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Characterization and Nucleotide Sequence of Two Herpes Simplex Virus 1 Genes Whose Products Modulate α-*trans*-Inducing Factor-Dependent Activation of α Genes

JENNIFER L. C. McKNIGHT, PHILLIP E. PELLETT,† FRANK J. JENKINS, AND BERNARD ROIZMAN*
The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

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Herpes simplex viruses encode a structural protein which induces, in *trans*, expression of α genes, the first set of genes to be expressed after infection of permissive cells. This protein, designated as the α-*trans*-inducing factor (α-TIF), maps within the *Bam*HI F fragment, and its gene has been sequenced. In the course of mapping the domain of the α-TIF gene, it was noted that the intact *Bam*HI fragment was consistently more effective than the complete domain of the α-TIF gene in inducing expression of α genes. Cotransfections of DNA fragments containing an α indicator gene and the α-TIF gene with various regions of the *Bam*HI F DNA fragment revealed that the sequences located 3′ to the α-TIF gene raised the activity of the α-TIF gene to nearly the same level as that of the intact *Bam*HI F fragment. The nucleotide sequence and S1 nuclease mapping analyses revealed the presence of two transcribed open reading frames capable of encoding polypeptides with translated molecular weights of 77,357 and 70,527. To determine whether the effect of these sequences in *trans* on α-TIF-mediated induction of α genes was due to expression of these genes or competition for transcriptional factors, we constructed plasmids that contained both genes. Into each or both of these genes we inserted, near the translation initiation sites, 14-base-pair linkers carrying translational stop codons (TAG) in all three reading frames. Analyses of these plasmids indicated that the gene encoding the 70,527-molecular-weight polypeptide reduced α-TIF-dependent induction of α genes, whereas the gene encoding the 77,357-molecular-weight polypeptide increased this activity. Insertion of the stop codons abolished these activities.

The herpes simplex virus 1 (HSV-1) genes form at least five groups (α, β_1, β_2, γ_1, and γ_2), whose expression is coordinately regulated and sequentially ordered in a cascade fashion (15). The five α genes (α0, α4, α22, α27, and α47) are expressed first and are operationally defined as capable of being transcribed in the absence of de novo protein synthesis (15, 16). Although the functions of the α genes are for the most part unknown, the products of at least three α genes (α4, α22, and α27) have been shown to be required for viral gene expression in productively infected cells (8, 18, 34, 36, 38).

A unique characteristic of HSV gene regulation is that a structural component of the virion induces α gene expression (3, 31). Specifically, chimeric genes consisting of the structural sequences of an indicator gene (e.g., the HSV-1 thymidine kinase [TK]) linked to the 5′ nontranscribed promoter-regulatory domain of α genes (e.g., α-TK) and resident in cells can be induced by infection of the cells with HSV-1 in the absence of de novo protein synthesis. The induced chimeric genes are regulated as α genes. The gene specifying the viral α-*trans*-inducing factor (α-TIF) has been mapped and sequenced (6, 7, 30). In the virion, it is located in the tegument, i.e., between the capsid and the envelope of the virus (3, 6).

α-TIF maps in the *Bam*HI F fragment of the HSV-1 genome. In the course of studies of the induction of α-chimeric genes in transient-expression systems, we noted that the intact fragment induced the chimeric genes more efficiently than did the intact domain of the α-TIF gene. Moreover, the portions of the *Bam*HI F fragment located 3′ to the α-TIF gene restored, in *trans*, the activity of the α-TIF gene to approximately the same level as that of the intact *Bam*HI F fragment (27). Neither fragment affected expression of the chimeric α genes in the absence of α-TIF.

Earlier studies by Hall et al. (13) have shown that *Bam*HI-F encodes several genes. These studies indicated that hybrid selected transcripts of the two genes located 3′ to the α-TIF gene specify, in an in vitro translation system, proteins with apparent molecular weights of 70,000 and 85,000. To explore further the observed phenomenon, we mapped the transcription initiation and termination sites and sequenced the two genes. The nucleotide sequences predict that the two genes specify proteins with translated molecular weights of 70,527 and 77,357. In this paper, we report that subfragments of the HSV-1 *Bam*HI F fragment containing an uninterrupted copy of the gene specifying the 77,357-molecular-weight protein enhanced, in *trans*, the activity of the α-TIF gene. In contrast, the subfragment containing an uninterrupted copy of the gene specifying the 70,527-molecular-weight protein reduced the activity of α-TIF, either alone or in the presence of the fragment specifying the 77,357-molecular-weight protein.

MATERIALS AND METHODS

Virus and cells. The properties and propagation of HSV-1(F), the prototype HSV-1 strain used in this laboratory, were described elsewhere (9). BHKtk$^-$ cells (BM0348A) were obtained from the Mutant Cell Repository, Camden, N.J.

RNA isolation. Cytoplasmic RNA was extracted at 14 h postinfection from Vero cells infected with 20 PFU per cell as previously described (17).

* Corresponding author.
† Present address: Centers for Disease Control, Atlanta, GA 30333.

TABLE 1. Recombinant plasmids used in these studies

Plasmid	Description[a]	DNA fragment no.	Reference
Plasmids containing α-TK chimeric genes[b]			
pRB314	*Bam*HI-Z inserted into the *Bgl*II site of *Bam*HI-Q in pBR322		31
pRB3354	363-bp *Bam*HI-*Sma*I fragment from *Bam*HI-N fused to the 1.6-kb *Eco*RI-*Nco*I fragment from *Bam*HI-Q in pUC9		27
*Bam*HI F-I′ fragment derivatives			
pRB158	*Bam*HI-F in pUC9		30
pRB502	*Sal*I-J′ in pBR322		
pRB3439	3.8-kb *Bam*HI-*Sal*I fragment of *Bam*HI-F in pUC9	8	27
pRB3441	4.4-kb *Pst*I-*Bam*HI fragment of *Bam*HI-F in pUC9	3	27
pRB3442	1.5-kb *Xho*I-*Bam*HI fragment of *Bam*HI-F in pUC9	5	27
pRB3443	6.6-kb *Bam*HI-*Xho*I fragment of *Bam*HI-F in pUC9	2	27
pRB3458	2.9-kb *Pst*I-*Xho*I fragment of *Bam*HI-F in pUC9	1	30
pRB3555	1.3-kb *Bam*HI-*Sac*I fragment of *Bam*HI-F in pUC9	7	
pRB3606	4.5-kb *Bam*HI-*Asu*II fragment of *Bam*HI-F in pUC9[c]	4	
pRB3608	2.4-kb *Bam*HI-*Mlu*I fragment of *Bam*HI-F in pUC9	6	
pRB3620	1.5-kb *Eco*RI-*Hin*dIII from *Hin*dIII-L in pUC9		
pRB3623	2.2-kb *Asu*II-*Xho*I fragment of *Bam*HI-F in pUC9	13	
pRB3708	*Spe*I linker inserted into *Sca*I site of pRB3724	11	
pRB3711	*Spe*I linker inserted into *Kpn*I site of pRB3724	10	
pRB3724	*Bam*HI-I′ inserted into the *Bam*HI site of pRB3606[d]	9	
pRB3728	*Spe*I linker inserted into *Kpn*I and *Sca*I sites of pRB3724	12	
pRB3734	990-bp *Pst*I fragment of *Bam*HI-F in pUC9		
pRB3735	*Bam*HI I′ in pUC9		
pRB3736	2.5-kb *Hin*dIII-*Kpn*I fragment of *Kpn*I-A into pUC19		

[a] Refer to Fig. 1 for fragment designations and relevant restriction enzyme sites.
[b] The α-TK chimeras are under promoter-regulatory control of the α4 gene.
[c] Actual right endpoint is 130 bp 3′ of the *Asu*II site at residue 31 (Fig. 2).
[d] Fragments are oriented as in the viral genome.

S1 nuclease mapping. DNA fragments were generated by restriction endonuclease cleavage of recombinant plasmid DNA. To label the 5′ end, we labeled DNA fragments with polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) as previously described (26). DNA fragments were labeled at the 3′ end with the Klenow fragment of *Escherichia coli* DNA polymerase and 100 μCi of [α-^{32}P]dCTP as previously described (26). End-labeled DNA was added to 150 μg of infected-cell cytoplasmic RNA and precipitated with 2 volumes of cold ethanol. The pellets were dissolved in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid], (pH 6.4), denatured by incubation at 70°C for 10 min, and hybridized overnight at 61°C. Hybridization was terminated by addition of 10 volumes of S1 buffer (250 mM NaCl, 1 mM $ZnSO_4$, 30 mM NaOAc, 5% glycerol, pH 4.6). S1 nuclease (167 Vogt units; Boehringer GmbH, Mannheim, Federal Republic of Germany) was added, and digestion carried out for 30 min at 45°C. The samples were extracted with phenol-chloroform and then chloroform. Carrier tRNA was added to each sample, followed by ethanol precipitation. Samples were separated by electrophoresis in denaturing 8% polyacrylamide gels.

DNA sequencing. Cloning, sequencing, and assembly of the DNA sequences and subsequent analysis of the completed sequence were done as previously described (29).

Plasmid constructs. The structures of all of the plasmids carrying HSV-1 DNA fragments used in the tests of α-TK chimeric gene expression are shown in Table 1. The two HSV-1 α-TK chimeric gene constructs used in these studies (pRB314 and pRB3354) are both under control of the α4 gene promoter-regulatory domain but differ in the size of the α4 domain fused to the TK gene (Table 1). pRB3724 was constructed by addition of the *Bam*HI I′ fragment (pRB3735) to pRB3606 to reconstruct the *Bam*HI-I′-F junction in the viral orientation. pRB3711, pRB3708, and pRB3728 were derived from pRB3724 by insertion of a 14-base-pair (bp) *Spe*I linker (New England BioLabs, Inc., Beverly, Mass.) into the *Kpn*I site of open reading frame A, the *Sca*I site of open reading frame B, or both sites, respectively (see Fig. 1). The *Kpn*I site was treated with T4 polymerase (New England BioLabs) before ligation, and both pRB3711 and pRB3708 were recut with *Spe*I (New England BioLabs) after colony purification to remove multimer linker insertions before religation. Plasmid DNA was isolated by the Triton X-100 procedure (26) and purified by two cesium chloride density gradient centrifugations. DNA used in transient-expression assays was greater than 80% supercoiled.

Transient-expression assays. Transient-expression assays were done as described elsewhere (20, 30). Briefly, BHKtk$^-$ cells were seeded at an initial density of 0.5×10^5 to 1.0×10^5 cells per well in 10-cm^2 six-well dishes (Costar, Cambridge, Mass.) and grown for 12 to 16 h in Dulbecco modified Eagle medium containing 5% fetal calf serum in an atmosphere of 5% CO_2–95% air. Cells were transfected in parallel on replicate dishes with plasmid DNA (adjusted to 5 μg of total DNA per well with pUC9 vector DNA) after calcium phosphate precipitation (12) and shocked 3.5 h posttransfection with 15% glycerol (11, 35). The cells were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. The cultures were replenished with fresh medium at 24 h and harvested at 40 to 48 h.

TK assays. TK assays were done as previously described

(20, 31). TK activity is expressed as counts per minute of [^{3}H]thymidine converted to thymidylate per microgram of cell lysate protein.

RESULTS

Sequences located 3′ to the α-TIF gene enhance α-TIF-mediated induction of the α-TK indicator gene. In the process of mapping the α-TIF gene, we noted that the intact *Bam*HI F DNA fragment reproducibly induced α-TK genes to a higher level of activity than did the DNA fragment encoding the structural sequences and the promoter-regulatory domain of the α-TIF gene (27). Figure 1 shows the DNA fragments derived from the HSV-1 genome and tested in this study. Table 2 illustrates this phenomenon and demonstrates that sequences mapping 3′ to the α-TIF gene enhance, in *trans*, α-TIF-dependent induction of α-TK genes specifically as follows. (i) Both the intact *Bam*HI F fragment, cloned as pRB158, and fragment 2 reproducibly induced the α-TK indicator gene to levels approximately twofold higher than those observed with fragment 1 or 3, which contained an intact α-TIF gene (experiments 1 and 2, Table 2). (ii) The DNA sequences 5′ from the α-TIF gene, represented as fragment 5, had no effect on α-TIF-mediated induction of the α-TK gene. In contrast, the sequences 3′ from the α-TIF gene (fragment 4) increased α-TIF-dependent induction of α-TK gene to levels higher than those observed with α-TIF alone. In the absence of α-TIF, this effect was abolished (experiment 3, Table 2). (iii) Hall et al. (13) reported the mapping of two genes in the segment of *Bam*HI-F, which in the experiments above are shown to increase the activity of the α-TIF gene. Preliminary studies on a subclone of fragment 4 (pRB3607), containing the proximal 3′ gene, suggested that it reduced the activity of the α-TIF gene (27). Experiment 4 (Table 2) indicated that the DNA fragment containing the distal 3′ gene (fragment 6) enhanced the activity of the α-TIF gene. Deletion of the 5′ end of the distal gene (fragment 7) abolished this effect when compared with results of using the α-TIF gene alone.

These observations raised the question of whether the effect of the sequences mapping 3′ from the α-TIF gene reflected competition for cellular factors able either to enhance or repress α-TIF activity or whether these sequences

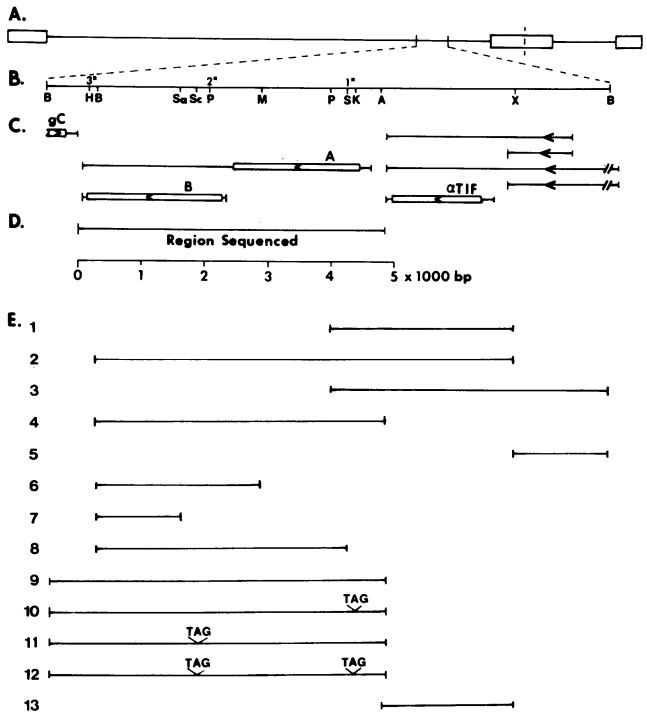

FIG. 1. (A) Schematic representation of the HSV-1 genome showing the region relevant to these studies. (B) Expansion of the region depicted in A which contains both the 0.7-kb *Bam*HI I′ and the adjacent 8.1-kb *Bam*HI F fragments of the HSV-1(F) strain. Numerals with asterisks indicate the locations of the 5′ and 3′ end-labeled probes used in S1 analyses. Only the relevant restriction endonuclease cleavage sites are indicated. Abbreviations: A, *Asu*II; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sc, *Sca*I; X, *Xho*I. (C) Domains (single and double lines), orientation (arrowheads), and coding sequences (double lines) of the mRNAs mapped to the *Bam*HI-I′-F region from open reading frames A and B. The line drawings with arrowheads to the right of α-TIF represent additional mRNAs encoded by the *Bam*HI F fragment which were mapped by Hall et al. (13). gC, Glycoprotein C. (D) Location of the 4,866-bp region sequenced in this study. This region extends from the 3′ terminus of the α-TIF gene previously sequenced (30) to the 3′ coterminus of the transcribed domains of the genes encoding the 70,527- and 77,357-molecular-weight polypeptides. (E) The domains of the cloned HSV-1 DNA fragments tested in transient expression systems in this study. The cloned DNA constructs are described in Table 1.

TABLE 2. Mapping of α-TIF-enhancing activity within the *Bam*HI F fragment of HSV-1

HSV-1 test sequence[a]	Induction level[b]
α-TK[c] plus:	
Expt 1	
None	1.0
*Bam*HI-F	20.0
Fragment 2	20.0
Fragment 3	13.0
Fragment 5	1.3
Expt 2	
None	1.0
Fragment 1	5.4
Fragment 2	14.0
Fragment 3	4.4
Fragment 5	1.0
Expt 3	
None	1.0
Fragment 1	3.6
Fragment 4	1.0
Fragment 6	1.4
Fragments 1 + 4	5.6
Fragments 1 + 6	12.0
Expt 4	
None	1.0
Fragment 1	10.0
Fragment 7	1.0
Fragment 8	1.0
Fragments 1 + 7	9.6
Fragments 1 + 8	14.0

[a] Refer to Fig. 1.

[b] Values are expressed as the ratio of the level of induced expression over the level of basal expression in transient-expression assays similar to those described in Fig. 5. Ratios are based on counts per minute of [^{3}H]thymidine converted to thymidylate per microgram of total cellular protein. Basal expression is given a value of 1.0. Values in this column should be compared with those obtained in the same experiment by using fragment 1 in the absence of additional fragments.

[c] The α-TK indicator gene used in these experiments was in either pRB314 (experiments 1, 2, and 4) or in pRB3354 (experiment 3).

encoded functions capable of modulating α-TIF activity. To answer these questions, we sequenced and analyzed the transcriptional domains of the DNA sequences downstream from the α-TIF gene.

The sequence of the *Bam*HI F fragment domain 3′ from the α-TIF gene. We sequenced (Fig. 1 and 2) a 4,866-bp DNA segment of HSV-1(F) DNA extending leftward in the prototype orientation from the polyadenylation signal shared by α-TIF and two other previously described mRNAs (13) to the polyadenylation signal for glycoprotein C (10). We included in the sequence 146 nucleotides (residues 1 to 146) previously reported at the 3′ end of the αTIF gene (30). In addition, the 5′ end of the sequence reported here overlaps a sequence previously reported for HSV-1(17) by 619 nucleotides (7). There is also an overlap of 72 nucleotides at the 3′ end of this sequence with the sequence reported for the 3′ end of glycoprotein C (10). The bulk of the nucleotide sequence was obtained from a panel of sonication-derived shotgun clones from the *Bam*HI F fragment which hybridized to the 3,652-bp *Bam*HI-to-*Sal*I fragment from *Bam*HI-F (Fig. 1). Additional clones were obtained as required to fill in gaps in the sequence and to sequence the portion of the genome extending into the *Bam*HI I′ fragment. All cloning was done in the pUC9 vector. Both strands of each clone were sequenced with the forward and reverse M13 sequencing primers, and the sequence shown was determined from double-stranded data. Codon usage analyses, done to minimize the possibility that the sequence contained reading frame errors, demonstrated that the nucleotide sequences of two genes have codon usage patterns typical of other sequenced HSV genes sequenced in this laboratory. In addition, since the sequence across the *Bam*HI-F-I′ junction was not determined, electrophoretic separations in high-resolution polyacrylamide gels were done to rule out the possibility that an additional small *Bam*HI fragment could be located between the *Bam*HI F and I′ fragments. Two plasmids were used for this analysis. pRB3715 contains the *Bam*HI I′ fragment cloned into the *Bam*HI site of pRB3443 to reproduce the junction in the viral orientation, and pRB3736 contains a 2.5-kilobase (kb) *Hin*dIII-*Kpn*I fragment which spans the junction. Both clones were cleaved with *Hin*dIII and *Acc*I to yield 228-bp fragments spanning the junction. Electrophoresis of the DNA fragments on a 21-cm 8% polyacrylamide gel failed to show any differences in their migration (data not shown). A minimum fragment of 6 bp would result from tandem *Bam*HI sites, and a shift of this size can be easily resolved on this gel.

Hall et al. (13) reported that this region encodes two mRNAs. One was estimated to be 4,700 nucleotides long (minus polyadenylation) and to encode a γ_2 protein with an apparent molecular weight of 70,000. The other mRNA was estimated to be 2,500 nucleotides long (minus polyadenylation) and to encode a γ_1 protein with an apparent molecular weight of 85,000. The nucleotide sequence (Fig. 2) indicates the presence of two long nonoverlapping reading frames with codon usage typical of HSV genes and corresponding to the transcripts described by Hall et al. (13). To verify the open reading frames, we mapped the 5′ terminus of the 4.7-kb transcript with the end-labeled probes indicated in Fig. 1. Hybridization of the late cytoplasmic RNA to pRB502 DNA that had been cleaved with *Sal*I (probe 1*, Fig. 1) and 5′ end labeled protected a DNA fragment 385 ± 5 bp long (Fig. 3) from S1 nuclease digestion. This located the 5′ end of the mRNA at nucleotide 232 ± 5, 28 nucleotides downstream from the sequence TATAAA beginning at position 204, which could serve as the TATA box (4). Two sequences, AGGGCGGCCC beginning at position 191 and GGGGCG GCGG beginning at position 250, differ by 3 and 2 nucleotides, respectively, from the consensus binding sequence $\underset{TA}{GGGGCG}\underset{TAAT}{GGGC}$ of the transcriptional factor SP1 (5). 5′ mapping of the γ_1 2.5-kb mRNA was done with the end-labeled probes shown in Fig. 1. Hybridization of late cytoplasmic RNA to pRB3734 DNA that had been cleaved with *Pvu*I (probe 2*, Fig. 1) and 5′ end labeled protected a DNA fragment 258 ± 3 bp long from S1 nuclease digestion (Fig. 3). This located the 5′ end of the transcript to position 2541 ± 3, which is 28 bp downstream of the sequence ATAAAAA, which could serve as the TATA box (4). Sequences which differ from the SP1 binding site consensus sequence by two nucleotides and one nucleotide were found beginning at positions 2218 and 2240, but on the opposite strand.

Previous work has shown that both transcripts are coterminal at the 3′ end (13). Hybridization of late cytoplasmic RNA to pRB3620 DNA that had been cleaved with *Hin*dIII (probe 3*, Fig. 1) and 3′ end labeled protected DNA fragments 118 to 119 bp long from S1 nuclease digestion (Fig. 3). The presence of bands differing by 1 nucleotide in length is due to imprecise digestion by the S1 enzyme and the high resolution of the gel in this region. This locates the 3′ terminus of both mRNA transcripts to nucleotide 4818 ± 3, which is 21 nucleotides downstream of the polyadenylation signal AATAAA at position 4798. The 5′ and 3′ termini of the two transcripts yielded values of 4.59 and 2.27 kb for the sizes of the nonpolyadenylated mRNA, and these correspond, respectively, to the 4.7- and 2.5-kb transcripts previously mapped to this region (13). A sequence, CGTGTTCT, beginning at position 4831 corresponds to the consensus sequence YGTGTTYY (Y = pyrimidine) which is found downstream of the polyadenylation signal of many eucaryotic genes and which has been shown to be a factor in efficient formation of 3′ termini (28).

The ATG codons predicted to initiate translation from the two open reading frames were located by comparison of the nucleotide sequence with the consensus sequence described by Kozak (19). In the case of the 4.59-kb RNA, the sequence CCACCATGT was located at position 427. Since it shares a five-of-six match with the consensus sequence CCRCC ATGG, this ATG is a likely candidate for translation initiation. The next ATG, located at position 512, shares only one of the six nucleotides with the consensus and, moreover, this codon is located in another reading frame. Mapping of the 5′ end of the message at nucleotide 232, combined with the predicted initiator ATG at nucleotide 427, gives the 4.59-kb RNA a 5′ nontranslated leader sequence 195 nucleotides long.

The first potential translation initiation codon downstream of the 5′ terminus of the 2.27-kb RNA is located at position 2586 and contains a four-of-six match with the consensus sequence (19). The next potential initiating codon is found 603 nucleotides downstream in a suboptimal environment for translation initiation, in frame with the upstream ATG at position 2586. Given that the ATG at position 2586 is likely to be the initiating codon, the 5′ nontranslated leader sequence would be 45 ± 3 nucleotides long.

The molecular weights and properties of the two polypeptides as predicted from the sequence analysis. The predicted translation initiation site for the 4.59-kb RNA located at position 427 is followed by an open reading frame extending to the next in-frame translation termination codon at position 2419. The polypeptide encoded in this open reading frame would consist of 664 amino acids with a translated molecular weight of 70,527 (open reading frame A,

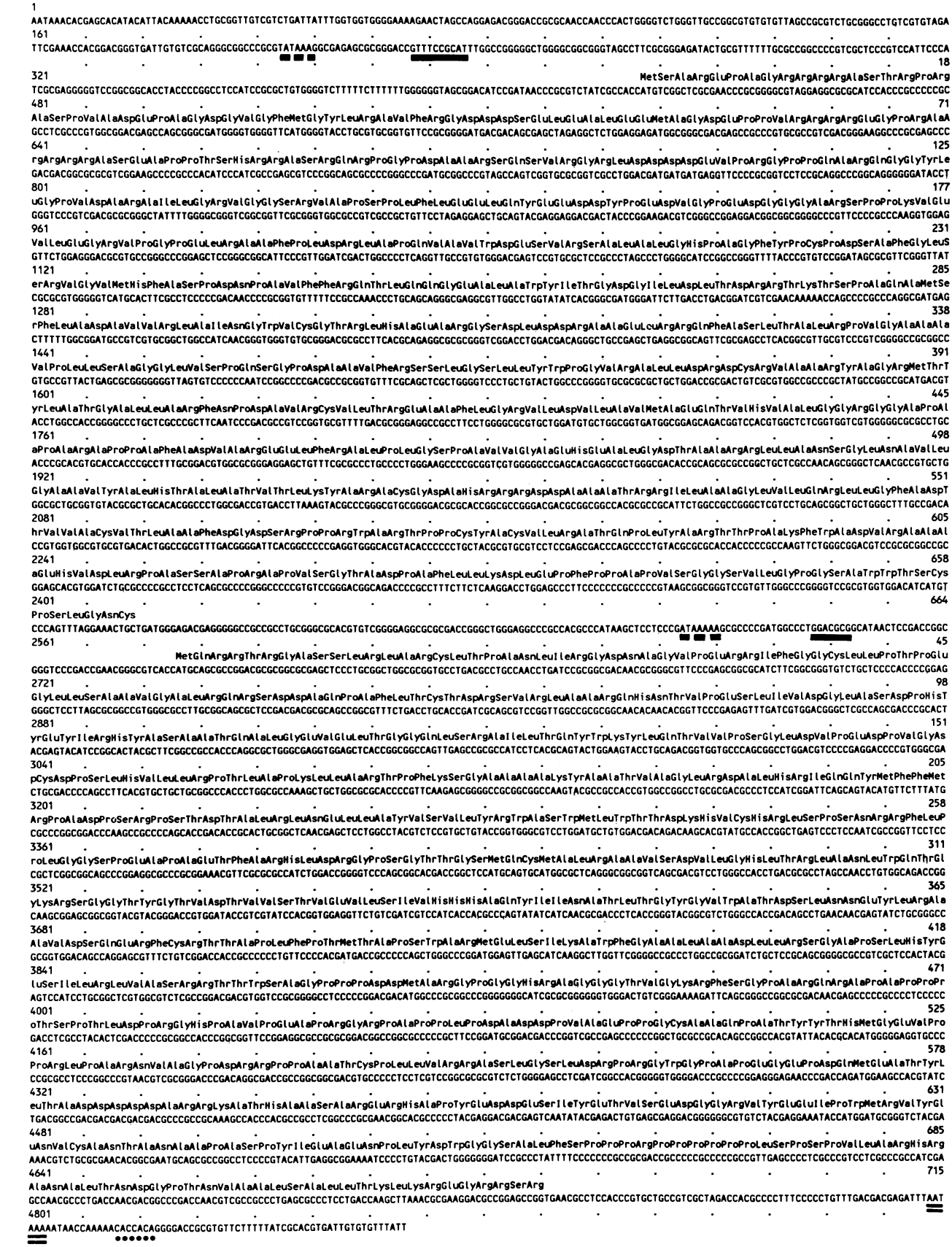

FIG. 2. Nucleotide and predicted amino acid sequence of open reading frames A and B encoding the 70,527- and 77,357-molecular-weight polypeptides. The solid lines indicate the approximate locations of capping sites determined from S1 nuclease analyses. The boldface dots indicate the 3′ coterminus of the mRNA encoded by open reading frames A and B. The broken lines indicate the positions of the TATA boxes upstream from the capping sites. The double solid lines indicate the position of the 3′ coterminal polyadenylation signal.

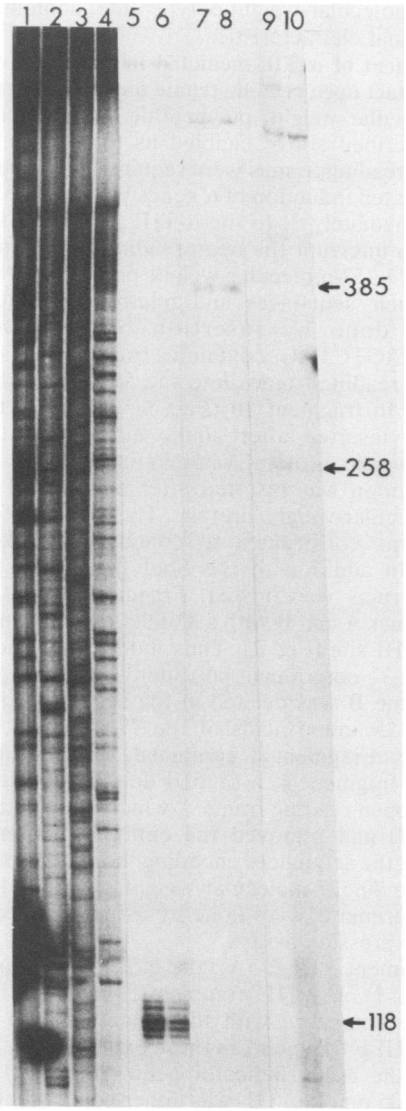

FIG. 3. Autoradiographic images of the labeled DNA probe fragments protected from digestion by S1 nuclease. The sizes of the protected fragments are indicated in nucleotides. 3′ or 5′ end-labeled probe DNA fragments were hybridized with γ (12 to 14 h postinfection) total cytoplasmic RNA and subjected to S1 nuclease digestion, and protected fragments were separated by electrophoresis in 8% denaturing polyacrylamide gels. Lanes: 1 to 4, a DNA sequencing ladder generated as described in Materials and Methods; 5, DNA fragments protected with 3′ end-labeled *Hin*dIII (probe 3*, Fig. 1)-digested pRB3670 DNA; 6, same as lane 5 except that the concentration of probe DNA in the hybridization mixtures was increased twofold; 7, DNA fragments protected with 5′ end-labeled *Sal*I (probe 1*, Fig. 1)-digested pRB502 DNA; 8, same as lane 7 except that the concentration of probe DNA in the hybridization mixture was increased twofold; 9, fragments protected DNA with 5′ end-labeled *Pvu*I (probe 2*, Fig. 1)-digested pRB3734 DNA; 10, same as lane 9 except that the concentration of probe DNA in the hybridization mixture was increased twofold.

Fig. 1). Hall et al. (13) estimated an apparent molecular weight of 70,000 for the polypeptide made in an in vitro translation system with the 4.7-kb mRNA as a template, in agreement with the predicted translated molecular weight. The N-terminal 175 amino acids of this polypeptide may be described as very hydrophilic on the average, with an average hydropathic value of −1.14 based on the values of Kyte and Doolittle (22), as compared with the grand average hydropathic value of −0.4 determined from a large data base of proteins. In this portion of the molecule 36% of the residues are charged, whereas in the carboxy-terminal 489 amino acids only 19% of the residues are charged. Of interest is the predicted arginine-rich domain located between amino acid positions 63 and 75, in which 9 of 13 predicted amino acid residues are arginine (Fig. 4A).

The first in-frame stop codon after the ATG predicted to initiate translation of the 2.27-kb RNA at position 2586 is located at position 4731. The open reading frame extending from position 2586 to 4730 encodes a polypeptide of 715 amino acids with a translated molecular weight of 77,357 (open reading frame B, Fig. 1). The difference between the translated molecular weight of this polypeptide and the reported 85,000 apparent molecular weight of the polypeptide translated in vitro from the 2.5-kb mRNA (13) may reflect the abundance of proline residues (10.6%). The hydropathic analysis is shown in Fig. 4B and, in contrast to

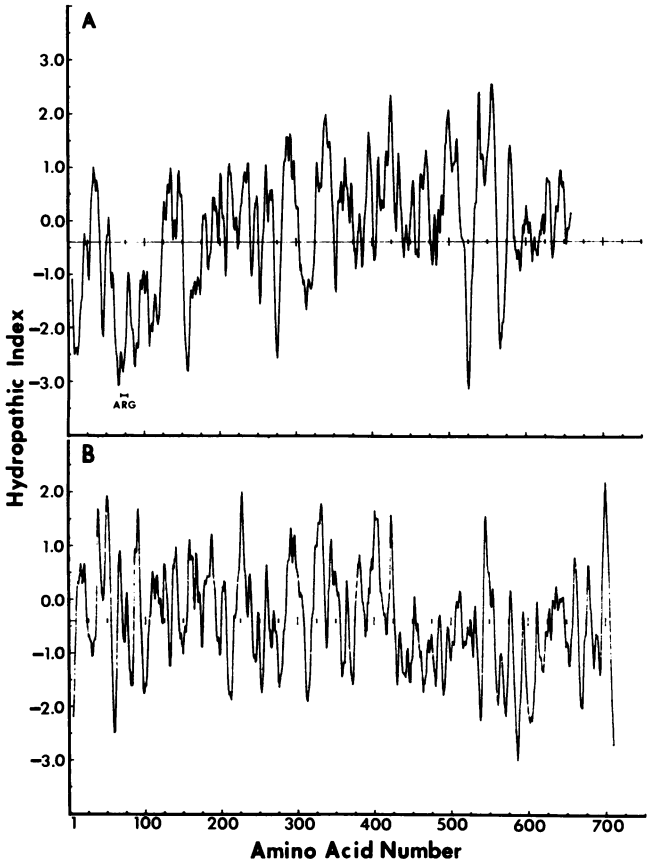

FIG. 4. Hydropathic analysis of the predicted amino acid sequence for the 70,527 (panel A)- and the 77,357 (panel B)-molecular-weight polypeptides. The hydropathic profile was obtained by using the algorithm of Kyte and Doolittle (22) with a moving window of seven residues. The subsequent profile was smoothed for plotting by taking its average in a moving three-residue-wide window. The X axis of the plot is drawn at the average hydropathicity value deduced by Kyte and Doolittle so that points above the line are of above-average hydrophobicity and those below the line are of above-average hydrophilicity. ARG marks the location of the arginine-rich region in the 70,525-molecular-weight polypeptide.

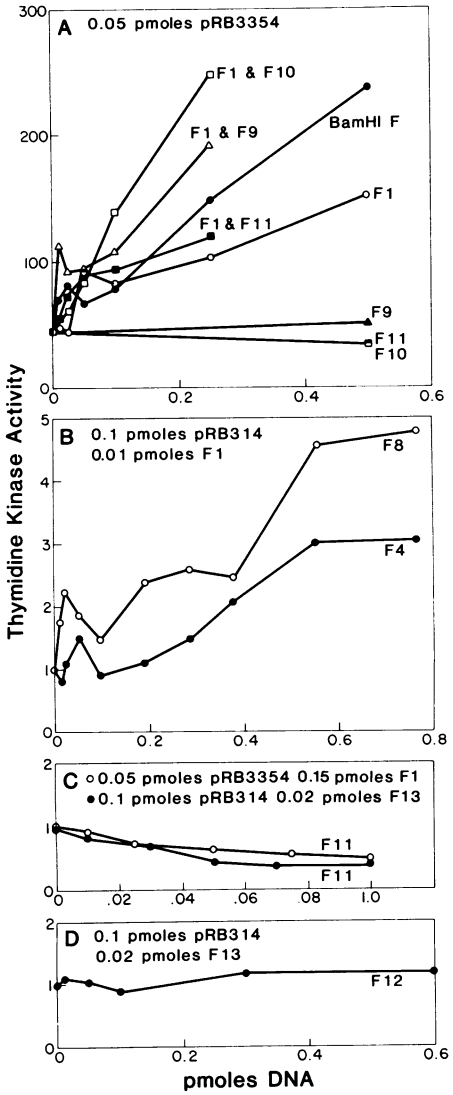

FIG. 5. Thymidine kinase activity in BHKtk⁻ cells transfected with mixtures of α-TK chimeric genes and fragments derived from the *Bam*HI I'-F fragment of HSV-1 DNA. All fragments used in these studies were cloned into a pUC vector and transfected as >80% supercoiled plasmid DNA. (A) Effect of increasing equimolar amounts of fragments 1 and 9, 10, or 11 cotransfected with 0.05 pmol of α-TK (pRB3354) per well. Fragments 9, 10, and 11 were also cotransfected in the absence of fragment 1 without effect on α-TK expression, as shown. The activity of thymidine kinase shown is the average of duplicate wells and is expressed in counts per minute per microgram of cell extract. (B to D) The effects of various cloned DNA fragments cotransfected with constant amounts of fragment 1 or 13 and α-TK chimeric genes (pRB314 or pRB3354, Table 1) on expression of the α-TK gene. The amounts of DNA fragments maintained at a constant concentration per well are indicated in each figure. Panel C shows a comparison of two different α-TK chimeric genes differing in the size of the promoter-regulatory domain fused to the TK gene (Table 1). The effects of various concentrations of fragments 11 and 12 on α-TK in the presence of constant amounts of fragment 13, shown in panels C and D, were obtained in the same experiment. As indicated in Fig. 1, fragment 13 contains the domain of the α-TIF gene, but it lacks the promoter-regulatory domain of open reading frame A present in fragment 1. The TK activity shown was normalized with respect to the activity of α-TK in the presence of the DNA fragment (1 or 13) containing the α-TIF gene. Data points shown are averages of duplicate experimental points. The TK activity of duplicate wells differed from 5 to 15% of actual counts. Background counts were subtracted before normalization.

the 70,527-molecular-weight polypeptide, demonstrates few, if any, unusual characteristics.

Enhancement of α-TIF-mediated induction of α genes requires an intact open reading frame for the gene encoding the 77,357-molecular-weight polypeptide. The nucleotide sequence described above enabled us to determine whether intact open reading frames were required for enhancement of α-TIF-mediated induction of α genes by the DNA sequences located immediately 3' to the α-TIF gene. The strategy we used was to interrupt the open reading frame encoding the 70,527- or 77,357-molecular-weight polypeptide while leaving both their sequences and relative orientation intact. This was done by insertion of an *Spe*I linker, CTAGACTAGTCTAG, containing translational stop codons in all three reading frames into the 5' translated portion of each gene. In fragment 10 (Fig. 1), the TAG termination codon was inserted after amino acid 35 of the 70,527-molecular-weight protein, whereas in fragment 11 (Fig. 1) the TAG codon was inserted after amino acid 130 of the 77,357-molecular-weight protein. The control, fragment 12 (Fig. 1), was constructed to contain the linker at both locations. In addition to the *Spe*I linker constructs, two other constructs were tested. Fragment 4 contained open reading frames A and B with a 3' deletion which extended up to the *Bam*HI site (Fig. 1). Thus, both open reading frames lacked the 3' coterminal polyadenylation site, and open reading frame B was deleted in the sequence encoding the C-terminal 55 amino acids of the 77,357-molecular-weight polypeptide. Fragment 8 contained, in addition to the 3' deletion of fragment 4, a second deletion located in the 5' portion of open reading frame A which extended to the *Sal*I site (Fig. 1) and removed the entire promoter-regulatory domain and the sequences encoding the N-terminal 63 amino acids of the 70,527-molecular-weight polypeptide. Four series of experiments were done by using these constructs in transient-expression assays.

In experiment 1 (Fig. 5A), the effects of fragments 9, 10, and 11 (Fig. 1) on α-TIF (fragment 1)-mediated induction of α-TK were compared with the induction of α-TK by the intact *Bam*HI F fragment. In these experiments, the concentration of the α-TK indicator gene (pRB3354) was maintained at 0.05 pmol. α-TIF was either cotransfected with the indicator gene alone or mixed with an equimolar amount of fragment 9, 10, or 11. Fragments 9, 10, and 11 were also cotransfected with the indicator gene in the absence of α-TIF.

In experiment 2 (Fig. 5B), we compared the effects of fragments 4 and 8 (Fig. 1) on α-TIF-mediated induction of α-TK. In these experiments, both the concentrations of the α-TK indicator gene (pRB314) and α-TIF (fragment 1) remained constant at 0.1 and 0.01 pmol, respectively.

In experiment 3, the effects of fragments 11 (Fig. 5C, closed circles) and 12 (Fig. 5D) on α-TIF-mediated induction of α-TK were compared. In this experiment, the concentrations of the α-TK indicator gene (pRB314) and α-TIF (fragment 13) were maintained at 0.1 and 0.02 pmol, respectively. Experiment 4 (Fig. 5C, open circles) was similar to the comparison of fragment 11 in experiment 3 but differed in both the amounts and structures of the α-TK and α-TIF fragments used. In experiment 4, the concentrations of the

α-TK indicator gene (pRB3354) and α-TIF (fragment 1) were maintained at 0.05 and 0.15 pmol, respectively. The results of experiment 4 (Fig. 5C and D) are shown in different panels for the sake of comparison, but each experiment used the same batch of cells and the same plasmid preparations and were done concurrently.

The conclusions of these experiments were as follows. (i) Consistent with previous results, the intact *Bam*HI F fragment induced α-TK activity approximately twofold greater than that induced by the α-TIF gene (Fig. 5A). The enhanced α-TIF-mediated induction observed with the intact *Bam*HI F fragment was also observed when the downstream sequences containing uninterrupted open reading frames A and B were physically separated from α-TIF (Fig. 5A, fragments 1 and 9).

(ii) The enhancement of α-TIF-mediated induction of α-TK in *trans* was higher when open reading frame A was interrupted than when it was intact. This conclusion is based on two experiments. In experiment 1 (Fig. 5A), the mixture of fragments 1 and 10 enhanced α-TK expression to a higher level than did the mixture of fragments 1 and 9. Experiment 2 (Fig. 5B) indicated that fragments 4 and 8, which lacked the 3′ terminus of open reading frame B, were both capable of enhancing α-TIF-mediated induction of α-TK. However, fragment 8, which lacked the promoter-regulatory domain and the 5′ region of open reading frame A, was more effective in enhancing α-TIF-mediated induction than was fragment 4, which contained an intact open reading frame A. Other experiments (Fig. 5A and C) are consistent with the conclusion that fragment 11, containing an intact open reading frame A and an interrupted open reading frame B, inhibits α-TIF-dependent induction of α-TK.

(iii) The mechanism responsible for modulation of α-TIF-mediated induction of α-TK can be attributed to expression of open reading frames A and B, which encode the 70,527- and 77,357-molecular-weight polypeptides. Interruption of both open reading frames A and B (fragment 12) abolished the effects observed when either one (fragments 10 and 11) or neither (fragment 9) of the two open reading frames was interrupted (Fig. 5A, C, and D).

(iv) Neither polypeptide was able to induce α-TK activity in the absence of α-TIF. Fragments 9, 10, and 11 transfected in the absence of α-TIF did not induce α-TK (Fig. 5A).

DISCUSSION

We found that the putative products of two open reading frames, A and B, of the HSV-1 genome located 3′ to the α-TIF gene domain affected its function; whereas one enhanced the activity of α-TIF, the other caused a reduction in the amount of α-TIF-dependent α gene expression. Several aspects of the results obtained in this paper merit further discussion.

Modulation of α-TIF-dependent induction of α-genes requires expression of the genes encoded in open reading frames A and B. Sequence analysis of the 4,866-bp DNA segment of the HSV-1 genome revealed the presence of two open, nonoverlapping reading frames starting immediately 3′ of the α-TIF gene and extending up to the poly(A) signal for the gene specifying glycoprotein C (10). S1 nuclease analysis of HSV-1 late cytoplasmic RNA identified two 3′ coterminal mRNAs corresponding to the two open reading frames. The sizes of the two RNAs, 4.59 and 2.28 kb, are in close agreement with the 4.7- and 2.5-kb RNAs previously mapped to this region (13). The translated, unmodified molecular weights of the two polypeptides corresponding to open reading frames A and B are 70,527 and 77,357, respectively, in good agreement with the previous estimates of 70,000 and 85,000 obtained by in vitro translation of the two RNA species mapped to this region (13). The significant finding is that the DNA fragments containing the two reading frames A and B act in *trans* to modulate α-TIF-mediated induction of α genes.

Modulation in *trans* of α-TIF activity by the DNA fragment containing the two open reading frames could occur by two different mechanisms; i.e., one which is sequence dependent but not product dependent and one which is gene product dependent. For example, the modulation could occur as a consequence of the competition of the *trans*-acting fragments for cellular repressors specific for DNA sequences contained in the domain of the α-TIF gene. Conversely, the modulation could be a function of products of the two genes affecting either the α-TIF protein or the physiological milieu of the transfected cell. Inasmuch as fragments carrying *Spe*I linkers inserted into the coding sequences of the two open reading frames eliminated the *trans*-acting effects on α-TIF-mediated induction of α-TK genes, we concluded that modulation of α-TIF activity was caused by the products of these genes.

Properties of the putative products of open reading frames A and B. Enhancement of α-TIF-dependent induction of α genes requires at least the 5′ domain of open reading frame B. The predicted amino acid sequence of the 77,357 predicted-molecular-weight polypeptide gives very little insight into its possible function. Computer-assisted analyses (23, 37) of available data bases have not revealed significant homology with other reported polypeptides.

DNA fragments containing open reading frame A decreased α-TIF-dependent induction of α-TK at relatively low concentrations, and this activity also required a 5′ uninterrupted open reading frame. The product of this open reading frame has a predicted translated molecular weight of 70,527, and the predicted amino acid sequence shares little overall homology with those of known proteins. Of interest is the small arginine-rich domain occurring near the amino terminus of the polypeptide. Significant homologies with the arginine-rich domain were present in the predicted amino acid sequence of the BBRF3 (2) open reading frame of Epstein-Barr virus and those of several protamine proteins present in the National Biomedical Research Foundation protein data base (data not shown).

The function of the putative products of open reading frames A and B in relation to α-TIF-dependent induction of α genes. The issues relevant to this section concern (i) the observation that genes affecting the function of α-TIF map so close to the gene or gene product on which they act and (ii) the mechanism by which these putative infected-cell proteins affect α-TIF-mediated induction of α genes.

With respect to the first issue, although functionally related genes do not necessarily map contiguously in a single cluster, subsets of these genes do form small clusters, Examples of such clusters are those formed by the genes specifying sets of α proteins (e.g., α4 and α22, and α47) (25, 33); glycoproteins G, D, and E and the small open reading frame encoding a putative glycoprotein gene located between D and E (1, 32, 33); and the cluster formed by the genes specifying the viral DNA polymerase and major DNA binding protein required for viral DNA synthesis (14). Thus, the clustering of genes related to α-TIF, either in its function as a structural component of the virion or in relation to induction of α genes, has precedent.

trans-acting factors regulating gene expression at the

transcriptional level could be expected to act by one of two mechanisms, i.e., by binding directly to DNA sequences of the regulated target gene or by acting on other transcriptional factors. α-TIF is a structural component of the virion contained in the tegument, i.e., between the capsid and the envelope of the virion (3, 6). α-TIF-dependent induction of α genes requires the presence of a *cis*-acting site in regulatory domains of α genes (20, 24, 27). Attempts to demonstrate that α-TIF binds directly to the promoter-regulatory domains of α genes, and specifically to the α-*trans* induction *cis*-acting site, have not been successful, but current studies indicate that the α-*trans* induction *cis*-acting site binds at least one and possibly several cellular proteins (21). These observations suggest that the polypeptides encoded by open reading frames A and B have one or more of several functions; i.e., (i) in the natural environment of the virion and the infected cell they form a complex with α-TIF and determine its functions, (ii) they affect the physiologic state of the cell in a fashion that is not specific for α-TIF, or (iii) they are transcriptional factors of undefined function in their own right. We cannot differentiate among these various possibilities at the present time, but two observations are of potential interest. (i) The reduction in α-TIF-mediated induction of α genes by relatively low ratios of DNA fragments encoding the 70,527-molecular-weight protein to α-TIF (Fig. 5C) is consistent with the possibility that the product of the gene interacts with α-TIF or other proteins in complexes differing in the ratio of the interacting proteins. (ii) The enhancement of α-TIF-mediated induction of α gene expression leveled off with increasing concentration of DNA fragments containing the intact open reading frame B (data not shown). This observation is consistent with the possibility that the 77,357-molecular-weight polypeptide either interacts with or modifies α-TIF. These observations are consistent with all three hypotheses. Further studies on the function of α-TIF and the products specified by open reading frames A and B are in progress.

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ADDENDUM IN PROOF

After this manuscript was accepted, we compared the sequences of α-TIF, open reading frames (ORFs) A and B with the complete nucleotide sequence of varicella-zoster virus (VZV) kindly provided by Andrew Davison, MRC Virology Unit, Glasgow, Scotland. In addition to the shared amino acid homology between VZV ORF 10 and α-TIF commented on by A. J. Davison (J. Gen. Virol. **67**:1759–1816, 1986), we noted that ORFs A and B of HSV-1 described in this paper share significant homology and identical order with ORFs 11 and 12 of VZV. The HSV-1 ORF A (70,527 M_r, 664 amino acids) shared significant homology with VZV ORF 11 (91,828 M_r, 819 amino acids) in two regions. One is a stretch of approximately 30 amino acids located between residues 350 and 400 of ORF A. The other occurs at the C-terminus of the protein and consists of broken stretches of approximately 10 and 20 amino acids located between residues 540 through 620 of ORF A. The HSV-1 ORF B (77,357 M_r, 715 amino acids) shared significant homology and contained large stretches of identity (20 to 40 amino acids) with VZV ORF 12 (74,271 M_r, 661 amino acids), beginning approximately 30 amino acids from the N-terminus and extending just beyond residue 400 of ORF B. The conservation of order and amino acid sequence homology suggests similar functions. The major divergence between ORFs B and 12 is in the C-terminal region, which, when absent (see Table 2 and Fig. 5) from the polypeptide encoded by the truncated HSV-1 ORF B, did not affect its function.

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