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An 85-Kilodalton Herpes Simplex Virus Type 1 α *trans*-Induction Factor (VP16)-VP13/14 Fusion Protein Retains the Transactivation and Structural Properties of the Wild-Type Molecule during Virus Infection

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The 65-kDa herpes simplex virus type 1 encoded α *trans*-induction factor (αTIF or VP16) has two important functions: it is required for the efficient transcriptional induction of the α or immediate-early genes, and it acts as an essential structural component of the virion. The transcription properties of αTIF have been well studied in vitro. The protein is a powerful inducer of RNA polymerase II-directed transcription and, similar to the cellular transcriptional transactivators GAL4 and CGN4, contains separable DNA binding and transactivation domains. In contrast, little is known about the structural function of αTIF because this function can be studied only during virus replication and structural mutants are lethal. The in vivo analysis of αTIF is further complicated by the likelihood that the transcription and structural functions are not entirely separable. In this study, we take an alternate approach toward the development of αTIF mutants and their subsequent characterization. Rather than analyzing the effects of intragenic mutations, we have examined the properties of a mutant virus which expresses an αTIF fusion protein containing 61 amino acids of another herpes simplex virus type 1 virion protein, VP13/14, fused to its C terminus. This is the first report which demonstrates that the C-terminus of αTIF can tolerate the addition of an adjacent protein domain without compromising its transactivation function in vivo. Moreover, the VP13/14 sequences do not interfere with the protein-protein interactions required for virion targeting and assembly.

The herpes simplex virus type 1 (HSV-1)-encoded α *trans*-induction factor (αTIF, VP16, Vmw65) is a powerful inducer of RNA polymerase II-directed transcription (4, 6, 11, 56, 57). The 490-amino-acid protein contains an N-terminal DNA-protein interacting domain which confers HSV-1 α gene promoter specificity and a C-terminal transactivating domain, located between amino acids 1 and 400 and amino acids 411 and 490, respectively (1, 9, 11, 20, 56, 66, 71). The two domains are separable and function independently; the C-terminal acid tail is active when fused to a heterologous DNA binding domain and conversely, the DNA-protein interacting domain retains the ability to form a transcription complex on its cognate site in the absence of the acid tail (49, 61). The DNA-protein interacting domain of αTIF recognizes a bipartite DNA element unique to HSV-1 α gene promoters. The bipartite element contains a cellular octamer element, ATG CTAAT, which overlaps the virus-specific α gene promoter element, TAATGARAT (30, 43, 44, 58, 60). Acting in concert, these elements recruit both viral and cellular transcription factors into a virus-specific transcription complex whose assembly follows a series of phosphorylation-dependent binding events that involve protein-protein and protein-DNA interactions between αTIF and the cellular transcription factors, Oct-1 and C1 (HCF, VCAF) (19, 21, 29, 31, 32, 50, 54, 59, 64, 74). The endpoint of these interactions is the formation of a mature $\sim 10^6$-Da αTIF-containing C1 complex which may interact with RNA polymerase II transcription factors TFIIB and TFIID through the transactivation domain of αTIF (27, 41, 65).

Superimposed on the transcription function and essential for viral replication is the role of αTIF as a major structural component of the virion tegument (2, 25, 63, 69). Between 1,000 to 2,000 molecules are present in each virion, making αTIF one of the most abundant of the virion proteins (25, 63, 75). Hence, the infecting virus has evolved an elegant mechanism to ensure efficient and immediate α gene induction without having to rely on de novo viral protein synthesis (4, 57). Aside from its copy number and essential nature, little is known about how αTIF actually functions as a structural protein. The lack of information may stem partially from the difficulty in constructing viable mutants in a bifunctional protein. Although the transcription and structural functions are mechanistically unrelated, it is difficult to introduce a mutation into one function without having an effect on the other. In fact, there are no αTIF deletion mutants reported to date that are able to grow without complementation, suggesting that the two functions may share critical amino acid sequences. As a result, separation of the transactivation and structural functions of αTIF necessitates either functional complementation in *trans* or the construction of mutants that do not alter the structural properties of the protein. Regarding the former, Weinheimer et al. were able to construct an αTIF deletion mutant which grows only when complemented in *trans* with wild-type αTIF (69). Regarding the latter, only one αTIF transactivation-null, replication-competent mutant has been constructed and tested in vivo. The *in*1814 mutant contains four amino acids inserted in frame at amino acid residue 397 (2). Interestingly, the loss of transactivation stems from a

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perturbation at the C-terminal end of the DNA-protein interacting domain.

In this report, we characterize the functional activities of an αTIF fusion protein, designated αTIF-f, which contains 61 amino acids of another HSV-1 protein, VP13/14, fused in frame to its C terminus. αTIF-f is expressed by a virus mutant (R[F]UL47Δ2) in lieu of the wild-type αTIF (76). We demonstrate that potential alterations in conformation and amino acid interactions resulting from this perturbation do not significantly alter αTIF-mediated C1 complex formation, transactivation, or virion assembly. The feasibility of adding extragenic domains onto αTIF and being able to analyze replication-competent mutant viruses that express the αTIF fusion proteins will facilitate further studies on the structure and functions of αTIF in vivo.

MATERIALS AND METHODS

Virus and cells. All viruses, cell lines, and conditions for viral infection, [^{35}S]Met labeling, and thymidine kinase assays have been described elsewhere (16, 30, 75, 76). The purity of each UL47 deletion mutant stock was confirmed before use by Southern blot analysis as described previously (75, 76).

Oligonucleotides. Synthetic oligonucleotide primers for PCRs were prepared on a Milligen/Biosearch Cyclone Plus automated DNA synthesizer by phosphoramidite chemistry (primer 48-A, 5′GCTCTGGATATGGCCGACTTCG3′; primer 47*Not*I, 5′ GGAAGGGCTCCAGGTCCTTGAG3′). Primers were purified by using NENSORB PREP cartridges (E. I. du Pont de Nemours & Co., Inc.) according to the manufacturer's instructions.

PCR. PCRs were performed by using GeneAmp PCR reagents and AmpliTaq DNA polymerase (Perkin-Elmer Cetus) according to the manufacturer's instructions, with minor modifications. Briefly, 7-deaza,2′-deoxyguanosine was substituted for dGTP in a 3:1 ratio in a final volume of 25 μl. Two or 4 ng of plasmid DNA was added to the PCR mixture and amplified for 5 cycles of 1 min at 97°C, 1 min at 55°C, and 2 min at 72°C followed by 25 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C. For chemical sequencing substrates, the PCRs were repeated under similar conditions except that one of the two primers was end labeled with [γ-^{32}P]-ATP. All PCR products were analyzed on 8% polyacrylamide gels.

Sequencing. Prior to sequencing, pUL47Δ2 was digested to completion with *Sac*I to obtain a 650-bp fragment containing the deletion endpoints. The fragment was isolated from a 0.7% agarose gel by using NA45 cation-exchange paper (Schleicher & Schuell), end labeled with [γ-^{32}P]ATP, and subsequently digested with *Bst*YI to give a 370-bp and a 280-bp fragment. The two fragments were electrophoretically separated on 1% SeaPlaque (FMC) low-melting-point agarose and purified from the gel (45). End-labeled fragments were chemically sequenced and resolved on 6% polyacrylamide gels containing 7 M urea (48).

SDS-PAGE and Western immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiograms, and Western blot analyses were done as described previously (76). Rabbit polyclonal antibodies against HSV-1 VP13/14 (R220/5), ICP4 (N15), and ICP8 (367) were the gifts of D. Meredith, N. DeLuca, and P. Kinchington, respectively. Antibodies R220/5 and 367 were raised against purified VP13/14 and ICP8, respectively. Antibody N15 was raised against an ICP4–β-galactosidase fusion protein containing amino acids 3 to 774 of ICP4. All antibodies were used at dilutions of 1:500.

Purification of virions. Cell-free and cell-associated virions were labeled to steady state with [^{35}S]Met (Tran^{35}S-label; ICN) and purified either from the growth medium or from infected cells at 18 h postinfection as previously described (75). Approximately 2 μg of protein was used for SDS-PAGE analysis. Autoradiograms of SDS-PAGE-separated Tran^{35}S-label (ICN)-labeled virion proteins obtained from HSV-1 (F) and R[F]UL47Δ2 were scanned with a Gilford Response I spectrophotometer programmed for peak area integration. Several exposures of each autoradiogram were scanned to rule out a nonlinear response caused by film saturation.

RNA analysis. Flasks (25 cm^2) containing 2 × 10^6 Vero cells were infected with 5 PFU of each virus per cell at 8°C in the

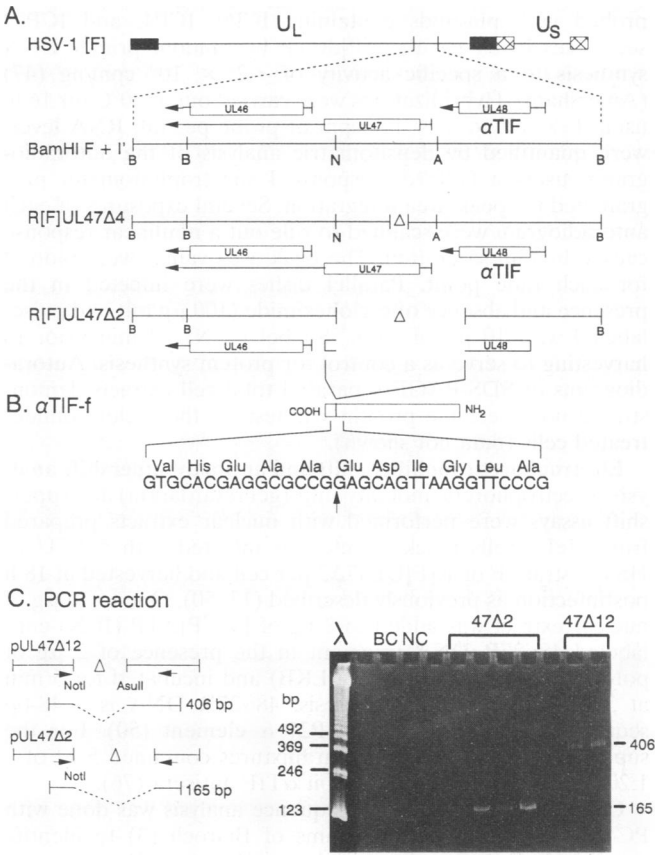

FIG. 1. Schematic representation of R[F]UL47Δ2 showing the construction strategy and relevant sequence information. (A) The HSV-1 genome depicting the unique long (U_L) and unique short (U_S) regions flanked by inverted repeats (boxes). Locations of UL46 (VP11/12), UL47 (VP13/14), and UL48 (αTIF, VP16) residing within the *Bam*HI I′/F fragment of the viral genome are shown, as well as the locations of the deletions in R[F]UL47Δ4 and R[F]UL47Δ2 (αTIF-f). (B) Sequence analysis of R[F]UL47Δ2. The R[F]UL47Δ2 deletion results from an in-frame collapse of Glu-487 (αTIF) to Ala-604 (VP13/14), giving rise to the αTIF-VP13/14 fusion protein. Sequencing details are presented in Materials and Methods. (C) Comparative PCR analysis of pUL47Δ2 and pUL47Δ12 showing the sizes and locations of the two deletions, the relative primer locations, and the PCR products. 47Δ12 results from the site-specific collapse of sequences between the *Asu*II (*Csp*45 I) site and the *Not*I site. The 48-A and 47*Not*I primers were used for both reactions. The reaction products were separated on an 8% acrylamide gel and stained with ethidium bromide. λ, 123-bp λ phage-derived ladder (Bethesda Research Laboratories); BC, buffer control; NC, negative control.

presence of 100 μg of cycloheximide per ml, adsorbed for 1 h in the cold, washed with fresh growth medium (199V), and returned to 37°C. Infections were terminated at 0, 1, 2, 3, and 4 h postinfection with guanidinium hydrochloride by the single-step procedure of Chomczynski and Sacchi (7). RNA was resuspended in 50 μl of diethylpyrocarbonate-treated Milli-Q-purified water (Milligen). Five microliters of each sample was separated on 1.2% agarose gels containing 1% formaldehyde and stained with ethidium bromide to assess recovery and quality. Uniform recovery was confirmed by the optical density at 260 nm (between 16 and 20 μg per sample). Poly(A) mRNA was purified from 10 μg of total RNA by using Hybond MAP paper as specified by the manufacturer (Amersham). One half of the mRNA was subjected to Northern (RNA) blot analysis, using GeneScreen Plus as specified by the manufacturer (DuPont) for formaldehyde gels. mRNA was probed with plasmids containing ICP0, ICP4, and ICP22 sequences labeled with [^{32}P]dCTP by random primer DNA synthesis to a specific activity of ~2 × 10^6 cpm/ng (47) (Amersham). Hybridizations were carried out at 60°C for 16 h, using 1 × 10^6 to 2 × 10^6 cpm of probe per ml. RNA levels were quantified by densitometric analysis of the autoradiograms, using a Gilford Response I spectrophotometer programmed for peak area integration. Several exposures of each autoradiogram were scanned to rule out a nonlinear response caused by film saturation. The peak area values were plotted for each time point. Parallel dishes were infected in the presence and absence of cycloheximide (100 μg/ml) and pulse-labeled with 10 μg of Tran^{35}S-label (ICN) 30 min prior to harvesting to serve as a control for protein synthesis. Autoradiograms of SDS-PAGE-separated total cell extracts demonstrated no detectable protein synthesis in the cycloheximide-treated cells (data not shown).

Electrophoretic mobility shift and antibody supershift analysis. Electrophoretic mobility shift (gel retardation) and supershift assays were performed with nuclear extracts prepared from HeLa cells mock infected or infected with 5 PFU of HSV-1 strain F or R[F]UL47Δ2 per cell and harvested at 18 h postinfection as previously described (13, 50). One to 10 μg of nuclear extract was added to 2 ng of [γ-^{32}P]ATP (ICN)-end-labeled 48α27R DNA fragment in the presence of 2 μg of poly(dI)-poly(dC) (Pharmacia-LKB) and incubated for 5 min at 37°C prior to electrophoresis. 48α27R DNA is a 48-bp sequence which spans the ICP27 α element (50). For the supershift analysis, the reaction mixtures contained 5 μl of a 1:20 dilution of polyclonal rabbit αTIF antisera (76).

Computer analysis. DNA sequence analysis was done with PC GENE, using the algorithms of Bairoch (3) to identify putative modification sites and those of Kyte and Doolittle (34) to calculate the hydropathic index (Intelligenetics, Inc., Mountain View, Calif.).

RESULTS

Viral expression of αTIF-f. We have previously described the construction of two UL47 deletion mutant viruses derived from the HSV-1 wild-type strain F (75, 76) (Fig. 1). RUL47Δ4 expresses the 65-kDa wild-type αTIF, whereas RUL47Δ2 expresses an 85-kDa αTIF fusion protein, termed αTIF-f (76). The kinetics of synthesis and turnover of αTIF-f and αTIF during infection were compared by pulse-labeling and parallel Western blot analysis as previously described (75, 76). Figure 2A shows that the appearance of αTIF-f in R[F]UL47Δ2 is coincident with the appearance of wild-type αTIF in HSV-1 strain F as a βγ gene (23), whereas Fig. 2B shows that the steady-state levels of αTIF and αTIF-f are comparable in cells

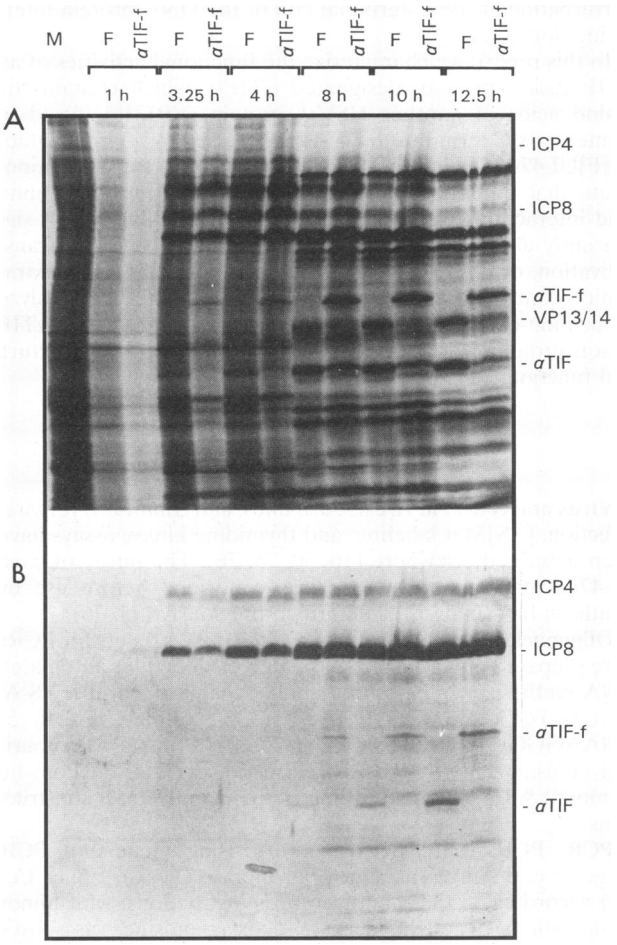

FIG. 2. Pulse-label and Western blot analysis of wild-type (F)- and R[F] UL47Δ2 (αTIF-f)-infected cell extracts. (A) Autoradiogram of SDS-PAGE-separated [^{35}S]Met-pulse-labeled cell extracts at 1, 3.25, 4, 8, 10, and 12.5 h postinfection. Flasks (25 cm^2) of Vero cells were infected with 5 PFU of the indicated virus per cell and pulse-labeled with 5 μCi of Tran^{35}S-label (ICN) 30 min prior to harvesting. The locations of αTIF and αTIF-f relative to other HSV-1 proteins are indicated. (B) Western blot analysis of the protein profile depicted panel A, using antibodies against HSV-1 ICP4, ICP8, and αTIF. M, mock infected; F, HSV-1 strain F; αTIF-f, R[F]UL47Δ2.

infected with the two viruses. The reduced levels of ICP4 and ICP8 in the 1- and 3.5-h lanes from the R[F]UL47Δ2-infected cell extracts relative to strain F reflect the reduced efficiency of α gene induction previously shown to result from the absence of the UL47 gene product, VP13/14 (75, 76).

Characterization of the αTIF-f protein. The RUL[F]47Δ2 mutant was constructed to delete the UL47 gene by the site-specific collapse of sequences lying between a unique *Asu*II (*Csp*45 I) site located 3′ of the αTIF-encoding gene, UL48 (11, 56), and a unique *Not*I site located near the 3′ terminus of the VP13/14-encoding gene, UL47 (51, 52, 56, 63) (Fig. 1A and B). Restriction enzyme analysis indicated that the deletion was slightly larger than predicted; therefore, the region flanking the deletion was amplified by PCR using a set of primers complementary to sequences located 299 bp 5′ of the *Asu*II site and 104 bp 3′ of the *Not*I site in UL47 (51, 56) (Fig. 1C). In contrast to the 406-bp amplification product obtained from an isogenic plasmid clone containing an unal-

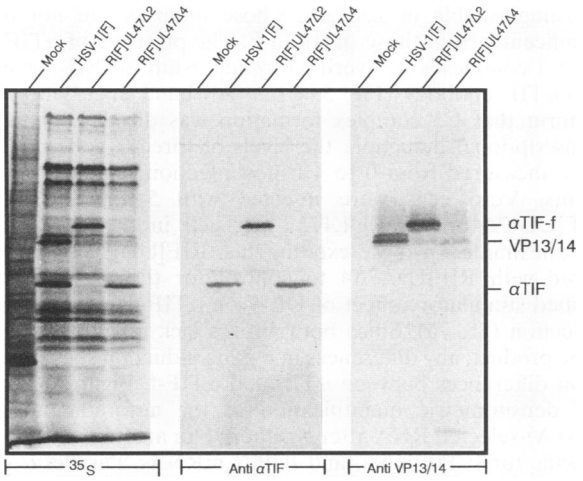

FIG. 3. Western blot analysis of the wild-type strain F- and R[F]UL47Δ2-infected cell extracts. Dishes (25 cm^2) containing ~4 × 10^6 Vero cells were mock infected or infected with 5 PFU of each virus per cell and labeled with Tran^{35}S-label (ICN) for 30 min prior to harvesting. Extracts were prepared, separated by SDS-PAGE, and transferred to nitrocellulose. The left-hand panel depicts the autoradiogram of the SDS-PAGE-separated infected cell extracts; the right-hand panel depicts the Western blot analysis of the same nitrocellulose filter, using antibodies against αTIF and VP13/14 (UL47). The locations of αTIF and αTIF-f relative to other HSV-1 proteins are indicated. Note that the VP13/14 antibody contains a small amount of gE antibody, which is the cause of the faint band in the vicinity of VP13/14 in the two UL47 deletion mutants.

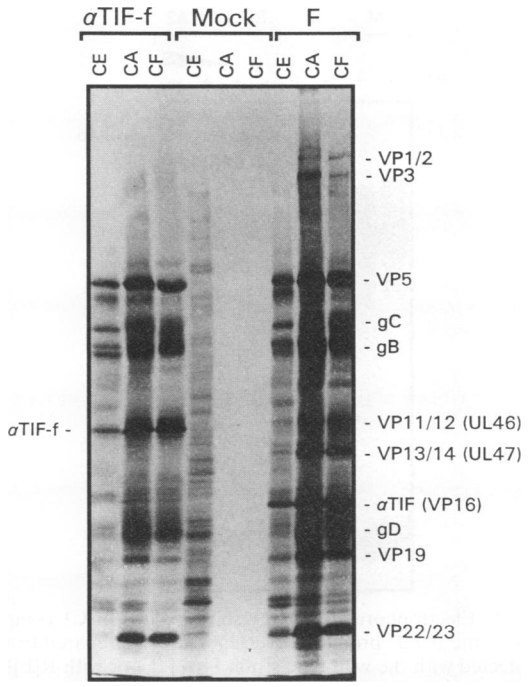

FIG. 4. Autoradiographic analysis of SDS-PAGE-separated virion proteins. HeLa cells were infected with 5 PFU of either wild-type (strain F) or R[F]UL47Δ2 (αTIF-f) per cell and metabolically labeled to steady state with Tran^{35}S-label (ICN). Eighteen hours postinfection, total cell extracts (CE), cell-free virions (CF), and cell-associated virions (CA) were prepared and purified in parallel as previously described (75). The locations of αTIF, αTIF-f, and relevant virion proteins are shown.

tered *Asu*II-*Not*I collapse (pUL47Δ12), amplification of the αTIF-f-containing construct (pUL47Δ2) resulted in a 165-bp fragment. The smaller fragment represented a loss of 231 nucleotides 5′ of the *Asu*II site (Fig. 1C). Sequence analysis of both the 165-bp PCR fragment and pUL47Δ2 predicted that the larger deletion resulted in the fusion of amino acid Glu-487 of αTIF to Ala-604 of VP13/14 (Fig. 1B) (see Materials and Methods for details).

To confirm that the αTIF fusion was indeed in frame with VP13/14, Western blot analysis of infected cell extracts was done with both αTIF and VP13/14 antibodies (Fig. 3). The analysis of extracts prepared from Vero cells infected with strain F, R[F]UL47Δ4 (TIF$^+$/UL47$^-$), and R[F]UL47Δ2 (αTIF-f$^+$/UL47$^-$) showed that the αTIF-f protein reacted with both αTIF and VP13/14 antibodies. Moreover, wild-type αTIF and VP13/14 were not detected in the R[F]UL47Δ2-infected cell extracts (Fig. 3). These data, combined with the nucleotide sequence, conclusively demonstrated that αTIF was fused to VP13/14, producing a chimeric αTIF-f molecule that was 58 amino acids larger than the wild type.

αTIF-f retains the structural properties of the wild-type molecule. It has been found that 1,000 to 2,000 molecules of αTIF are essential for virion assembly and, once packaged, are transported by the virus into the cell, where they act immediately to induce α gene expression (4, 24, 57, 63, 69, 75). Clearly, αTIF-f was able to substitute for the missing αTIF in R[F]UL47Δ2, since the virus was viable and able to grow to within 1 log titer of the wild type (75, 76). However, it was possible that the VP13/14 sequences interfered with virion assembly and that αTIF-f was processed to a smaller size prior to tegument packaging. To determine whether R[F]UL47Δ2 packaged an intact αTIF-f molecule, Tran^{35}S-label (ICN)-labeled virions were purified from the growth medium of HSV-1 strain F- and R[F]UL47Δ2-infected cells. Autoradiograms prepared from SDS-PAGE analysis of purified virions confirmed that the 85-kDa αTIF-f protein was assembled into progeny R[F]UL47Δ2 virions and that the levels of αTIF-f packaged into the R[F]UL47Δ2 virions were comparable to the levels of αTIF present in strain F virions (Fig. 4).

There is indirect evidence that the function of αTIF may be regulated by its phosphorylation state. First, it was originally identified as a phosphoprotein (ICP25) whose phosphorylation state varies during infection (72); second, C1 complex formation is driven by the phosphorylation state of its components (33). The ability of αTIF-f to undergo phosphorylation by the virion-associated kinase was determined in parallel reactions carried out on the wild-type, R[F]UL47Δ4, and R[F]UL47Δ2 virions as described previously for the analysis of UL47 deletion mutants (75). No significant differences were observed, suggesting that the VP13/14 sequences do not grossly interfere with the phosphorylation of αTIF (data not shown). Note that this region of VP13/14 does not harbor any sequence-predicted posttranslational modifications (3, 53, 75).

αTIF-f is transactivation competent and forms a complex on the ICP27 promoter sequence. The ability of αTIF to transactivate α genes has been associated with its presence in the C1 complex. Although complex formation is not a functional assay, mutant molecules of αTIF that lack DNA-protein binding properties do not transactivate in vitro (66). We therefore examined the ability of αTIF-f to drive C1 complex formation on the α element located within the ICP27 promoter (Fig. 5). Nuclear extracts obtained from HeLa cells infected with R[F]UL47Δ2 were able to form C1 complexes that were

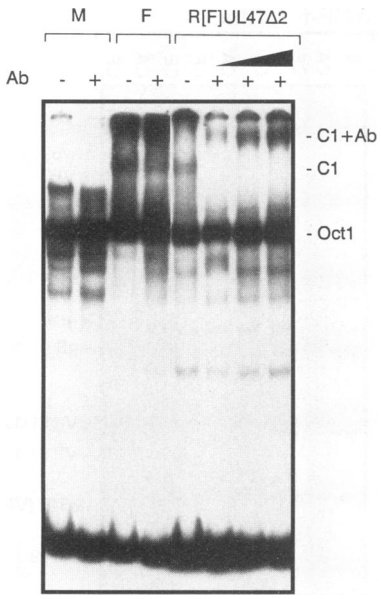

FIG. 5. Electrophoretic mobility shift analysis of C1 complex formation on the ICP27 promoter. Nuclear extracts prepared from HeLa cells infected with the wild-type strain F (αTIF) or with R[F]UL47Δ2 (αTIF-f) were used in electrophoretic mobility shift assays as previously described (50), using the 48-bp ICP27 α element, 48α27R. Supershifts (+) were carried out with an αTIF-specific antibody (Ab) (76). Prior to the gel shift assay, Western blot analysis was carried out on equivalent amounts of nuclear extract (80 μg) to determine relative levels of αTIF and αTIF-f present in the extract. Levels were within twofold of each other by this criterion. Five micrograms of mock (M)- or strain F (F)-infected and 10 μg of R[F]UL47Δ2-infected nuclear extract was used in each assay except for the antibody supershift analysis using R[F]UL47Δ2-infected cell extract. In this case, extract was titrated (from left to right) with 1, 5, and 10 μg of nuclear extract. C1 + Ab, location of the antibody-supershifted C1 complex; C1, location of the C1 complex; Oct1, location of the Oct-1 cellular complex.

indistinguishable in size and whose intensity did not differ significantly from those of strain F. The presence of αTIF-f in the C1 complexes was verified by supershift analysis using an anti-αTIF antibody (Fig. 5, C1 + Ab band in + lanes). To confirm that C1 complex formation was directly related to transcriptional induction, the levels of three α gene mRNAs were measured from 0 to 4 h postinfection. In these experiments, Vero cells were infected with 5 PFU of either R[F]UL47Δ4 or R[F]UL47Δ2 per cell in the presence of cycloheximide. In these experiments, R[F]UL47Δ2 was compared with R[F]UL47Δ4 to control for the previously described stimulatory effect of UL47 on αTIF-mediated α gene induction (75, 76). Since both viruses lack a wild-type UL47 gene product, any differences in α gene induction should result from differences between αTIF and αTIF-f. Figure 6 depicts the densitometric quantification of the autoradiograms of poly(A)-selected RNA after Northern blot analysis with probes specific for ICP0, ICP4, and ICP22 mRNAs. Parallel α gene induction profiles were observed between the two viruses, demonstrating unequivocally that αTIF-f transactivates as well as the wild-type αTIF. Similar results were obtained with total cellular RNA (data not shown).

Viral thymidine kinase levels were also measured over a 24-h time period to compare the efficiency of the transition from α to β gene expression between the two isogenic UL47 deletion mutants. Since expression of the α genes, primarily ICP4, is required for β gene expression, it follows that the kinetics of appearance of thymidine kinase activity should reflect the efficiency of α gene induction by αTIF (12, 14, 26, 68, 75, 76). No significant differences were observed between the two viruses (data not shown).

DISCUSSION

In this report, we describe the characterization of R[F]UL47Δ2, a viral mutant that expresses an 85-kDa αTIF-VP13/14 fusion protein. We have demonstrated that R[F]UL47Δ2 expresses αTIF-f at the same time postinfection (βγ) and at levels comparable to wild-type levels. The αTIF-f protein, notwithstanding the presence of the VP13/14 sequences, was packaged into mature virions at wild-type levels and was present in the ICP27 promoter C1 complex. We also

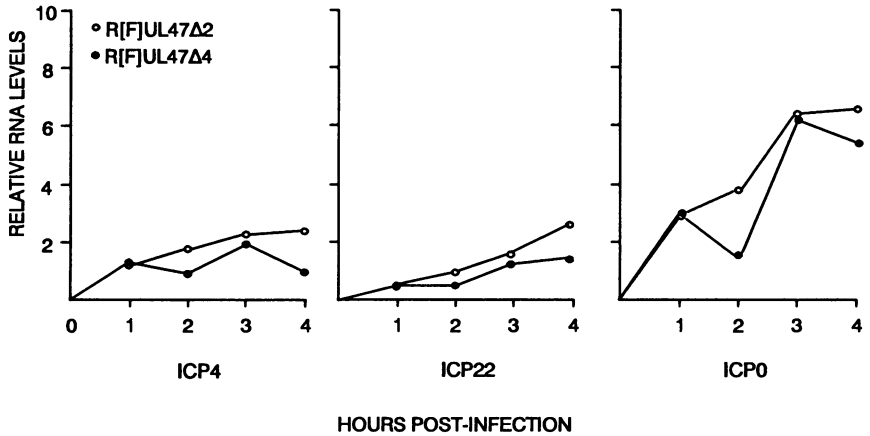

FIG. 6. Northern blot analysis of ICP0, ICP4, and ICP22 mRNA expression. Depicted is a graphical representation of the densitometric analysis of the autoradiogram comparing mRNA expression over a 4-h time course between R[F]UL47Δ2 (αTIF-f) and its isogenic UL47$^-$/αTIF$^+$ counterpart, R[F]UL47Δ4 (αTIF). Poly(A)-selected mRNA was obtained from 2 × 10^6 Vero cells infected with 5 PFU of either R[F]UL47Δ2 or R[F]UL47Δ4 per cell in the presence of cycloheximide and subjected to Northern blot analysis using DNA probes specific for ICP0, ICP4, and ICP22 mRNA (7, 47) (see Materials and Methods for details).

demonstrated that during infection, αTIF-f induces the expression of α gene mRNAs as well as the wild-type αTIF.

To address the impact of these findings on the transcriptional functions of αTIF, in vitro studies of acidic transactivators have resulted in several models of how this group of activators enhance gene expression (reviewed in references 18, 22, 38, 46, and 62). In vitro binding assays have suggested that the acid tail of αTIF interacts with the transcription factors TFIIB and TFIID and, more recently, with the cellular replication factor A (24, 27, 39, 41, 65). GAL4-αTIF fusions have been used to identify specific amino acid residues and secondary structure(s) associated with RNA polymerase II transcription factors and their associated TATA-binding protein-associated factors in transient assays (5, 9, 10, 28, 35, 36, 40, 61, 73). However, sufficient differences have arisen among these studies to raise the possibility of system dependence and to question whether mechanisms that operate in vitro or in transient assays occur during virus infection. In other words, interpretation of the in vitro studies may be complicated by the interaction of the C terminus of αTIF and associated coactivator molecules with the DNA binding domain and its associated binding cofactors, some of which clearly differ between the wild-type αTIF molecule and those that contain a heterologous DNA binding domain. An example of altered interactions conferred by a heterologous DNA binding domain was demonstrated in a related system by Fitzpatrick et al., who reported that GAL4-FTZ (*fushi-tarazu*) fusions exhibit homeodomain-independent activity in *Drosophila* embryos (17). Relevant to the DNA binding domain of αTIF, Kristie and Sharp have recently shown that in addition to Oct-1, a family of C1 proteins are required for stable C1 complex formation on the HSV-1 α element (33). The acid tail is not required for this complex series of interactions, since C-terminal deletion mutants form C1 complexes (49); however, it is important to note that the impact of the C1 complex on the transactivation domain has not been tested. It is tempting to speculate at this point that the interactions among Oct-1, the C1 peptides, and the DNA-protein interacting domain of αTIF may affect the conformation of the acid tail and that amino acid residues critical for transactivation in this context may differ from those observed in heterologous constructs. For example, Cohen recently demonstrated that only 14 amino acids of the αTIF acid tail (residues 449 to 462) are required to substitute for the internally positioned glutamine-rich activating domain (residues 439 to 451) of the Epstein-Barr virus-encoded transactivator, EBNA2 (8). Interestingly, residues 449 to 462 of αTIF lie between the two predicted transactivation domains and are not sufficient for the transactivation of α promoters (1, 15, 19, 20, 66), suggesting that the context of the acid domain may influence its activity. Furthermore, acidic activation domains do not appear to share consistent secondary structures in vitro. For example, the transactivating domain of GAL4-AH has an amphipathic α helix, whereas αTIF, GCN4, Fos/Jun, and Tat contain less structured acidic regions that become structured into either α helices or β sheets, depending on their surrounding environment (37, 41, 42, 55, 67, 70). At this time, it is difficult to predict which secondary structures are critical for αTIF-mediated transactivation, since the direct association of a given secondary structure with transcriptional activation has yet to be established.

Returning to this report, the addition of 7 kDa of VP13/14, a predicted hydrophobic domain, onto the C terminus of αTIF introduces the possibility that the conformational constraints of this transcription factor are somewhat flexible in its authentic context, insofar as C1 complex formation and transactivation were not detectably altered. One explanation for the lack of effect could be that the VP13/14 sequences form a separate domain which does not interfere with complex assembly. Alternatively, RNA polymerase II coactivator molecules and C1 peptides may target specific amino acid sequences in αTIF and, in combination with an induced fit mechanism of transcriptional activation, tolerate or move the interfering domain aside. It is also possible that αTIF tolerates the additional domain because it is composed of VP13/14 sequences. We have previously shown that VP13/14 enhances αTIF-mediated transactivation (75, 76); although a direct association between the two proteins has not been shown, such an event could explain the lack of effect of the VP13/14 sequences on αTIF-f. The latter possibility is currently being tested through the construction of non-VP13/14-containing αTIF fusion proteins. Regardless, our data are the first to demonstrate that the acid tail of αTIF is functional as an internal domain, i.e., that a free C terminus is not required for transactivation in vivo.

Perhaps of even greater significance is the impact of these studies on HSV-1 tegument assembly. Little information is available on the protein-protein interactions required for proper tegument targeting and assembly partially because many of the tegument proteins have yet to be identified and/or characterized. To date, αTIF (VP16) is the only essential tegument protein to be reported. Pertinent to the lack of effect of αTIF-f on tegument assembly, we have reported previously that the 87- to 93-kDa VP11/12 and the 79- to 81-kDa VP13/14 tegument proteins are present in roughly equal numbers to αTIF but are not essential for virus replication (75, 76). Together, VP11/12 and VP13/14 constitute between 15 and 18% of the total predicted virion mass, yet a VP11/12-VP13/14 double-deletion mutant exhibited less than a 10-fold decrease in plaque formation relative to wild-type virus (25, 75, 76). In addition, VP11/12 and VP13/14 appear to be able to substitute for one another in the tegument (75). As a result of these observations, we had proposed a model of tegument assembly in which αTIF provides the major framework around which other tegument proteins are assembled or packaged with a certain degree of flexibility. However, this model assumes that very specific protein-protein interactions are required among the proteins essential for proper αTIF-driven tegument assembly. It is evident from this report that the VP13/14 domain does not interfere with these interactions. Taken together, our observations introduce the possibility that the protein-protein interactions associated with the assembly of both essential and nonessential tegument proteins possess a reasonable degree of flexibility that does not interfere with specificity. Alternatively, the fact that the fused sequences are those of another HSV-1 major tegument protein could explain why αTIF tolerates the additional domain without a detectable effect on virion assembly. The VP13/14 sequences could behave as a separate domain that is recognized by other tegument proteins and does not interfere with αTIF, or it may simply be packaged as a neutral passenger domain. The disparity between the sequence-predicted and apparent molecular masses of the in vitro-translated unmodified αTIF molecule (53 and 65 kDa, respectively) suggests that αTIF may be asymmetric (11, 50, 56). If an asymmetric αTIF molecule is necessary for proper tegument structure, the scarcity of αTIF mutants might be explained by a low tolerance for conformational alterations. Interestingly, although the sequence-predicted molecular mass of the C-terminal 61 amino acids of VP13/14 is 7 kDa (51), αTIF-f migrated at an apparent molecular mass of 85 kDa, not 72 kDa, suggesting that the asymmetry of the wild-type molecule may be conserved in αTIF-f. The preceding notion must be viewed as speculation until the biophysical properties of the intact wild-type protein are analyzed. On a somewhat less

speculative note, it may simply be that the absence of wild-type VP13/14 from the tegument creates sufficient space to permit the packaging of the larger αTIF-VP13/14 fusion protein. Studies are in progress to determine the size restriction and specificity of these events.

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