 viral DNA with 1 μ g of the corresponding purified linearized plasmid DNA, pRB3622 or pRB3787 (Fig. 1B). All of the above were gifts from B. Roizman. Similarly, RUL46 Δ 3, RUL47 Δ 2, and RUL46/47 Δ 2 were constructed by the cotransfection of 1 to 2 μ g of purified cytoplasmic R3787, R3622, and R3787 viral DNAs with 1 μ g of the corresponding purified linearized plasmid DNA; pUL46 Δ 3, pUL47 Δ 2, or pUL46/47 Δ 2, respectively (Fig. 1C to E). Viral DNA was purified as previously described (42), with the following modification: following proteinase K digestion, the cytoplasmic fraction was extracted once with an equal volume of phenol, twice with an equal volume of phenol-chloroform (1:1), three times with an equal volume of chloroform, and two or three times with an equal volume of ether. DNA was then precipitated with ethanol and resuspended in water. The plasmid DNA used in the cotransfection was purified by either cesium chloride gradient centrifugation or by fast protein liquid chromatography (FPLC) over an HR16/50 column packed with Superose-12 (preparative grade) by following the manufacturer's suggested protocol (Pharmacia-LKB) (30). Viral and plasmid DNAs were cotransfected into 25-cm² flasks containing approximately 2×10^6 143 TK⁻ cells by using DEAE-dextran as previously described (42). R3622 and R3787 were subjected to three rounds of plaque purification in the presence of hypoxanthine-aminopterin-thymidine (HAT) medium, and their purity was confirmed by Southern blot analysis (Fig. 2 and 3). Each isolate of RUL46 Δ 3, RUL47 Δ 2, and RUL46/47 Δ 2 was subjected to three rounds of plaque purification in the presence of 5-bromo-2'-deoxyuridine (10 μ g/ml), and their purity was confirmed by Southern blot analysis (Fig. 2 and 3). Viral stocks were prepared as described previously (46).

Cell lines. African green monkey kidney (Vero) cells, Syrian baby hamster (BHK-21) cells, human epidermal carcinoma (HEp-2) cells, and human lung carcinoma (A549) cells were obtained from the American Type Culture Collection, Rockville, Md. and maintained in Dulbecco modified Eagle medium (DMEM; GIBCO) containing 10% newborn calf serum (GIBCO) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. TK-deficient human osteosarcoma (143 TK⁻) cells and TK⁻ baby hamster kidney (BHK TK⁻) cells were obtained from the Mutant Cell Repository, Camden, N.J. Both the 143 TK⁻ and BHK TK⁻ cells were maintained as above, except that 2% fetal calf

TABLE 1. Plasmids used in these studies

Plasmid	Description	Reference
pSV2 <i>neo</i>	SV40 ^a promoter-driven neomycin-G418 resistance shuttle vector	50
pRB20	0.8-kb <i>Bam</i> HI- <i>Bg</i> II fragment of pRB103 (TK promoter) replacing the SV40 promoter in pSV2 <i>neo</i>	
pRB103	<i>Bam</i> HI-Q in pBR322	40
pRB158	<i>Bam</i> HI-F in pUC9	38
pRB201	<i>Hind</i> III-D in pBR322	53
pRB3092	α 27-regulated TK chimera in pUC8	22
pRB3193	Insertion of the α 27-regulated TK from pRB3092 into the <i>Hind</i> III site of pRB20	
pRB3367	1.8-kb α TK in pUC8	26
pRB3443	6.6-kb <i>Bam</i> HI- <i>Xho</i> I fragment of <i>Bam</i> HI-F into pUC9	34
pRB3458	2.9-kb <i>Pst</i> I- <i>Xho</i> I fragment of <i>Bam</i> HI-F into pUC9	38
pRB3621	Insertion of the α TK of pRB3367 into the <i>Sma</i> I site of pUC18	
pRB3622	Insertion of the 1.8-kb α 27TK from pRB3367 into the <i>Asu</i> II site of pRB3458	
pRB3708	<i>Spe</i> I linker inserted into the <i>Sca</i> I site of pRB3724	34
pRB3715	6.6-kb <i>Xho</i> I- <i>Bam</i> HI fragment of <i>Bam</i> HI-F fused in the proper orientation to <i>Bam</i> HI-I' in pUC9	
pRB3787	Insertion of the 1.8-kb α TK from pRB3621 into the <i>Spe</i> I site of pRB3708	
pUL46 Δ 3	1-kb <i>Bal</i> 31 deletion flanking the <i>Spe</i> I site in pRB3708 (see Fig. 1C)	
pUL47 Δ 2	<i>Asu</i> II- <i>Not</i> I collapse of pRB3443 (see Fig. 1D)	
pUL46/47 Δ 2	<i>Asu</i> II- <i>Nco</i> I collapse of pRB3715 (see Fig. 1E)	
pTIFA-1	1,217-bp <i>Sal</i> I fragment of α TIF in pRIT2T	
pRIT2T	<i>S. aureus</i> protein A fusion expression vector	35

^a SV40, Simian virus 40.

serum (FCS; GIBCO) and 8% Serum Plus (S+; Hazelton) was used in lieu of the 10% newborn calf serum. The I3193 transfectant cell line was constructed by the transfection of approximately 10^7 143 TK⁻ cells with 60 μ g of linearized pRB3193, followed by selection in the presence of 400 μ g of geneticin (G418; GIBCO) per ml as specified by a previously described protocol (50). The I3193 cells were maintained as above, only in 10% FCS and 200 μ g of G418 per ml.

Plasmids. A summary of all plasmids used in these studies is given in Table 1. All plasmids with the prefix pRB were the gift of B. Roizman. pRB3193 was constructed by replacing the simian virus 40 promoter in pSV2*neo* with the *Bam*HI-*Bg*II fragment of pRB103, which contains the promoter-regulatory region of the HSV-1 *tk* gene, to give pRB20. This was followed by the insertion of an α -regulated TK (α TK) construct, excised from pRB3092 by using *Eco*RI and *Hind*III, into the *Hind*III site of pRB20. pRB3622 was constructed by the insertion of the α TK chimera from pRB3367 into the *Asu*II site of pRB3458. pRB3787 was constructed by inserting the α TK chimera from pRB3621 into the *Spe*I site of pRB3708. The orientations of the α TK insertions relative to UL46 and UL47 are shown in Fig. 1B. pUL46 Δ 3 was constructed by *Bal*31 digestion of *Spe*I-digested pRB3708.

The fine mapping of this plasmid is shown in Fig. 4. pUL47Δ2 is an *AsuII-NorI* collapse of pRB3443. pUL46/47Δ2 is an *AsuII-NcoI* collapse of pRB3715. pRB3715 is the 6.7-kb *XhoI-BamHI* fragment of *BamHI-F* fused in the proper orientation to the *BamHI* I' fragment of HSV-1. pTIFA-1 was constructed by insertion of the 1,217-bp *Sall* fragment (nucleotides 850 to 2104 in the numbering of Pellett et al. [38]) of αTIF into the *Sall* site of pRIT2T (Promega), resulting in an in-frame fusion between αTIF and *Staphylococcus aureus* protein A.

Southern blot analysis. Southern blots were carried out by the procedure described by Chen et al. (7) with the following modification: prior to prehybridization, membranes were subjected to a 1-h wash in 2× SSPE (0.3 M NaCl, 23 mM NaH₂PO₄, 2 mM EDTA [pH 7.4]) at 65 to 68°C to reduce nonspecific binding.

Virus infections. For infection of I3193 cells, six-well dishes were seeded at a density of 1×10^5 to 2×10^5 cells per well, and the cells were grown overnight at 37°C in DMEM containing 10% FCS and antibiotics. At densities of 2×10^5 to 5×10^5 cells per well, the I3193 cells were infected with 1 to 50 PFU of the indicated virus per cell at 8°C. The virus was allowed to adsorb for 60 min, washed twice with cold phosphate-buffered saline (PBS), overlaid with 1 ml of medium 199 (Hazleton) containing 1% FCS, and shifted to 37°C for 1 h before addition of an additional 1 ml of medium 199 containing (in addition to 1% FCS) 0.1% pooled human immunoglobulin G (IgG) (Calbiochem). Wells were harvested at the indicated time for TK assays. Mock-infected controls were run in parallel. Experiments involving *ts502Δ305* were carried out similarly, except that cells were seeded into 25-cm² flasks and infected with 5 PFU of virus per cell and parallel dishes were shifted to either 34 or 39°C. Human IgG was not used in these experiments. Plaque assays were performed on confluent monolayers of the indicated cells, seeded in six-well dishes. Duplicate wells were infected with serial dilutions of the indicated viral stocks into medium 199 containing 1% FCS, the viruses were allowed to adsorb for 1 h at room temperature, and then the cells were aspirated, overlaid with 2 ml of medium 199 containing 1% FCS and 0.1% pooled human IgG, and shifted to 37°C. The cells were washed twice with PBS, fixed for 1 to 5 min in methanol, and stained with either Giemsa or gentian violet.

Pulse-labeling of infected cells. Flasks (25 cm²) containing a confluent layer of Vero cells were infected with 5 PFU of the indicated virus stock per cell, under the conditions described above for the plaque assays. At 1 h before labeling, medium was aspirated and replaced with methionine-deficient DMEM (DMEM – Met; Sigma), and the flasks were returned to 37°C. The DMEM – Met was then replaced with 1 ml of DMEM – Met containing 2 μCi of [³⁵S]Met (NEN), and the flasks were returned to 37°C for 30 min. The Vero cell monolayer was removed from the dishes by scraping, washed twice in cold PBS, overlaid with 300 μl of solubilization buffer, and sonicated, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (5). For the 0.5-h time point in Fig. 9, infections were carried out in DMEM – Met and labeling was performed immediately after adsorption.

TK assays. TK assays were performed as previously described (22).

Antibodies. Competent N4830 bacterial cells (Pharmacia-LKB) were transformed with either pTIFA-1 or the protein A-expressing vector, pRIT2T, and bacteria containing these constructs were grown at 25, 37, and 42°C (30). Protein

extracts were prepared and screened for expression of either the αTIF-protein A fusion or protein A by IgG Sepharose-6 FF affinity purification, by following the protocol supplied by the manufacturer (Pharmacia-LKB). Optimal expression of pTIFA-1 occurred at 37°C as determined by SDS-PAGE separation of the affinity-purified proteins on 11% acrylamide gels (28% acrylamide, 0.73% bisacrylamide) (25). One-liter volumes of bacteria containing either pTIFA-1 or pRIT2T were grown to the stationary phase overnight at 37°C, and extracts were prepared and purified by FPLC. Briefly, bacteria were pelleted at $10,000 \times g$ for 10 min, resuspended in 10 ml of TST buffer (50 mM Tris hydrochloride [pH 7.6], 150 mM NaCl, 0.05% Tween-20), and sonicated for 5 min by using a Branson sonifier equipped with a microtip. The sonicated extract was centrifuged for 10 min at $12,000 \times g$, and the supernatant was diluted to a final volume of 50 ml with TST buffer. The supernatant was cycled over an HR5/10 column packed with IgG Sepharose-6 FF (equilibrated as recommended by the manufacturer) at approximately 1 ml/min by using a peristaltic pump. The column was washed and bound protein was eluted as recommended by the manufacturer. Fractions containing either protein A or the αTIF-protein A fusion were analyzed by SDS-PAGE. At weeks 0, 3, 5, 7, 9, 11, and 14, two New Zealand White rabbits were immunized with 1 ml of the RIBI adjuvant system (RIBI ImmunoChem Research) containing 120 μg of the αTIF-protein A fusion protein (0.3 ml intradermal [back and neck], 0.4 ml intramuscular [thigh], and 0.2 ml [peritoneal]); every 4 to 6 weeks thereafter, they were given maintenance immunizations consisting of 1 ml of RIBI adjuvant system containing 25 μg of acid-hydrolyzed (70% formic acid at 25°C for 60 h [39]) αTIF-protein A fusion protein injected at the same sites. Rabbit serum was screened by Western immunoblot analysis of HSV-1-infected cell extracts starting 3 months postimmunization.

Western blot analysis. Western blot analysis was carried out as previously described (5).

RESULTS

The gene products of UL46 or UL47 either separately or in combination are not essential for viral growth in cell culture. To determine whether the gene products of UL46 and UL47 were required for viral growth in cell culture, we constructed five mutant viruses (see Materials and Methods) (Fig. 1). R3622 and R3787 resulted from the homologous recombination of pRB3622 and pRB3787 with Δ305 DNA to provide a TK⁺ parental virus for the subsequent deletion of UL46 and UL47 genes. The locations and orientation of the TK insertion are summarized in Fig. 1, and the purity of these mutants was confirmed in the Southern blot analyses shown in the upper panels of Fig. 2 and 3. Both were probed with the HSV-1 *BamHI* F fragment in pUC9. The insertion of the 1.8-kb αTK fragment into the *AsuII* site results in a shift of the 8.1-kb *BamHI* F fragment of Δ305 to 9.9 kb in R3622 (Fig. 2, upper panel, lane 5), which is indistinguishable from the modified *BamHI* F fragment observed in the corresponding plasmid, pRB3622 (Fig. 2, upper panel, lane 4). The insertion of a 1.8-kb αTK fragment, containing a 3' *BamHI* site, into the *SpeI* site (modified from the original *Scal* site [34]) results in two modified *BamHI* F fragments. The first extends from the *BamHI* site on the right end of the *BamHI* F fragment to the *SpeI* site and includes the 1.8-kb TK fragment, resulting in a fragment of 8.3 kb. The second fragment extends from the *SpeI* site to the left end of *BamHI*, giving a second fragment, of 1.6 kb (Fig. 3, upper

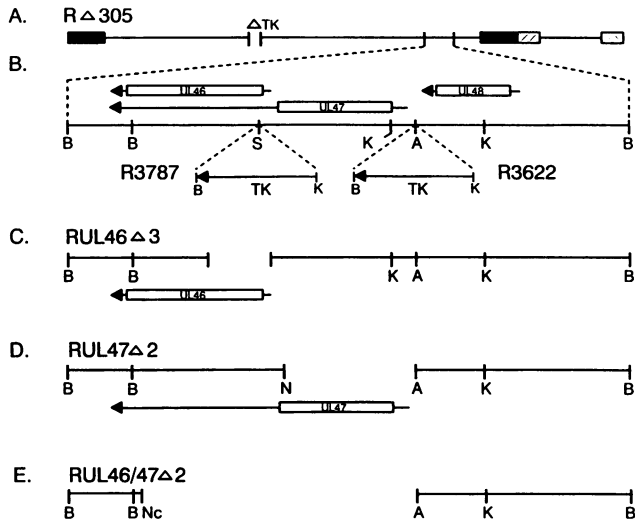


FIG. 1. Schematic representation of the HSV-1 (F) genome showing the regions relevant to this study. (A) Location of the TK deletion (Δ TK) and locations of UL46, UL47, and UL48 (α TIF) within the *Bam*HI F and I' regions of the viral genome in R Δ 305. The solid lines flanked by the filled rectangles and the solid line flanked by the hatched rectangles represent the unique and repeated regions of the long and short components of the viral genome, respectively. (B to E) An expansion of the *Bam*HI F and I' regions of the viral genome. Panel B shows the locations of UL46, UL47, and UL48, the site of the α TK insertions in R3787 and R3622, and relevant restriction sites. The arrows indicate the transcriptional orientation and location of the mRNAs relative to the HSV-1 genome prototype orientation, and the rectangles represent the open reading frames of UL46, UL47, and UL48. Panels C to E show the locations of the UL46, UL47, and UL46/47 deletions in RUL46 Δ 3, RUL47 Δ 2, and RUL46/47 Δ 2, respectively. Abbreviations: A, *Asu*II; B, *Bam*HI; K, *Kpn*I; N, *Not*I; Nc, *Nco*I; S, *Spe*I.

panel, lane 7), which is indistinguishable from the 1.6-kb fragment in pRB3787 (note that since pRB3787 was constructed from pRB3708, it does not contain the complete *Bam*HI F fragment of R Δ 305). The 8.1-kb *Bam*HI F fragment of R Δ 305 is shown in Fig. 2 and 3, lanes 1 and 3, respectively.

RUL46 Δ 3 contains a 1.0 kb deletion extending from immediately 5' of the *Pvu*I site at nucleotide 3584 to immediately 3' of the *Sac*I site at nucleotide 2610 (according to the nucleotide designations of McKnight et al. [34]) (Fig. 1C and 4B). Since RUL46 Δ 3 resulted from the homologous recombination between pUL46 Δ 3 plasmid and R3787 viral DNA, and since the deletion was the result of a limited *Bal*31 digest, pUL46 Δ 3 DNA sequences were fine-mapped with a number of restriction enzymes to determine the extent of the deletion. The results of the restriction mapping are shown in Fig. 4A and can be compared with the corresponding map of this region depicted in Fig. 4B. Note, in particular, the loss of the 788-bp *Pvu*I and 1,544-bp *Sma*I fragments in lanes 4 and 11 in pUL46 Δ 3 when compared with lanes 3 and 10, respectively, containing the intact construct, pRB3708. In addition to the loss of these fragments are the shifts to lower molecular weights of the 1,587-bp *Pvu*II-*Mlu*I (approximately 1 kb) and the 5.6-kb *Sac*I fragments in lanes 6 and 9 when compared with lanes 5 and 8, respectively. Two mutants were independently isolated and were designated RUL46 Δ 3.1 and RUL46 Δ 3.2. Southern blot analysis of the RUL46 Δ 3 viral DNAs to characterize the recombination of

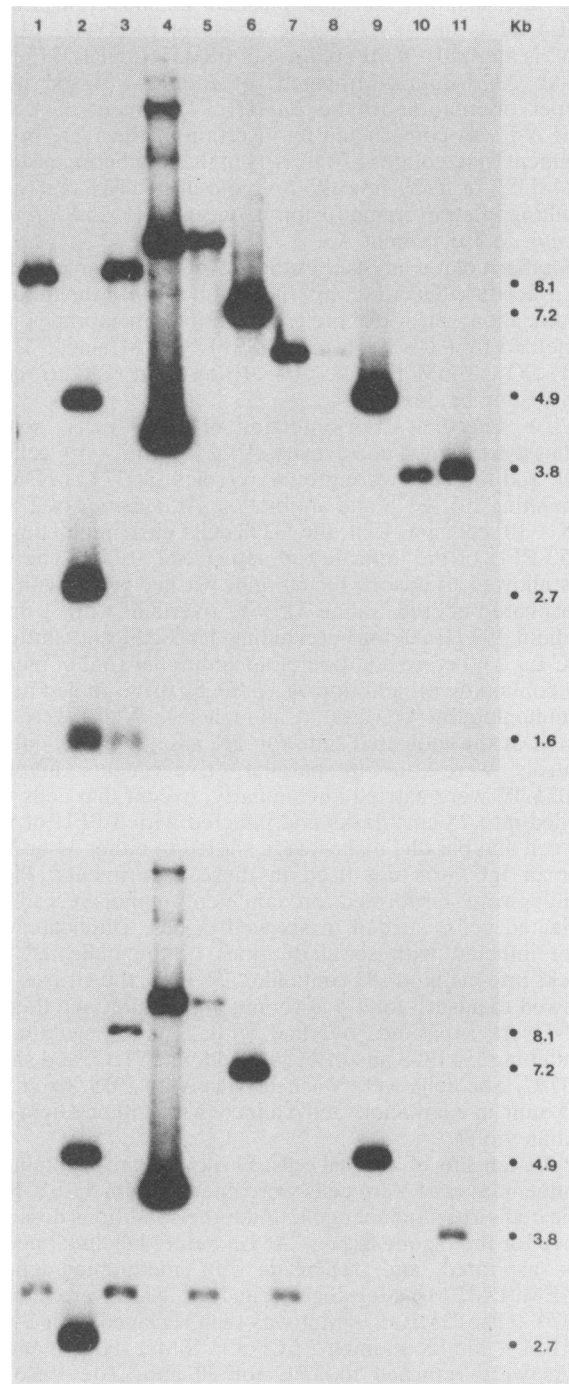


FIG. 2. Southern blot analysis of RUL47 Δ 2.1, RUL47 Δ 2.2, and RUL46/47 Δ 2. In the upper panel, 5 ng of plasmid or 0.2 μ g of viral DNA (except for lane 8, which contains 0.02 μ g) was digested overnight with *Bam*HI (except marker DNA in lane 11), electrophoresed through a 0.8% agarose gel, transferred to GeneScreen Plus, and hybridized to the *Bam*HI F fragment of HSV-1 (pRB158). Lane 11 contains pRB158 marker DNA, digested with *Sal*I. The fragment sizes are indicated in kilobases. Lanes 1 to 10 contain R Δ 305, pRB3787, R3787, pRB3622, R3622, pUL47 Δ 2, RUL47 Δ 2.1, RUL47 Δ 2.2, pUL46/47 Δ 2, and RUL46/47 Δ 2, respectively, digested with *Bam*HI. In the lower panel, the membrane in the upper panel was stripped and re-probed with the 1.5-kb *Bgl*III-*Nco*I fragment of TK in pUC9. Lanes are as indicated for the upper panel.

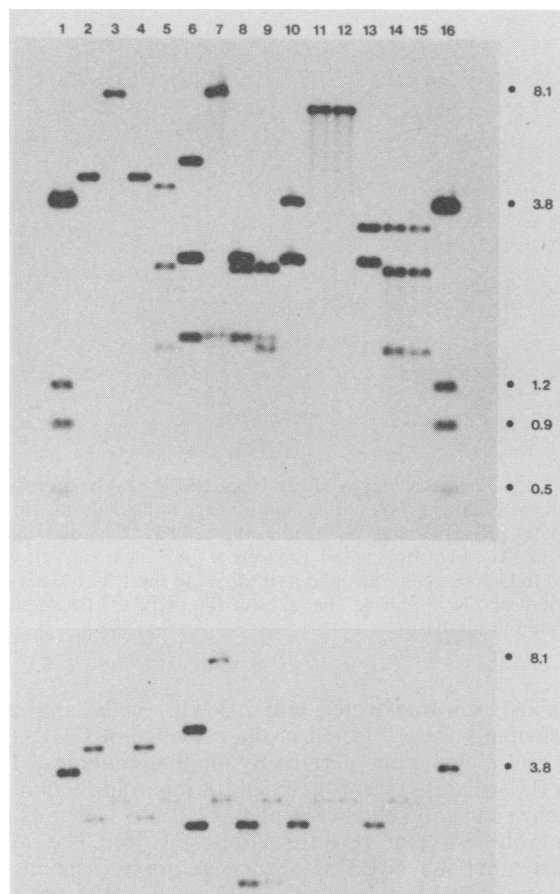


FIG. 3. Southern blot analysis of RUL46 Δ 3.1 and RUL46 Δ 3.2. In the upper panel, 5 ng of plasmid or 0.2 μ g of viral DNA was digested overnight with restriction endonucleases, electrophoresed through a 0.8% agarose gel, transferred to GeneScreen Plus, and hybridized to the *Bam*HI F fragment of HSV-1 (pRB158). Lanes 1 and 16, pRB158 marker DNA, digested with *Sall*; lanes 2 and 3, pRB Δ 305 and R Δ 305, respectively, digested with *Bam*HI; lanes 4 and 5, pRB Δ 305 and R Δ 305, respectively, digested with *Bam*HI and *Kpn*I; lanes 6 and 7, pRB3787 and R3787, respectively, digested with *Bam*HI; lanes 8 and 9, pRB3787 and R3787, respectively, digested with *Bam*HI and *Kpn*I; lanes 10, 11, and 12, pUL46 Δ 3, RUL46 Δ 3.1, and RUL46 Δ 3.2, respectively, digested with *Bam*HI; lanes 13, 14, and 15, pUL46 Δ 3, RUL46 Δ 3.1, and RUL46 Δ 3.2, respectively, digested with *Bam*HI and *Kpn*I. The fragment sizes are indicated in kilobases. In the lower panel, the membrane in the upper panel was stripped and reprobed with the 1.5-kb *Bgl*II-*Nco*I fragment of TK in pUC9. Lanes are as indicated for the upper panel.

pUL46 Δ 3 into R3787 and to confirm the purity of the mutant viruses is shown in Fig. 3. The upper panel was probed with the *Bam*HI F fragment in pUC9. Note first the decrease in size of the R Δ 305 *Bam*HI F fragment from 8.1 to 7.1 kb in both RUL46 Δ 3.1 and RUL46 Δ 3.2 (lanes 3, 11, and 12, respectively) and second the decrease in size of the R Δ 305 *Bam*HI-*Kpn*I fragment from 4.0 to 3.0 kb in both RUL46 Δ 3 mutants (lanes 5, 14 and 15, respectively). In addition, the 3.0-kb bands in RUL46 Δ 3 comigrate with those of the plasmid pUL46 Δ 3 (lanes 13, 14, and 15). The bottom panel of Fig. 3 shows the same membrane probed with the 1.5-kb *Bgl*II-*Nco*I fragment of TK in pUC9 and confirms the presence of the Δ 305 TK deletion in RUL46 Δ 3.1 and

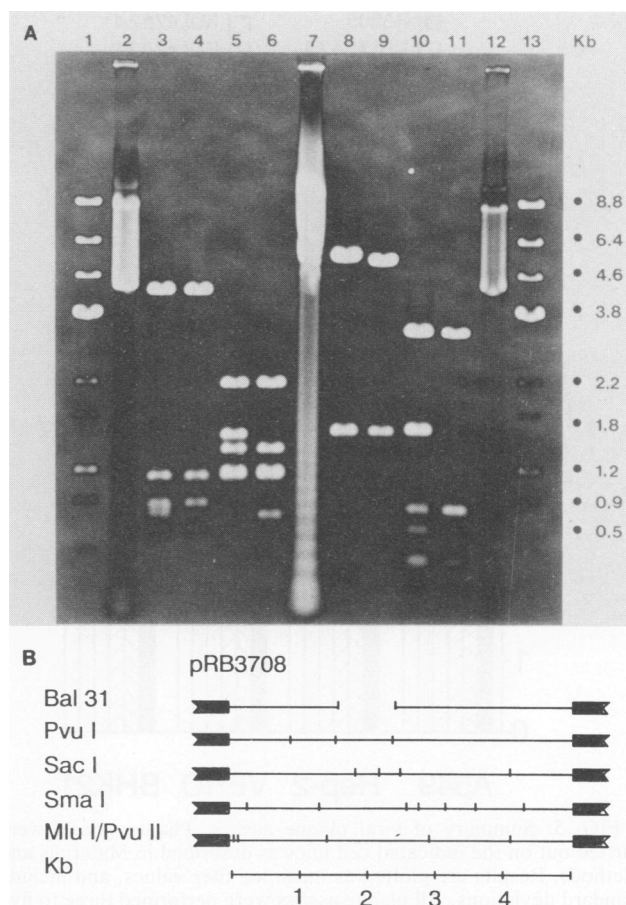


FIG. 4. Fine mapping of the UL46 deletion. (A) A 1- μ g portion of pRB3708 and pUL46 Δ 3 plasmid DNA was digested overnight with restriction endonucleases, electrophoresed through a 0.7% agarose gel, and stained with ethidium bromide. Lanes 1 and 13, pRB201 and pRB158 marker DNA digested with *Bam*HI and *Sall*, respectively; lanes 2, 7, and 12; 123-bp λ phage-derived marker DNA ladder (Bethesda Research Laboratories); lanes 3 and 4, pRB3708 and pUL46 Δ 3, respectively, digested with *Pvu*I; lanes 5 and 6, pRB3706 and pUL46 Δ 3, respectively, digested with *Pvu*II and *Mlu*I; lanes 8 and 9, pRB3708 and pUL46 Δ 3, respectively, digested with *Sac*I; lanes 10 and 11, pRB3708 and pUL46 Δ 3, respectively, digested with *Sma*I. The sizes of the fragments are indicated in kilobases. (B) Schematic representation of pRB3708 showing the location of the restriction sites used for the fine mapping of pUL46 Δ 3. The location of the UL46 deletion relative to these sites is indicated.

RUL46 Δ 3.2 (lanes 11, 12, 14, and 15) when compared with R Δ 305 (lanes 3 and 5). Lanes 2 and 4 contain a *Bam*HI Q deletion (*Bgl*II-*Sac*I) about 200 bp larger than in Δ 305.

RUL47 Δ 2 and RUL46/47 Δ 2 resulted from the homologous recombination of pUL47 Δ 2 and pUL46/47 Δ 2 into R3622 and R3787, respectively (Fig. 1D and E). Two mutants were independently isolated from the pUL47 Δ 2-R3622 cotransfections and were designated RUL47 Δ 2.1 and RUL47 Δ 2.2. The Southern blot analysis of these mutants is shown in Fig. 2. The upper panel was probed with the *Bam*HI F fragment in pUC9. Lanes 7 and 8 show the novel 6.0-kb *Bam*HI F fragment resulting from the *Asu*II-*Not*I collapse (compare with lane 1) in RUL47 Δ 2.1 and RUL47 Δ 2.2, respectively. Lane 10 shows the novel 3.8-kb *Bam*HI F fragment resulting from the *Asu*II-*Nco*I collapse (compare with lane 1) in

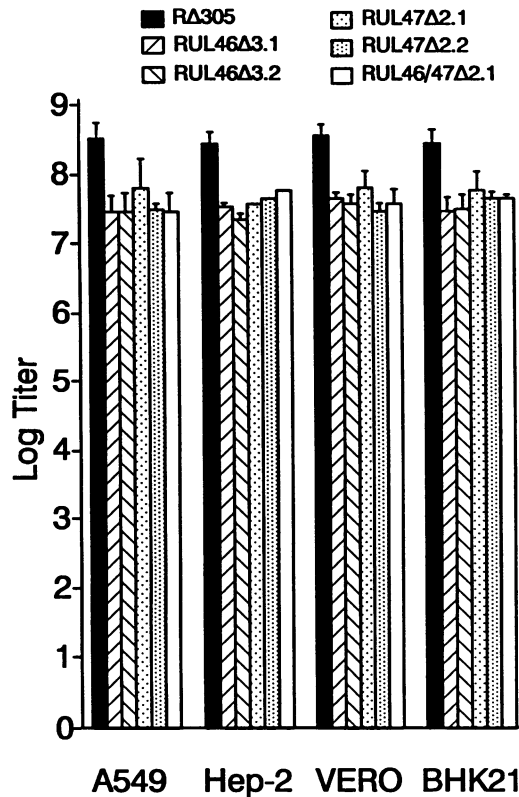


FIG. 5. Summary of viral plaque assays. Plaque assays were carried out on the indicated cell lines as described in Materials and Methods. Results are plotted as mean log titer values, and include standard deviations. All plaque assays were performed three to five times, in duplicate, with the exception of the assays of the UL47 and UL46/47 deletion mutants on HEp-2 cells, which were carried out in duplicate only.

RUL46/47Δ2. The bottom panel of Fig. 2 shows the same membrane as the upper panel, and, similarly to Fig. 3, was probed with the 1.5-kb *Bgl*III-*Nco*I fragment of TK in pUC9; this confirms the presence of the Δ305 deletion in all mutant viruses used in these studies (compare lanes 1, 3, 5, 7, 8, and 10).

The UL46Δ3, UL47Δ2, and UL46/47Δ2 mutant viruses, being isogenic derivatives of the TK⁻ parental strain, RΔ305, were then compared with RΔ305 for their ability to form plaques in human-, monkey-, and hamster-derived cell lines. The results of the plaque assays (detailed in Materials and Methods) are summarized in Fig. 5 and are as follows. First, neither UL46 nor UL47, either separately or in combination, is required for viral growth in cell culture. Second, there do not appear to be any host range restrictions in the cell lines tested. Third, all mutants formed plaques to within a single log of RΔ305.

The gene products of UL46 and UL47 differ in their ability to modulate αTIF-dependent induction of an αTK reporter gene resident in I3193 cells. Previous work has suggested a role for UL46 and UL47 in the modulation of the αTIF-dependent induction of αTK reporter genes in transient-expression assays (33, 34). To further characterize the roles of the two putative modulators within the context of the virus, 143 TK⁻ cells were stably transformed with a previously characterized αTK reporter gene (22) and assayed for αTIF-mediated induction. pRB3193 (see Materials and

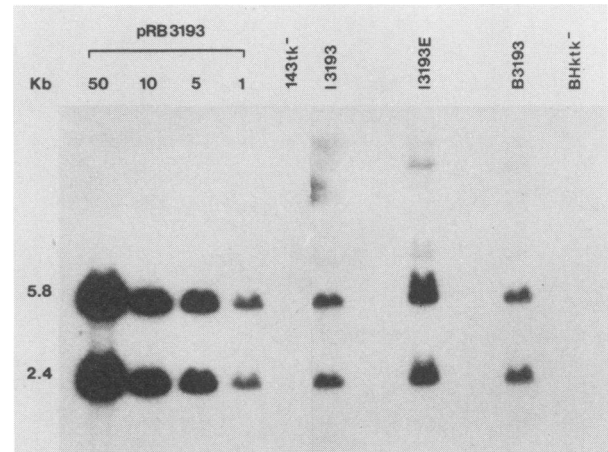


FIG. 6. Southern blot analysis of pRB3193 stably transformed cell lines. Cellular DNA (20 μg) and, where indicated, 50, 10, 5, or 1 copy of pRB3193 were included in the cellular DNA and digested with *Eco*RI, electrophoresed through a 0.8% agarose gel, transferred to GeneScreen Plus, and hybridized to the 1.5-kb *Bgl*III-*Nco*I fragment of TK in pUC9. The sizes of the pRB3193 fragments are indicated in kilobases.

Methods) was transfected into 143 TK⁻ cells, and stable transfectants were selected in the presence of G418. I3193 cells and a clonal line derived by limiting dilution of I3193 cells (I3193E cells) were the result of this transfection. The presence and copy number of pRB3193 DNA were analyzed in a Southern blot reconstruction (Fig. 6). The results indicate that the pRB3193 sequences are present in both I3193 and I3193E cells (lanes I3193 and I3193E) and range from three to five copies per cell. Little difference was apparent between the mixed and clonal cell lines, suggesting that a relatively homogeneous cell type is present in I3193 cells. Parallel transfections were done with BHK TK⁻ cells and produced similar results (lanes B3193 and BHK).

To determine the extent of αTIF-specific induction of the αTK reporter gene in the I3193 cells, we carried out parallel infections at 34 and 39°C by using the previously characterized ICP4 *ts* mutant virus, *ts*502Δ305 (41). Since this mutant does not produce functional ICP4 at the nonpermissive temperature, induction of the αTK resident in I3193 cells at 39°C should be a function of virion input αTIF, an assay similar to those which originally identified the virion transactivator (4, 29, 41). A representative analysis is shown in Fig. 7A and demonstrates the following. First, from 0 to 8 h postinfection there is no significant difference in the levels of αTK induction between 34 and 39°C, confirming the prediction that the TK levels observed over this period result from αTIF-specific induction. Second, a significant divergence occurs between 8 and 12 h postinfection. This divergence was reproducible and most probably results from the action of the de novo-synthesized αTIF on the resident αTK at the permissive temperature.

I3193 cells were infected with 5 PFU of either RΔ305, RUL46Δ3.1, or RUL46Δ3.2 per cell, and TK assays were performed over a 24-h time course. A representative time course is shown in Fig. 7B. Our results demonstrate that in the context of this assay, the deletion of UL46 has no apparent effect on the ability of αTIF to induce expression of the αTK reporter gene resident in I3193 cells.

In contrast, when similar experiments were carried out by using the UL47 deletion mutants RUL47Δ2.1 and

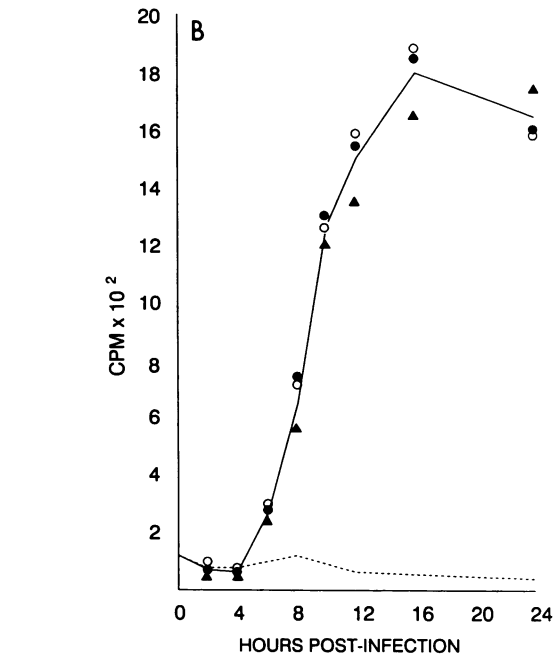
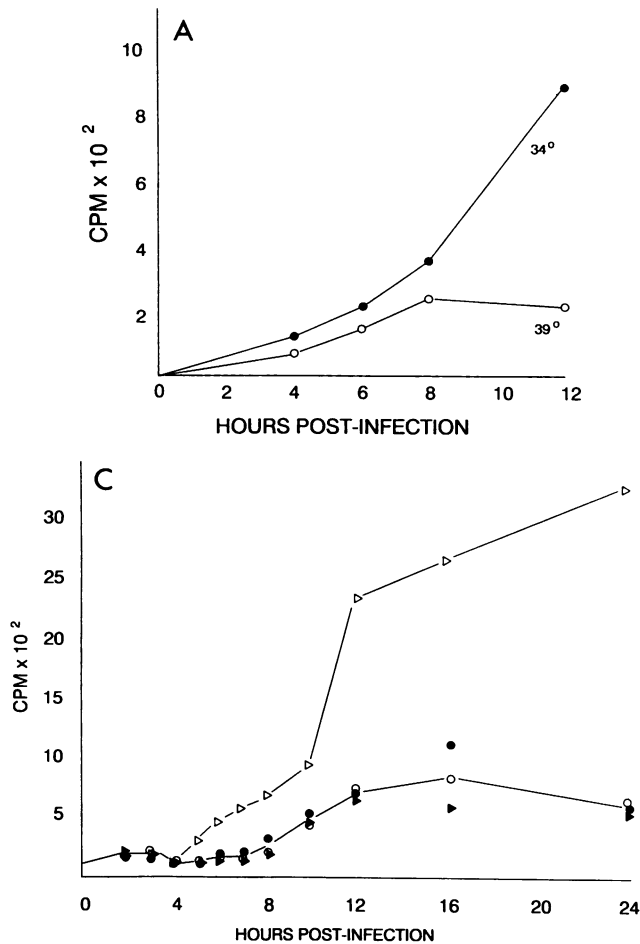


FIG. 7. α TIF-specific induction of I3193 cells. (A) Characterization of the α TK chimera resident in I3193 cells. I3193 or 143 TK⁻ cells (2×10^6) were seeded into 25-cm² dishes and grown overnight. Infections with *ts502Δ305* and TK assays were carried out as described in Materials and Methods. TK activity is expressed as counts per minute of [³H]thymidine converted to thymidylate per microgram of cell lysate protein as determined by the method of Lowry et al. (27). Counts per minute were normalized to mock-infected I3193 cells. (B) Induction of I3193 cells by the UL46 deletion mutants. I3193 cells (2×10^5) were seeded into 9-cm² well cluster dishes and grown overnight. Infections and TK assays were carried out as described in Materials and Methods. TK activity is expressed as in panel A. Symbols: ----, mock-infected I3193 cells; ●, RUL46Δ3.1; ○, RUL46Δ3.2; ▲, RΔ305. (C) Induction of I3193 cells by the UL47 and UL46/47 deletion mutants. Experiments were carried out as described for panel B. Symbols: Δ, RΔ305; ●, RUL47Δ2.1; ○, RUL47Δ2.2; ▲, RUL46/47Δ2. The y-axis intercept represents the basal TK level of mock-infected I3193 cells.

RUL47Δ2.2 and the UL46/47 double-deletion mutant, RUL46/47Δ2, the effect on α TK induction when compared with that by RΔ305 was significant. This result is shown in Fig. 7C and suggests a biphasic difference consistent with the inductions carried out with *ts502Δ305* (Fig. 7A). Induction by all three deletion mutants lagged behind that by RΔ305 by approximately 4 h, after which time the α TK was induced with a slope which paralleled that of RΔ305. Beginning at about 10 h postinfection, when de novo-synthesized α TIF reached significant levels (see Fig. 8 and 9), a second, more significant divergence occurred between RΔ305 and the deletion mutants. No significant difference was observed between the UL47 deletion mutants and the UL46/47 deletion mutant. The experiment shown in Fig. 7C was representative of experiments in which the I3193 cells were infected with 2 to 5 PFU per cell. Higher input multiplicities (10 to 50 PFU per cell) also demonstrated a reduced induction of α TK, but differences were only two- to threefold and were observed only at the later time points, suggesting that the enhancement of α TIF-specific induction by UL47 can be compensated for, to various degrees, by the amount of input virion α TIF (data not shown).

Does UL46 and/or UL47 effect the synthesis or steady-state levels of α TIF? The most direct effect of UL46 or UL47 on α TIF would be to modulate its levels within the cell. To determine whether the deletion mutants had an effect on the synthesis or steady-state levels of α TIF, anti- α TIF antibodies were made against an *S. aureus* protein A-containing

α TIF fusion protein. Figure 8 shows a Coomassie blue-stained SDS-PAGE gel of the IgG Sepharose-6 FF affinity-purified α TIF fusion protein expressed by pTIFA-1 in N4830 cells at the permissive temperature (see Materials and Methods). The fusion protein has a predicted molecular mass of approximately 74 kDa. Present in the lane marked pTIF (37°C) is a 74-kDa protein band corresponding to the size predicted for the α TIF-protein A fusion product. Note that this band is not present in cells grown at the nonpermissive temperature (lane pTIF, 25°C). N4830 control extracts do not produce any detectable affinity-purified proteins (lane N4830), and N4830 cells that were transformed with only the pRIT2T vector result in the affinity purification of the 33-kDa protein A expressed by this plasmid at 37°C. Western blot analyses of HSV-1 (F) 24-h-infected cell extracts, using serum obtained from rabbits after 4 months of immunization with this fusion protein (see Materials and Methods), demonstrated an α TIF-specific band which was absent in both mock-infected cell extracts and infected-cell extracts incubated with preimmune serum (data not shown). The α TIF band comigrated with the 64-kDa α TIF band previously

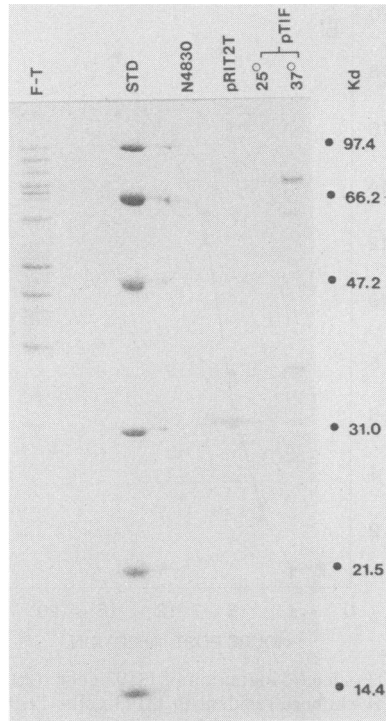


FIG. 8. IgG Sepharose-6 FF purification of the α TIF-protein A fusion protein. Extracts of N4830 cells alone or containing pRIT2T or pTIFA-1 were prepared, purified by FPLC over an IgG Sepharose-6 FF column, and subjected to SDS-PAGE as described in Materials and Methods. F-T, Flowthrough. The sizes of the molecular mass standards (lane STD) are indicated (97.4 kDa, rabbit muscle phosphorylase; 66.2 kDa, bovine serum albumin; 47.2 kDa, hen egg white ovalbumin; 31.0 kDa, bovine carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 kDa, hen egg white lysozyme [Bio-Rad]).

characterized (32) with an anti- α TIF peptide antibody (data not shown).

This serum was used in the following series of experiments (Fig. 8 and 9) to compare the levels of α TIF expression between R Δ 305 and the deletion mutants. Vero cells were infected with 5 PFU of R Δ 305 or the indicated mutants per cell and pulse-labeled with [35 S]methionine for 30 min before being harvested. The infected cells were harvested at representative time points over a 24-h period, and cell extracts were prepared, separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose. Autoradiograms and Western blots were prepared in parallel by using the same membrane. The comparison of the synthesis and steady-state levels of α TIF in R Δ 305 with those in the UL46 deletion mutants is shown in Fig. 9. The autoradiogram of the pulse-labeled cell extracts depicted in the upper panel reveals no significant difference in the time course of synthesis of α TIF among the different viruses over the 24-h period. α TIF first becomes visible approximately 4 to 6 h postinfection, and its synthesis continues through the remainder of the time course. The bottom panel depicts the parallel Western blot with the anti- α TIF antibody and shows the accumulation of α TIF from approximately 8 h postinfection through the remainder of the time course. Similar to the pulse-label shown in the upper panel, there is no detectable difference in the steady-state levels of α TIF between R Δ 305

(see lanes marked Δ 305) and the two UL46 deletion mutants (see lanes marked Δ 46).

A similar experiment comparing R Δ 305 with the UL47 and UL46/47 deletion mutants is shown in Fig. 10. As observed for the UL46 deletion mutants, the absence of both UL46 and UL47 (see lanes marked Δ 46/47) has little effect on the synthesis (upper panel) or steady-state levels (lower panel) of α TIF when compared with R Δ 305 (see lanes marked Δ 305). This appears to be the case for the UL47 deletion as well, if the novel α TIF-containing protein (indicated by the solid circle), as defined by the anti- α TIF antibody, is considered. It is apparent from both the pulse-label and Western blot analysis that deletion of UL47 results in the disappearance of the 64-kDa α TIF band concomitant with the appearance of a novel α TIF-specific band migrating at approximately 85 kDa. The appearance of the novel α TIF-containing protein, particularly in the complete absence of the 64-kDa α TIF protein band, was unexpected, and possible mechanisms for the synthesis of this novel protein will be discussed below.

DISCUSSION

In this report we present a preliminary characterization of functional properties of the proteins encoded by the UL46 and UL47 genes of HSV-1. We have demonstrated that neither gene product is essential for viral growth in cell culture, independent of whether the genes were deleted separately or in combination. All of the deletion mutants grew well, showing only a slight decrease in the yield of virus obtained during the preparation of viral stock when compared with the isogenic TK⁻ control virus, R Δ 305. The plaque size and morphology were not significantly different, and there were no observable differences in host range among human-, monkey-, and hamster-derived cell lines. Although an argument could be made for a synergistic role of the TK deletion in these mutants, we have recently constructed a parallel series of TK⁺ UL46, UL47, and UL46/47 deletion mutants and compared these mutants with the isogenic TK⁺ parental strain, HSV-1 (F). Our preliminary results indicate that although the stock titers of the TK⁺ UL46 and UL47 deletion mutants increased by approximately 1 log, they are still approximately 1 log lower than those for HSV-1 (F) (31a). Therefore, the mutants maintain the same relative titers to their isogenic parental viruses, independent of TK, which argues against possible synergism in the R Δ 305-derived UL46, UL47, and UL46/47 deletion mutants.

In contrast to previous results which analyzed the function of UL46 in a transient-expression assay (34), a similar characterization of UL46 within the context of the virus indicates that UL46 has no apparent effect on the ability of α TIF to induce the α TK reporter gene in I3193 cells. This may be a result of stoichiometry, genomic context, cell background, or other viral gene products; however, it does provide an additional cautionary note for the interpretation of transient-expression assays. The use of the I3193 cells as an assay system may also produce its own artifact, and the role of UL46, if any, in the modulation of α TIF-mediated induction may not be elucidated until the appropriate viral construct (for example, a UL46 deletion mutant carrying an α -regulated reporter gene) has been tested or animal studies have been carried out. Preliminary experiments suggest that the stoichiometry of UL46 relative to α TIF may be important, since differences in α TK induction levels between R Δ 305 and the UL46 deletion mutants were observed in an

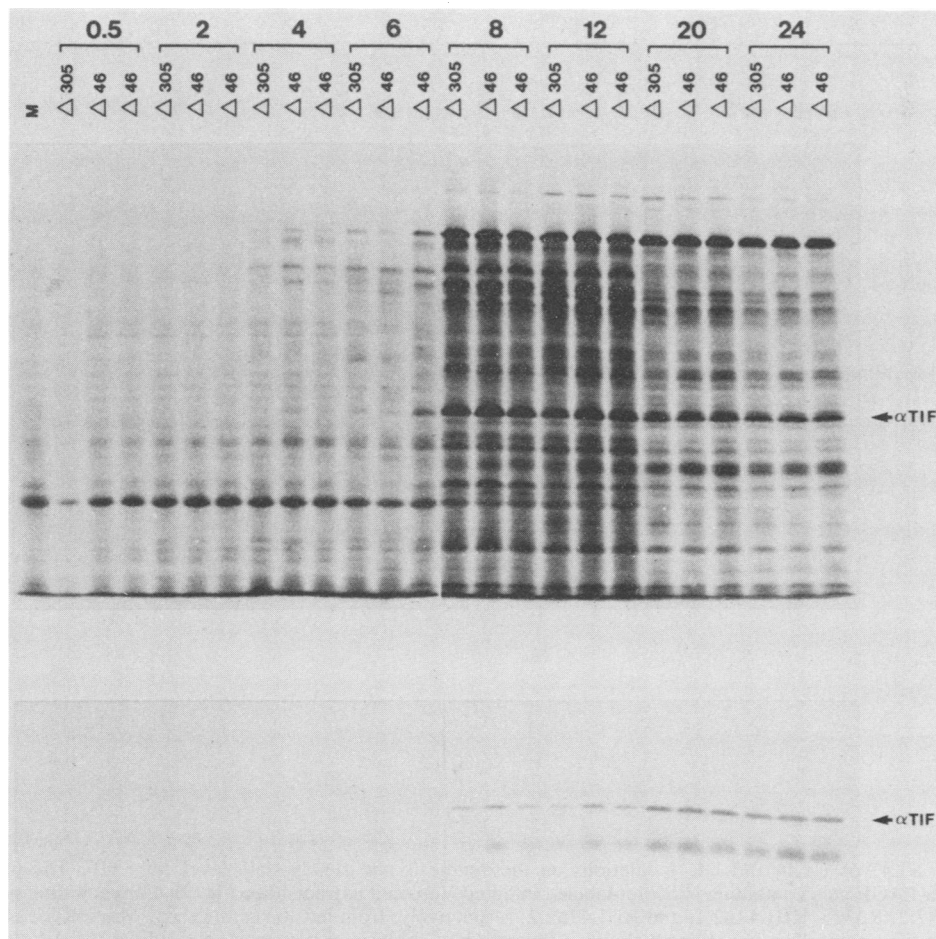


FIG. 9. Effect of UL46 deletion on the synthesis and steady-state levels of α TIF. Mock- and virus-infected Vero cells were pulse-labeled 30 min prior to harvesting and harvested at the indicated time (in hours), and extracts were prepared and electrophoretically separated on a 9.3% acrylamide gel as described in Materials and Methods. The upper panel shows an autoradiogram of SDS-PAGE gels containing [35 S]Met-labeled infected Vero cell extracts. For each time point the virus strains are as follows: R Δ 305, RUL46 Δ 3.1, and RUL46 Δ 3.2, respectively, from left to right. The lower panel shows a Western blot analysis of the same gel used for the autoradiogram in the upper panel, with the heterologous rabbit anti- α TIF antibody made against the α TIF-protein A fusion protein. Lanes are the same as in the upper panel.

experiment in which the input multiplicity was less than 1 PFU per cell (data not shown).

In contrast, deletion of UL47 had a significant effect on the ability of α TIF to induce expression of the α TK resident in I3193 cells. This effect also depends upon the multiplicity of infection. At a low input multiplicity (2 to 5 PFU per cell), we observed an 80% reduction in α TK levels in RUL47 Δ 2-infected I3193 cells when compared with R Δ 305-infected cells. At higher input multiplicities (10 to 50 PFU per cell), α TK inductions were still reduced in RUL47 Δ 2-infected cells, but only up to 40 to 50% and only at late times after infection (data not shown). This latter observation may be particularly relevant to the biphasic induction observed when using an input multiplicity of 5 PFU per cell (Fig. 7C). In these experiments, the initial slope of induction paralleled that for R Δ 305, but lagged behind R Δ 305 by approximately 4 h. Since no detectable α TIF is synthesized during this time, the lag probably results from a reduction in the ability of α TIF to effect transactivation, suggesting that UL47 functions to enhance the efficiency of α TIF-mediated transactivation during the infection process. The second phase of induction occurs between 8 and 10 h postinfection, when

newly synthesized α TIF is available. It is during this phase of induction that the effect of the UL47 deletion is the most pronounced. Note that at this time postinfection, host factors involved in this process may be limiting as a result of host shutoff, making the function of UL47 more evident. As in the case of UL46, the results obtained with the UL47 deletion mutants do not reflect the previous observations made in the transient-expression assays (34). Similar arguments to those presented above would also be valid in this case. Viral DNA recovery, the pulse-label results, and the fact that three isolates were indistinguishable in these assays are not consistent with a significant difference in particle numbers between the deletion mutants and R Δ 305.

The predicted levels of interaction between UL46 and UL47 and α TIF can be divided into two classes, those which involve the synthesis and/or stability of α TIF and those which occur posttranslationally (excluding stability). The most direct effect of either UL46 or UL47 on α TIF would be on the synthesis or steady-state levels of the protein, which would be consistent with the greater effect seen during the second phase of induction. The pulse-labeling and Western blot analyses demonstrate that neither UL46 nor UL47 acts

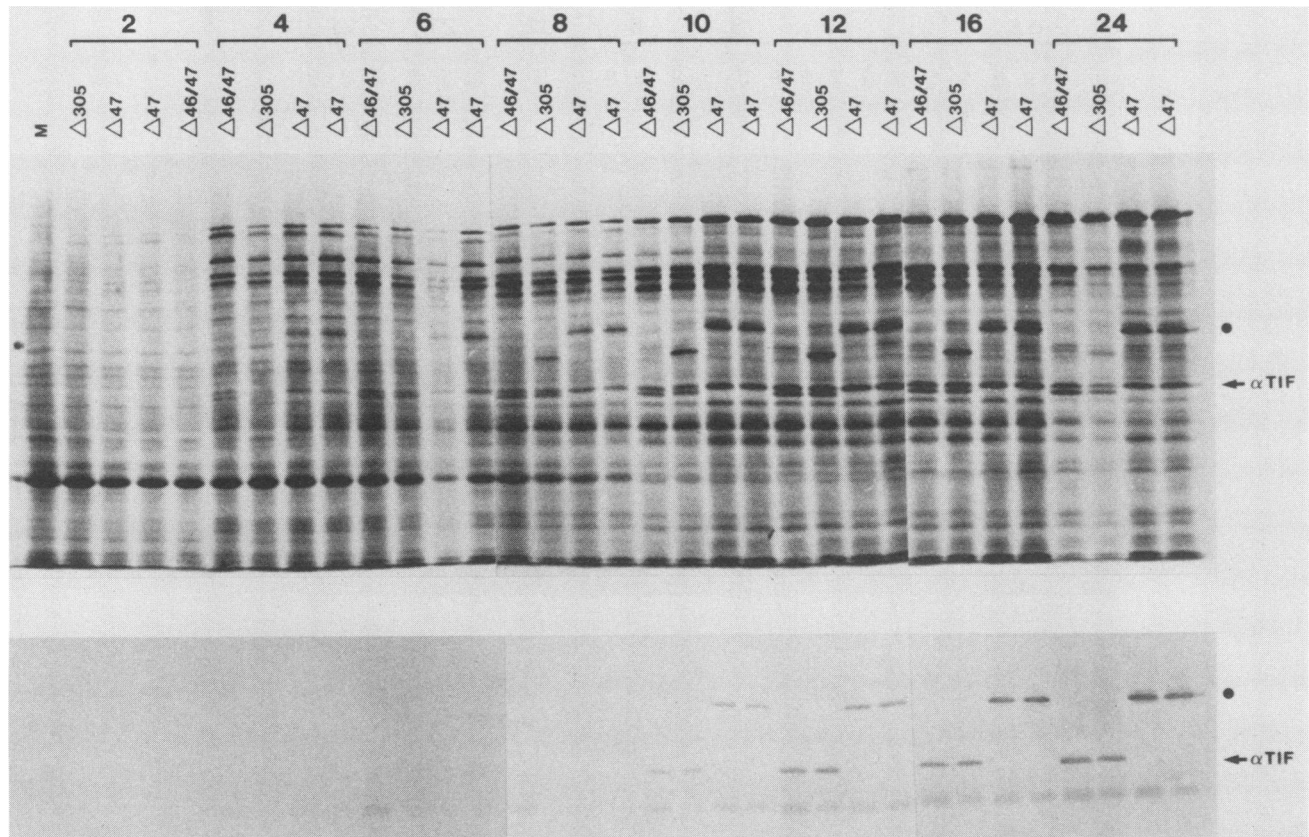


FIG. 10. Effect of UL7 or UL6 and UL7 deletions on the synthesis and steady-state levels of α TIF. The upper panel shows an autoradiogram of SDS-PAGE gels containing [35 S]Met-labeled infected Vero cell extracts (see Fig. 9). For each time point, virus strains are as follows: RUL46/47.2, R Δ 305, RUL47 Δ 2.1, and RUL47 Δ 2.2, respectively, from left to right, except where RUL46/47.2 is placed after RUL47 Δ 2.2 in the 2-h time point. The lower panel shows a Western blot analysis of the same gel used for the autoradiogram in the upper panel, with the heterologous rabbit anti- α TIF antibody made against the α TIF-protein A fusion protein. Lanes are the same as in the upper panel.

at this level, indicating that they are not involved in regulating the transcription, mRNA stability, or proteolytic turnover of α TIF.

If the effect of UL6 and UL7 is posttranslational and involves either modification, protein-protein association, or interactions with cellular factors, it might explain the inconsistencies between our current results and those observed previously (34) in a transient-expression system. It is conceivable that additional viral and/or host factors (cell specific) are involved in the UL6- and/or UL7-dependent modulation of α TIF which were absent (or present) in the transient-expression system. Again, this is particularly relevant in light of the host shutoff during viral infection. Alternatively, if coordinate transcription of UL6 and UL7 occurs, the stoichiometry of these proteins relative to each other could be important. Since the transient-expression assays, unlike the current experiments, did not transcriptionally separate UL6 and UL7, but rather interrupted protein expression through the insertion of translational stop codons, the effect of putative coordinate transcription of these 3' coterminal mRNAs would not have been measured (15, 34).

Of particular interest were the results observed with the UL7 deletion mutants. The behavior of these mutants was indistinguishable from that of the UL6/47 double-deletion mutant, even in the absence of wild-type α TIF. It is not

surprising that the UL7 and UL6/47 mutants behave similarly with regard to UL6, since this gene product does not appear to have a measurable effect in this system. However, the fact that the novel α TIF-containing protein induces the α TK as well as the 64-kDa α TIF present in RUL46/47 Δ 2 is unexpected. There are several explanations for the novel α TIF-containing protein. First, the *AsuII-NotI* collapse which generated this deletion may have resulted in a fusion of sequences within UL6 to UL48 (α TIF). If it is a fusion protein, it would mean that both the α TIF 3' poly(A) and the UL6 5' TATA sequences (which would not have been affected by the collapse) are nonoperational, since we were unable to detect any wild-type α TIF. It also suggests that the 3' TAG in the α TIF mRNA is nonfunctional. Since the novel band is too small to represent a fusion of α TIF (64 kDa) with UL6 (77 kDa), the α TIF fusion protein would, in all likelihood, contain a protein sequence fused to its COOH terminus which is normally not expressed by HSV-1. Since the COOH-terminal domain of α TIF contains the acid tail transactivation domain, the addition of 20 kDa of protein sequence to this domain does not affect transactivation (8, 52). A second possibility is that we have generated a novel splice site. If this is the case, it, too, does not appear to affect the transactivation domain of α TIF and could contain a legitimate portion of some other HSV-1 open reading frame, not necessarily UL6. A third possibility is that α TIF

associates with another protein or forms a dimer and that UL46 and UL47 are involved in this process. Since there is no detectable wild-type α TIF, the association would have to be completely resistant to denaturing gel electrophoresis, suggesting the formation of a covalent intermediate. In addition, experiments (not shown) involving mixed infections of RUL47 Δ 2.1 and RUL47 Δ 2.2 with R Δ 305 did not result in either the reduction or disappearance of the novel α TIF band; therefore, UL46 and UL47 functions provided in *trans* do not affect this novel protein.

Although general functions have been ascribed to a number of HSV-1 gene products, the biochemical mechanisms involved in these functions have been characterized in relatively few of these proteins. In part, the lack of characterization of HSV-1 gene function is due to the complexity of host-virus interactions, the inconsistent results generated by different model systems, and the absence of an in vitro latency system (reviewed in reference 47). Clearly, host factors present in cells passaged in vitro are able to compensate for viral gene products which may be involved in host range specificity and/or latency. In addition, it was demonstrated previously that the genomic environment plays an important role in the regulation of viral gene expression, so that mechanisms of action require a system interpretation as well (reviewed in reference 33). Numerous studies have been carried out to characterize the transactivation function of α TIF in vitro (1, 11, 13, 14, 48). However, the precise function of α TIF in the context of the virus remains unclear. α TIF, to date, remains essential for viral growth, since attempts at constructing deletion mutants have been unsuccessful (31a). The inability to construct deletion mutants, however, is more likely to be due to the structural role of this protein rather than its ability to transactivate. The previous statement is based on the reports by Ace et al. and Steiner et al. which demonstrate that mutant viruses, impaired in the transactivation function of α TIF, reduce, but do not eliminate, the ability of the virus to replicate, establish latency, and reactivate (2, 51). The fact that α TIF requires host proteins for transactivation suggests that the availability of these proteins determines the efficiency with which a productive infection will occur. The inherent flexibility of this system is evident, particularly in light of the observations of Kristie et al. (21) that the POU domain alone may be sufficient for the formation of an α TIF-protein-DNA complex and that the components of the complex may be cell type specific. It is reasonable to predict, then, that additional viral factors may be important in regulating α TIF complex formation during the establishment of and reactivation from a latent state, since a large family of POU-domain proteins are expressed in mammalian neural tissue (16). Consistent with this hypothesis are preliminary results obtained from BALB/c mice infected with the above-mentioned TK⁺ UL46 and UL47 deletion mutants. In these studies, although the UL46 and UL47 deletion mutants and HSV-1 (F) exhibited a similar degree of ocular pathogenicity, the degree of neural pathogenicity exhibited by both sets of deletion mutants was significantly reduced when compared with HSV-1 (F) (12a).

Our observations that neither UL46 nor UL47 is required for viral growth in cell culture, that neither gene product appears to affect the host range of the virus, and that UL47 has an effect on the efficiency of α TIF-mediated induction support an indirect regulatory role for at least one of these proteins. The argument can be made that the effect is not at the level of transcriptional or posttranslational stability since neither the synthesis nor the steady-state levels of α TIF are affected by the deletions. This result would be consistent

with the hypothesis that UL46 and UL47 are structural proteins which may associate with α TIF in the tegument and mediate their effect during infection. Newly synthesized UL46 and UL47 could also play a role in directing de novo-synthesized α TIF into virion assembly and modulate transactivation through compartmentalization. Alternatively, they could affect or compensate for the cellular proteins which are associated with α TIF. Recent evidence indicates that α TIF is able to bind to its cognate *cis* site, but that independent binding occurs with an extremely low affinity (20a). This observation suggests a role for the host factors in a conformationally dependent binding of α TIF, consistent with the observations of Kristie et al. that domains other than the POU domain influence the formation of the α TIF-protein-DNA complex (21). We are continuing our characterization of these deletion mutants, both in vitro and in animals, to further elucidate their biological function within the context of viral infection.

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ADDENDUM IN PROOF

We would like to point out a recent publication wherein a UL46/47 double deletion mutant, similar to the TK⁺ RUL46/47 Δ 2 deletion mutant discussed above, was also shown to be nonessential for growth in cell culture (D. E. Barker and B. Roizman, *Virology* 177:684-691, 1990).

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