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# The Promoter and Transcriptional Unit of a Novel Herpes Simplex Virus 1 α Gene Are Contained in, and Encode a Protein in Frame with, the Open Reading Frame of the α22 Gene

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**The herpes simplex virus type 1 genome encodes a set of genes (α genes) expressed in the absence of de novo viral protein synthesis. Earlier studies have shown that the product of the α22 gene, a member of this set, is nucleotidylylated by casein kinase II and phosphorylated by viral protein kinases encoded by $U_L$13 and $U_S$3. Mutants lacking the carboxyl-terminal domain starting with amino acid 200 exhibit reduced capacity to replicate in primary human cell strains or in cells of rodent derivation and also exhibit reduced expression of a subset of γ or late genes. We report that the domain of the α22 gene is transcribed by two 3′-coterminal mRNAs. The longer transcript reported encodes the 420-amino-acid α22 protein, whereas the shorter transcript reported here encodes a protein containing the carboxyl-terminal 273 amino acids of the α22 protein. The shorter gene is designated $U_S$1.5. The $U_S$1.5 mRNA is synthesized in cells infected and maintained in the presence of cycloheximide and under other conditions which restrict viral gene expression to α genes. In-frame insertion of linkers encoding 18, 21, or 22 amino acids after codon 200 or 240 of the α22 protein did not affect the known functions or phenotype associated with the wild-type α22 gene or its product. Earlier studies have placed the nucleotidylylated sequences in the amino-terminal portion of the protein. The results of these studies indicate that the $U_S$1.5 gene encodes the functions associated with replication in human primary or rodent cells and optimal expression of α0 and γ genes. This finding brings the number of genes known to map in the unique short region of the herpes simplex virus type 1 DNA to 14 and the total number of different genes to 78.**

Current literature contains references to 77 different genes encoded by herpes simplex virus type 1 (HSV-1) (37). These genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion. The α genes are expressed first (12, 13). Their hallmark is the presence of a homolog of the consensus sequence 5′NCGyATGnT AATGArATTCyTTGnGGG in the 5′ nontranscribed domain of the gene (16, 21). This sequence serves as the response element of the virion protein VP16, which, in conjunction with cellular proteins, binds to it and transactivates the downstream gene after infection (16, 22). The operational definition of the α genes is transcription of the gene in cells infected and maintained in the presence of inhibitors of protein synthesis (12, 13). At present, five open reading frames (ORFs) qualify as α genes (12, 13). These are α0, α27, α4, α22, and α47. α4 codes for the major regulatory protein encoded by the virus. The protein encoded by this gene binds to viral DNA and regulates viral genes both positively and negatively (6, 7, 10, 15, 18, 23, 24, 26). α0 encodes a promiscuous transactivator (9, 27, 31). α27 encodes a protein which regulates the processing of viral RNA (33). α47 encodes a protein reported to inhibit the presentation of antigenic peptides to $CD8^+$ cells (39). Lastly, to α22, the subject of this article, have been ascribed several functions critical for replication of the virus in cells that it normally infects. Specifically, mutants carrying a deletion in the carboxyl-terminal half of the α22 gene multiply as well as wild-type virus in Vero and HEp-2 cell lines but not in rodent cells or primary human cell strains (28, 35). In the restricted cells $\alpha22^-$ virus exhibits a reduction in the expression of a subset of late genes, and there is a decrease in the amounts of both α0 mRNA and protein accumulating in these cells (29).

The product of the α22 gene, infected-cell protein no. 22 (ICP22), consists of 420 amino acids, is extensively processed, and forms five bands on electrophoresis in denaturing polyacrylamide gels (2). Posttranslational processing includes phosphorylation related to the viral protein kinases encoded by $U_L$13 and $U_S$3 (30) and nucleotidylylation ascribed to casein kinase II (4, 5, 25). Some of the functions described above, especially the expression of a subset of late genes and full expression of the α0 gene, appear to be related to the modifications of ICP22 by the $U_L$13 gene inasmuch as the phenotype of the $U_L13^-$ viruses cannot be differentiated from that of R325, a recombinant HSV-1 strain with a deletion in the carboxyl terminus of the α22 gene (28, 29).

In this article we report the existence of a transcript which is 3′ coterminal with the mRNA encoded by the α22 gene and which directs the synthesis of a protein containing amino acids 147 to 420 of the ICP22 protein. We have designated the 273-codon ORF expressed by this transcript $U_S$1.5. We also report that the in-frame insertion of 18, 21, or 22-amino-acid linkers after amino acid 200 or 240 did not affect the wild-type phenotype of ICP22 as described above. This suggests, in conjunction with earlier data, that the product of the α22 gene expresses at least two sets of functions and that the functions responsible for wild-type expression of the α0 gene and that of

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a subset of late genes map downstream of the site of the linker insertions.

## MATERIALS AND METHODS

**Cell lines and viruses.** Vero and HEp-2 cell lines were from the American Type Culture Collection. The 143TK$^-$ cells were obtained from Carlo Croce. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (8); like many isolates subjected to few serial passages in cells in culture, it carries temperature-sensitive α4 genes, and genetically engineered recombinants derived from HSV-1(F) retain this phenotype. At 39.5°C cells infected with HSV-1(F) express predominantly α genes. Recombinant virus HSV-1(F)Δ305 lacks the 501-bp *Sac*I-*Bgl*II fragment from the *Bam*HI Q fragment encoding the viral thymidine kinase (*tk*) gene (28). R321, derived from HSV-1(F)Δ305, carries the HSV-1(F) *tk* gene inserted into the *Pvu*II site of the α22 gene (28).

**Construction of plasmids.** Plasmid pRB4271 contained the *Sac*I-to-*Kpn*I fragment of the domain of the α22-$U_S2$ genes (Fig. 1, line 4) cloned into the *Sac*I and *Kpn*I restriction endonuclease sites of pGEM3z. The orientation is such that the SP6 polymerase transcribes $U_S2$ and the T7 polymerase transcribes $U_S1$. Plasmid pRB138 (28) carries the HSV-1 *Bam*HI N fragment inserted into the *Bam*HI site of pBR322. The *Pvu*II site in the vector of pRB138 was destroyed to make the *Pvu*II site in the HSV-1(F) sequences unique. Plasmid pRB4648 contained the *Eco*NI-to-*Aat*II fragment of pRB138 blunted with T4 polymerase and cloned into *Sma*I-cut pGEM3Z. The orientation is such that the SP6 polymerase transcribes $U_S1$ whereas the T7 polymerase transcribes $U_S2$ (Fig. 1B). pRB4719 was constructed by insertion in frame of the oligonucleotide created by hybridization of the single-stranded oligonucleotides 5′-CTGACGAGTACGACGACGCAGCC GACGCCGCCGGCGACCGGGCCCCGAAGCTTG-3′ and 5′-CAAGCTT CGGGGCCCGGTCGCCGGCGGCGTCGGCTGCGTCGTCGTACTCGTCA G-3′ into the *Pvu*II site of pRB138. The double-stranded oligonucleotide encodes an ICP4 epitope that reacts with a monoclonal antibody described previously (14) and a *Hin*dIII restriction endonuclease cleavage site. pRB4844 was constructed by the in-frame insertion of an oligonucleotide created by hybridization of the sequences 5′-GAAGGGACAGAAGCCCAACCTGCTAGAC CGACTGCGACACCGCAAAAACGGGTACCGACACTT-3′ and 5′-AAGT GTCGGTACCCGTTTTTGCGGTGTCGCAGTCGGTCTAGCAGGTTGGG CTTCTGTCCCTTC-3′ into the *Pvu*II site of pRB138. The double-stranded oligonucleotide encodes a human cytomegalovirus (CMV) epitope that reacts with a monoclonal antibody described previously (20) and a *Kpn*I restriction endonuclease cleavage site. Plasmid pRB4862 was constructed by in-frame insertion of the oligonucleotide created by hybridization of the sequences 5′-GAAGGGACAGAAGCCCAACCTGCTAGACCGACTGCGACACCGCAA AAACGGGTACCGACACACGTG-3′ and 5′-GTGTGTCGGTACCCGTTTT TGCGGTGTCGCAGTCGGTCTAGCAGGTTGGGCTTCTGTCCCTTCC AC-3′ into the *Dra*III site of pRB138. The oligonucleotide encodes the CMV epitope described above and a *Kpn*I restriction endonuclease cleavage site. The nucleotides surrounding the sites of insertion of all oligonucleotides were sequenced to verify that the amino acid sequences were inserted in frame. All oligonucleotides were synthesized on an Applied Biosystems model 308B DNA synthesizer.

**Construction of recombinant viruses.** Recombinant viruses R4719, R4844, and R4862 were constructed as described in detail elsewhere (28). Briefly, R4719, R4844, and R4862 were constructed by cotransfection of intact R321 DNA and plasmid DNA of pRB4719, pRB4844, and pRB4862, respectively, into rabbit skin cells. The progeny of the transfections were plated on 143TK$^-$ cells in the presence of bromodeoxyuridine. In the presence of the drug, only the recombinant *tk*-minus viruses were able to replicate and form plaques. Individual plaques were picked and plaque purified again, and their DNAs were tested for the presence of the oligonucleotide insert by hybridization of electrophoretically separated *Bam*HI and *Hin*dIII (R4719) or *Sac*I and *Kpn*I (R4844 and R4862) restriction endonuclease digests.

**RNA analyses.** Rabbit skin or HEp-2 cells grown in 150-cm$^2$ flasks were exposed to 10 PFU of wild-type or mutant virus per cell and harvested at the times indicated in Results. For infections in the presence of cycloheximide, 100 μg of drug (Sigma) per ml of medium was present both during exposure of the virus to cells and during subsequent incubation. Cytoplasmic RNA was extracted as previously described (36) and digested with amplification grade DNase (Gibco-BRL) according to the manufacturer's instructions. For Northern (RNA) analyses, approximately 20 μg of RNA was loaded per lane and separated in a 1.5% agarose gel with formaldehyde and ethidium bromide. The gels were then photographed under UV light, and the RNA was transferred to Zeta-probe membrane (Bio-Rad) in 10× SSC (1.5 M NaCl plus 0.15 M trisodium citrate). The blots were air dried and baked at 80°C in a desiccating oven. Nick-translated probes were generated by using the plasmids indicated below and the Renaissance nick translation system (Dupont NEN). Riboprobes were generated by using the plasmid templates indicated below and polymerase with the Gemini riboprobe system according to the manufacturer's directions (Promega). Hybridizations were done at 65°C with nick-translated DNA probes or at 85°C with riboprobes overnight in hybridization solution (1 mM EDTA, 0.5 M $NaH_2PO_4$ [pH 7.2], 7% sodium dodecyl sulfate [SDS]).

For primer extension analyses 5 μg of RNA was mixed with 300 ng of labeled primer no. 89 containing the sequence 5′-GGCGGTTGGCACTGCCGT-3′. The samples were then boiled for 5 min and quick-chilled on ice. To the reaction mixtures were added 40 U of RNasin (Promega); 1 mM (each) dATP, dCTP, dGTP, and dTTP; buffer (final concentrations, 50 mM KCl, 10 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$); and 1 μl of mouse mammary tumor virus reverse transcriptase (Gibco-BRL) in a final volume of 20 μl. The reaction was carried out

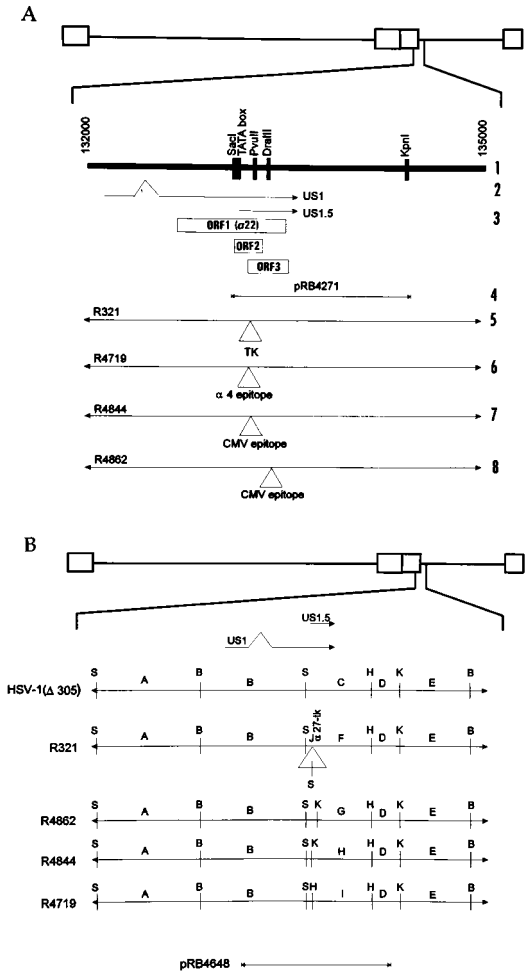


FIG. 1. Schematic diagrams of the HSV-1 region analyzed in these studies. (A) The top line represents the HSV-1 genome. The rectangles represent the inverted repeats flanking the unique (single line) long and unique short sequences. Line 1, DNA sequences comprising the domain of the α22 gene and showing key restriction endonuclease cleavage sites and the TATA box immediately 5′ to the transcription initiation site of the $U_S1.5$ transcript; line 2, the positions of the α22 transcript and the $U_S1.5$ transcript (the wavy line represents the intron upstream of the translation initiation site of the α22 gene); line 3, representation of the locations of the three largest ORFs from the three reading frames contained in the $U_S1.5$ transcript; line 4, position of the HSV-1 sequences of pRB4271 represented in the riboprobe used for analyses of viral mRNAs; line 5, position of the α27-*tk* gene insertion into R321, the parental recombinant virus for each of the epitope-tagged recombinant viruses in lines 6 to 8, described below; line 6, schematic representation of R4719 containing an α4 epitope inserted in frame with ORF 3 of the $U_S1.5$ transcript; line 7, schematic representation of R4844 containing a CMV epitope in frame with ORF 2 of the $U_S1.5$ transcript; line 8, schematic representation of R4862 containing a CMV epitope in frame with ORF 1 (α22) of the $U_S1.5$ transcript. (B) Representation of the restriction endonuclease sites and corresponding restriction endonuclease DNA fragments pertaining to the domain of the α22 gene. The letters above vertical lines represent cleavage sites. The letters on horizontal lines between vertical lines represent DNA fragments generated by cleavage of the DNA. The fragment designations shown here are identical to those described in the text and shown in Fig. 5. The last line indicates the location of the DNA sequences cloned in pRB4648 and used for analyses of recombinant viral DNAs. Abbreviations for restriction endonucleases are as follows: S, *Sac*I; B, *Bam*HI; H, *Hin*dIII; and K, *Kpn*I.

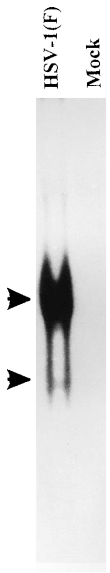


FIG. 2. Autoradiographic image of denatured, electrophoretically separated RNA hybridized with the HSV-1 sequences located as shown in Fig. 1, line 4. Cytoplasmic RNAs extracted from mock-infected or HSV-1(F)-infected cells at 12 h after infection were electrophoretically separated under denaturing conditions and hybridized with an SP6-directed riboprobe from the template pRB4271. Arrows indicate the two viral RNA species which reacted with the riboprobe.

at 42°C for 1 h. A 10-μl portion of sequencing stop solution (United States Biochemicals) was added to each reaction mixture. A 3-μl portion of each reaction mixture was loaded per lane of a 6% polyacrylamide–7 M urea gel following boiling of the samples for 5 min. After electrophoresis, the gel was dried and exposed to Kodak X-Omat film.

**Analyses of proteins.** Rabbit skin cells in a 25-cm-diameter flask were infected with 10 PFU of the viruses indicated below per cell. For infections at 39.5°C, cells were exposed to virus for 1 h at 4°C and then placed in fresh medium at 39.5°C. For infections at 37°C, the cells were exposed to the virus and maintained at 37°C. The cells were harvested by scraping and pelleted by centrifugation at times after infection indicated in Results, resuspended in 100 μl of disruption buffer (12.5 mM Tris HCl [pH 6.8], 0.5% SDS, 2.5% glycerol, 5% β-mercaptoethanol), and boiled immediately for 10 min. The solubilized proteins were then subjected to electrophoresis in denaturing polyacrylamide gels (50 μl per lane), transferred to a nitrocellulose membrane (Schleicher & Schuell), and reacted with appropriate antibody, and the bound antibody was visualized with antibody conjugated to alkaline phosphatase (Bio-Rad). Monoclonal antibodies to ICP4 (H943) (14) or to ICP0 (H1083) (1) or ICP27 (H1117) (29) were purchased from the Goodwin Institute. The monoclonal antibody to $U_S11$ (CL28) was described elsewhere (32). The polyclonal rabbit antiserum R77 directed against an amino-terminal peptide of ICP22 was described previously (2).

## RESULTS

**Detection of a novel transcript within the α22 gene domain.** In an effort to detect low-abundance transcripts in the domain of the α22 gene, strand-specific riboprobes were hybridized with electrophoretically separated, denatured cytoplasmic RNAs extracted from HEp-2 cells at 12 h after infection. In Fig. 1, line 4 shows the position of the sequences contained in the pRB4271 probe and line 2 shows the position of the α22 mRNA with which this probe was expected to hybridize (38). The SP6-driven riboprobe generated from the pRB4271 template detected the $U_S1$ transcript as well as a novel transcript of approximately 1 kb as determined from the relative migration of rRNA (Fig. 2). This second transcript was very low in abundance compared with that of α22 at 12 h after infection. The T7-driven riboprobe detected only the $U_S2$ transcript (data not shown). For reasons indicated later in the text, the novel transcript was designated $U_S1.5$.

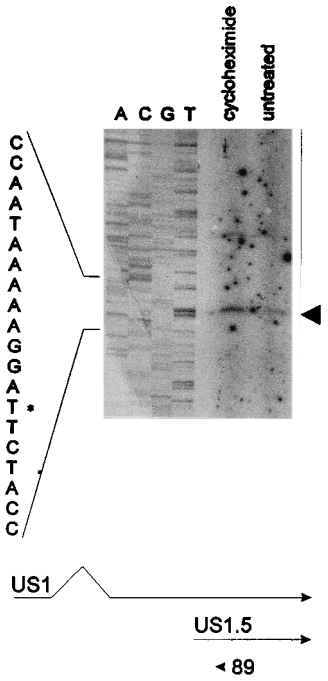


FIG. 3. Autoradiographic images of electrophoretically separated DNA from primer extension analyses to determine the 5′ end of the $U_S1.5$ transcript. Lanes A, C, G, and T represent a sequencing reaction using pRB4648 DNA and primer 89. Cycloheximide-treated and untreated HSV-1(F)-infected cytoplasmic RNAs extracted at 6 h after infection were used for the primer extension reactions with primer 89. The arrow indicates the extended species seen in both lanes. The sequence of the immediate area of the extended species is shown on the left with the asterisk indicating the start site. The diagram below the gel illustrates the position of the primer relative to α22 and $U_S1.5$.

**Fine mapping and temporal pattern of synthesis of the $U_S1.5$ mRNA.** The 5′ terminus of the novel transcript was determined by primer extension analyses. Preliminary Northern analyses had indicated that the $U_S1.5$ transcript appeared with the same kinetics as α22 mRNA (i.e., in the presence of cycloheximide; data not shown). Therefore, primer extension analyses were done on cytoplasmic RNA prepared from both untreated and cycloheximide-treated cells harvested at 6 h after infection. As shown in Fig. 3, extension of primer no. 89, described in Materials and Methods and located approximately 80 bases upstream of the *Dra*III site (Fig. 1, line 1), yielded a single species of approximately 215 nucleotides in reactions with both cycloheximide-treated and untreated RNAs. Sequence analyses of the region upstream of this site for characteristics of a promoter showed a putative TATA box 10 bases upstream of the mapped start site, one Sp1 binding site further upstream, and one Sp1 binding site downstream (Fig. 4) (8). S1 analyses to identify the 3′ terminus of the RNA revealed a single 3′ terminus identical to that for the α22 transcript, suggesting that the novel transcript and the α22 mRNAs are coterminal (data not shown). On the basis of these results, the novel transcript was designated $U_S1.5$.

**Construction of recombinant viruses to detect a protein product associated with the $U_S1.5$ transcript.** To determine whether $U_S1.5$ encodes a protein, a sequence encoding an epitope tag was inserted into the largest ORF of each of its three reading frames (Fig. 1, lines 3 and 6 to 8). Recombinant virus R4862 contained a CMV epitope tag in frame with the ICP22 ORF at the *Dra*III site. The net effect of this insertion was the insertion in frame with ICP22 of the amino acids

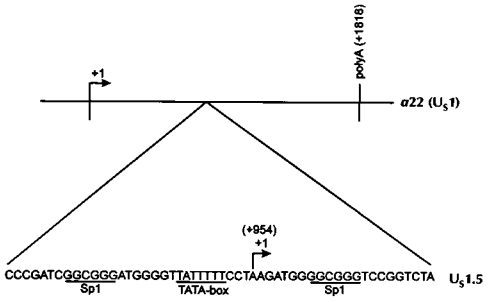


FIG. 4. Schematic diagram and sequence of the proposed transcription initiation site of $U_S$1.5. The upper line shows the α22 ($U_S$1) gene with its transcription initiation site marked as +1. The polyadenylation (polyA) signal for α22 is at position +1818. The mapped region of the $U_S$1.5 transcription initiation site has been expanded and is shown on the lower line. The transcription initiation site (+1) for $U_S$1.5 is indicated and corresponds to nucleotide +954 of the α22 transcript. Potential TATA box and Sp1 transcription factor binding sites are underlined.

LysGlyGlnLysProAsnLeuLeuAspArgLeuArgHisArgLysAsn GlyTyrArgHisThrTrp after amino acid Trp-240. Recombinant virus R4844 contained a CMV epitope tag in frame with ORF 2 at the *Pvu*II site resulting in the insertion in frame with ICP22 of the amino acids GluGlyThrGluAlaGlnProAlaArg ProThrAlaThrProGlnLysArgValProThrLeu after amino acid Gln-200. Recombinant virus R4719 contained an α4 epitope tag in frame with ORF 3 at the *Pvu*II site resulting in the insertion in frame with ICP22 of amino acids LeuThrSerThr ThrThrGlnProThrProProAlaThrGlyProArgSerLeu after amino acid Gln-200. The structures of the recombinant viruses were verified by hybridization of electrophoretically separated DNA digests, as described in Materials and Methods, with nick-translated pRB4648 as a probe (Fig. 1B). The *Sac*I-*Kpn*I double digests of R4862 and R4844 DNAs showed the loss of the bands F+D and J containing the *tk* gene inserted in the parental virus R321 and the gain of a *Kpn*I restriction site as shown by the presence of bands H+D and G+D, respectively, in place of the C+D band of the parent HSV-1(Δ305) virus (Fig. 1B and 5). It is noteworthy that only a small fraction of the probe hybridized with the band A+B. A+B bands were present in the *Sac*I-*Kpn*I digests of R4862 and R4844 DNAs, but they were of lower intensity than those in the corresponding digests of HSV-1(Δ305) and R321 DNAs because there was less overall DNA in the former digests. For recombinant R4719, analysis of the *Bam*HI-*Hin*dIII double digest showed the disappearance of the *tk*-containing band B+J+F seen in the parental virus R321 and the appearance of a new band I (Fig. 5) that migrated more rapidly than the corresponding DNA band of the parental HSV-1(Δ305) virus (data not shown).

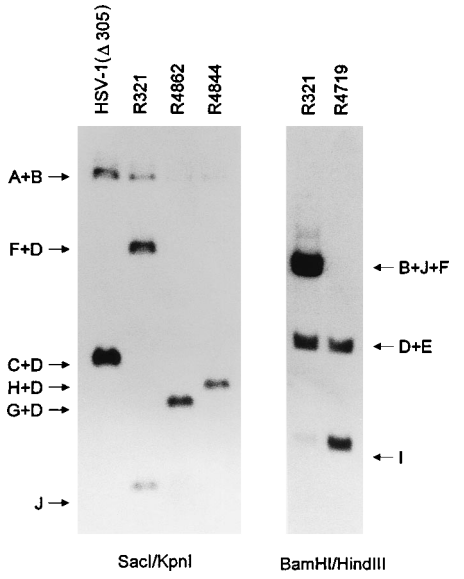


FIG. 5. Autoradiographic images of electrophoretically separated restriction endonuclease digests of parent HSV-1(F)Δ305 or of recombinant viruses schematically depicted in Fig. 1A, lines 6 to 8, hybridized with the probe depicted on the bottom line of Fig. 1B. The letters on the right and left of the two panels refer to the designations of the DNA fragments generated by restriction endonuclease cleavages.

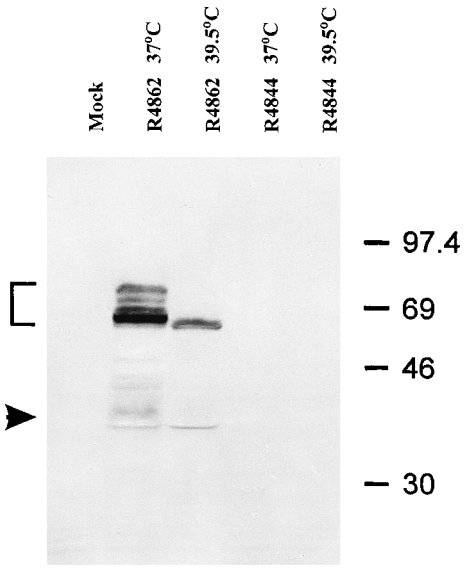


FIG. 6. Photographs of polypeptides electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with mouse monoclonal antibody to the CMV epitope. The cells were either mock infected or infected for 18 h with parent or recombinant viruses and incubated at either 37 or 39.5°C as indicated. The procedures were as described in Materials and Methods. The arrow points to the position of the $U_S$1.5 protein bands, whereas the bracket indicates ICP22. Note that at the permissive temperature (37°C) both ICP22 and the $U_S$1.5 proteins were processed and formed several bands with lower electrophoretic mobility. The proteins were not processed and formed a single sharp band in cells incubated at the nonpermissive temperature.

The predicted $M_r$s of proteins generated from the three tagged ORFs were 30,400 for ORF 1, 9,000 for ORF 2, and 11,300 for ORF 3. Analyses of electrophoretically separated lysates of cells harvested at 18 h after infection with the recombinant viruses showed the presence of a novel protein product from the virus R4862 (ORF 1; Fig. 6). This novel protein product migrated with an apparent $M_r$ of 35,000. Since HSV-1(F) proteins, including ICP22, are known to have mobilities greater than those expected from their amino acid sequences, we predict that Met-147 serves as the initiation methionine for protein encoded by $U_S$1.5. Met-147 is the first methionine after the start site mapped by primer extension. Since HSV-1(F) and its derivatives express mainly α proteins at 39.5°C, the accumulation of the $U_S$1.5 protein in infected cells maintained at 39.5°C is consistent with the synthesis of the $U_S$1.5 transcript in the presence of cycloheximide. Also of note are the small numbers of bands representing ICP22 and $U_S$1.5 at 39.5°C because of the absence of β and γ viral gene products which extensively modify these proteins. Novel protein bands

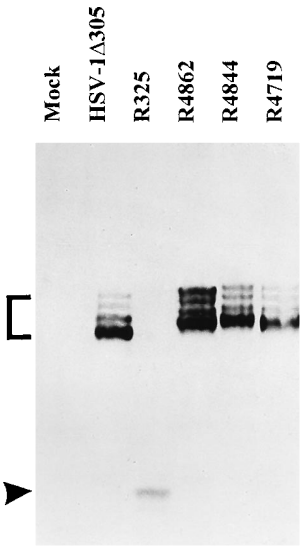


FIG. 7. Photograph of polypeptides electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with rabbit polyclonal antibody R77 prepared against the amino-terminal domain of ICP22. The cells were harvested at 18 h after infection with parent or recombinant viruses or mock infection. The procedures were as described in Materials and Methods. The bracket on the left indicates the group of five bands containing posttranslationally processed ICP22 species. The arrow on the left points to the truncated ICP22 species created as a consequence of the deletion of the 3′ domain of the α22 gene in recombinant virus R325.

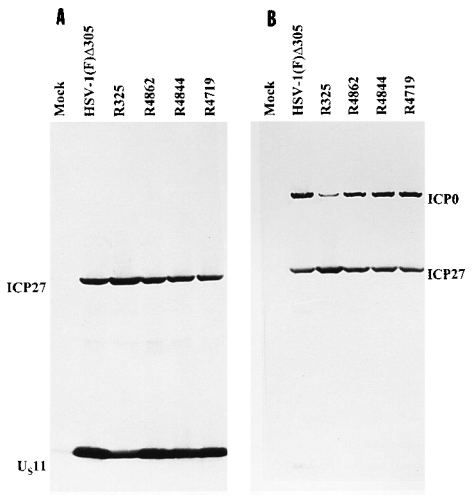


FIG. 8. Photographs of polypeptides electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with monoclonal antibodies to ICP27 and to either $U_S11$ (A) or ICP0 (B). ICP27 served to indicate that equivalent amounts of proteins were loaded in each lane. The procedures are described in Materials and Methods.

were not detected with antibodies against the epitope insertions at either temperature for R4844 (Fig. 6) or R4719 (data not shown).

**The insertion of the amino acid linkers in the recombinant viruses does not alter the processing and function of ICP22.** Since each of the three recombinant viruses contained in-frame inserts of relatively long amino acid sequences after amino acid 200 or 240, it was of interest to determine whether the wild-type phenotype associated with the α22 gene was affected by the insertions. To test the phenotype of the ICP22 proteins in the mutant viruses, two series of experiments were done.

The first was based on the observation noted in the introduction that ICP22 contained in lysates of cells harvested late (18 h) after infection with wild-type virus forms five bands in denaturing gels. The more slowly migrating bands represent ICP22 posttranslationally processed by $U_L13$ and $U_S3$ protein kinases inasmuch as these bands are not formed by ICP22 extracted from cells infected with the protein kinase deletion mutants (30). Analyses of electrophoretically separated lysates of cells harvested at 18 h after infection with parent or mutant viruses showed the presence of five polypeptide bands reactive with the anti-ICP22 polyclonal rabbit serum in the profile of each infected cell lysate (Fig. 7). As expected, the ICP22 polypeptide bands of recombinant viruses migrated more slowly than the wild-type polypeptides inasmuch as they contained inserts ranging from 18 to 22 amino acids. We conclude from this experiment that ICP22 of the mutant viruses was processed posttranslationally, at least at a superficial level, in a manner analogous to that of ICP22 of wild-type virus.

The second series of the experiments was based on the observation that in the absence of ICP22, the expression of ICP0 and of a subset of late proteins represented by $U_S11$ is reduced (29). This phenotype is most dramatic in infected rodent cells (29). To determine if the insertions made in the recombinant viruses impair this function of ICP22, electrophoretically separated lysates of wild-type or mutant infected cells were reacted with monoclonal antibodies to ICP0 (H1083) and to $U_S11$ (CL28). The results shown in Fig. 8 indicate that the levels of both ICP0 and $U_S11$ protein in lysates of cells infected with $\alpha22^-$ virus (R325) were reduced but that those in lysates of cells infected with the recombinant viruses were comparable to those in lysates of wild-type infected cells. In these studies, the control for equivalent loading of proteins in the gel consisted of ICP27 measured with monoclonal antibody H1117. The synthesis of this protein was previously shown to be unaffected by ICP22 (29); therefore, the increase in ICP27 seen in the R325 lanes was due to the loading of more protein in those lanes. We conclude that the linker insertions at amino acids 200 and 240 of ICP22 did not affect the known phenotype of wild-type infected cells related to the function of ICP22.

## DISCUSSION

**The properties and synthesis of α22 and $U_S1.5$ gene products.** In this report we have demonstrated the presence of a hitherto unreported HSV-1 transcript. This transcript is 3′ coterminal with the transcript encoding ICP22. It originates within the coding sequences of the α22 gene, and therefore its promoter must lie within the gene. The $U_S1.5$ transcript is made in the presence of cycloheximide. Since its synthesis does not require de novo viral protein synthesis, it fulfills the expectations of an α mRNA. This report brings the number of α genes to 6 and the number of genes encoded in the unique short region to 14.

The novel transcript encodes a polypeptide representing the carboxyl-terminal 273 amino acids (i.e., amino acids 147 to 420) of ICP22. Given its position and sequence content, we have designated the gene $U_S1.5$. For reasons that are not immediately apparent but that are discussed below, HSV-1 encodes both a full-length ICP22 protein and a truncated ICP22 protein. It is noteworthy that HSV-1(F) carries a temperature-sensitive lesion in the α4 gene, like many HSV-1 isolates with a limited history of passages outside the human host. At 39.5°C, cells infected with this virus make predomi-

nantly, if not exclusively, α proteins and small quantities of proteins whose genes are repressed by ICP4 (17). The $U_S$1.5 protein is made at both permissive and nonpermissive temperatures, consistent with its classification as an α protein.

**Functional segregation of ICP22 and $U_S$1.5 protein.** To facilitate a discussion of the functional significance of the $U_S$1.5 gene, it is convenient and necessary to review the structures of ICP22 and the existing mutants and the localization of the functions associated with ICP22. Schwyzer et al. (34) proposed that ICP22 can be divided into four separate domains on the basis of the nature of the amino acids and sequence conservation in homologs of this gene in other herpesviruses. These domains include a domain unique to HSV-1 and possibly HSV-2 (amino acids 1 to 160), a highly conserved domain shared by many of the herpesviruses sequenced to date (amino acids 161 to 292), an acidic domain (amino acids 293 to 324), and a carboxyl-terminal basic domain (amino acids 387 to 420). The sequences encoded by the $U_S$1.5 protein contain all of the conserved, carboxyl acidic, and basic domains.

ICP22 is extensively posttranslationally modified. The protein is nucleotidylylated by casein kinase II (5, 25) and phosphorylated at least by the protein kinases $U_S$3 and $U_L$13 (30).

The mutants studied extensively to date include two series of recombinant viruses. The first series, reported previously, includes the HSV-1 recombinants R321 and R325. In recombinant R321 a chimeric HSV-1 *tk* gene was inserted into the α22 gene immediately after codon 200. In recombinant R325, the sequences encoding amino acids 200 to 420 were deleted. Both recombinants direct the synthesis of truncated amino-terminal polypeptides of ICP22 (28). As previously reported, the phenotype of this series of mutants includes the following characteristics: (i) in cells of rodent derivation and in human primary cell strains the yield of virus is reduced (35), and (ii) α0 mRNA and protein accumulations, as well as the accumulation of a subset of late proteins exemplified by $U_S$11, are also reduced in these cells (29). The same phenotype has been observed in cells infected with a recombinant virus carrying a wild-type α22 gene but lacking the $U_L$13 gene encoding a protein kinase (29). One site of nucleotidylylation ascribed to casein kinase II is in the amino-terminal domain between amino acids 78 and 81 (4, 25). The precise location of the sequences phosphorylated by the $U_S$3 and $U_L$13 protein kinases are not known. The second set of mutants are the insertional mutagenesis mutants described in this report. Surprisingly, the insertion of sequences specifying 18 to 22 amino acids at codon 200 or 240 had no effect on any of the properties associated with the wild-type ICP22. These studies suggest that ICP22 encodes at least two sets of functions. The first maps in the amino-terminal domain of ICP22; it may be activated or suppressed by nucleotidylylation, and it is not expressed by the $U_S$1.5 protein. The second function maps in the carboxyl-terminal half of the protein, within the domain of $U_S$1.5 between amino acids 240 and 420, and is responsible for the features of the wild-type phenotype missing from cells infected with the R325 and R321 deletion mutants. It is conceivable that the two sets of functions are related and expressed by amino acid sequences interacting in *trans* rather than in *cis.*

It is noteworthy that precedents exist for functionally related, overlapping genes sharing nucleotides and encoded amino acid sequences in the HSV-1 genome. Investigators from this laboratory described another instance in which two coterminal transcripts encoded polypeptides with identical carboxyl-terminal sequences but differed in size. Thus, the domain of the $U_L$26 gene is transcribed by two 3′-coterminal transcripts designated $U_L$26 and $U_L$26.5 mRNAs (20). The promoter of $U_L$26.5 is within the coding domain of $U_L$26, and the initiating methionine of the $U_L$26.5 protein is Met-307 of the $U_L$26 protein. The two proteins are functionally related but are different: whereas the $U_L$26 protein gives rise by autoproteolytic cleavage to a protease and a component of the scaffolding for capsid assembly, the smaller $U_L$26.5 protein acts exclusively as a scaffolding protein and as a substrate for the protease (19). Subsequently, Baradaran et al. described two overlapping transcripts, $U_L$8.5 and $U_L$9, which share coding domains (3). The $U_L$9 transcript codes for the origin-binding protein, whereas the $U_L$8.5 transcript codes for the carboxyl-terminal domain of origin-binding protein (3). The precise interaction of ICP22 with the $U_S$1.5 protein remains to be determined.

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## REFERENCES

1. **Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman.** 1984. Characterization of herpes simplex virus 1 α proteins 0, 4, and 27 with monoclonal antibodies. J. Virol. **52:**108–118.
2. **Ackermann, M., M. Sarmiento, and B. Roizman.** 1985. Application of antibody to synthetic peptides for characterization of the intact and truncated α22 protein specified by herpes simplex virus 1 and the R325 α22$^-$ deletion mutant. J. Virol. **56:**207–215.
3. **Baradaran, K., C. E. Dabrowski, and P. A. Schaffer.** 1994. Transcriptional analysis of the region of the herpes simplex virus type 1 genome containing the $U_L$8, $U_L$9, and $U_L$10 genes and identification of a novel delayed-early gene product, OBPC. J. Virol. **68:**4251–4261.
4. **Blaho, J. A., C. Mitchell, L. McCormick, and B. Roizman.** Unpublished data.
5. **Blaho, J. A., C. Mitchell, and B. Roizman.** 1993. Guanylylation and adenylylation of the α regulatory proteins of herpes simplex virus require a viral β or γ function. J. Virol. **67:**3891–3900.
6. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense polypeptides. Nucleic Acids Res. **15:**4491–4511.
7. **Dixon, R. A., and P. A. Schaffer.** 1980. Fine structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **37:**189–203.
8. **Ejercito, P., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. J. Gen. Virol. **2:**357–364.
9. **Everett, R. D.** 1984. A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by immediate-early gene products are not early-gene specific. Nucleic Acids Res. **12:**3037–3055.
10. **Faber, S. W., and K. W. Wilcox.** 1988. Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 transcription initiation site. Nucleic Acids Res. **16:**555–570.
11. **Faisst, S., and S. Meyer.** 1992. Compilation of vertebrate-encoded transcription factors. Nucleic Acids Res. **20:**3–26.
12. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14:**8–19.
13. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72:**1276–1280.
14. **Hubenthal-Voss, J., R. A. Houghten, L. Pereira, and B. Roizman.** 1988. Mapping of functional and antigenic domains of the α4 protein of herpes simplex virus. J. Virol. **62:**454–462.
15. **Kristie, T. M., and B. Roizman.** 1986. α4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of α genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83:**3218–3222.
16. **Kristie, T. M., and B. Roizman.** 1988. Differentiation and DNA contact points of host proteins binding at the *cis* site for virion-mediated induction of α genes of herpes simplex virus 1. J. Virol. **62:**1145–1157.
17. **Lagunoff, M., and B. Roizman.** 1994. Expression of a herpes simplex virus 1 open reading frame antisense to the $\gamma_1$34.5 gene and transcribed by an RNA 3′ coterminal with the unspliced latency-associated transcript. J. Virol. **68:** 6021–6028.
18. **Leopardi, R., N. Michael, and B. Roizman.** 1995. Repression of the herpes simplex virus 1 α4 gene by its gene product (ICP4) within the context of the viral genome is conditioned by the distance and stereoaxial alignment of the ICP4 DNA binding site relative to the TATA box. J. Virol. **69:**3042–3048.

19. **Liu, F., and B. Roizman.** 1991. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65:**5149–5156.
20. **Liu, F., and B. Roizman.** 1991. The promoter, transcriptional unit, and coding sequence of herpes simplex virus 1 family 35 proteins are contained within and in frame with the $U_L26$ open reading frame. J. Virol. **65:**206–212.
21. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44:**939–949.
22. **McKnight, J. L. C., T. M. Kristie, and B. Roizman.** 1987. Binding of the virion protein mediating α gene induction in herpes simplex virus 1 infected cells to its cis site requires cellular proteins. Proc. Natl. Acad. Sci. USA **84:**7061–7065.
23. **Michael, N., and B. Roizman.** 1989. Binding of the herpes simplex virus major regulatory protein to viral DNA. Proc. Natl. Acad. Sci. USA **86:**9808–9812.
24. **Michael, N., and B. Roizman.** 1993. Repression of the herpes simplex virus 1 α4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. Proc. Natl. Acad. Sci. USA **90:**2286–2290.
25. **Mitchell, C., J. A. Blaho, and B. Roizman.** 1994. Casein kinase II specifically nucleotidylylates *in vitro* the amino acid sequence of the protein encoded by the α22 gene of herpes simplex virus 1. Proc. Natl. Acad. Sci. USA **91:**11864–11868.
26. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. **61:**858–865.
27. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. **67:**2365–2380.
28. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227–232.
29. **Purves, F. C., W. O. Ogle, and B. Roizman.** 1993. Processing of the herpes simplex virus regulatory protein α22 mediated by the $U_L13$ protein kinase determines the accumulation of a subset of α and γ mRNAs and proteins in infected cells. Proc. Natl. Acad. Sci. USA **90:**6701–6705.
30. **Purves, F. C., and B. Roizman.** 1992. The $U_L13$ gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein α22. Proc. Natl. Acad. Sci. USA **89:**7310–7314.
31. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5:**957–963.
32. **Roller, R. J., and B. Roizman.** 1992. The herpes simplex virus 1 RNA binding protein $U_S11$ is a virion component and associates with ribosomal 60S subunits. J. Virol. **66:**3624–3632.
33. **Sandri-Goldin, R., and G. Mendoza.** 1992. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. Genes Dev. **6:**848–863.
34. **Schwyzer, M., U. V. Wirth, B. Vogt, and C. Fraefel.** 1994. BICP22 of bovine herpesvirus 1 is encoded by a spliced 1.7 kb RNA which exhibits immediate early and late transcription kinetics. J. Gen. Virol. **75:**1703–1711.
35. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the α22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. **55:**338–346.
36. **Spector, D., F. Purves, and B. Roizman.** 1990. Mutational analysis of the promoter region of the α27 gene of herpes simplex virus 1 within the context of the viral genome. Proc. Natl. Acad. Sci. USA **87:**5268–5272.
37. **Ward, P. L., and B. Roizman.** 1994. *Herpes simplex* genes: the blueprint of a successful human pathogen. Trends Genet. **10:**267–274.
38. **Watson, R. J., M. Sullivan, and G. F. Vande Woude.** 1981. Structures of two spliced herpes simplex virus type 1 immediate-early mRNA's which map at the junctions of the unique and reiterated regions of the virus DNA S component. J. Virol. **37:**431–444.
39. **York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson.** 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell **77:**525–535.