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Herpes Simplex Virus 1 ICP22 Regulates the Accumulation of a Shorter mRNA and of a Truncated U_S3 Protein Kinase That Exhibits Altered Functions

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The U_S3 open reading frame of herpes simplex virus 1 (HSV-1) was reported to encode two mRNAs each directing the synthesis of the same protein. We report that the U_S3 gene encodes two proteins. The predominant U_S3 protein is made in wild-type HSV-1-infected cells. The truncated mRNA and a truncated protein designated $U_S3.5$ and initiating from methionine 77 were preeminent in cells infected with a mutant lacking the gene encoding ICP22. Both the wild-type and truncated proteins also accumulated in cells transduced with a baculovirus carrying the entire U_S3 open reading frame. The $U_S3.5$ protein accumulating in cells infected with the mutant lacking the gene encoding ICP22 mediated the phosphorylation of histone deacetylase 1, a function of U_S3 protein, but failed to block apoptosis of the infected cells. The $U_S3.5$ and U_S3 proteins differ with respect to the range of functions they exhibit.

The U_S3 open reading frame (ORF) of herpes simplex virus 1 (HSV-1) encodes a 481-residue protein kinase (31). As is the case with many HSV proteins, the U_S3 protein kinase is associated with multiple disparate functions. It is required for egress of virus particles from nuclei (37), to activate cyclic AMP-dependent protein kinase A, and either alone or with activated protein kinase A to block apoptosis induced by viral mutant or activated proapoptotic genes (5, 6, 14, 15, 22, 23, 25). In recent studies, the U_S3 protein kinase was shown to be required for the interaction of infected-cell protein no. 22 (ICP22) with cyclin-dependent kinase 9 (11).

The organization of genes in the segment of the HSV-1 genome containing the U_S3 gene is illustrated schematically in Fig. 1A. Early studies have shown that the U_S3 ORF yields two transcripts of 2,544 and 2,319 residues, respectively (20, 21), and suggested that both transcripts encode the same protein. In preliminary studies, we noted that cells infected with the mutant R7802 lacking the α22 gene encoding ICP22 accumulated a truncated form of the U_S3 protein designated $U_S3.5$. As reported here, this protein also accumulated in cells transduced with a baculovirus carrying the U_S3 ORF driven by the human cytomegalovirus (CMV) immediate-early promoter. The accumulation of this protein was unaffected by protease inhibitors, and further studies indicated that the $U_S3.5$ protein shared the carboxyl-terminal domain with the U_S3 protein. It comigrated with a polypeptide band present in lysates of cells transduced with a baculovirus encoding U_S3 driven by the CMV promoter. This polypeptide band could not be detected in lysates of cells transduced with the U_S3 gene in which methionine codon 77 was replaced with the alanine codon. Consistent with this observation, cells infected with the R7802 mutant accumulated larger amounts of the shorter U_S3 mRNA than cells infected with either wild-type or repaired virus. Finally, we noted that $U_S3.5$ accumulated in cells infected with the R7802 mutant retained some but not all of the functions associated with the U_S3 protein kinase.

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MATERIALS AND METHODS

Cells and viruses. Vero cells were obtained from the American Type Culture Collection, and rabbit skin cells (RSC) were originally obtained from J. McClaren. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (RSC) or 5% newborn calf serum (Vero cells). HSV-1(F) is the prototype HSV-1 strain used in this laboratory (12). Mutant viruses R7041(ΔU_S3), R7356 (ΔU_L13), R7353 (ΔU_L13/ΔU_S3), R325 (Δα22 C-terminal 220 amino acids), R7802 (Δα22), and R7808 (Δα22 N-terminal 170 amino acids) and α22-repaired viruses R7804 and R7828 were described previously (1, 26, 30, 31, 32, 33, 41). The structures of other ICP22 recombinant viruses used in this study are shown schematically in Fig. 1B and had been described elsewhere (26, 28). Baculovirus expressing U_S3 protein (Bac-U_S3) was described previously (22).

Generation of recombinant baculoviruses. Recombinant baculoviruses were generated using the PharMingen baculovirus expression system as described previously (13, 22, 29). Briefly, a DNA fragment containing the wild-type HSV-1(F) or mutant U_S3 coding sequence was cloned into baculovirus transfer vector pAc-CMV (43) and the subsequent plasmid was cotransfected into Sf9 insect cells together with the BaculoGold baculovirus DNA (PharMingen) according to the manufacturer's instructions. Supernatant containing the recombinant virus was collected and cleared by centrifugation at 2,500 rpm for 10 min 4 to 6 days after transfection, and virus was amplified in Sf9 cells grown in a 150-cm^2 flask. Mutant U_S3 ORFs, each with simple in-frame methionine codons at 77, 164, 182, or 189 replaced, respectively, with alanine, glycine, glycine, and alanine codons, were generated by site-directed mutagenesis as previously described (28).

Preparation of cell lysates, electrophoretic separation of proteins, and immunoblotting. Replicate cell cultures in 25-cm^2 flasks were either mock infected or infected with 5 or 10 PFU of HSV-1 per cell and maintained at 37°C in medium 199V consisting of a mixture of 199 supplemented with 1% calf serum. Cell cultures infected with Bac-U_S3 were maintained in DMEM supplemented with 5% newborn calf serum in the presence of 6 or 9 mM sodium butyrate (Sigma). In some experiments, cells were exposed to 10 μM proteasome inhibitor MG132 (Biomol) or caspase inhibitors (2 μM DEVD or 50 μM Z-VAD-FMK from Biomol). Cells were harvested at either an early (6 h) or a late (19 h to 24 h) time after infection, rinsed three times with phosphate-buffered saline containing protease inhibitor cocktail (Roche), and then solubilized in 100 or 150 μl of disruption buffer (50 mM Tris-HCl [pH 7], 2% sodium dodecyl sulfate, 710 mM β-mercaptoethanol, 3% sucrose). Fifty-microliter aliquots of lysates were boiled for 5 min, and the solubilized proteins were subjected to electrophoresis in a 9%

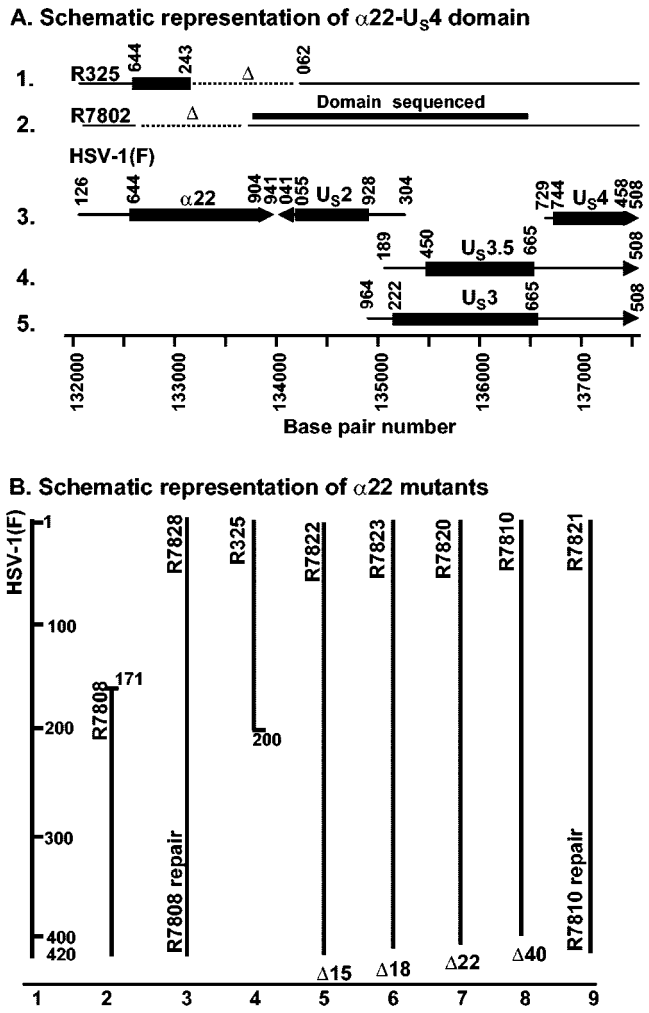

FIG. 1. (A) Schematic representation of α22 (U_S1)-U_S4 domain. Lines 3, 4, and 5 show the structural organization of wild-type HSV-1(F). The arrows represent the transcripts and the rectangles the coding domains. The base pair numbers are those mapped previously (20, 21). Abridged numbers (lacking the first three digits) appear at appropriate points on top of lines 1 to 5. Those of $U_S3.5$ as predicted from this study are indicated in line 4. Lines 1 and 2, the α22 domain present in α22 deletion mutant viruses R325 and R7802, respectively (26, 30). The deleted regions are indicated by Δ. The DNA fragment which was sequenced in HSV-1(F), and R7802 is shown in line 2. (B) Schematic representation of ICP22 proteins present in wild-type and α22 mutant viruses. Line 1, wild-type ICP22 protein encoded by HSV-1(F) containing 420 amino acids. Lines 2 and 4, ICP22 proteins encoded by deletion mutants R7808 and R325 containing the C-terminal 250 and N-terminal 200 amino acids, respectively. The N-terminal deletion was repaired in R7828 (line 3). Lines 5 to 8, ICP22 proteins encoded by C-terminal deletion mutants. Line 9, ICP22 protein encoded by R7821 which was derived from R7810 in which the C-terminal 40-amino-acid deletion had been repaired.

or 11% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, blocked with 5% nonfat milk, reacted with primary antibody, followed by an appropriate secondary antibody conjugated to alkaline phosphatase (Bio-Rad), and visualized according to the manufacturer's instructions.

Extraction of total RNA and Northern blotting. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 10 PFU of virus per cell. The cells were harvested at 9.5 h after infection. Total RNA was extracted by using Trizol reagent (Gibco BRL). Aliquots of 15 μg of total RNA were separated on 1% formaldehyde agarose gels, transferred to membranes, and hybridized with ^{32}P-labeled plasmid pRB5252 (28) or pAc-U_S3 (22) to detect α22 or U_S3 transcript, respectively. The bound probe was visualized by autoradiography.

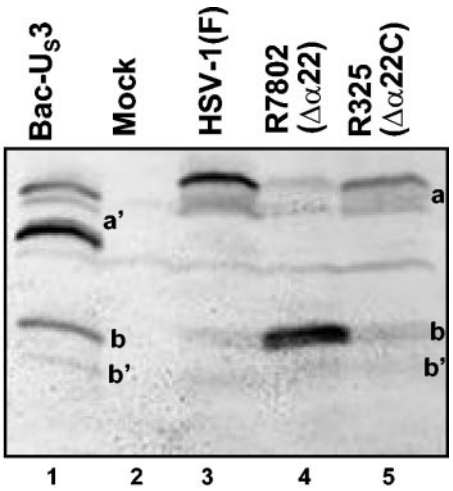

FIG. 2. Electrophoretic profiles of U_S3 proteins in RSC infected with baculovirus expressing U_S3 (Bac-U_S3) and wild-type or mutant HSV-1. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected (lane 2) or infected with 10 PFU of Bac-U_S3 (lane 1), wild-type HSV-1(F) (lane 3), or deletion mutant R7802 (Δα22; lane 4) or R325 (Δα22 C-220; lane 5) virus per cell. The cells were harvested at 20 h after infection. Proteins were solubilized in 150 μl of disruption buffer, and 50-μl aliquots were electrophoretically separated in a 9% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, blocked with 5% nonfat milk, and reacted with polyclonal antibody to U_S3 as described in Materials and Methods.

Antibodies. Rabbit polyclonal antibodies against U_S3, U_L31, and the carboxyl-terminal region of ICP22 (W2) were described previously (9, 10, 16, 22). Monoclonal antibodies to ICP0 and ICP4 were purchased from the Goodwin Cancer Research Institute (Plantation, Fla.). The mouse monoclonal antibody against Us11 was described previously (39). The polyclonal antibody against histone deacetylase 1 (HDAC1) and monoclonal antibodies for actin and the Flag epitope were purchased from Sigma, and the polyclonal antibody for poly-ADP ribose polymerase (PARP) was purchased from Cell Signaling Technology Inc.

RESULTS

The accumulation of a truncated U_S3 protein in cells transduced by baculovirus expressing the U_S3 ORF or infected with an HSV-1 mutant lacking the entire α22 ORF. In this series of experiments, RSC were harvested 20 h after transduction with baculovirus expressing the U_S3 ORF or mock infection or infection with HSV-1(F), R7802 (Δα22), or R325 (Δα22C); solubilized; subjected to electrophoresis in denaturing gels; and probed with rabbit anti-U_S3 polyclonal antibody. The results shown in Fig. 2 were as follows. As reported elsewhere, the U_S3 protein kinase from lysates of wild-type virus-infected cells formed several bands (lane 3, bands designated a) in denaturing polyacrylamide gels. The average apparent M_r was approximately 70,000. A fainter set of bands (b and b′) with an average apparent M_r of approximately 50,000 was also present and reacted with the anti-U_S3 antibody. A similar set of U_S3 protein bands interacting with the anti-U_S3 antibody was present in lysates of cells infected with the R325 mutant virus (lane 5). In cells infected with the Δα22 mutant R7802, the bands containing full-length U_S3 protein were faint. The preeminent band reacting with the anti-U_S3 antibody was the fast-migrating M_r 50,000 protein (band b, lane 4) and fainter

bands designated b′. Finally, the cells transduced with the U_S3 baculovirus accumulated both sets of bands. The M_r 50,000 bands comigrated with those present in lysates of R7802-infected cells or those present in lesser amounts in lysates of wild-type virus-infected cells. The slow-migrating forms (bands marked a′) migrated faster than those formed by lysates of wild-type virus-infected cells. As described later in Results, the electrophoretic mobility of these bands reflects the absence of U_L13 protein kinase, which mediates the posttranslational modification of the U_S3 protein.

The experiments described above were repeated in part with Vero cells. In this experiment, we omitted the transduction with baculoviruses but included replicate cultures infected with R7804 derived by replacement of the α22 gene deleted in R7802 or R7808 lacking the amino-terminal 170 codons or R7828 in which the codons missing from R7808 were replaced. The cells were harvested 24 h after infection and processed as described in Materials and Methods. The results of this experiment again show the preeminent accumulation of a truncated form of U_S3 protein (band b) in cells infected with the R7802 mutant lacking the entire α22 ORF (Fig. 3, lane 3). As expected from earlier results showing that Vero cells support the replication of the Δα22 mutant, Fig. 3 shows that the patterns of accumulation of ICP0, ICP4, and U_S11 proteins in R7802 mutant virus-infected cells cannot be differentiated from those of wild-type virus-infected cells.

The focus of this report is on the rapidly migrating forms of the U_S3 protein accumulating in R7802-infected cells. In anticipation of the results presented below, we designated this protein $U_S3.5$.

Analyses of α22, U_S3, and U_L13 mutants for expression of the $U_S3.5$ protein. In this series of experiments, replicate cultures of Vero cells (Fig. 4A) or RSC (Fig. 4B) were mock infected or exposed to 10 PFU of wild-type or mutant virus per cell. The cells were harvested at 19 h (Vero cells) or 22 h (RSC) after infection, processed as described in Materials and Methods, and reacted with anti-U_S3 polyclonal rabbit serum. The results were as follows. (i) As expected from Fig. 2 and 3, cells infected with the R7802 (Δα22) mutant accumulated primarily the truncated $U_S3.5$ protein (Fig. 4A, lane 1, and B, lane 7). This protein formed at least four bands. The two slower-migrating bands designated b were significantly more abundant than the faster-migrating doublet designated b′. In contrast, cells infected with the wild-type virus accumulated predominantly the full-length, highly processed forms of the U_S3 protein (Fig.4A, lane 3, and B, lane 2; bands a and a′). The truncated $U_S3.5$ bands, while present, were not preeminent. Neither the truncated $U_S3.5$ bands nor the U_S3 bands were formed by lysates of mock-infected cells or cells infected with the ΔU_S3 mutant viruses R7041 and R7353 (Fig. 4B, lanes 1, 4, and 5). (ii) Cells infected with the ΔU_L13 mutant virus accumulated faster-migrating forms of full-length U_S3 protein (Fig. 4B, lane 3, band a′). Cells infected with this mutant accumulated the faster-migrating forms of the $U_S3.5$ protein (band b′). These results suggest that the U_L13 protein kinase mediates the posttranslational modification of both the U_S3 and the $U_S3.5$ proteins. (iii). Cells infected with the mutants lacking the carboxyl-terminal amino acids (Fig. 4A, lanes 5 to 8) accumulated hyperphosphorylated forms of the U_S3 protein. However, since the virus lacking 40 amino acids of ICP22 (R7810) and the repaired virus (R7821) yielded virtually indistinguishable profiles of U_S3, it is likely that the accumulation of the very slow-migrating forms of U_S3 does not reflect properties of the α22 gene.

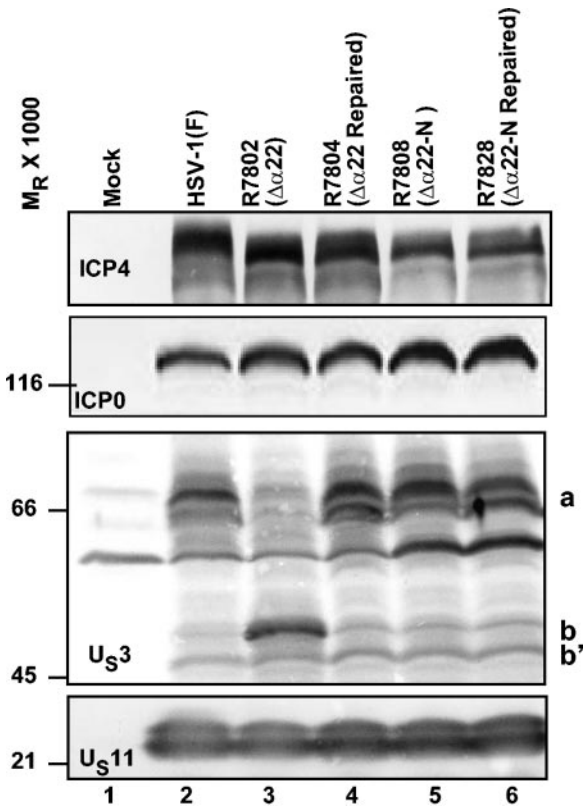

FIG. 3. Electrophoretic profiles of U_S3 proteins in Vero cells infected with wild-type and α22 deletion mutant viruses. Replicate cultures of Vero cells in 25-cm^2 flasks were either mock infected or infected with 10 PFU of wild-type HSV-1(F), deletion mutant R7802 (Δα22) or R7808 (Δα22 N-170), or repaired recombinant R7804 or R7828 virus per cell. The cells were harvested at 24 h after infection. Proteins were solubilized in 150 μl of disruption buffer, and 50-μl aliquots were electrophoretically separated in an 11% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, blocked with 5% nonfat milk, and reacted with polyclonal antibody to U_S3 or monoclonal antibody for ICP4, ICP0, or Us11 as described in Materials and Methods.

The synthesis of the $U_S3.5$ protein is not a consequence of a mutation introduced during the construction of the Δα22 mutant virus R7802. Several lines of evidence indicate that the synthesis of the $U_S3.5$ protein is not the consequence of a mutation in the U_S3 gene. The method of constructing the recombinants used in these studies is based on double-recombination events that led to the excision of the α22 ORF or the rescue of the deletion mutant, again by double recombination with mutated copies of the gene. In this system, there is a risk that nucleotide sequences may be inserted or deleted in regions of homologous recombinations. Although the rescuing fragment did not extend into the coding sequence of the U_S3 ORF, it was nevertheless necessary to consider this possibility. In the initial studies, we sequenced the U_S3 genes in HSV-1(F), R7802, R7804, R7808, and R7828 beginning with nucleotide −41 relative to the translation initiation site and

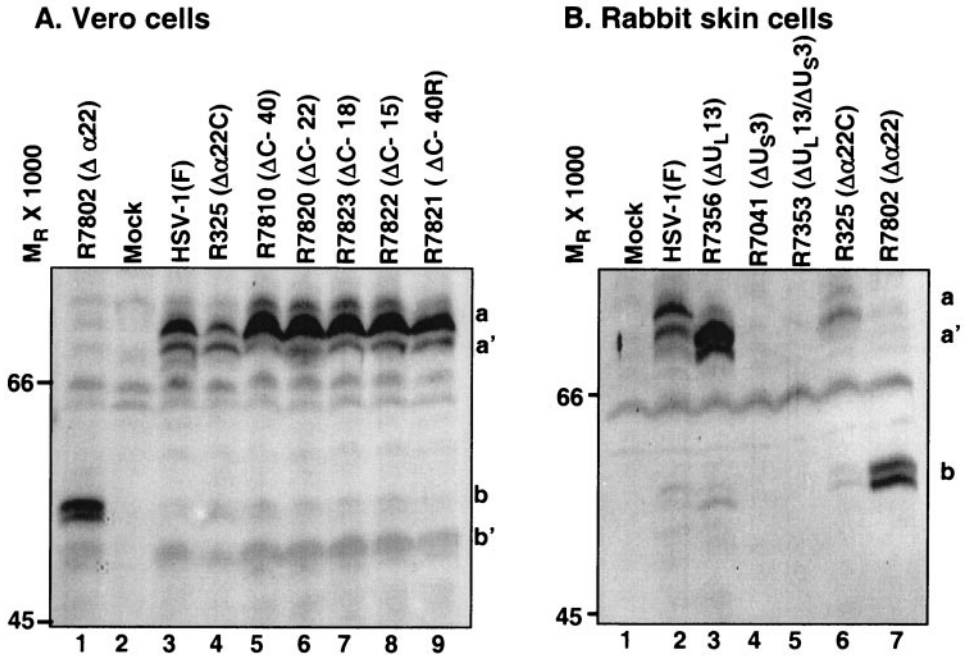

FIG. 4. Electrophoretic profiles of U_S3 proteins in Vero cells and RSC infected with wild-type and deletion mutant viruses. (A) Replicate cultures of Vero cells in 25-cm^2 flasks were either mock infected or infected with 10 PFU per cell of wild-type HSV-1(F) virus or α22 deletion mutant R7802 (Δα22), R325 (Δα22 C-220), R7810 (Δα22 C-40), R7820 (Δα22 C-22), R7823 (Δα22 C-18), R7822 (Δα22 C-15), or R7821, in which the C-terminal 40-amino-acid deletion in R7810 had been repaired. Cells were harvested at 19 h after infection. Proteins were solubilized in 150 μl of disruption buffer. (B) RSC in 25-cm^2 flasks were either mock infected or infected with 5 PFU of wild-type HSV-1(F) or mutant R7356 (ΔU_L13), R7041 (ΔU_S3), R7353 ($\Delta U_L13/\Delta U_S3$), R7802 (Δα22), of R325 (Δα22 C-220) virus per cell. The cells were harvested at 22 h after infection. Proteins were solubilized in 100 μl of disruption buffer. In both panels A and B, 50-μl aliquots were electrophoretically separated in 9% denaturing polyacrylamide gels, transferred to nitrocellulose sheets, blocked with 5% nonfat milk, and reacted with polyclonal antibody to U_S3.

extending to the end of the coding sequence. Subsequently, we sequenced the entire region from the stop codon of the α22 gene to the end of the U_S3 coding sequence in HSV-1(F) and R7802. The sequence of the U_S3 coding sequence and 5′ untranslated region is shown in Fig. 5 and Table 1. Our results were as follows. (i) The HSV-1(F) U_S3 gene differs from the published sequence of strain 17 with respect to three amino acids (Gly62 [GGC] in place of Ser [AGC], Asp63 [GAT] in place of Glu [GAG], and Pro122 [CCC] in place of Thr [ACC]). In addition, there are seven silent substitutions in codons 74, 160, 210, 240, 276, 325, and 361. (ii) The sequenced U_S3 domains in R7802 (Δα22), R7804 (repaired virus), R7808 (Δα22-N), and R7828 (repaired virus) contained no deletions, no new stop codons, and no loss of the initiator codons. We did find two amino acid substitutions in all three recombinant viruses. These were in codons 40 (Ala [GCC] to Thr [ACC]) and 417 [Glu [GAA] to Lys [AAA]).

The implication of these results is that two mutations resulting in amino acid substitution were introduced into the U_S3 coding sequence in the course of isolation of the α22 mutant viruses. The mutations do not account for the failure of the U_S3 protein to accumulate in cells infected with the R7802 mutant since the same mutations were present in the coding sequence of the repaired virus (R7804), the mutant virus R7808, and the repaired virus R7828. None of these viruses induce the accumulation of the $U_S3.5$ protein in infected cells (Fig. 3, lanes 4, 5, and 6). We conclude that the mutations introduced into the U_S3 gene cannot account for the accumulation of the $U_S3.5$ protein in cells infected with the R7802 mutant virus.

The $U_S3.5$ protein accumulates in cells treated with protease inhibitors. Replicate cultures of RSC were either mock infected or infected with HSV-1(F), R7802, or R325 virus. After 1 h of incubation, the medium was replaced with either fresh medium and either mock-treated or exposed to the proteasome inhibitor MG132 (10 μM), the caspase inhibitor Z-VAD-FMK (50 μM), or DEVD (2 μM). The cells were harvested at 20 h after infection, processed as described above, and reacted with anti-U_S3 antibody. The results shown in Fig. 6A indicate that the protease inhibitors had no effect on the accumulation of either U_S3 or $U_S3.5$ protein.

The second experiment in this series examined the possibility that the U_S3 protein is subject to cleavage in the absence of phosphorylation by the U_L13 protein kinase. In this instance, the experiment described above was repeated except that the cells were mock infected or exposed to the wild-type or ΔU_L13 or $\Delta U_L13/\Delta U_S3$ mutant virus. The results shown in Fig. 6B indicate that the U_S3 proteins accumulating in U_L13 mutant-infected cells migrated faster than those accumulating in wild-type virus-infected cells were not subject to proteolytic cleavage.

Cells infected with the R7802 mutant virus accumulate increased amounts of a truncated U_S3 mRNA relative to those of wild-type virus-infected cells. In this series of experiments, replicate cultures of RSC in 25-cm^2 flasks were mock infected or exposed to 10 PFU of wild-type HSV-1(F), deletion mutant

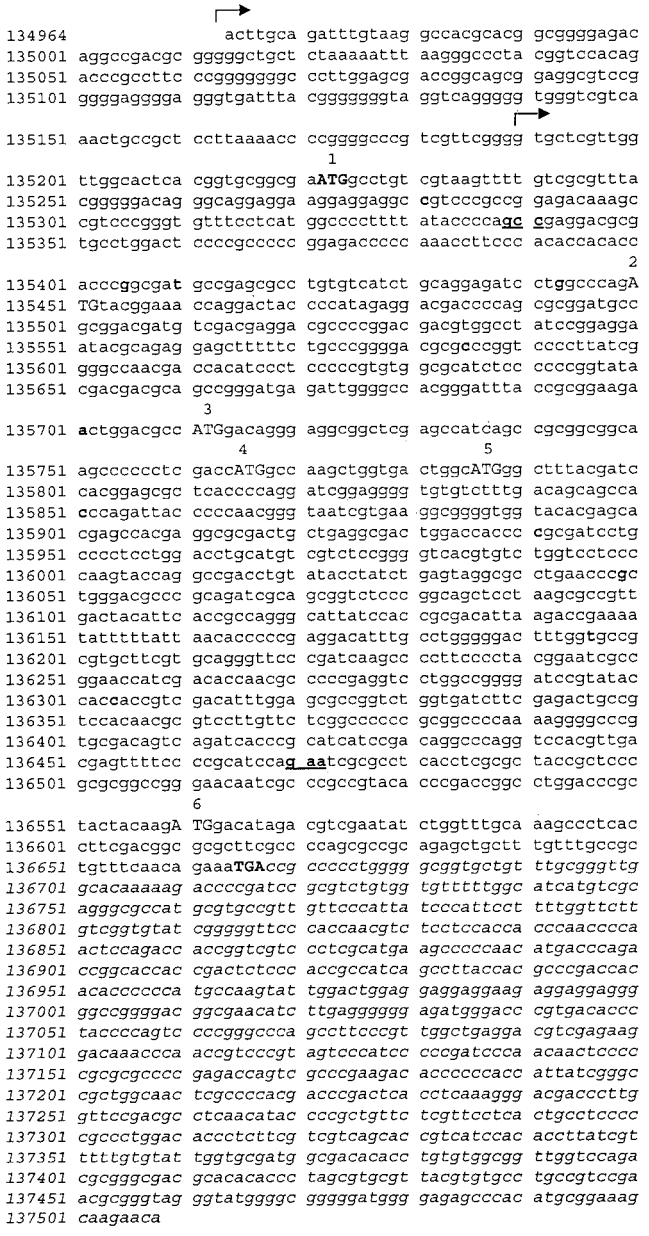

FIG. 5. Sequences of HSV-1(F) U_S3 transcripts extend from nucleotide 134964 to nucleotide 137508 and from nucleotide 135189 to nucleotide 137508. The coding sequence extends from nucleotide 135222 to nucleotide 136665. The upstream region from nucleotide 133900 to the end of the U_S3 coding domain at nucleotide 136665 was verified. The initiation sites of the two predicted transcripts are marked by arrows, and initiation (ATG) and stop (TGA) codons for the U_S3 protein are capitalized and in bold. In-frame methionine codons 1, 77, 164, 182, 189, and 447 are numbered 1 to 6, respectively. Codons 40 and 417, which show variations in mutant viruses, are underlined. Nucleotide variations from the HSV-1(17) strain are shown in Table 1. We also noted some variations in the HSV-1(F) sequence upstream of the U_S3 transcripts, but in all cases those differences are reflected in the R7802 mutant virus (data not shown).

R7802 (Δα22) or R7808 (Δα22 N-170), or repaired recombinant R7804 or R7828 virus per cell. The cells were harvested at 9.5 h after infection. Total RNA was extracted with Trizol reagent (Gibco BRL). Aliquots of 15 μg of total RNA were

TABLE 1. Codon variations in U_s3 sequences

Codon	HSV-1 (F)[a]	HSV-1 (17)
62	**GGC (Gly)**	**AGC (Ser)**
63	**GAT (Asp)**	**GAG (Glu)**
74	CTG (Leu)	CTT (Leu)
122	**CCC (Pro)**	**ACC (Thr)**
160	GAA (Glu)	GAG (Glu)
210	CAC (His)	CAT (His)
240	CCC (Pro)	CCG (Pro)
276	CCG (Pro)	CCA (Pro)
325	GGT (Gly)	GGC (Gly)
361	ACC (Thr)	ACG (Thr)

[a] The coding sequences of mutant viruses R7802 (and repaired virus R7804) and R7808 (and repaired virus R7828) are the same as that of HSV-1 (F) except in codon 40 (GCC for Ala in wild-type virus to ACC for Thr in mutants) and codon 417 (GAA for Glu in wild-type virus to AAA for Lys in mutants). These two codons are underlined in the HSV-1 (F) U_s3 sequence in Fig. 5. Boldface type indicates codon variations resulting in different compositions of amino acids in U_S3 proteins of HSV-1 strains F and 17.

separated on 1% formaldehyde agarose gels, transferred to membranes, and hybridized with ^{32}P-labeled plasmid pRB5252 (for α22) or pAc-U_S3 (for U_S3). The bound probe was visualized by autoradiography. The results were as follows. The preeminent viral mRNA hybridizing with the α22 probe was absent from mock-infected or R7802 mutant virus-infected cells (Fig. 7A, lanes 1 and 3). As expected, the corresponding mRNA extracted from cells infected with the R7808 mutant lacking the amino-terminal 170 amino acids of ICP22 migrated faster than the wild-type mRNA (Fig. 7A, lane 5). The mRNAs extracted from R7804 and R7828 (corresponding to viruses R7802 and R7808, in which the α22 gene was restored) could not be differentiated from that of wild-type HSV-1(F) (Fig. 7A, compare lanes 4 and 6 with lane 2).

As illustrated in Fig. 1A, the full-length and truncated mRNAs of the U_S3 ORF were reported to be 2,544 and 2,319 bases long, respectively (20, 21). The preeminent mRNA accumulating in cells infected with wild-type HSV-1(F) hybridizing with the U_S3 probe corresponds in size to that reported for the U_S3 mRNA. A faster-migrating band accumulated at higher levels in R7802-infected cells compared to either wild-type or repaired virus-infected cells (Fig. 7B, top, compare lanes 2, 4, 5, and 6 with lane 3).

The truncated $U_S3.5$ protein initiates at Met77. The results presented in this section represent two series of experiments. In the first, we tagged the carboxyl terminus of the U_S3 ORF in HSV-1(F) with the Flag epitope and isolated the recombinant baculovirus Bac-U_S3(Flag). Lysates of cells exposed to R7802 or transduced with Bac-U_S3(Flag) were electrophoretically separated in a denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with the anti-U_S3 antibody (Fig. 8A, lanes 1 and 2). The electrophoretically separated proteins from lysates of cells transduced with Bac-U_S3(Flag) also reacted with the anti-Flag antibody (Fig. 8A, lane 3). The images obtained with the anti-Flag antibody were less intense than those obtained with the anti-U_S3 antibody. The results nevertheless indicate that the bands seen with the anti-Flag antibody correspond to prominent bands illuminated by the anti-U_S3 antibody. In particular, the band marked b in lane 3 has the same electrophoretic mobility as the marked bands in lanes 1 and 2. These results are consistent with the

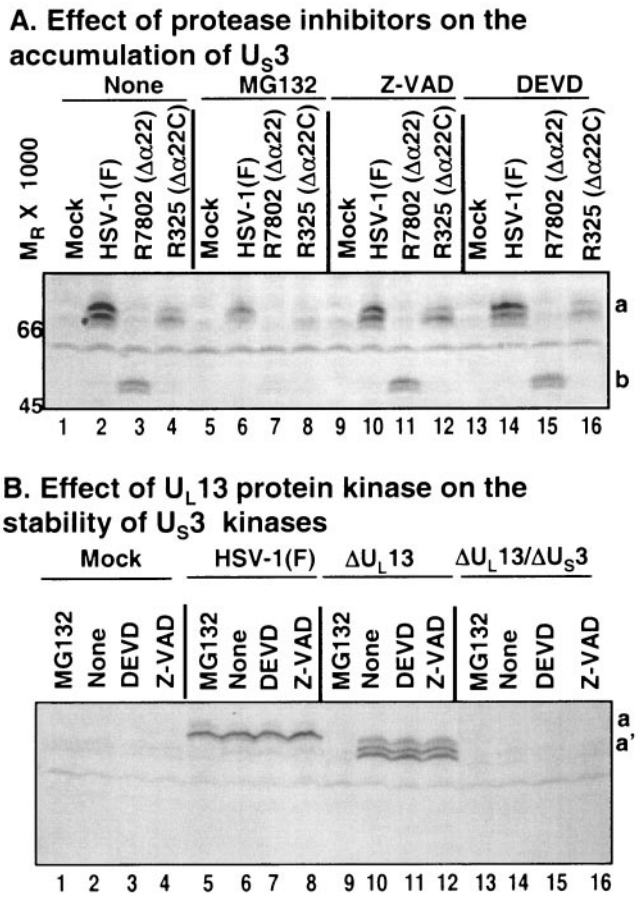

FIG. 6. Effects of protease inhibitors on U_S3 accumulation and processing. (A) U_S3 accumulation in α22 deletion mutant virus-infected cells. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 5 PFU of wild-type HSV-1(F) or mutant R7802 (Δα22) or R325 (Δα22C) virus per cell. At 1 h after infection, one set of cultures was replenished with fresh untreated medium (lanes 1 to 4), whereas a second set was replenished with medium containing 10 μM MG132 (lanes 5 to 8), a third set was replenished with medium containing 50 μM Z-VAD-FMK (lanes 9 to 12), and another set was replenished with medium containing 2 μM DEVD (lanes 13 to 16). The cells were harvested at 20 h after infection. Proteins were solubilized in 100 μl of disruption buffer, and 50-μl aliquots were electrophoretically separated in 9% denaturing polyacrylamide gels, transferred to nitrocellulose sheets, blocked with 5% nonfat milk, and reacted with polyclonal antibody to U_S3. (B) Processing of U_S3 in U_L13 deletion mutant virus-infected cells. The experiment was repeated using U_L13 deletion mutants R7356 (ΔU_L13) and R7353 (ΔU_L13/ΔU_S3). RSC were infected with 10 PFU of virus per cell and harvested at 19 h after infection, and proteins were solubilized in 150 μl of disruption buffer, processed as described above, and reacted with polyclonal antibody to U_S3.

hypothesis that the truncated $U_S3.5$ protein accumulating in R7802 mutant virus-infected cells shares the carboxyl-terminal domain with the full-length U_S3 protein.

In the second series of experiments, we transduced RSC with baculoviruses carrying the wild-type gene or a mutant U_S3 gene in which the methionine codon at position 77, 164, 182, or 189 was replaced with a glycine or alanine codon. Lysates of transduced cells along with those of cells infected with HSV-1(F) or R7802 were electrophoretically separated on a dena-

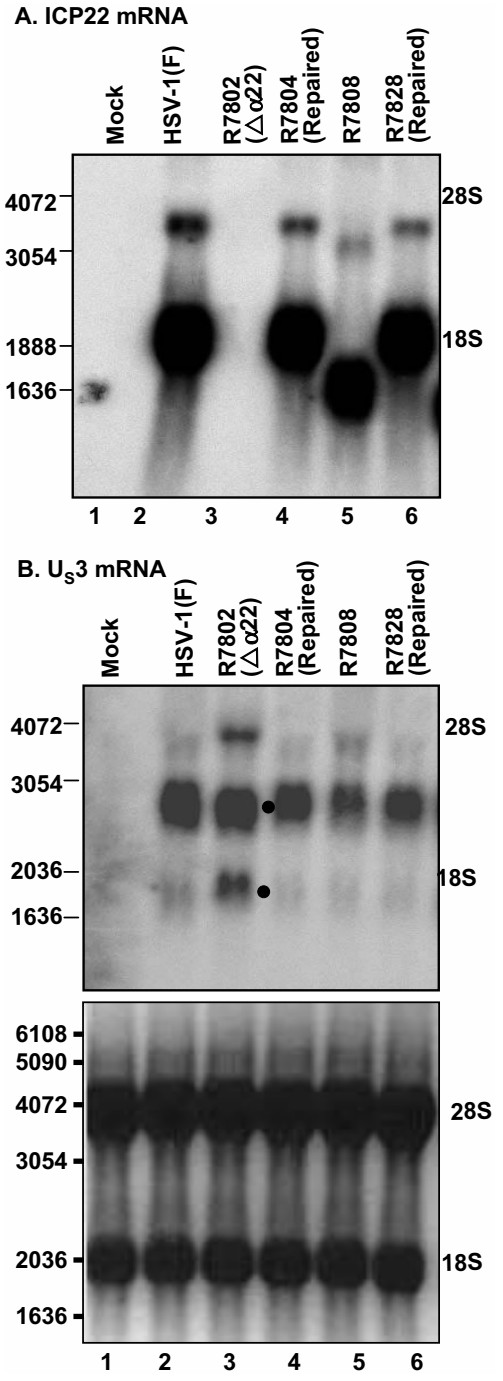

FIG. 7. Photographs of RNAs separated in formaldehyde agarose gel and autoradiographic images of the U_S3 (B) and α22 (A) transcripts. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 10 PFU of wild-type HSV-1(F), deletion mutant R7802 (Δα22) or R7808 (Δα22 N-170), or repaired recombinant R7804 or R7828 virus per cell. The cells were harvested at 9.5 h after infection. Total RNA was extracted by using Trizol reagent (Gibco BRL). Aliquots of 15 μg of total RNA were separated on 1% formaldehyde agarose gels, transferred to membranes, and hybridized with ^{32}P-labeled plasmid pRB5252 (for α22) or pAc-U_S3 (for U_S3). The bound probe was visualized by autoradiography. The values on the left are in base pairs.

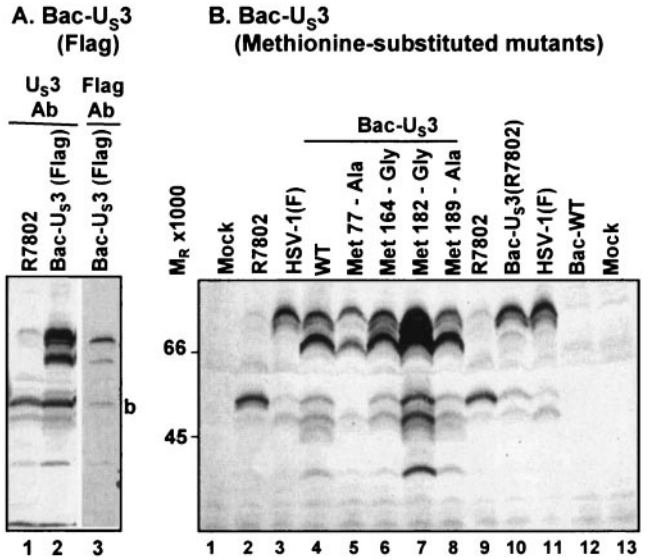

FIG. 8. Identification of the initiation codon for U_S3.5 protein. (A) Expression of U_S3 proteins by recombinant baculovirus Bac-U_S3(Flag). Replicate cultures of RSC in 25-cm^2 flasks were either infected with mutant R7802 ($\Delta\alpha$22) or transduced with a recombinant baculovirus containing U_S3 tagged with the Flag epitope in the carboxyl terminus. Cells were harvested 24 h after infection. Proteins were electrophoretically separated in an 11% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, blocked with 5% nonfat milk, and reacted with a polyclonal antibody (Ab) to U_S3 (lanes 1 and 2) or a monoclonal antibody for the Flag epitope (lane 3). (B) Expression of U_S3 proteins by recombinant baculoviruses carrying a U_S3 gene in which the in-frame methionine at codon 77, 164, 182, or 189 was replaced with an alanine or glycine codon. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 10 PFU per cell of wild-type (WT) HSV-1(F) or mutant R7802 ($\Delta\alpha$22) (lanes 1, 2, 3, 9, and 11) or transduced with baculoviruses (lanes 4 to 8, 10, 12, and 13). Cells were harvested 23 to 24 h after virus infection or transduction and processed as described above. Proteins were electrophoretically separated in an 11% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, blocked with 5% nonfat milk, and reacted with a polyclonal antibody to U_S3.

turing gel, transferred to a nitrocellulose sheet, and reacted with the anti-U_S3 antibody. The results were as follows. As shown in experiments described earlier in the text, lysates of the R7802 virus-infected cells accumulated a truncated protein that was also present in smaller amounts in lysates of wild-type virus-infected cells (Fig. 8B, compare lanes 2 and 9 with lanes 3 and 11). This protein was also present in lysates of cells transduced with baculoviruses carrying the wild-type U_S3 gene or a U_S3 gene in which the codon for Met164, Met182, or Met 189 was replaced (Fig. 8B, lanes 4, 6, 7, and 8). This protein was absent from lysates of cells transduced with the baculovirus carrying the U_S3 gene in which Met77 was replaced (Fig. 8B, lane 5) or in lysates of mock-infected cells or cells transduced with wild-type baculovirus (Fig. 8B, lanes 1, 12, and 13). We conclude that the truncated protein designated U_S3.5 initiates at codon Met77 and shares its carboxyl terminus with that of the wild-type U_S3 protein.

The functions of the U_S3.5 protein. U_S3 acts as a protein kinase to mediate the phosphorylation of numerous proteins in infected cells. A function that appears to be related to its protein kinase activity is to block apoptosis induced by viral gene products or exogenous agents. The experiments described below were designed to determine whether the truncated form accumulating in R7802-infected cells functioned in two assays as the U_S3 protein. In the first series of experiments, replicate cultures of RSC growth in 25-cm^2 flasks were either mock infected or exposed to 10 PFU of HSV-1(F) or mutant virus R7802 ($\Delta\alpha$22), R7356 (ΔU_L13), R7041 (ΔU_S3), or R7353 (ΔU_L13/ΔU_S3) per cell. The lysates of cells harvested at 24 h after infection were solubilized and electrophoretically separated on an 11% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, blocked with 5% nonfat milk, and reacted with polyclonal antibody to HDAC1 as described in Materials and Methods. Elsewhere, we reported that the U_S3 protein kinase mediates the phosphorylation of HDAC1 (27). The results of the experiment shown in Fig. 9A (lanes 2 to 6) are consistent with the earlier report that the U_S3 protein kinase is essential for the phosphorylation of HDAC1. The results also show that HDAC1 is phosphorylated in cells infected with the R7802 mutant virus, indicating that U_S3.5 also mediates the phosphorylation of HDAC1. The same results were obtained using the Vero, SK-N-SH, and pHEL cell lines (data not shown).

In the second series of experiments, replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 5 PFU per cell of wild-type HSV-1(F), mutant R7802 ($\Delta\alpha$22) or R7041 (ΔU_S3) virus, or repaired virus R7804 12 h after transduction with wild-type baculovirus or baculovirus expressing the U_S3 gene. Lysates of cells harvested at 25 h after infection were solubilized, subjected to electrophoresis in a denaturing gel, transferred to a nitrocellulose filter, and reacted with anti-PARP antibody. The immunoblot of proteins from lysates of mock-infected cells transduced with wild-type baculovirus or the baculovirus encoding the U_S3 protein is shown in Fig. 9C. The results were as follows. Cleaved fragments of PARP were detected in lysates of cells transduced with wild-type baculovirus and infected with the R7802 ($\Delta\alpha$22) or R7041 (ΔU_S3) mutant. The cleaved PARP products were not seen in infected cells transduced with the baculovirus expressing the U_S3 protein kinase (Fig. 9B, compare lanes 3 and 5 with lanes 8 and 10). Finally, the control experiment (Fig. 9C) shows that the U_S3 protein kinase was expressed in the transduced RSC. The same set of lysates was tested for the expression of U_L34, a known substrate of U_S3 protein kinase (34, 35, 40), and U_L31, an interacting partner of U_L34 (36, 42). U_L31 present in each of the infected-cell lysates showed a significant shift in electrophoretic mobility, whereas U_L34 did not (data not shown).

Results from a similar experiment using Vero cells are shown in Fig. 9D. U_L31 from R7041 (ΔU_S3)-infected cells migrated faster than that from wild-type HSV-1(F) virus-infected cells (Fig. 9D, compare lane 5 with lane 2), and prior transduction with Bac-U_S3 led to accumulation of the slow-migrating form (Fig. 9D, lane 9). This result indicates that U_L31 is modified by U_S3 protein kinase. Slow-migrating U_L31 accumulated in R7802-infected cells (lane 3), indicating that U_S3.5 also mediates the modification of U_L31.

We conclude from these experiments that the truncated U_S3.5 protein kinase expressed by the R7802 mutant lacking the α22 gene mediates the phosphorylation of HDAC1 and viral protein U_L31 as the full-length U_S3 protein kinase does.

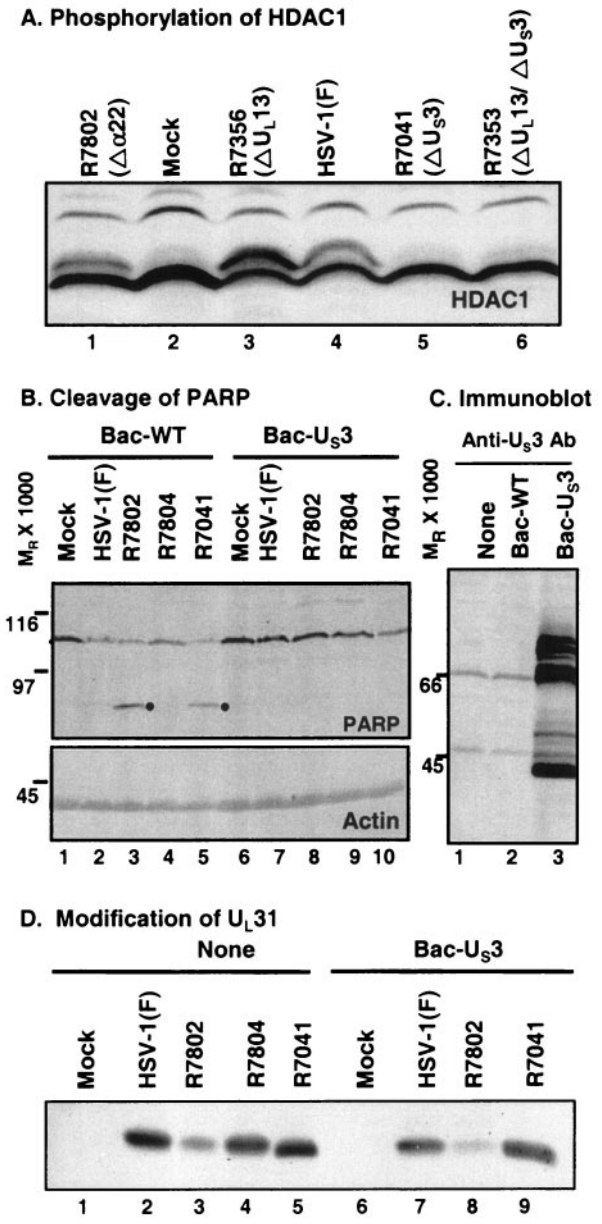

FIG. 9. Identification of functions performed by the alternatively expressed U_S3 protein. (A) Modification of HDAC1. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 10 PFU per cell of wild-type HSV-1(F) or mutant R7802 ($\Delta\alpha22$), R7356 (ΔU_L13), R7041 (ΔU_S3), or R7353 ($\Delta U_L13/\Delta U_S3$). Cells were harvested at 24 h after infection. Proteins were solubilized in 150 μl of disruption buffer. Fifty-microliter aliquots were electrophoretically separated in 11% denaturing polyacrylamide gels, transferred to nitrocellulose sheets, blocked with 5% nonfat milk, and reacted with polyclonal antibody to HDAC1 as described in Materials and Methods. Similar results were obtained using Vero, SK-N-SH, and pHEL cells (data not shown). (B) Protection of cells from PARP cleavage. Replicate cultures of RSC in 25-cm^2 flasks were either mock infected or infected with 5 PFU per cell of wild-type HSV-1(F), mutant R7802 ($\Delta\alpha22$) or R7041 (ΔU_S3), or repaired virus R7804. Cultures were exposed to 10 PFU per cell of Bac-WT (lanes 1 to 5) or Bac-U_S3 (lanes 6 to 10) 12 h before HSV-1 infection, and cells were maintained in DMEM supplemented with 5% newborn calf serum and 9 mM sodium butyrate. Cells were harvested 25 h after HSV-1 infection. Proteins were solubilized in 150 μl of disruption buffer. Fifty-microliter aliquots were electrophoretically separated in 11% denaturing polyacrylamide gels, transferred to nitrocellulose sheets, blocked with 5% nonfat milk, and reacted with a polyclonal antibody (Ab) to PARP and a monoclonal antibody for actin as described in Materials and Methods. Expression of U_S3 proteins by Bac-U_S3 is shown in panel C. (D) Modification of U_L31 protein. Replicate cultures of Vero cells in 25-cm^2 flasks were either mock infected or infected with 5 PFU per cell of wild-type HSV-1(F), mutant R7802 ($\Delta\alpha22$) or R7041 (ΔU_S3), or repaired virus R7804. One set of cultures was exposed to Bac-U_S3 9.5 h before HSV-1 infection (lanes 6 to 9). Cells were harvested 20 h after virus infection and processed as described above. Fifty-microliter aliquots of protein extracts were electrophoretically separated in 11% denaturing polyacrylamide gels, transferred to nitrocellulose sheets, blocked with 5% nonfat milk, and reacted with polyclonal antibody to U_L31.

However, $U_S3.5$ does not block apoptosis induced by the mutant virus in RSC. In essence, the truncated $U_S3.5$ protein retains some, but not all, of the functions of U_S3 protein kinase.

DISCUSSION

The salient features and the significance of this report are as follows. We have shown that cells infected with a mutant lacking the α22 gene accumulate a truncated form of the U_S3 protein. The truncated form designated $U_S3.5$ shares its carboxyl terminus with the full-length U_S3 protein. Both the U_S3 and $U_S3.5$ proteins accumulated in lysates of cells transduced with baculoviruses carrying the U_S3 ORF driven by the CMV immediate-early promoter. A protein corresponding in electrophoretic mobility to the $U_S3.5$ protein was present and accumulated in cells transduced with baculoviruses carrying U_S3 ORFs in which methionine codons 164, 182, and 189 were replaced with either glycine or alanine but not in cells transduced with a baculovirus carrying the U_S3 ORF encoding a substitution of methionine 77. In other experiments, we excluded a role of proteases in the generation of the $U_S3.5$ protein. We conclude that the U_S3 and $U_S3.5$ proteins consist of 481 and 405 residues and initiate at methionine codons 1 and 77, respectively.

The shorter transcript of the U_S3 ORF was reported to initiate at residue 135189. The translation initiation codon of the U_S3 protein is at residue 135222, i.e., 33 nucleotides from the transcription initiation site (20). It is more likely that the product of the shorter transcript is the $U_S3.5$ protein rather than the full-length U_S3 protein. Consistent with this interpretation of available data, cells infected with the R7802 mutant lacking the α22 gene accumulated significantly larger amounts of the shorter mRNA than either the wild-type virus or the R7804 recombinant virus in which the missing α22 gene had been restored.

The role of ICP22 in regulating accumulation of viral proteins is not novel. ICP22 has been shown to be required for optimal accumulation of a subset of late (γ2) proteins exemplified by the U_S11, U_L38, and U_L41 proteins (24, 26, 28, 32). This function maps in the carboxyl-terminal domain of ICP22, and two distinct functions mapping to that site may account for this activity. First, this laboratory has shown that ICP22, in conjunction with the U_L13 protein kinase, mediates the degradation of cyclins A and B and the acquisition by cdc2 of a new partner, the U_L42 DNA polymerase accessory protein.

The cdc2-U_L42 complex recruits topoisomerase IIα in an ICP22-dependent manner (2, 3, 4). Second, independent studies have shown that ICP22, in conjunction with the U_L13 protein kinase, mediated the phosphorylation of RNA polymerase II (19, 38). The detailed mechanisms by which these activities of ICP22 enable optimal synthesis of the subset of late proteins remain to be worked out. The enablement of synthesizing optimal amounts of the subset of late proteins and of synthesizing full-length U_S3 protein do not appear to be covariant properties inasmuch as cells infected with α22 mutants are disabled with respect to the accumulation of optimal amounts of late proteins (e.g., R325) and appropriate levels of the U_S3 protein. This function remains to be mapped. We should also note that in earlier studies this laboratory reported that ICP22 regulated the accumulation and size of ICP0 mRNA although the significance of this observation is not known (8, 32).

The synthesis of carboxyl-coterminal, truncated proteins from independently regulated mRNAs appears to be an HSV strategy reflected also in the case of the α22 and U_L26 ORFs (7, 17, 18). This strategy has several advantages, especially in the case of proteins that perform multiple functions in different subcellular compartments. Fundamentally, the function of proteins is commonly regulated by posttranslational modifications and indeed U_S3 is phosphorylated by at least the U_L13 protein kinase and possibly by other cellular kinases. Another form of imposed functional differentiation is the synthesis of truncated proteins that could be more readily directed to a different compartment or alternative posttranslational modifications. To test the hypothesis that the U_S3.5 protein may differ functionally from the U_S3 protein, we tested two disparate functions of the U_S3 protein. Thus, in earlier studies this laboratory reported that the U_S3 protein kinase mediates the phosphorylation of HDAC1 (27) and blocks apoptosis induced by mutant viruses or by activated proapoptotic cellular proteins (5, 22, 23). In the studies reported here, we showed that in cells infected with the R7802 mutant virus lacking the α22 gene HDAC1 was phosphorylated. However, in RSC infected with either the R7802 mutant virus or the R7041 mutant virus lacking the U_S3 gene, the PARP protein was cleaved, an indication that the U_S3.5 protein kinase encoded by the R7802 mutant was unable to block the activation of caspases. PARP was not cleaved in cells infected with wild-type virus or in mutant virus-infected cells that had been transduced with the U_S3 gene prior to infection. These findings indicate that the U_S3 and U_S3.5 proteins exhibit different sets of partially overlapping functions. The full range of U_S3 and U_S3.5 protein kinase functions remains to be discovered.

A central question of obvious interest is the range of functions expressed by HSV-1. The sum total of independently regulated mRNAs encoded by HSV-1 appears to be 84. But this number is misleading inasmuch as most of the proteins examined to date in this laboratory appeared to be multifunctional and hence the actual number of diverse functions is probably far greater.

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