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Characterization of the Protease and Other Products of Amino-Terminus-Proximal Cleavage of the Herpes Simplex Virus 1 U_L26 Protein

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The herpes simplex virus 1 U_L26 open reading frame encodes a protease which cleaves a small carboxyl-terminal peptide of itself and its substrate encoded by an overlapping, 3′-coterminal transcriptional unit, designated $U_L26.5$. The translational product of $U_L26.5$ is infected-cell protein 35c,d (ICP35c,d) (F. Liu and B. Roizman, J. Virol. 65:206–212, 1991; F. Liu and B. Roizman, J. Virol. 65:5149–5156, 1991). The protease activity maps at the amino terminus of U_L26 translation product designated Pra. Cleavage of Pra to remove the carboxyl-terminal 25 amino acids converts the protein to Prb (F. Liu and B. Roizman, Proc. Natl. Acad. Sci. USA 89:2076–2080, 1992). Other studies reported a second, amino-terminus-proximal cleavage in U_L26 gene products made in *Escherichia coli* (I. C. Deckman, M. Hagen, and P. J. McCann III, J. Virol 66:7362–7367, 1992; C. L. DiIanni, D. A. Drier, I. C. Deckman, P. J. McCann III, F. Liu, B. Roizman, R. J. Colonno, and M. G. Cordingley, J. Biol. Chem., 368:2048–2051, 1993). We report the following results. (i) The amino-terminus-proximal cleavage of U_L26 protein in eukaryotic cells generates two polypeptides, an apparent M_r-25,000 amino-terminal polypeptide designated Prn and a carboxyl-terminal polypeptide which corresponds in electrophoretic mobility to ICP35a. Cleavage of the carboxyl-terminal 25 amino acids by the U_L26 protease converted ICP35a to ICP35b. (ii) Replacement of Ala-247–Ser-248 with Arg-Pro precluded the amino-terminus-proximal cleavage. (iii) Prn, the amino-terminal product of the cleavage reaction at amino acid 247 functions as a protease. (iv) Additional amino acid substitutions in the putative domain of the protease yielded results consistent with the hypothesis that U_L26 encodes a serine protease. (v) The domain of the U_L26 protein whose modification confers the formation of double bands for all products (Pra, Prb, ICP35a, ICP35b, ICP35c,d, and ICP35e,f) except Prn maps in the domain shared by U_L26 and $U_L26.5$, between codons 307 and 417.

Herpes simplex virus 1 (HSV-1) capsids comprise three major groups. These are capsids devoid of cores (A capsids), capsids containing a toroidal structure (B capsids), and capsids containing DNA (C capsids). The B capsids contain two proteins, VP21 and VP22a, that are absent from A capsids. The C capsids lack VP22a (8, 9). VP22a was linked to a family of HSV-1 proteins designated infected-cell protein 35 (ICP35). These proteins form a set of at least six major bands, designated ICP35a, -b, -c, -d, -e, and -f, in one-dimensional gels and numerous spots in two-dimensional separations (3, 4). All of the proteins in this family were shown to react with the same monoclonal antibody. Pulse-chase experiments indicated that the polypeptides in bands designated ICP35c,d were chased into ICP35e,f by posttranslational processing (3, 4).

McGeoch et al. (14) assigned the ICP35 family of proteins to the U_L26 open reading frame on the basis of an observation that at the nonpermissive temperature, ICP35 is not processed in cells infected with a temperature-sensitive (*ts*) mutant whose lesion maps in U_L26 (Fig. 1A, lines 1 and 2) (17). The nature of the mutation has not been reported. Of interest to us was the fact that U_L26 had the capacity to encode a protein of 635 amino acids, whereas the average size of the ICP35 polypeptides was at least 40% smaller. To probe the coding domain of the U_L26 open reading frame, we inserted, at several different sites in frame, a small oligonucleotide which encoded the epitope of a monoclonal antibody which reacted with glycoprotein B of human cytomegalovirus (11). S1 nuclease mapping assays and analyses of proteins reacting with the anti-ICP35 monoclonal antibody as well as those tagged by the "traveling" epitope established that two transcriptional units exist within the domain of U_L26. The larger transcriptional unit encodes a protein with an apparent molecular weight (M_r) of 75,000. The smaller transcriptional unit initiates within the U_L26 open reading frame and is 3′ coterminal with it (Fig. 1A, line 3). The promoter of the smaller unit is within the 5′-terminal coding domain of U_L26. The translation initiation codon of the smaller unit corresponds to Met-307 of the larger unit; that is, the amino acid sequence of the smaller protein is identical to amino acids 307 to 635 of the larger protein. We have designated the open reading frame of the smaller transcriptional unit $U_L26.5$ (11).

In a subsequent study, we demonstrated that the product of U_L26 is a protease (12). The translational product of the open reading frame was designated as the protease *a* band, or Pra. Pra cleaves itself and ICP35c,d at exactly the same position, 25 amino acids from the carboxyl terminus, to yield Prb and ICP35e,f bands, respectively (Fig. 1B). The cleavage of Pra to Prb occurred after translation of the U_L26 open reading frame and incubation of the translated protein in vitro. Studies of the cleavage reaction indicated the following. (i) The protease activity mapped at the amino-terminal domain of the U_L26 gene product, i.e., in a region not shared with ICP35. (ii) The first nine amino acids and the sequences

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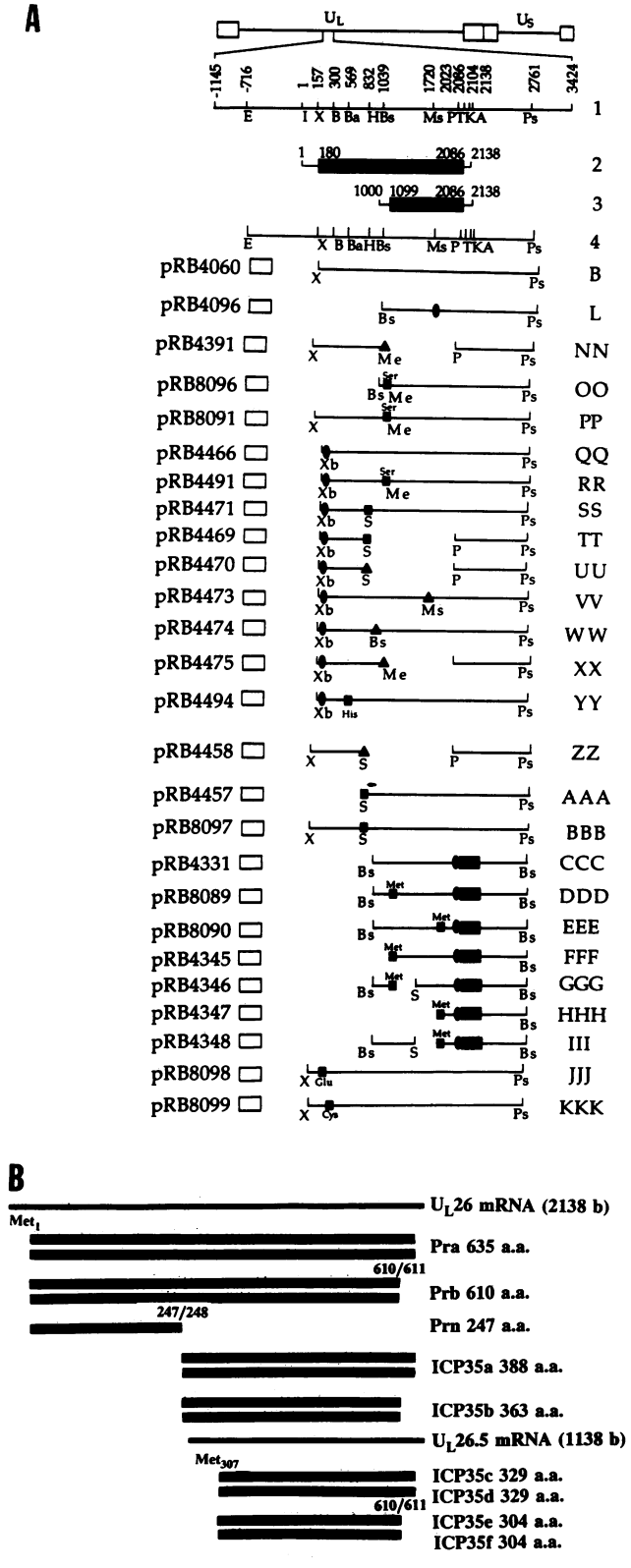

FIG. 1. Schematic representation of the U_L26 and $U_L26.5$ open reading frames and their products tested in these studies. (A) Line 1, nucleotide numbers relative to the approximate transcription initiation site of U_L26 (I represents nucleotide +1), restriction endonuclease sites of the HSV-1 *Eco*NI-*Pst*I DNA fragment containing the U_L26 and the $U_L26.5$ open reading frames, and positions of the 3′ of the translation initiation site of ICP35 were not essential for the proteolytic activity of the gene product. (iii) Mutagenesis of the putative protease domain and studies with inhibitors yielded results consistent with the hypothesis that U_L26 encoded a serine protease (13).

Using an *Escherichia coli* expression system, Deckman et al. (5) identified a second cleavage site amino terminal to the one described by Liu and Roizman (12). DiIanni et al. (6) purified the cleavage products of U_L26 made in *E. coli*. Sequencing analyses of the *E. coli* products established that the protease cleaves between Ala-247 and Ser-248 and between Ala-610 and Ser-611.

A protease corresponding to U_L26 in cytomegalovirus (CMV) was also reported by Welch et al. (18). In this instance, the four open reading frames overlap (19), and the largest encodes a 590-amino-acid protease which cleaves itself at amino acids 249 and 557.

This report focuses on the properties of the cleavage products of the protease at the site proximal to the amino terminus. We report the following. (i) The cleavage generates two polypeptides, an amino-terminal polypeptide designated Prn and a carboxyl-terminal polypeptide which corresponds to ICP35a. Replacement of Ala-247–Ser-248 with Arg-Pro precludes the amino-terminus-proximal cleavage. The cleavage reaction does not require the participation of

translation initiation site of ICP35, of the translation termination site (T), and of the single poly(A) additional signal (A) that serve both the U_L26 and $U_L26.5$ RNAs. Lines 2 and 3, nucleotide positions of transcription initiation, translation initiation and termination, and polyadenylation signal for the U_L26 and $U_L26.5$ open reading frames, respectively. All numbers shown are relative to the transcription initiation site of the U_L26 mRNA at +1. The thick horizontal bar represents the coding sequence. Line 4, restriction endonuclease map with reference to plasmids B through KKK, which are schematic representations of the U_L26 and $U_L26.5$ open reading frames used in studies reported here. The construction of plasmids is described in detail in Materials and Methods. Open rectangles represent the *Bam*HI-Z fragment used as the source of the α4 gene promoter and inserted in the proper transcriptional orientation relative to that of the U_L26 and $U_L26.5$ open reading frames; solid ovals, the 20-amino-acid CMV epitope described elsewhere (11); solid triangles, the inserted stop codon; solid rectangles, mutations introduced by in vitro mutagenesis. In plasmids OO (pRB8096) and PP (pRB4091), the Met-307 codon was replaced with a serine codon, creating a new *Bst*BII site. In plasmid BBB (pRB8097), the Ala-247 and Ser-248 codons were replaced with codons specifying Arg and Pro, respectively. The base substitution created a new *Stu*I site. B, *Bam*HI; Ba, *Bal*I; Bs, *Bst*EII; E, *Eco*NI; H, *Hpa*I; K, *Kpn*I; Ms, *Mst*II; P, *Pml*I; Ps, *Pst*I; S, *Stu*I; X, *Xcm*I; Xb, *Xba*I; Me, methionine translation initiation codon of the $U_L26.5$ open reading frame. The arrow on the drawing of plasmid AAA represents the site of the methionine initiation codon. (B) Schematic representation of the polypeptide products of the U_L26 and $U_L26.5$ open reading frames. The thin lines represent the U_L26 and $U_L26.5$ mRNAs. The heavy lines represent the primary translation products (Pra and ICP35c,d) of the mRNAs and the products of cleavage as deduced from previous studies and those reported here (5, 6, 11–13). With the exception of Prn, all polypeptides form double bands in denaturing polyacrylamide gels. The double bands formed by the translation products of the $U_L26.5$ open reading frame are designated by individual letters (e.g., ICP35c and ICP35d, ICP35e and ICP35f) (4). The same designation applies to each of the double bands formed by the products of the U_L26 open reading frame. Thus, ICP35a and ICP35b each form a double band on electrophoresis in denaturing gels. The subscripts identify the initiating methionine. The sites of cleavage of Pra and ICP35c,d are identified by amino acid (a.a.) numbers above the lines. b, bases.

amino acid sequences carboxyl terminal to amino acid 287. (ii) Prn, the amino-terminal product of the cleavage reaction at amino acid 247 functions as a protease. (iii) Extension of the mutagenesis studies reported earlier indicates that U_L26 encodes a serine protease. (iv) ICP35b arises by carboxyl-terminal cleavage of ICP35a. (v) The doublet band formation characteristic of Pra, Prb, ICP35a, ICP35b, ICP35c,d, and ICP35e,f but not of Prn (Fig. 1B) is a function of the amino acid sequences shared by U_L26 and $U_L26.5$.

MATERIALS AND METHODS

Viruses and cells. HSV-1(F) is the prototype HSV-1 strain used in this laboratory. Thymidine kinase-negative hamster kidney (BHKTK$^-$) cells were propagated as described elsewhere (1, 7).

Monoclonal antibodies. Monoclonal antibodies H725, reacting with the ICP35 family of proteins, and CH28-2, reacting with the 20-mer CMV epitope, were obtained from Lenore Pereira and have been described elsewhere (3, 4, 11). The polyclonal antibody Gy13-6, made against the 14 carboxyl-terminal amino acids of $U_L26.5$, was a gift from Bristol-Myers Squibb and has been described elsewhere (6).

Construction of plasmids. Plasmids pRB4060, pRB4096, and pRB4391 have been described elsewhere (11). The $U_L26.5$ open reading frame in plasmid pRB4093 (11) and the U_L26 open reading frame in pRB4245 (13) were mutagenized to give rise to constructs pRB8089, pRB8090, pRB8091, pRB8096, pRB8097, pRB8098, and pRB8099 with the aid of the Muta-Gene kit (Bio-Rad) in accordance with the manufacturer's recommendations. The 40-mer oligonucleotides used for this purpose were synthesized in an Applied Biosystems model 380B DNA synthesizer. The mutations in these plasmids are shown in Fig. 1. In plasmids pRB8091 and pRB8096, the U_L26 codon Met-307 was changed to a serine codon. In plasmid pRB8089, the U_L26 codons Gln-413-Pro-Ala-Gly-Asp were replaced with a sequence encoding His-Leu-Asp-Met-Val and new *Xba*I and *Sal*I sites. In plasmid pRB8090, the U_L26 codons Pro-583-Thr-Glu-Pro were replaced with a sequence encoding Asp-Met-Val-Asp and new *Xba*I and *Sal*I sites. In plasmid pRB8097, the U_L26 codons Ala-247–Ser-248 were replaced with a sequence encoding Arg-Pro and a new *Stu*I site. In plasmids pRB8098 and pRB8099, the U_L26 codons Glu-114–Glu-115 and Cys-152 were replaced with a sequence encoding Gly and Ala codons and a new *Apa*LI site and a sequence encoding an Ala codon containing a new *Nhe*I site, respectively.

Short double-stranded DNA fragments were made by synthesizing complementary DNA strands in an Applied Biosystems 380B DNA synthesizer and allowing them to anneal. Sequence D (5′-CTAGAACCATGGAGAAGGGA CAGAAGCCCAACCTGCTGACCGACTGCGACACCG CAAAAACGGGTACCGACACT-3′) and its complement encode the epitope of the monoclonal antibody CH28-2 and contain a translational initiation codon and a *Kpn* site for convenient verification of insertion of oligonucleotides into the plasmids. pRB4466 was constructed by inserting sequence D and its complement into the *Xba*I site of pRB4310 (13). pRB4491, pRB4471, and pRB4494 were derived from pRB8091, pRB4466, pRB8077, and pRB8097. pRB4469 was derived from pRB4466 by deleting sequences between the *Stu*I and *Pml*I sites. pRB4473, pRB4474, and pRB4475 were derived from pRB4466 by inserting the translational termination codon (*Spe*I linker; New England Biolabs) into the *Mst*II, *Bst*EII, and $U_L26.5$ translational initiation site, respectively. pRB4470 and pRB4458 were generated from pRB4471 and pRB8097, respectively, by replacing the sequence between the *Stu*I and *Pml*I sites with the translational termination codon (*Spe*I linker; New England Biolabs). pRB4457 was derived from pRB8097 by deletion of the sequence between the *Xba*I and *Stu*I sites. Plasmids pRB4331, pRB4345, and pRB4346 were derived from pRB4093 and pRB8089.

Transfections and infection of cells transfected with plasmid DNAs. Transfections were done as detailed by Kristie and Roizman (10). In most experiments, the transfected cells were exposed for 18 to 20 h posttransfection to 10 PFU of HSV-1(F) per cell, as stated in Results. After 2 h of exposure of the cells to virus at 10°C, the inoculum was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and the cells were incubated at either 34 or 39°C for 20 h. In the experiments which did not involve viral infection, the cells were incubated at 37°C and harvested at 40 to 42 h posttransfection. The harvested cells were washed once with phosphate-buffered saline, pelleted by centrifugation at 4,000 rpm for 5 min in a Du Pont Sorvall SS34 rotor spun in a Du Pont centrifuge, suspended in disruption buffer (0.05 M Tris [pH 7.0], 8.5% [vol/vol] sucrose, 5% [vol/vol] 2β-mercaptoethanol, 2% [vol/vol] sodium dodecyl sulfate [SDS]), sonicated for 20 s in ice, and boiled for 1 min before electrophoretic separation in denaturing gels.

Electrophoretic separation and staining of viral protein bands with antibody. The denatured, solubilized polypeptides from cell lysates were separated on 12% (vol/vol) polyacrylamide–SDS gels cross-linked with *N*,*N*′-diallyltartardiamide (3, 4). The separated polypeptides from BHKTK$^-$ cells were transferred electrically to nitrocellulose membranes and reacted in an enzyme-linked immunoassay with either monoclonal antibody H725 against HSV-1 ICP35, CH28-2 against the CMV epitope, or rabbit polyclonal antibody Gy13-6 against a polypeptide mapping at the carboxyl terminus of the translation product of the U_L26 open reading frame and then with the appropriate anti-mouse or anti-rabbit immunoglobulin G (IgG) antibody conjugated with alkaline phosphatase, as described previously (4).

RESULTS

Characterization of product of cleavage of the translation product of U_L26 at the amino-terminal cleavage site. (i) Experimental design. Because the U_L26 and $U_L26.5$ (ICP35) open reading frames overlap and have identical sequences at the carboxyl-terminal domain of the U_L26 gene product, the experimental design for these studies (shown in Fig. 1) required that the plasmids encoding the protease be modified to exclude or mark the expression of one or the other open reading frame.

To exclude ICP35 expression, the ICP35 translation initiation codon Met-307 was replaced with a serine codon in both $U_L26.5$ (plasmid OO) and U_L26 (plasmid PP). To mark specifically the protease specified by U_L26, the nine amino-terminal codons of U_L26 previously shown to be dispensable for proteolytic activity (13) were replaced in frame with 20 codons specifying the CMV epitope described previously (11). The location of the inserted CMV epitope enabled us to trace the metabolism of the protease by its reactivity with the monoclonal antibody to the CMV epitope. Plasmids QQ, RR, SS, TT, UU, VV, WW, XX, YY, and ZZ were designed to map functional domains of the protease by carrying additional mutations within either the wild-type or the PP plasmid construct. In some plasmids, e.g., SS, the predicted

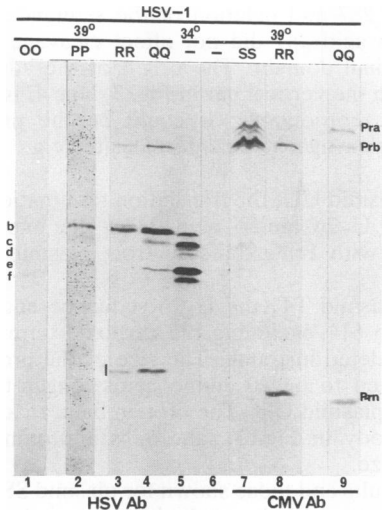

FIG. 2. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs, infected with HSV-1(F), and maintained at 34 or 39°C. The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and reacted first with monoclonal antibody H725 to HSV-1 ICP35 (lanes 1 to 6, HSV AB) or monoclonal antibody CH28-2 to the CMV epitope (lanes 7 to 9, CMV Ab) and then with goat anti-mouse IgG antibody coupled to alkaline phosphatase. The experimental procedure is described in Materials and Methods. The letters above the lanes identify the plasmid constructs with which the cells were transfected. A dash indicates that the cells were infected but not transfected. Prb and Prn represent cleavage products of Pra, the precursor form of the CMV-U_L26 fusion protein. Letters b, c, d, e, and f refer to the ICP35 bands described by Braun et al. (4). See text for explanation of the band marked by the vertical bar in lane 3.

cleavage site at Ala-247–Ser-248 was replaced with codons specifying Pro-247–Arg-248. The stop codons or deletions introduced into other plasmids are shown in Fig. 1.

(ii) Cleavage of U_L26 at its amino-terminal domain. BHKTK$^-$ cells were transfected with plasmids, infected with HSV-1(F), and maintained at 39°C. The cell lysates were electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and treated with either monoclonal antibody H725, against HSV-1 ICP35 protein, or CH28-2, against the CMV epitope. Because HSV-1(F) contains a *ts* lesion in the α4 gene, specifying the major HSV-1 regulatory protein, it does not express its own U_L26 protease or the ICP35 substrate at 39°C (2, 12, 16). The results (Fig. 2) were as follows.

(i) As expected from the previous studies, the $U_L26.5$ gene resident in the HSV-1(F) genome expressed protein bands reactive with the HSV monoclonal antibody at 34°C (Fig. 2, lane 5) but not at 39°C (Fig. 2, lane 6). Furthermore, the presence of the products of proteolytic cleavage (Fig. 2, bands e and f) indicated that the viral protease U_L26 was expressed at 34°C.

(ii) Because the initiator Met-307 codon of $U_L26.5$ was replaced by a serine codon, it could be predicted that cells transfected with plasmid OO would not express ICP35. The absence of a protein band reactive with the ICP35 antibody in Fig. 2, lane 1, indicated that this was in fact the case. The same mutation precluded the expression of ICP35c through ICP35f in cells transfected with plasmid PP. In this instance (Fig. 2, lane 2), a protein with an apparent M_r of 50,000 comigrated with ICP35b (lane 5). We conclude from this experiment that the M_r-50,000 protein is a product of the U_L26 open reading frame and, as demonstrated later in the text, resulted from cleavage of the M_r-75,000 translation product of the U_L26 open reading frame.

(iii) In plasmid QQ, the CMV epitope was inserted in frame with and replaced the first nine amino acids of U_L26 (Fig. 1). Pra and Prb, the full-length and the 25-amino-acid carboxyl-terminally truncated U_L26 proteins, respectively, were expressed from cells transfected with this plasmid and reacted with the CMV antibody (Fig. 2, lane 9). The CMV antibody also reacted with a polypeptide of M_r 25,000 (Prn) (Fig. 2, lane 9). The CMV antibody also reacted with a polypeptide of M_r 25,000 (Prn) (Fig. 2, lane 9). The anti-ICP35 HSV antibody reacted with Prb, ICP35c, -d, -e, and -f, and the M_r-50,000 polypeptide which comigrated with ICP35b expressed from HSV-1-infected cells (Fig. 2, lane 4). The replacement of Met-307 in plasmid RR precluded the expression of ICP35c through ICP35f (Fig. 2, lane 3). In this instance, the CMV antibody reacted with Prb and Prn, whereas the HSV antibody reacted with Prb and the ICP35b-comigrating protein (Fig. 2, lanes 3 and 8). It is noteworthy that, in this series of experiments, Pra was not detected in lanes 3, 4, and 8, most likely because of rapid conversion to Pra. Prn was detected by the CMV antibody only, whereas the polypeptide which comigrated with ICP35b reacted with the HSV antibody only. These results indicate that the sequences encoding Prn are located at the 5′ terminus of the U_L26 open reading frame, whereas the sequences encoding the ICP35b-comigrating polypeptide include the domain of the ICP35 protein containing the epitope to the HSV antibody, and therefore the sequences encoding it are located at or near the 3′ terminus of the U_L26 open reading frame. Neither of these polypeptides is a product of ICP35c,d, since these polypeptides are not made in cells transfected with plasmid RR.

(iv) In plasmid SS, the predicted cleavage site at Ala-247–Ser-248 was replaced with codons specifying Pro-247–Arg-248. The objective was to block the amino-terminal cleavage of the translation product of the U_L26 open reading frame encoded by this plasmid. Cells transfected with plasmid SS yielded Pra, the translational product of U_L26, and Prb, the carboxyl-terminally cleaved product of Pra (Fig. 2, lane 7). Prn was not detected in the lysate of the transfected cells. Staining of the same samples with the HSV-1 antibody to ICP35 revealed that, although the ICP35c through ICP35f bands were present, ICP35b was not, indicating that the M_r-50,000 protein is not derived by translation initiated from a cryptic internal translation initiation site (data not shown).

The key conclusion to be derived from these studies is that Prn is the amino-terminal cleavage product of the protease. As a general observation, it appeared that Prn was more abundant than Pra and Prb in all our assays. In addition, Prb was detected predominantly by both the HSV and CMV antibodies and appeared to be much more abundant than Pra, suggesting that the carboxyl-terminal cleavage of U_L26 is very efficient. The cleavage of Prb at the Ala-247–Ser-248 site, generating the amino-terminal Prn polypeptide, would also generate a carboxyl-terminal polypeptide whose size should correspond to that of ICP35b. This polypeptide would be expected to react with the HSV anti-ICP35 antibody but not with the CMV antibody because this epitope is in the domain of the Prn polypeptide. We should also note that in some but not all assays, a protein with an apparent M_r of 27,000 (marked by a vertical bar) was also detected by the HSV antibody. This protein may be the translation product

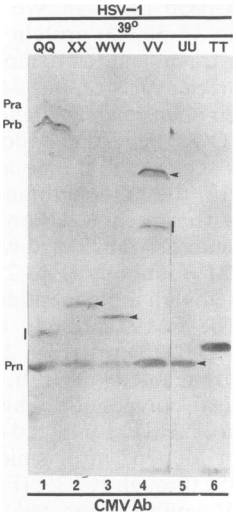

FIG. 3. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs, infected with HSV-1(F), and maintained at 39°C. The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and treated first with monoclonal antibody CH28-2 to the CMV epitope (CMV Ab) and then with goat anti-mouse IgG antibody coupled to alkaline phosphatase. The letters above the lanes identify the plasmid constructs with which the cells were transfected. Prb and Prn represent the cleavage products of Pra, the translational product of the U_L26 gene tagged by the sequence encoding the CMV epitope. The arrows identify the U_L26 protein expressed from the deletion (UU and TT) and stop codon insertion (XX, WW, and VV) plasmids. See text for explanation of the bands marked by vertical bars.

of $U_L26.5$ arising from an internal initiation site (e.g., Met-482 or Met-493). This protein (Fig. 2, lanes 3 and 4) did not comigrate with Prn (Fig. 2, lane 8), a conclusion supported by the fact that the electrophoretic separations were done on the same gel.

(iii) Sequence requirements for amino-terminal cleavage of the U_L26 protein in eukaryotic cells. A series of plasmids (QQ, TT, UU, VV, WW, and XX, Fig. 1A) containing a stop codon insertion or deletion within the U_L26 open reading frame were tested for their capacity to express the U_L26 products Pra, Prb, and Prn. In all these plasmids, the sequence encoding the CMV epitope was inserted in frame in place of the first nine amino acids of U_L26. In this series of experiments, cells were transfected with a plasmid, infected with HSV-1(F), and maintained at 39°C. Analyses were made of the electrophoretically separated, electrically transferred polypeptides with monoclonal antibody CH28-2 against the CMV epitope (Fig. 3).

(i) As expected from the results shown in Fig. 2, lane 9, cells transfected with plasmid QQ expressed Pra, Prb, and Prn (Fig. 3, lane 1). The additional bands marked by a vertical bar in this lane are not reproducible and may be spurious: they are not present in Fig. 2, lane 9.

(ii) In plasmids VV, XX, and WW, the translation termination codon was inserted at position 514, 306, and 287 of U_L26, respectively (Fig. 1). In cells transfected with these plasmids, the expressed epitope-tagged U_L26 migrated faster, corresponding in size to the truncated protein product (Fig. 3, lanes 2, 3, and 4). In addition, Prn was present in all lysates, indicating that the cleavage site was upstream from amino acid 287 and deletion of the sequence downstream from amino acid 287 did not affect cleavage at the U_L26 amino-terminal domain. The origin of the additional band marked with the vertical bar in Fig. 3, lane 4, is unclear, and it was not reproducible; it could be the product of an additional cleavage at an internal site (e.g., Ala-331–Ser-332).

(iii) In plasmid UU, the translation termination codon was inserted at U_L26 amino acid 247. The expressed U_L26 comigrated with Prn expressed from plasmid QQ (Fig. 3, lane 5).

(iv) In plasmid TT, the U_L26 sequence encoding amino acids 248 to 614, including the carboxyl-terminal cleavage site, was deleted in frame. The size of the protein product was predicted to be 20 amino acids longer than that expressed by plasmid UU. The protein band reacting with the CMV antibody in Fig. 3, lane 6, is approximately of the predicted size.

These results and those shown for plasmid SS (Fig. 2, lane 7) are consistent with the prediction from studies with *E. coli* that the U_L26 open reading frame is cleaved between amino acids 247 and 248 (5, 6). It is noteworthy that the mutations in codons Ala-247 and Ser-248 which block cleavage at that site do not affect the function of the protease, because the protease expressed from plasmid SS still catalyzes the carboxyl-terminal cleavage of 25 amino acids to yield Prb.

(iv) U_L26 Prn polypeptide is a functional protease and is the only viral factor required for both amino-terminal and carboxyl-terminal cleavages. Two series of experiment were done to determine whether Prn can function as a protease. The objective of the first series of experiments described in this section was to determine whether the Prn polypeptide retains proteolytic activity and whether it requires other proteins for its activity. The HSV-1 open reading frames in all three plasmids used in this study were controlled by the α4 promoter, consistent with the experimental design of previous studies showing that U_L26 open reading frames controlled by this promoter are readily expressed in transfected cells in the absence of infection (11, 12). Plasmid B contained an intact U_L26 open reading frame. In plasmid ZZ, the coding domain downstream from Ala-247 was deleted. In an earlier study, replacement of the U_L26 His-148 by Ala abolished proteolytic activity (13). In plasmid construct YY, the U_L26 open reading frame was modified as follows: the amino-terminal nine codons were replaced by 20 codons specifying the CMV epitope and His-148 was replaced with Ala (Fig. 1). The electrophoretically separated cell lysates were tested with CMV antibody, and the results, shown in Fig. 4, were as follows. Cells transfected with plasmid YY alone accumulated Pra only (Fig. 4, lane 1). In cells cotransfected with YY and B or ZZ, Prb and Prn as well as Pra could be detected by the CMV antibody against the epitope. The additional band marked by a vertical bar (Fig. 4, lanes 2 and 3) may be the result of an additional cleavage at Ala-331–Ser-332.

The second series of experiments were designed to test whether Prn could cleave the carboxyl-terminal 25 amino acids from ICP35c,d. The plasmids used in this series of experiments included plasmids L and AAA in addition to those described above. Plasmid L, described previously (12), contains the coding sequence of the CMV epitope inserted in frame into the $U_L26.5$ open reading frame. The gene product serves as the test substrate for the protease. In plasmid AAA, the sequences between codons 1 and 247 have been deleted. This plasmid was expressed from the α4 promoter, and the available initiation codon was codon

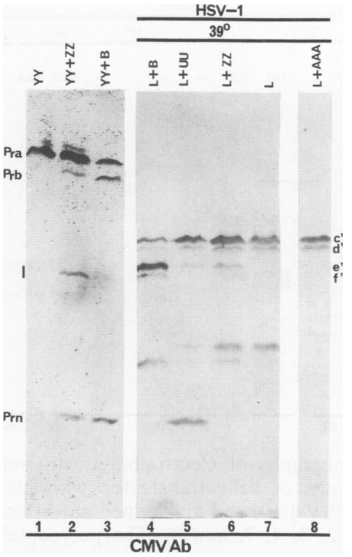

FIG. 4. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs and then either mock infected (lanes 1 to 3) or infected with HSV-1(F) and maintained at 39°C (lanes 4 to 8). The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and treated first with monoclonal antibody CH28-2 to the CMV epitope (CMV Ab) and then with goat anti-mouse IgG antibody coupled to alkaline phosphatase. The letters above the lanes identify the plasmid constructs with which the cells were transfected. Prb and Prn represent the cleavage products of Pra, the translational product of the U_L26 gene tagged with the sequence encoding the CMV epitope. c', d', e', and f' refer to the ICP35 bands containing the CMV epitope, as described previously (12). See text for explanation of the bands marked by vertical bars.

Met-252 of the U_L26 open reading frame. In this series of experiments, the cells were transfected with these plasmids, infected with HSV-1(F), and maintained at 39°C. Cell lysates were separated on SDS-polyacrylamide gels, electrically transferred to a nitrocellulose membrane, and stained with monoclonal antibody CH28-2 against the CMV epitope. The salient features of the results were as follows (Fig. 4).

(i) Cells transfected only with plasmid L expressed the ICP35 precursor forms ICP35c and ICP35d as expected. Both bands reacted with monoclonal antibody CH28-2 against the CMV epitope (Fig. 4, lane 7). In lysates of cells cotransfected with plasmids L and B, the CMV epitope-tagged ICP35 contained both the precursor (ICP35c,d) and the cleavage (ICP35e,f) products (Fig. 4, lane 4).

(ii) Only ICP35c and ICP35d were detected in cells cotransfected with plasmids L and AAA, indicating that the U_L26 protein sequence from amino acids 248 to 635 did not possess proteolytic activity (Fig. 4, lane 8).

(iii) ICP35c, -d, -e, and -f, i.e., both precursor and cleavage products, were detected in cells cotransfected with plasmids L and UU or ZZ (Fig. 4, lanes 5 and 6). These results indicate that Prn, containing the first 247 amino acids of U_L26 protein, is capable of cleaving ICP35. It is noteworthy that more ICP35c and ICP35d were converted into ICP35e and ICP35f in cells transfected with plasmid ZZ than in cells transfected with plasmid UU, suggesting that the substitution of the first 10 amino acids with the 20-amino-acid CMV epitope sequence at the amino terminus of the U_L26 protein reduced its enzymatic activity.

TABLE 1. Mutant U_L26 genes used in these studies[a]

Plasmid	Wild-type sequence affected	Mutated sequence
DDD	Gln-413-Pro-Ala-Gly-Asp	His-Leu-Asp-Met-Val
EEE	Pro-583-Thr-Glu-Pro	Asp-Met-Val-Asp
JJJ	Glu-114-Glu	Gly-Ala
KKK	Cys-152	Ala

[a] The substituted sequences (new restriction sites) were as follows: plasmid DDD, GGTCAGCCGGCCGCGGGAGAC with GGTCATCTAGACATGG TCGAC (*Xba*I and *Sal*I); plasmid EEE, CTACCGACGGACCCCGCG with CTAGACATGGTCGACGCG (*Xba*I and *Sal*I); plasmid JJJ, CGGGAGG AGCGC with CGGGGTGCACGC (*Apa*LI); and plasmid KKK, GCGCTGT GCGCG with GCGCTAGCCGCG (*Nhe*I).

These results indicate the following. (i) The truncated polypeptide specified by plasmid ZZ and equivalent to the proteolytically generated Prn was sufficient to cleave both the U_L26 protein rendered incapable of proteolytic activity and the ICP35c,d precursor protein. (ii) As in the case of the carboxyl-terminal cleavage of ICP35c,d by the products of the U_L26 open reading frame demonstrated earlier, the cleavage of the U_L26 products by Prn does not require other viral gene products. The additional bands marked by vertical bars in Fig. 4 may represent translational products of $U_L26.5$ arising from an internal initiation codon, i.e. Met-482 or Met-493.

(v) Cys-152 is not essential for proteolytic activity. Earlier studies have shown that the properties of U_L26 are consistent with those of a serine protease (13). A sequence homology search for U_L26 and its homologs in all herpesviruses suggested that U_L26 may be either a cysteine protease with an active site at His-61, Glu-114 or Glu-115, or Cys-152 or a serine protease with an active site at His-61, Glu-114 or Glu-115, Ser-198, or Ser-215 (19). Earlier studies on the protease (13) have shown that His-61 and His-148 are essential for proteolytic activity. In order to determine whether Cys-152, Glu-114, and Glu-115 are essential for proteolytic activity, the codons specifying these amino acids were replaced with a codon specifying glycine or alanine (plasmids KKK and JJJ, respectively) (Fig. 1 and Table 1). In plasmid L, the $U_L26.5$ open reading frame contains the CMV epitope inserted in frame and is controlled by an $\alpha 4$ promoter. Cells were cotransfected with a plasmid containing either the wild-type or mutated protease and plasmid L, infected with HSV-1(F), and maintained at 39°C. Analyses of the electrophoretically separated polypeptides in denaturing gels with antibody to the CMV epitope, which reacts only with the products of the L plasmid, were as follows (Fig. 5).

(i) As expected, and consistent with previous results, cells transfected only with plasmid L expressed the ICP35 precursor ICP35c,d but not the processed product, ICP35e,f (Fig. 5, lane 1). ICP35e,f and ICP35c,d were present in cells cotransfected with plasmid L and plasmid B, which contains the intact U_L26 sequence (Fig. 5, lane 4).

(ii) Both the precursor (ICP35c,d) and cleavage (ICP35e,f) products were present in cells cotransfected with plasmids L and KKK. These experiments indicated that the replacement of Cys-152 did not affect the proteolytic activity of the U_L26 protease (Fig. 5, lane 3).

(iii) The precursor ICP35c,d but not the cleavage product ICP35e,f was present in cells cotransfected with plasmids L and JJJ. These results indicated that the replacement of Glu-114 and Glu-115 abolished proteolytic activity (Fig. 5, lane 2).

Characterization of carboxyl-terminal product of cleavage

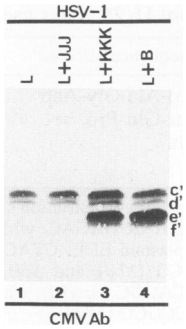

FIG. 5. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs, infected with HSV-1(F), and maintained at 39°C. The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and treated first with monoclonal antibody CH28-2 to the CMV epitope (CMV Ab) and then with goat anti-mouse IgG antibody coupled to alkaline phosphatase. The letters above the lanes identify the plasmid constructs with which the cells were transfected. c', d', e', and f' refer to the ICP35 bands containing the CMV epitope, as described previously (12).

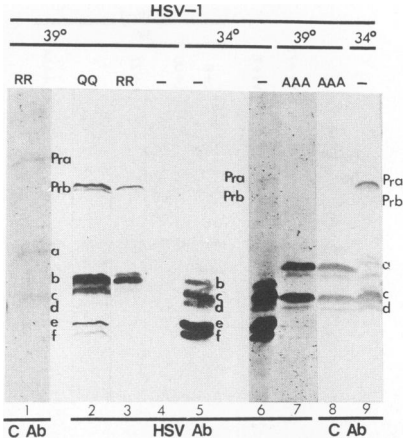

FIG. 6. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs, infected with HSV-1(F), and maintained at 34 (lanes 5, 6, and 9) or 39°C (lanes 1, 2, 3, 4, 7, and 8). The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and reacted first with monoclonal antibody H725 to HSV-1 ICP35 (lanes 2 to 7, HSV AB) or polyclonal antibody to the 14-amino-acid sequence of the $U_L26.5$ carboxyl-terminal domain (lane 1, 8, and 9, C Ab) and then with an appropriate IgG antibody coupled to either alkaline phosphatase or horseradish peroxidase. The experimental procedure is described in Materials and Methods. The letters above the lanes identify the plasmid constructs with which the cells were transfected. A dash indicates that the cells were infected but not transfected. Prb represents the cleavage product of Pra, the precursor form of U_L26 protein. Letters a, b, c, d, e, and f refer to the ICP35 bands as described by Braun et al. (4).

of the U_L26 protein at the amino-terminal cleavage site. (i) Experimental design. Braun et al. (3, 4) described two additional proteins, ICP35a and ICP35b, as members of the ICP35 family characterized by common epitopes. Previous studies have shown that ICP35a and -b are not the products of the $U_L26.5$ open reading frame, although these proteins share with ICP35c and -d, the products of this open reading frame, the epitope recognized by monoclonal antibody H725 (12). In a preceding section, we reported that one of the products of the amino-terminal cleavage comigrated with ICP35b. In this section, we show that the carboxyl-terminal product of the cleavage is the carboxyl domain of the U_L26 protein and that this domain corresponds to ICP35a. Cleavage of the carboxyl-terminal 25 amino acids from ICP35a yields ICP35b. To demonstrate that ICP35a is the product of the amino-terminal cleavage of Pra, the product of the U_L26 open reading frame, we show that it contains the carboxyl-terminal sequences encoded by U_L26. We used for this purpose the rabbit polyclonal antibody Gy13-6 made against a 14-amino-acid sequence contained in the 25-mer sequence cleaved by the protease from the carboxyl terminus of ICP35c,d. The antibody was obtained from Bristol Myers Squibb and has been described elsewhere (6).

(ii) Antibody Gy13-6 reacts with U_L26 and $U_L26.5$ gene products containing the carboxyl-terminal 25-amino-acid peptide cleaved off by the U_L26 protease. Previous studies have shown that the translation products Pra of U_L26 and ICP35 of $U_L26.5$ are cleaved at the carboxyl terminus by the product of the U_L26 gene (12). The immunoblot in Fig. 6, lane 9, shows that the polyclonal Gy13-6 serum made against a carboxyl-terminal peptide reacts with Pra and ICP35c,d but not with the cleaved forms, Prb and ICP35e,f, respectively.

(iii) ICP35a and ICP35b are derived from the amino-terminal cleavage of U_L26. In previous studies and earlier in the text, it was shown that Pra made either in *E. coli* or in eukaryotic cells is cleaved by itself at two positions, between amino acids 610 and 611 and at an amino-terminus-proximal position between amino acids 247 and 248. The products of the amino-terminal cleavage are Prn, a functional protease with an apparent M_r of approximately 25,000, and a larger protein (apparent M_r of 50,000) corresponding in electrophoretic mobility to the ICP35a,b product. In this series of experiments, we show that a protein which initiates from amino acid 252, i.e., four amino acids after the cleavage site at amino acid 247, exhibits properties similar to those of ICP35a,b described by Braun et al. (4). In plasmid AAA, the amino acid sequence of U_L26 downstream from residue 247 was controlled by the α4 promoter. Plasmids QQ and RR, shown in Fig. 1, contained the CMV epitope at the amino terminus of the U_L26 open reading frame. In addition, in plasmid RR, the Met-307 codon, which serves as a translation initiation codon for ICP35, was mutated into a serine codon, and this mutation was previously shown to block synthesis of ICP35c through ICP35f. Cells were transfected with plasmids, infected with HSV-1, and maintained at 39°C. The results of analyses of these constructs (Fig. 6 and 7) show the following.

(i) As shown in earlier studies, the $U_L26.5$ gene resident in the HSV-1(F) genome expressed protein bands reactive with the HSV-1 anti-ICP35 monoclonal antibody at 34°C (Fig. 6, lane 5) but not at 39°C (Fig. 6, lane 4), because HSV-1(F) contains a *ts* lesion in the α4 gene and late genes are not expressed at the nonpermissive temperature.

(ii) Because the Met-307 codon was substituted with a serine codon, ICP35c through ICP35f were not expressed in cells transfected with plasmid RR (Fig. 6, lane 3), whereas plasmid QQ which lacks this substitution, did express these polypeptides (Fig. 6, lane 2). The carboxyl-terminal portion of amino-terminal cleavage of the U_L26 product (ICP35b) appeared to be a doublet and comigrated with ICP35b from HSV-1-infected cells (Fig. 6, lane 3). Staining of the same

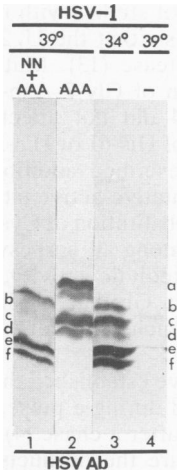

FIG. 7. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs, infected with HSV-1(F), and maintained at 34°C (lane 3) or at 39°C (lanes 1, 2, and 4). The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and reacted first with monoclonal antibody H725 to HSV-1 ICP35 (HSV Ab) and then with goat anti-mouse IgG antibody coupled to alkaline phosphatase. The procedures are described in Materials and Methods. The letters above the lanes identify the plasmid constructs with which the cells were transfected. A dash indicates that the cells were infected but not transfected. Prb represents the cleavage product of Pra, the translation product of the U_L26 open reading frame. a, b, c, d, e, and f refer to the ICP35 bands as described by Braun et al. (4).

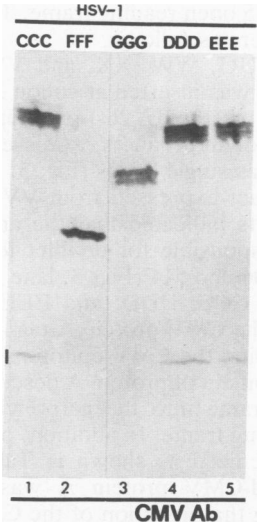

FIG. 8. Photographs of electrophoretically separated polypeptides from lysates of cells transfected with plasmid constructs and then infected with HSV-1. The polypeptides were electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and treated first with monoclonal antibody CH28-2 to the CMV epitope (CMV Ab) and then with goat anti-mouse IgG antibody coupled to alkaline phosphatase. The letters above the lanes identify the plasmid constructs with which the cells were transfected. Prb and Prn represent the cleavage products of Pra, the translational product of the U_L26 gene tagged with the sequence encoding the CMV epitope. See text for explanation of the band marked by a vertical bar.

lysate with Gy13-6 (Fig. 6, lane 1) revealed that no ICP35b was detected. Prolonged staining of the transferred electrophoretically separated proteins made apparent three faint bands corresponding to Pra, ICP35a, and ICP35c.

(iii) Cells transfected with plasmid AAA, infected with HSV-1, and maintained at 39°C expressed ICP35c and ICP35d (Fig. 6, lanes 7 and 8) since, as described previously (12), the $U_L26.5$ promoter resident in the plasmid is activated by viral infection at 39°C. In addition, a doublet band comigrating with ICP35a from HSV-1-infected cells was detected with HSV-1 anti-ICP35 antibody (Fig. 6, lane 7) and the Gy13-6 antibody (Fig. 6, lane 8). It is noteworthy that the top band (ICP35a) of HSV-1-infected cells in lane 9 migrated slower than the top band in lanes 7 and 8 in Fig. 6. As indicated above, the translation product of the truncated U_L26 open reading frame encoded by plasmid AAA was predicted to be 283 amino acids long, i.e., five amino acids shorter than the predicted product of ICP35a arising from cleavage of Pra, the translation product of U_L26 after amino acid 247. These results indicate that the carboxyl-terminal portion of the U_L26 protein after cleavage at amino acid 247 is the doublet ICP35a.

(iv) In construct NN, the entire $U_L26.5$ open reading frame was deleted and the truncated U_L26 sequence encoding amino acids 1 to 306 was placed under the control of the $\alpha 4$ promoter. The translation product of the truncated U_L26 in plasmid NN cannot react with antibodies against ICP35. Because the truncated protein specified by NN is proteolytically active (13), it could be expected that ICP35c,d would be cleaved into ICP35e,f. This in fact was the case (Fig. 7, lane 1). ICP35b and ICP35e,f were predominantly detected when large amounts of plasmid NN DNA were transfected with plasmid AAA, whereas only ICP35a and ICP35c,d were detected in cells transfected with plasmid AAA only (Fig. 7, lane 2). These results indicated that ICP35b was derived from ICP35a by cleavage of the carboxyl-terminal 25 amino acids.

Therefore, we conclude from this series of experiments that (i) ICP35a and ICP35b each form two bands, (ii) ICP35b is derived from ICP35a by cleavage of the carboxyl-terminal 25 amino acids, and (iii) ICP35a and ICP35b are derived from the carboxyl-terminal portion of U_L26 by cleavage of Pra, the translation product of U_L26 after amino acid 247.

(iv) Protein modification reflected in the formation of double bands by ICP35a, ICP35b, ICP35c,d, ICP35e,f, Pra, and Prb maps in the domain of the $U_L26.5$ open reading frame. It was reported elsewhere that the U_L26 proteins Pra and Prb made in eukaryotic cells each form doublet bands in denaturing polyacrylamide gels (see Fig. 6 in reference 11). One hypothesis presented at that time was that the double bands arose by initiation at both methionine 1 and methionine 10. In plasmid QQ, the CMV epitope sequence and a translation initiation codon were inserted in frame and replaced the first nine codons of U_L26. Cells transfected with this plasmid and infected with HSV-1(F) expressed both Pra and Prb doublets that reacted with CMV monoclonal antibody (Fig. 3, lane 1). However, Prn formed a single band (Fig. 3, lane 1), indicating that the modification responsible for doublet formation was carboxyl terminal to amino acid 247.

In order to map the site at which the modification responsible for doublet formation occurs, we analyzed the electrophoretic mobility of the products of plasmid constructs containing either a stop codon insertion or a deletion within

the U_L26 or $U_L26.5$ open reading frame. The results, shown in Fig. 3 and 8, were as follows.

(i) In plasmids UU, WW, XX, and VV, the translation termination codon was inserted at codon 247, 287, 306, and 514, respectively, of the U_L26 open reading frame. The truncated U_L26 protein products expressed from UU, WW, and XX appeared as single bands (Fig. 3, lanes 2, 3, and 5), whereas the product expressed from VV formed a double band. These results indicated that the amino acids whose modification is responsible for doublet formation map between positions 306 and 514 (Fig. 3, lane 4).

(ii) In plasmids CCC, DDD, and EEE (Fig. 8), the sequence encoding the CMV protein A tag—a 276-amino-acid polypeptide encoding the CMV epitope and five repeats of the IgG-binding domain of protein A described earlier (12)—was inserted in frame into the carboxyl terminus of the $U_L26.5$ open reading frame. In addition, plasmids DDD and EEE were mutagenized as shown in Table 1. The fusion protein $U_L26.5$c,d-CMV protein A was expressed as a doublet, indicating that insertion of the CMV protein A tag and of the additional mutations did not affect doublet formation (Fig. 8, lanes 1, 4, and 5).

(iii) In plasmid GGG, the U_L26 sequence between codons 417 and 462 was deleted in frame (Fig. 1). The presence of the ICP35c,d-CMV protein A doublet bands indicated that the deletion did not affect the modification (Fig. 8, lane 3).

(iv) Plasmid FFF contains a deletion in the $U_L26.5$ coding sequences from U_L26 codons 307 to 417 (Fig. 1). In this instance, the chimeric $U_L26.5$-protein A product of the transfected cells formed a single band, indicating that the deletion abolished the modification (Fig. 8, lane 2).

We conclude from these experiments that (i) the sequence responsible for doublet band formation in denaturing polyacrylamide gels maps in the $U_L26.5$ open reading frame and contributes to the doublet appearance of Pra, Prb, ICP35a,b, and ICP35c through ICP35f but not Prn and (ii) the sequences which impart this property to the proteins map between amino acids 307 and 417, because the protein lacking this sequence forms a single band (Fig. 8, lane 2).

DISCUSSION

The translational product of the HSV-1 U_L26 open reading frame, Pra, is cleaved in eukaryotic cells at two positions, at a site proximal to the carboxyl terminus and in the amino-terminal half of the molecule. These sites correspond to the positions of Ala-611–Ser-612 and Ala-247–Ser-248 mapped by DiIanni et al. (6). Neither the carboxyl-terminal nor the amino-terminus-proximal cleavage requires the participation of other viral proteins.

The products of the amino-terminus-proximal cleavage are proteins with apparent M_rs of 25,000 and 50,000. We have designated the smaller protein Prn, for protease amino terminus. Prn has retained its proteolytic activity, as predicted from mapping studies showing that the protease activity maps at the amino terminus of the U_L26 protein (13). In the design of this experiment, we took advantage of the observation that His-148 is essential and its replacement with alanine destroyed the protease activity. The protein with the His-148 substitution became a very useful substrate for the Prn. It is noteworthy that the amino-terminal cleavage does not require the sequences carboxyl terminal to amino acid 287, which is only 40 amino acids distant from the cleavage site. Attempts to date to define the nature of the HSV-1 protease rest on the use of inhibitors and on substitutions of critical sites within the mapped domain of the protease. The results of studies with inhibitors were consistent with the hypothesis that the U_L26 open reading frame encodes a serine protease (13). Mutagenesis studies indicated that substitution of Gly-7–Asp-8–Arg-9, Asp-31–Ser-32–Gly-33, or Asp-34 did not affect proteolytic activity, whereas substitution of His-61 or His-148 abolished activity. In this report, we described additional studies based on mutagenesis of the putative active sites of the protease. In the current studies, substitution of Cys-152, the single highly conserved cysteine among all herpesvirus U_L26 homologs, did not affect the proteolytic activity, whereas the replacement of Glu-114 and Glu-115 abolished activity. These results support the conclusion that U_L26 encodes a serine protease.

Previous studies have established that ICP35c and ICP35d bands are synthesized during a pulse, whereas ICP35e and ICP35f bands appear after a chase (4). We have shown that ICP35c and ICP35d are the products of the $U_L26.5$ open reading frame and that these proteins are cleaved by the U_L26 protease at the carboxyl terminus to yield ICP35e and ICP35f (12). The studies presented in this report are consistent with the hypothesis that ICP35a and -b correspond to carboxyl-terminal products of the amino-terminus-proximal cleavage of Pra and Prb, respectively. Pra and Prb are the designations we have given to the U_L26 protease translation product and its processed form after cleavage of the carboxyl-terminal 25 amino acids, respectively (12, 13). It is possible that only ICP35b was detected because of the rapid processing of Pra to Prb. It is noteworthy that Pra and Prb are processed rapidly and efficiently to Prn and ICP35a,b. These observations shed light on the relative paucity of Pra and Prb in infected-cell extracts reported in earlier studies (11–13).

A curious observation reported in previous studies (4, 11–13) was that all of the bands were doublets. In the case of the faster-migrating bands, e.g., ICP35c through ICP35f, the doublets were well enough resolved to be designated individually (e.g., ICP35c and ICP35d, ICP35e and ICP35f). In the case of the slower-migrating bands, the individual bands forming the doublets were not individually identified, although the doublet nature of the bands was recognized. In the case of Pra and Prb proteins, the existence of two methionine codons in frame and only 10 codons apart suggested the possibility that the doublets arise by translation initiation from the 1st and 10th codons to yield the doublet bands constituting both Pra and Prb. Elimination of one of the methionine codons did not affect the formation of doublets, and in any event, the same doublet origin could not be attributed to the ICP35c,d doublet. In the studies reported here, we show that Prn forms a single band, and therefore doublet formation must be the function of a sequence encoded 3′ of codon 247. Mapping studies reported here indicate that sequences whose modification confers the capacity to form doublets map in the domain shared by the U_L26 and $U_L26.5$ open reading frames, more specifically, between U_L26 codons 307 and 417. The nature of the modification which results in doublet formation is not known. It is conceivable that Pra, Prb, and ICP35a through f exist as multimers, and only a specific fraction of the molecules in the multimeric protein are differentially modified.

A central and key question that remains unresolved at present is the significance of the cleavages observed in this and earlier studies. The question arises from the observation that coreless (A) capsids lack toroidal structure and VP22a, whereas capsids with the inner toroidal structure contain

VP22a. Treatment of capsids with guanidine hydrochloride extracts both VP22a and the toroidal structure (15). VP22a corresponds in size and has been linked to the ICP35c and -d forms. It could be expected that the cleavages we have observed and mapped occur in the process of capsid assembly. In fact, cleavages are demonstrable in cells in the absence of other viral proteins, suggesting that the presence of self-substrate is sufficient for cleavage. It is possible that (i) both carboxyl- and amino-terminal cleavages are necessary for capsid assembly to take place or (ii) the cleavages we have observed are aberrant; they occurred because other structural components of the capsid are absent. The question remains unresolved and is under investigation. We should note, however, that Prn corresponds in size to VP24, which remains unassigned to a specific open reading frame. It is conceivable that VP24 is the protease and that it is retained as a component of the virion.

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