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Genetics

Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of *trans*-inducing α genes

(transcriptional factors/regulation)

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ABSTRACT **The five α genes of herpes simplex virus 1 are the first set of genes to be expressed after infection. Previous studies have shown that α genes resident in eukaryotic cells are induced by infection with herpes simplex virus 1 or 2 but not by other herpesviruses and indicate that the α *trans*-inducing factor was a structural component of the virion. This factor induces genes linked to a bona fide promoter and containing at the 5′ end a small sequence derived from the promoter-regulatory domains of α genes. We report the sequence of a small DNA fragment shown previously to be capable of expressing the α *trans*-inducing factor in transient expression systems. The only gene encoded in its entirety in this fragment is predicted to specify a 479 amino acid protein with a M_r of 53,053. The precise termini of the 1.74-kilobase mRNA specifying this protein were determined in our 5′ and 3′ S1 nuclease protection studies.**

In this paper we report the nucleotide sequence of a gene whose product, a structural protein of herpes simplex virus 1 (HSV-1), appears to induce the transcription of α genes. Relevant to the studies reported here are the following.

(*i*) The HSV-1 genes form several major groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (1). The α genes are the first group to be expressed after infection and are transcribed in the absence of *de novo*, viral protein synthesis by the host RNA polymerase II. At least one α protein, α4, is required for the expression of the β_1 and β_2 genes and, along with other viral factors, for the expression of the later γ_1 and γ_2 genes (2–4).

(*ii*) In an attempt to determine how the host transcriptional machinery differentiates between α and subsequent groups, Post *et al.* (5) constructed a chimeric gene consisting of the 5′ transcribed noncoding sequence and coding sequence of the thymidine kinase (*TK*) gene, a β gene, fused to the capping site and 5′ promoter regulatory domains of the α4 gene. This chimeric *αTK* gene was recombined into the viral genome and shown to be regulated as an α gene. The unexpected finding was that in cells converted from TK^- to TK^+ phenotype with the *αTK* chimera, the TK activity was induced by superinfection with a TK^- HSV-1 mutant, indicating the existence of an α *trans*-inducing factor (αTIF). Furthermore, the induction of the *αTK* chimera resident in the converted cells occurred also in the absence of viral *de novo* protein synthesis. Inasmuch as the α genes are the first set of genes to be expressed, the αTIF had to be introduced into cells during infection. Subsequent studies by Batterson and Roizman (6) indicated that at multiplicities from 0.1 to 10 plaque-forming units per cell, αTIF activity correlated with multiplicity of infection, that other herpesviruses did not induce HSV-1 *αTK* chimeras resident in cells, and that HSV-1(HFEM)tsB7 was one of the most potent inducers. In cells infected with HSV-1(HFEM)tsB7 at the nonpermissive temperature, the capsids containing the DNA migrated to the nuclear pore but the DNA was not released, suggesting that the αTIF was located in the virion outside the capsid.

(*iii*) Analyses of the promoter-regulatory domains of α genes revealed that the promoter and regulatory domains were separable and independently movable and could confer upon both viral and host genes the capacity to be expressed or to be regulated as α genes (7, 8). The regulatory domains of α genes share two types of sequences (9)—i.e., an (A+T)-rich sequence, which confers upon bona fide promoters the ability to be induced as α genes by the αTIF, and (G+C)-rich sequences associated with perfect inverted repeats, which confer upon promoters a higher level of constitutive expression but not the capacity to be induced by the αTIF.

Recently, Campbell *et al.* (10) reported that a HSV-1 DNA fragment induced *αTK* chimeric genes in transient expression systems and concluded that the inducer was a structural protein designated previously as VP16 (11), which is found either in the envelope or in the tegument, an architectural component of the virion situated between the capsid and the envelope (12). In this paper we report the nucleotide sequence of a gene contained in a small HSV-1 DNA fragment that expresses αTIF activity in transient expression systems. The predicted amino acid sequence of the 53,053 M_r protein encoded in its entirety in this fragment does not correspond to the recently published partial sequence of a protein also identified as VP16 (13).

MATERIALS AND METHODS

Virus. The properties and propagation of HSV-1(F), the prototype HSV-1 strain used in this laboratory, were described elsewhere (14).

DNA Sequencing. A panel of random clones from *Bam*HI F fragment was obtained by using the strategy of Deininger (15), except that the vector was pUC9 instead of an M13 vector. The DNA clones were selected for sequencing from the *Bam*HI F clone library by colony hybridization using as probes the *Sal* I fragments 3–5 (Fig. 1). Other clones were obtained as described (16). DNA sequencing was done as described (17).

Computer Analyses. All analyses were done on a Hewlett-Packard 9000 with previously described software (17–20).

RESULTS

Mapping of a Minimal DNA Sequence Capable of Inducing a Chimeric *αTK* Gene in Transient Expression Assays. Campbell *et al.* (10) reported that a HSV-1 fragment con-

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Abbreviations: HSV-1, herpes simplex virus 1; TK, thymidine kinase; bp, base pair(s); αTIF, α *trans*-inducing factor.

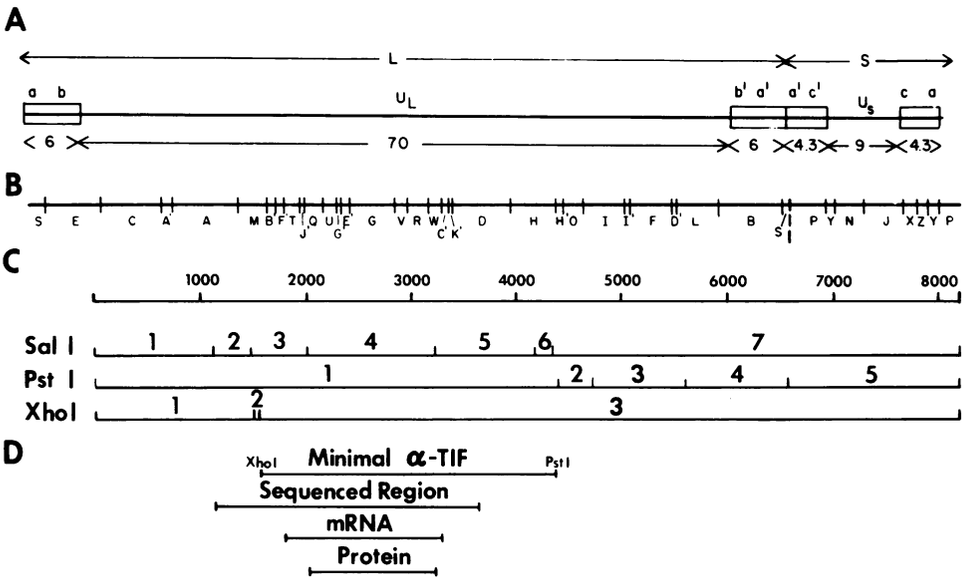

FIG. 1. Schematic diagram of the sequence arrangement in HSV-1 DNA, relevant restriction endonuclease maps, and domains of the αTIF gene. (*A*) Sequence arrangement in HSV-1 DNA showing the location of the unique sequences of the L and S components (U_L and U_S) and of the inverted repeats (ab, b'a'a'c', and ca) flanking the unique sequences (reviewed in ref. 21). The numbers are expressed as $M_r \times 10^{-6}$. (*B*) *Bam*HI restriction endonuclease map of the prototype arrangement of HSV-1(F) DNA. (*C*) Relevant restriction endonuclease maps of the 8.3-kilobase-pair *Bam*HI F fragment. The orientations of this map and all that follow are reversed relative to the prototype orientation. The fragments are labeled with arabic numbers. The scale at the top is in base pairs. (*D*) The domain of the fragment shown to induce αTIF activity, the position of the sequenced region, and the domains of the mRNA and protein of the gene entirely contained in that fragment, shown in relation to the restriction endonuclease maps in *C*.

tained within the domain of *Bam*HI F DNA fragment (Fig. 1) induced an indicator gene fused to an α promoter-regulatory domain in a transient expression system. We have subcloned the *Bam*HI F fragment and found that only those subclones that contain the 2879-base-pair (bp) *Xho* I–*Pst* I fragment from *Bam*HI F (Fig. 1) are capable of inducing a chimeric *αTK* gene in transient expression systems. A representative experiment illustrating the capacity of that fragment to induce the *αTK* chimeric gene is shown in Fig. 2.

In this series of experiments the BHKtk$^-$ cells were transfected with increasing amounts of either the chimeric *αTK* gene or the natural *βTK* gene to determine the level of noninduced expression of the genes as a function of the molarity of the DNA (Fig. 2*A*). Concentrations of the plasmids containing the *αTK* chimera and the natural *βTK* that gave low constitutive levels of expression were then mixed with increasing concentrations of the plasmid carrying the *Pst* I–*Xho* I fragment and transfected into BHKtk$^-$ cells. As shown in Fig. 2*B*, the plasmid containing the *Xho* I–*Pst* I fragment from *Bam*HI F induced expression of the *αTK* chimera but not of the natural *βTK* gene. As a positive control a construct carrying the gene encoding the α4 protein (26), known to induce transient expression of *αTK* genes (ref. 27; unpublished data), was used in cotransfection experiments with both the *αTK* and the *βTK* genes and was observed to induce the expression of the natural *βTK* gene but not the *αTK* chimera (data not shown).

Nucleotide Sequence of the HSV-1(F) Fragment Expressing αTIF Activity in Transient Expression Systems. The *Bam*HI F fragment DNA purified from a cloned plasmid was ligated to itself, sonicated, and separated electrophoretically according to size, and fragments 250–300 bp in length were cloned into the plasmid vector pUC9. The amplified clones were hybridized to probes made from individual *Sal* I fragments from

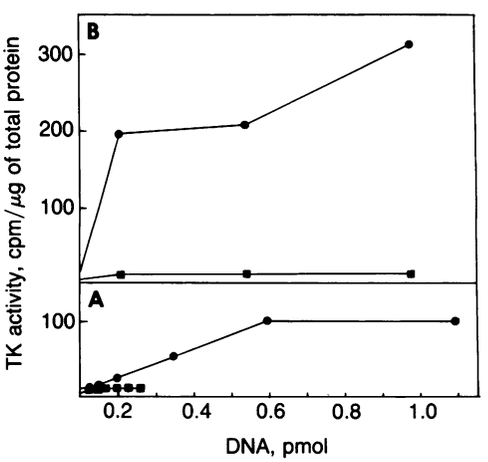

FIG. 2. Expression and induction of *TK* genes in BHKtk$^-$ cells. All experiments shown were done in parallel on replicate six-well dish cultures of BHKtk$^-$ cells (BM0348A, obtained from Mutant Cell Repository, Camden, NJ) containing $\approx 10^6$ cells per well. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The appropriate amounts of supercoiled test DNA made up to 5 μg per well with pUC9 vector DNA were transfected into each well by the calcium phosphate procedure (22, 23). The cells were shocked by the addition of glycerol 3.5 hr after the addition of DNA (24), maintained at 37°C in an atmosphere of 95% air/5% CO_2, and replenished with fresh medium at 24 hr. TK activity was assayed 48 hr after transfection as described (5, 9) and was expressed as cpm of [^{3}H]thymidine converted to thymidilate per μg of total cellular protein. Points indicated with filled circles were obtained by using as the test sequence the *αTK* plasmid pRB3354, which contains the *Sma* I–*Bam*HI promoter-regulatory sequence of the α4 gene (−330 to +33) fused to +50 of HSV-1 TK and extending to the *Nco* I site at +1477, cloned into pUC9 (9). Points indicated by filled squares were obtained by using as the test sequence the *βTK* plasmid pRB103, which carries the *Bam*HI Q fragment containing the natural HSV-1 *βTK* gene (25). (*A*) Transient expression of *αTK* and *βTK* genes. The cultures were transfected with increasing amounts of either pRB3354 or pRB103 DNA to determine the level of TK activity expressed in the absence of αTIF. (*B*) Induction of the natural *βTK* and the *αTK* genes by αTIF. Nonsaturating levels of pRB3354 and pRB103 DNAs (0.05 and 0.1 pmol, respectively), as determined from the experiment described in *A*, were added to separate wells in duplicate cotransfections with increasing amounts of pRB3458 DNA, which carries the *Xho* I–*Pst* I fragment from *Bam*HI F (Fig. 1).

*Bam*HI F and those hybridizing with *Sal* I fragments 3–5 (Fig. 1) were selected for sequencing. Both strands of each clone were sequenced by using both the forward and reverse M13 sequencing primers. The entire sequence was determined on both strands and there was an average of approximately five determinations for each nucleotide. Codon usage analyses were done to assist in ensuring that the open reading frames were not artifacts of faulty sequencing and deoxyinosine was used in sequencing reactions as required to eliminate gel compressions.

The Domain of the Intact Gene Contained in the Fragments Expressing the αTIF. The nucleotide sequence of a DNA segment extending 2520 bp from the leftmost *Sal* I site rightward is shown in Fig. 3. The sequencing data indicated that the *Xho* I–*Pst* I fragment encoding the αTIF contained in its entirety only one large reading frame capable of encoding a complete protein. The minimal coding sequences of this protein extend from an ATG initiating codon at nucleotide 871 to the stop codon at nucleotide 2308. This reading frame is embedded in a network of transcriptional and translational regulatory signals, which could enable the expression and define the domain of the gene. Because the fragment contains only a single intact gene and because a frame shift mutation in the fragment at the position 1404 abolishes the transactivation of *αTK* chimeras (10), this gene is likely to be the one specifying the αTIF.

αTIF Gene Transcription Initiation and Termination Sites. Five mRNA species were reported previously to arise entirely or in part from the domain of the *Xho* I–*Pst* I fragment. Of these, the 1.7-kilobase mRNA (mRNA no. 3, Fig. 4) mapping entirely within the sequenced region encoded a polypeptide with an apparent M_r of 65,000. The 5′ terminus of this mRNA was mapped to a site ≈900 bp to the left of a *Pvu* II site at nucleotide 1402, whereas the 3′ terminus was mapped to the right of the junction between *Sal* I fragment 4 and *Sal* I fragment 5 (28).

The strategies for mapping the 5′ and 3′ termini of the αTIF mRNA are shown in Fig. 4. For mapping of the 5′ terminus, cytoplasmic RNAs extracted at 5.5 and 13 hr after infection were hybridized to pRB3458 DNA that had been cleaved with *Nar* I and 5′ end-labeled with ^{32}P. Of the four hybrid fragments protected from S1 nuclease digestion (Fig. 5*A*), the 748-nucleotide fragment resulted from self-hybridization of the probe, the 577-nucleotide fragment resulted from hybridization with mRNAs designated as nos. 2 and 4 (Fig. 4), the 826-nucleotide fragment resulted from either self-hybridization or hybridization with mRNAs designated nos. 1–3 (Fig. 4), and the 306-nucleotide fragment likely represents the fragment protected by the 5′ terminus of αTIF mRNA. The 306-nucleotide fragment placed the capping site to nucleotide 656 ± 2, located 19–23 nucleotides from a sequence TTAAAT, which could serve as the "TATA" box. A number of

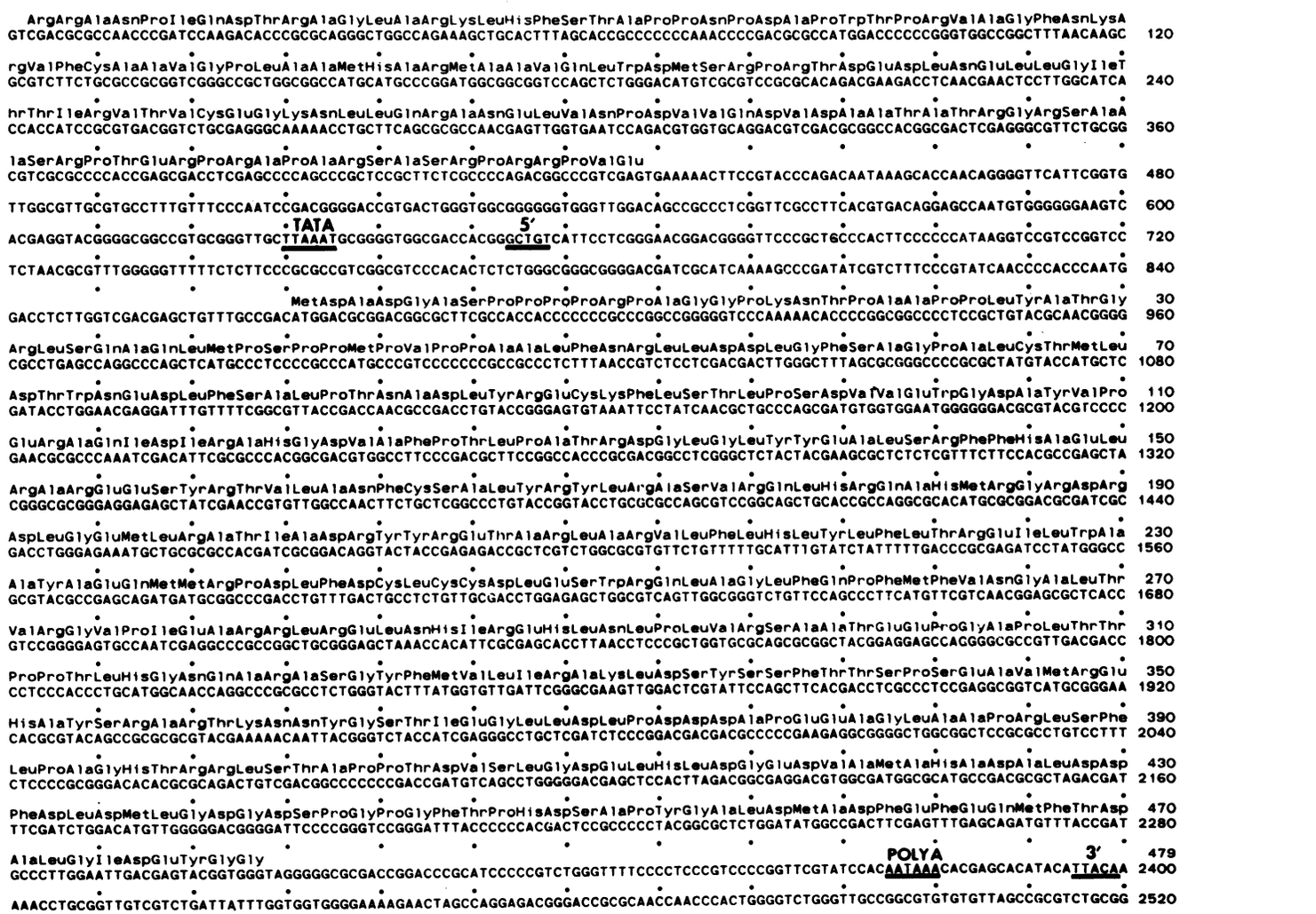

FIG. 3. Nucleotide and predicted amino acid sequence of the domain of the αTIF gene. 5′, mRNA capping site; 3′, 3′ terminus of the transcribed domain of the mRNA; TATA, the putative "TATA" sequence upstream from the capping site; POLY A, polyadenylylation signal. The amino acid sequence upstream from the TATA site is the predicted carboxyl terminus of the protein with an apparent M_r of 42,000 reported to map upstream of the αTIF (28) and to be encoded by mRNAs designated nos. 1 and 2 in Fig. 4.

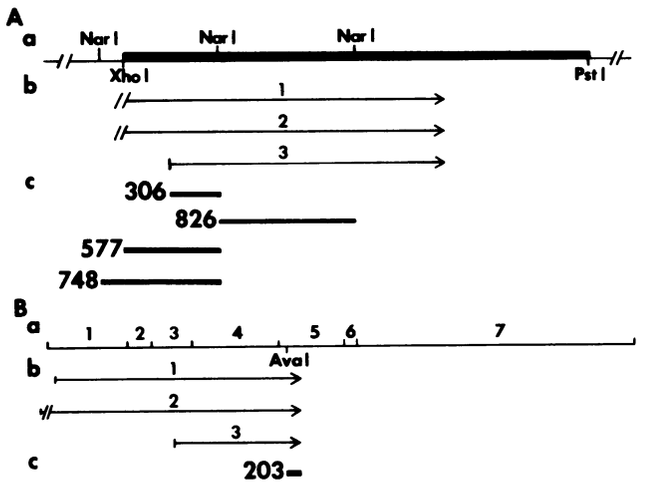

FIG. 4. Fine mapping of the 5′ and 3′ termini of αTIF mRNA. (*A*) Mapping of the 5′ terminus of the αTIF mRNA. a, *Nar* I restriction endonuclease map of the *Xho* I–*Pst* I DNA fragment cloned in vector pUC9. The heavy line represents the HSV-1 sequences. The *Nar* I fragments were 5′ end-labeled with ^{32}P, hybridized with cytoplasmic RNA extracted from infected cells, and digested with S1 nuclease as described (29). b, Previously reported domains of mRNAs mapping in this region of HSV-1 DNA (28). The map location of mRNA no. 3 is that determined in this study. c, Location and size in nucleotides of the 5′ end-labeled DNA probe fragments protected from S1 nuclease digestion shown in Fig. 5. (*B*) Mapping of the 3′ terminus of the αTIF mRNA. a, *Sal* I restriction endonuclease map of *Bam*HI F DNA showing the location of the *Ava* I cleavage site in *Sal* I fragment 5. b, Previously reported domains of mRNAs mapping in and to the left of *Sal* I fragment 5 (shown also in *A*, group b) (28). c, Location and size in nucleotides of the 3′ end-labeled probe DNA fragment protected from digestion by S1 nuclease. The probe consisted of the *Sal* I clone digested with *Ava* I and 3′ labeled with ^{32}P as described (29).

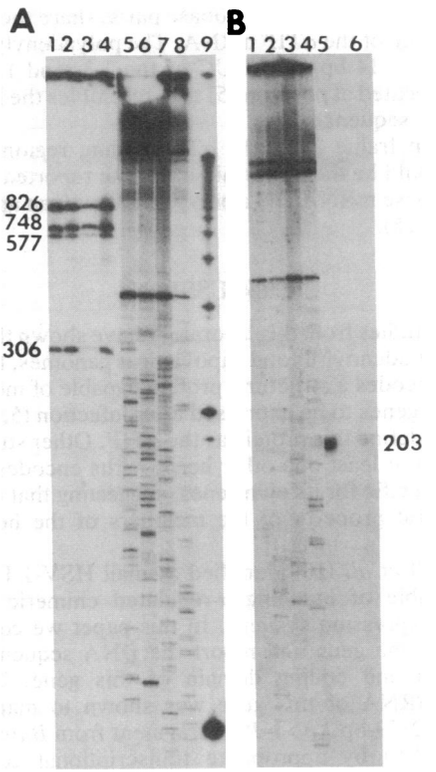

FIG. 5. Autoradiographic images of the labeled probe fragments protected from digestion by S1 nuclease. The molecules protected from digestion with S1 nuclease due to hybridization with cytoplasmic mRNA from infected cells with the 5′ and 3′ end-labeled probes described in the legend to Fig. 4 were electrophoretically separated in denaturing 8% polyacrylamide gels along with size markers. The sizes of the protected fragments are indicated in nucleotides. (*A*) Determination of the location of the 5′ terminus of the αTIF mRNA. Lane 1, 5′ probe fragments protected by 5.5-hr cytoplasmic RNA; lane 2, same as lane 1 except that the concentration of the probe DNA in the hybridization mixture was increased 2-fold; lane 3, 5′ probe fragments protected by 13-hr cytoplasmic RNA; lane 4, same as lane 3 except that the concentration of probe DNA in the hybridization mixture was increased 2-fold; lanes 5–8, a DNA sequencing ladder generated as described in the text; lane 9, an end-labeled 123-nucleotide ladder. (*B*) Determination of the location of the 3′ terminus of the αTIF mRNA and of two additional co-terminal mRNAs. Lanes 1–4, a DNA sequencing ladder; lane 5, 3′ probe fragments protected by 5.5-hr cytoplasmic RNA; lane 6, 3′ probe fragments protected by 13-hr cytoplasmic RNA.

DNA sequences immediately upstream of the TATA box bear strong resemblance to transcriptional regulatory signals found in the promoter-regulatory domains of other herpes simplex virus genes. Specifically, the hexanucleotide core sequence CCGCCC described by McKnight *et al.* (30) was found at position 552 and in an inverted orientation at position 611. The latter of the two is part of a sequence beginning at position 604, AGGTACGGGGCGGCC, which is similar to the first proximal signal, CAGTCGGGGCGGCG, of the *TK* gene (30). The CCAAT sequence at nucleotide 583 could serve as the "CAAT box" found upstream of many eukaryotic genes (31).

For mapping the 3′ terminus, the mRNA was hybridized to a 3′ end-labeled DNA fragment beginning at position 2194. The 203-nucleotide fragment (Fig. 5*B*) protected from S1 nuclease digestion positioned the encoded 3′ terminus of the mRNA molecule to nucleotide 2397 ± 2. Consistent with this finding, a polyadenylylation site was found at position 2375—i.e., 65 nucleotides downstream of the stop codon. This polyadenylylation site would also serve the 3.2-kilobase β and the 3.8-kilobase γ_1 mRNAs designated nos. 1 and 2 (Fig. 4). The nucleotide sequence at and downstream of this site—i.e., AAUAAA...(12 bp)...CAUUA...(0 bp)...CAAA—closely resembles the consensus sequence AAUAAA...(11 bp)...CAYUG...(1 bp)...NAAA found in the vicinity of other polyadenylylation sites by Berget (32).

αTIF Translation Initiation Site. Two potential translation initiating ATG codons are found in the 5′ domain of the mRNA. Comparison of the nucleotides surrounding the initiating codons with the consensus sequence CCRCC*ATG*G derived by Kozak for efficient initiators of translation (33) indicates that the sequence aCcCa*ATG*G at position 838 has a three out of six match, whereas the sequence CCGaC*ATG*G at position 871 has a five out of six match with the consensus, suggesting that the ATG at position 871 is the primary translation initiation site of αTIF. The conclusion that the ATG at nucleotide 871 is the primary site of translation initiation is consistent with the observation that insertion of an 8-bp linker into the *Sal* I site at position 850 did not disrupt the induction of α genes in a transient expression system (10).

Stop Codon. The open reading frame beginning with the ATG at position 871 continues for 1437 nucleotides until a stop codon is encountered at position 2308. The open reading frame encodes a polypeptide of 479 amino acids with a M_r of 53,053.

Other Genes Mapping in the Vicinity of αTIF. Within the domain of the sequenced DNA fragment and 5′ to the αTIF gene there is present an open reading frame terminating at nucleotide 428. This reading frame very likely represents the 3′ terminus of the gene specifying the polypeptide with an apparent M_r of 42,000 translated from a family of four colinear mRNA species (28). As noted earlier, the longer two

RNA species, 3.2 and 3.8 kilobase pairs, share the polyadenylylation site of the αTIF mRNA. The polyadenylylation site AAUAAA...(14 bp)...CAUUC of the 1.3- and 1.8-kilobase mRNAs located at position 451 also resembles the Berget (32) consensus sequence.

An open frame 3′ to the αTIF coding region (data not shown) could be the 5′ domain of a gene reported to encode a 4.7-kilobase mRNA and a polypeptide with an apparent M_r of 70,000 (28).

DISCUSSION

Previous studies from this laboratory have shown that, unlike the smaller adenovirus and papovavirus genomes, the HSV-1 genome encodes a structural protein capable of inducing the first set of genes to be expressed after infection (5, 6). In this paper, we define this protein as the αTIF. Other studies have shown that at least one other herpesvirus encodes a similar product specific for its own genes, suggesting that αTIF may be a general property of the members of the herpesvirus family.*

Campbell *et al.* (10) identified a small HSV-1 DNA fragment capable of inducing α-regulated chimeric genes in transient expression systems. In this paper we confirm the location of the gene and report the DNA sequence of the transcribed and coding domain of this gene. The 1.74-kilobase mRNA of this gene was shown to map entirely within the 2879-bp *Xho* I–*Pst* I fragment from *Bam*HI F and to be flanked by appropriate transcriptional control sequences. The predicted 479 amino acid protein does not have an unusual amino acid composition or predicted secondary structure and its function is not readily deduced from its sequence. Because the αTIF requires for induction the presence of a specific small sequence in the promoter-regulatory domain and because of its virus specificity, it is likely that it acts by DNA sequence recognition.

The early studies on the nature of this protein suggested that it is a located in the virion outside the capsid—i.e., in the tegument or the envelope of the virus. Campbell *et al.* (10), using a monoclonal antibody, identified the *in vitro* translation product of RNAs homologous to a sequence equivalent to the *Xho* I–*Pst* I fragment used in our studies as the infected cell polypeptide no. 25. This polypeptide corresponds to virion protein no. 16 (VP16). The authentic VP16 is present in 390–580 molecules per virion (34); it appears to be associated with viral membrane glycoproteins in virosomes derived from virion envelopes (35). Recently, however, Welling-Wester *et al.* (13) reported the amino-terminal sequence of a 57,000 M_r protein purified by high-pressure liquid chromatography that reacts with a monoclonal antibody to VP16 derived independently from the monoclonal antibody used by Campbell *et al.* (10). The reported sequence of this protein does not correspond to the predicted amino acid sequence of the αTIF protein obtained in our study nor to any protein able to be encoded in any of the reading frames of the sequenced DNA fragment. The availability of the αTIF sequence should permit rapid identification of the protein and its location in the virion.

*Stinski, M. F., Roehr, T. J. & Jeang, K.-T., *Ninth International Herpesvirus Workshop*, August 24–29, 1984, Seattle, WA, p. 177 (abstr.).

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