The Human Cytomegalovirus 86-Kilodalton Immediate-Early 2 Protein: Synthesis as a Precursor Polypeptide and Interaction with a 75-Kilodalton Protein of Probable Viral Origin

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The immediate-early 2 (IE2) 86-kDa polypeptide, a major immediate-early gene product of human cytomegalovirus, regulates transcription both positively and negatively. We report two new properties of the IE2 86-kDa polypeptide in infected cells. Immunoprecipitation of infected cell proteins from human embryonic lung cells by antipeptide or monoclonal antibodies specific for IE2 epitopes revealed three closely migrating polypeptide species. The slowest, p86, behaved as expected for the mature 86-kDa IE2 polypeptide. The middle species, p80, was immunoprecipitated from denatured as well as native samples and labeled to steady state rapidly. Pulse-chase analysis demonstrated directly that p80 was a metabolic precursor to p86. The fastest-migrating species, p75, was not detected by probing blots of the immunoprecipitated proteins with IE2-specific antisera; p75 was not precipitated from denatured protein samples; and the products of partial proteolysis of p75 were distinct from those of p86. These properties established p75 as an unrelated coprecipitated polypeptide complexed with p86. The p75 proteins coprecipitated from cells infected with two different strains of human cytomegalovirus, AD169 and Towne, had different mobilities. p75 was detected as early as 6 h and as late as 72 h after infection, but it was not synthesized in cells released from a cycloheximide block. Thus, it is likely that p75 is an early viral protein.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an important human pathogen that causes congenital abnormalities in neonates and life-threatening infections in immunocompromised individuals (for a review, see reference 42). The viral genome is a double-stranded linear DNA molecule of approximately 230 kbp (12, 16, 18, 32). HCMV infection of permissive human fibroblast cells results in a temporally regulated pattern of sequential gene expression divided into three phases: immediate-early (IE), early, and late (11, 13, 39, 68, 69). IE gene expression is restricted to a few regions of the genome and occurs in the absence of viral protein synthesis (11, 28, 39, 59, 61, 68, 69, 71).

The coding sequences for the most abundant HCMV IE products map to two adjacent regions designated IE1 and IE2 and are expressed under control of the major IE enhancer-promoter (MIEP) located 5' of IE1 (3, 58, 58, 61, 65). Expression of these two IE regions is regulated both transcriptionally and posttranscriptionally (19, 20, 25, 37, 45, 53, 55, 56, 60) and gives rise to differentially spliced mRNAs ranging in size from 1.4 to 2.25 kb (55, 57, 58, 61). An abundant 72-kDa IE1 protein is encoded by a 1.95-kb mRNA consisting of exons 1 to 4 from the IE1 region (57, 61) (see Fig. 1). The predominant IE2 gene product has an apparent size of about 86 kDa and is encoded by a 2.25-kb mRNA consisting of exons 1 to 3 and unspliced exon 7 from the IE2 region (37, 55, 58, 61). IE2 exons 5 and 6, spliced to the shared 5' exons 1 to 3, give

rise to a 1.7-kb transcript that codes for a 55-kDa protein (26, 44, 55). These IE1 and IE2 proteins are phosphorylated and localize to the nucleus of infected cells (26, 31, 44). The region also encodes other IE2 proteins including a 40-kDa species, a nonphosphorylated 28-kDa protein (26, 44, 55), and several polypeptides whose structures have not been defined precisely.

Whereas the 72-kDa IE1 and the 86-kDa IE2 proteins are synthesized throughout infection, the 55-kDa IE2 protein has been observed only under IE conditions (26, 37, 44, 55, 58). The 40-kDa IE2 product is synthesized from mRNA expressed from a separate late promoter in the IE2 region (44, 47, 55).

IE1 and several of the IE2 proteins regulate transcription of homologous as well as heterologous viral and cellular genes (1, 4, 6, 9, 10, 21, 22, 26, 37, 45, 48, 56, 63). Depending on the target promoter, these proteins activate transcription alone or in combination (14, 21, 22, 56, 67). Whereas specific promoter sequences are required for activation by IE1, either independently or in cooperation with IE2, no specific IE2 target sequences have been identified (6, 14, 21, 54, 67). In addition to its activation function, the 86-kDa IE2 protein represses transcription from the MIEP (5, 25, 43, 45). This negative autoregulation is mediated by a specific *cis*-acting repression signal located immediately 5' of the transcription initiation site in MIEP (5, 35, 43).

Although the molecular mechanisms by which IE1 and IE2 function in transcription control *in vivo* are not known, recent *in vitro* studies provide some clues with respect to IE2. The 86-kDa IE2 protein interacts with the general transcription factor TBP (TATA-binding protein) *in vitro* (22). Purified bacterial fusion proteins containing either the entire 86-kDa IE2 protein or the carboxy-terminal region bind specifically to the *cis*-acting repression signal and repress *in vitro* transcription mediated by the MIEP (34, 36). The IE2 protein and TBP can occupy their binding sites simultaneously, although binding of one impairs the subsequent binding of the other (29). These

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studies suggest that the binding of the IE2 protein to the *cis*-acting repression signal accomplishes autoregulation by physically inhibiting the interaction of other transcription factors near or at the site of transcription initiation. IE2 protein activates transcription *in vitro* at least in part through relief of histone H1-mediated repression (30). The IE2 protein interacts with itself as well as with several cellular proteins ranging in size from more than 200 to 14 kDa (7, 17). Identification of these proteins should help elucidate the mechanisms by which IE2 functions during virus replication.

The synthesis of the 579-amino-acid 86-kDa IE2 polypeptide in cells infected by the Towne strain of HCMV has been analyzed by using monoclonal antibodies and defined antipeptide antisera. After immunoprecipitation or immunoblotting, a band with an apparent size of 80 to 86 kDa in sodium dodecyl sulfate (SDS)-polyacrylamide gels commonly is observed (26, 37, 40, 44, 55). In some cases, the band observed after immunoprecipitation is diffuse (37, 55), although possible heterogeneity has not been analyzed. Multiple immunoprecipitable species could arise from posttranslational modifications or interactions with other proteins of a similar size, either of which could be important for IE2 protein function.

We report here the resolution of three polypeptides from 75 to 86 kDa by one-dimensional electrophoresis after immunoprecipitation by IE2-specific antisera from cells infected with HCMV strain AD169 or Towne. Two of these proteins are a precursor (p80) and mature form (p86) of the IE2 protein. The third species is an unrelated protein (p75) of probable viral origin isolated in a protein-protein complex with p86.

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung (HEL) cells (kindly supplied by Brian Wigdahl) were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, 0.075% sodium bicarbonate, and 10% fetal bovine serum (FBS) (GIBCO or HyClone Laboratories, Inc.) and incubated at 37°C in 5% CO₂. HEL cells were free of mycoplasma as determined by a commercial biological test (Mycotect; Life Technologies, Inc.) and the absence of 23S and 16S RNA species in cytoplasmic RNA preparations.

The source of HCMV strain AD169 has been described previously (2). Strain Towne was a gift of A. Colberg-Poley. Stocks of HCMV were prepared by infecting subconfluent monolayers of HEL cells with 0.01 PFU per cell. After viral adsorption for 1 h at 37°C, the cells were provided with DMEM supplemented with 5% heat-inactivated FBS, 0.225% sodium bicarbonate, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2 mM glutamine and incubated at 37°C in a humidified 5% CO₂ atmosphere. In some cases, 0.4 µg of dexamethasone per ml was added (2). Medium was changed every 4 to 5 days. When 80% of the cells demonstrated cytopathic effect, the volume of the medium was reduced by half. After 1 to 2 days or when 100% cytopathic effect was observed, the cells and extracellular fluid were collected and transferred to 50-ml centrifuge tubes. The infected cell suspension was subjected to three cycles of freeze-thaw, sonicated for 45 s in a water bath sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), and centrifuged to pellet cell debris. Virus stocks also were prepared from extracellular fluid only. Virus was stored at -70°C and used directly for infections. Virus was quantified by plaque titration on HEL cells (70).

Antibodies. Polyclonal antiserum 901, a generous gift of S. S. Tevethia, recognizes the large-tumor (large-T) antigen of simian virus 40 (64). Monoclonal antibodies 810 (Chemicon International, Inc., El Segundo, Calif.), also called monoclonal antibody E-13 (Biosoft, Paris, France), and NEA 9221 (Du Pont/New England Nuclear) both recognize epitopes in exon 2 shared by IE1 and some IE2 gene products (38, 46). Antipeptide antibodies 8528, 2183, 8575, 1219, and 1218 were generously provided by Jay A. Nelson. Antibody 8528 was raised against a peptide from exons 2 and 3 (55). Antisera 2183 and 8575 are IE1 specific and recognize the same exon 4 peptide (IE1-2) (42a, 55). Antibodies 1219 and 1218 are IE2 specific and recognize epitopes encoded by exons 5 and 6, respectively (26, 55).

Infections and metabolic labeling. Cells growing in 100-mm dishes at about 90% confluence (about 2×10^7 cells per dish) were infected with 1 ml of AD169 or 0.25 to 1 ml of Towne virus stock. The multiplicities of infection ranged from 0.5 to 10. The monolayers were washed twice with medium without serum prior to infection. Following adsorption for 1 h at 37°C, fresh DMEM-10% FBS was added.

Prior to radiolabeling, monolayers were washed twice with prewarmed phosphate-buffered saline (PBS) and 20 ml of methionine-free DMEM-2% dialyzed FBS was added. After 1 h of starvation at 37°C, the medium was replaced with 1 ml of the same medium supplemented with 100 to 150 mCi of [³⁵S]methionine (NEN-DuPont) per plate. Cultures were incubated at 37°C for 1 to 2 h with constant rocking. Labeled cells were washed twice with ice-cold PBS, scraped from the plate, and pelleted at 4°C. Cell pellets were either processed immediately or stored at -70°C.

Preparation of cell extracts and protein analysis. To prepare cell extracts, cell pellets were thawed rapidly, resuspended in 1 ml of IP lysis buffer (50 mM Tris [pH 8], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride from freshly prepared 100 mM stock in ethyl alcohol) (27) supplemented with 20 µg of aprotinin (Sigma) per ml, and incubated for 30 min at 0°C. After lysis, extracts were sonicated for 30 s and clarified by centrifugation.

To prepare the denatured cell extracts, SDS was added to the lysates prepared as described above to a final concentration of 2%. The samples were boiled for 15 min and diluted 20-fold with NET-GEL buffer (150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Nonidet P-40, 50 mM Tris [pH 7.5]) (23). The detergent-treated lysates in NET-GEL buffer were used directly for immunoprecipitations.

(i) Immunoprecipitation. Cell lysates were preadsorbed for at least 2 h at 4°C with 35 µl of a 50% suspension of Sepharose-immobilized staphylococcal protein A (Sigma) covalently conjugated with bovine serum albumin (BSA) (fraction V; Sigma). For each sample, total incorporation of [³⁵S]methionine into the infected cell proteins was determined by trichloroacetic acid precipitation of an aliquot of the precleared lysate and counting by liquid scintillation. When appropriate, sample volumes were equalized for trichloroacetic acid-precipitable radioactivity. Equalized samples were mixed with antibody, 30 µl of a 30% suspension of protein A-Sepharose, and enough IP lysis buffer to bring the volume to 500 µl. Immunoprecipitation was at 4°C for at least 2 h with constant rocking. The precipitates were collected by centrifugation, washed three times with SNTE buffer (50 mM Tris [pH 7.4], 5 mM EDTA, 500 mM NaCl, 5% sucrose, 1% Nonidet P-40) (27) and once with RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) (26). The washed immunoprecipitates were resuspended in 30 µl of sample buffer (160 mM Tris [pH

6.8], 4% SDS, 200 mM dithiothreitol, 20% sucrose, 0.02% bromophenol blue, 10% 2-mercaptoethanol) and boiled for 5 min to elute precipitated proteins from the Sepharose bead complexes. Eluted proteins were separated by electrophoresis in SDS–7.5% polyacrylamide gels alongside ^{14}C -labeled protein molecular weight standards (GIBCO BRL). Gels were fixed with 25% methanol and 7% acetic acid for 45 min, soaked in Amplify (Amersham) for 1 h, and dried. Dried gels were exposed to Kodak XAR-5 film. For quantitative analysis, autoradiograms were scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.). To determine relative incorporation of [^{35}S]methionine into p80 and p86, absorbance of either protein band was divided by the combined absorbance of the two bands and expressed in percent.

(ii) Immunoblot analysis. Labeled proteins immunoprecipitated and resolved on gels as described above were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell) in buffer containing 20 mM Tris, 220 mM glycine, 1% SDS, and 20% methanol. Transfer was performed at room temperature for 1 h at 1.20 A by using the Bio-Rad Trans-Blot System. Filter membranes were treated with 5% BSA in TBST (50 mM Tris [pH 7.7], 150 mM NaCl, 0.05% Tween 20) for 1 h with gentle agitation and probed with primary antibody diluted in 5% BSA–TBST for 2 h at room temperature. Filters were washed twice with 5% BSA–TBST, probed for 1 h with an alkaline phosphatase-conjugated secondary antibody (Promega Corp.) diluted in 5% BSA–TBST, and developed according to the manufacturer's recommendations for the ProtoBlot AP System (Promega Corp.). After color development, the membranes were wrapped with clear plastic and exposed to film for autoradiography.

V8 protease mapping. One-dimensional partial protease mapping was performed essentially as described by Cleveland (8) with slight modifications. Proteins were resolved on SDS-polyacrylamide gels, dried without being fixed, and detected by autoradiography. With the autoradiograph as a guide, gel slices containing the two proteins to be digested were excised and allowed to equilibrate in buffer containing 125 mM Tris (pH 6.8), 1 mM EDTA, and 0.1% SDS for 1 h at 4°C with occasional swirling. The rehydrated gel slices were positioned on the bottom of sample wells so that the proteins migrated perpendicularly to the initial direction of electrophoresis and were overlaid with V8 protease diluted in digestion buffer (125 mM Tris [pH 6.8], 1 mM EDTA, 0.1% SDS, 10% glycerol, 0.3% β -mercaptoethanol, 0.01% bromophenol blue). Digestion occurred in the stacking gel prior to separation in a 15% resolving gel. Gels were processed for autoradiography as described above.

RESULTS

Immunoprecipitation of 75- and 86-kDa polypeptides from HCMV-infected cells by IE2-specific antisera. Studies by others, primarily using the Towne strain of HCMV, mapped abundant polypeptides to individual IE1 and IE2 transcripts (Fig. 1). In a time course analysis of cells infected with HCMV strain AD169, we resolved two closely migrating bands with apparent molecular masses of about 75 and 86 kDa in SDS–7.5% polyacrylamide gels (Fig. 2). The two proteins, designated p75 and p86, were precipitated either by IE2-specific antisera (1218) or by antisera that recognize epitopes shared with IE1 (9221), but not by antisera specific for IE1 (2183) or by heterospecific antisera (901). The proteins were detected from 6 to 72 h postinfection (hpi). Less p75 than p86 was detected at the earliest time points; however, the labeling of

FIG. 1. Molecular organization of the HCMV major IE gene region. The arrows indicate the direction of transcription. Exon structures (1 to 7) of abundant IE1 and IE2 transcripts are shown with the mRNA sizes in kilobases and the apparent molecular masses in kilodaltons (kd) of the corresponding translation products. The unshaded boxes below indicate the approximate locations of epitopes recognized by the monoclonal antibodies (9221 and 810) and anti-peptide sera (8528, 2183, 8575, 1219, and 1218) used for protein detection. m.u., map unit.

p75 increased as the infection progressed, and by 24 hpi it reached a level similar to that of p86.

As expected, the abundant 72-kDa IE1 polypeptide, with an apparent molecular mass in our gel system of 65 kDa, was recognized both by the IE1-specific antisera and by antisera specific for shared epitopes. Detection of the IE1 protein with the latter (9221) was less efficient than with the former (2183), presumably because of a difference in equilibrium binding by the two antisera. The labeling of IE1 protein declined throughout the time course but still was detected in the latest samples. In addition to the IE1 protein, the 2183 antisera precipitated some less abundant polypeptides with apparent molecular masses of about 50 and 55 kDa. Other precipitated species, not shown in Fig. 2, included an abundant 40-kDa species with the properties of a previously characterized translation product of late IE2 mRNA (44, 47, 55) and a polypeptide with the reactivity profile predicted for the 55-kDa translation product of the 1.7-kb IE2 mRNA (44, 55). Both of these proteins were detected at 48 and 72 hpi (data not shown).

Subsequently, with more extended electrophoretic separation, we resolved a third IE2-specific polypeptide with an apparent molecular mass of about 80 kDa (p80) migrating between p86 and p75. p80 was the most abundant species in

FIG. 2. Time course of the appearance of polypeptides immunoprecipitated from infected cells by antisera against IE1 and IE2 epitopes. HEL cells infected with HCMV (AD169) were labeled with [^{35}S]methionine for 1-h intervals prior to being harvested at the times after infection indicated. Lanes are labeled according to the antisera used for immunoprecipitation. Antibody 901, specific for the simian virus 40 large-T antigen, was used as the control antibody. The positions of the p86, p75, and IE1 proteins are indicated on the right. The sizes of molecular mass standards (in kilodaltons [kD]) shown in the marker lane (M) are indicated on the left.

FIG. 3. Detection of p80 in cells labeled for short periods. HEL cells infected with HCMV (AD169) for 48 h were labeled with [35 S]methionine for the time intervals indicated above the lanes prior to being harvested and immunoprecipitated with IE2-specific antisera 1218. Mock-infected cells were labeled for 60 min. The positions of p86, p80, and p75 proteins are indicated on the left. The positions of molecular mass standards (in kilodaltons [kD]) shown in the marker lane (M) are indicated on the right.

infected cells labeled for 5 or 10 min (Fig. 3). With longer labeling times, p80 sometimes was detected in low abundance, although often it was obscured by the intense labeling of p86 and p75.

Direct test for reactivity of p86 and p75 with IE2-specific antisera. Several possible sources of the p75 and p86 proteins were considered. The use of various combinations of the proteolysis inhibitors aprotinin, phenylmethyl sulfonyl fluoride, tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK), and leupeptin did not alter the ratio of p75 to p86 (data not shown). Therefore, it was unlikely that p75 was a proteolytic fragment of p86. If the two proteins were related in sequence, then either they were primary translation products of differentially spliced mRNAs or one was a precursor to the other. If the two proteins were unrelated in sequence, then only one was

derived from IE2 and the second probably was coprecipitated in a complex with the IE2 species. Since both proteins were recovered by using antisera with at least three different IE2 specificities (Fig. 4), it was very unlikely that one was an unrelated protein containing an IE2-specific epitope.

To determine the antigenic relationship of the two proteins, we compared the binding of antibodies to denatured p75 and p86. Immunoprecipitated labeled proteins, separated by electrophoresis, were blotted to nitrocellulose membranes. Autoradiograms of the blots (Fig. 4, left panels) revealed the labeled, denatured immunoprecipitated proteins, whereas antibody probing of the blots (Fig. 4, right panels) defined their immunological reactivity. As expected, p75 and p86 were recovered in blots after immunoprecipitation with antibodies specific for three different regions of the IE2 protein: a region shared with IE1 as well as two unique regions in the amino- and carboxy-terminal portions of IE2 (Fig. 4, left panels). The IE1 protein was immunoprecipitated by antisera with shared or IE1-specific recognition properties. In probed blots (Fig. 4, right panels), IE1 protein was recognized by 9221 antibody, which recognizes epitopes common to IE1 and IE2. Similarly, p86 always was recognized by antibody 9221 and the IE2-specific antibodies 1218 and 1219 (data not shown). However, blotted p75 was not recognized by any of the antibodies. These results unambiguously identified p86 as the IE2 species observed previously by immunoblotting (44, 55). Since p75 lacked a series of epitopes distributed throughout the larger product and was of similar length, p75 was not a breakdown product of p86. Rather, p75 must have been detected because it coprecipitated with p86.

Two other properties of p75 and p86 in the complexes were examined. The labeling characteristics of the two proteins (Fig. 3) suggested that p86 and p75 were relatively stable in infected cells. In pulse-chase experiments, both p86 and the complexed p75 had half-lives on the order of a few hours (data not shown). In vitro, the interaction between the two proteins was

FIG. 4. Direct immunological detection of precipitated p86 but not p75 in immunoblots. HEL cells infected with HCMV (AD169) were labeled with [35 S]methionine for 2 h at 48 hpi. The infected cells were harvested after labeling, and cell extracts were prepared for immunoprecipitation with the antisera indicated above the lanes. After electrophoretic separation of the immunoprecipitated radiolabeled proteins, they were blotted to nitrocellulose and probed with antibodies 9221 (A) and 1218 (B), which recognize epitopes in the amino and carboxy termini of p86. The probed blots are shown on the right, and the corresponding autoradiograms are shown on the left. The positions of p86, p75, and IE1 proteins are indicated in the middle of the figure. The sizes of molecular mass standards (in kilodaltons [kD]) shown in the marker lane (M) are indicated on the left.

FIG. 5. Analysis of products of partial proteolysis of p86 and p75. Partial proteolytic mapping of p86 and p75 was performed as described in Materials and Methods. The amounts of V8 protease added were 0 ng (lane a), 17.5 ng (lane b), 35 ng (lane c), 70 ng (lane d), and 140 ng (lane e). In each lane, the p86 proteolytic products are on the left track and the p75 products are on the right track. The positions of molecular mass standards are indicated on the left.

resistant to washing of the immunoprecipitates with 1 M NaCl (data not shown).

Comparative peptide mapping of p86 and p75. The immunological studies of p75 and p86 provided strong evidence that the two polypeptides are distinct. Their sequence relationship was compared directly by analysis of the products of partial proteolysis by V8 protease. The primary amino acid sequence of the IE2 protein contains 68 potential V8 cleavage sites. Digestion to completion should produce 12 methionine-containing peptides, the largest of which should contain only 54 amino acids. We predicted that the large number of digestion intermediates would make resolution of individual products difficult. Figure 5 shows that the few distinguishable products of V8 digestion of p86 were of either very high or very low molecular weight; the latter products were observed only with high concentrations of protease. Proteolysis of labeled p75 produced a different profile at each V8 concentration used. These results provided direct evidence that the two proteins are distinct and unrelated in primary amino acid sequence.

Precursor-product relationship of p80 and p86. In the course of these experiments, we observed a decreased mobility of p86 relative to p75 in gels with a reduced percentage (0.125%) of bisacrylamide (see Fig. 6, 8, and 9 for examples). p80 also had a decreased mobility, which made detection easier and facilitated further characterization. The result also provided evidence that p80 was related in sequence to p86, in which case the former may have escaped detection in immunoblots (Fig. 4) because of its low abundance.

If p80 is related to p86, then both proteins should be recovered even when labeled polypeptides are denatured prior to immunoprecipitation. Accordingly, proteins from parallel immunoprecipitations of native cell extracts were separated alongside those from denatured samples (Fig. 6). Both p86 and

FIG. 6. Immunoprecipitation of native or denatured proteins by IE protein-specific antisera. HEL cells infected with HCMV (AD169) were labeled at 48 hpi with [35 S]methionine for 1 h prior to harvest and preparation of cell lysates for immunoprecipitation. One-half of each lysate was denatured as described in Materials and Methods, and denatured and native samples were processed for immunoprecipitation with the antisera indicated above the lanes. The epitopes recognized by 8528 and 9221 may be sensitive to denaturation of IE2 (8528) or both IE1 and IE2 proteins (9221). The positions of p86, p80, p75, and IE1 proteins are indicated on the right. Molecular mass standards are indicated on the left.

p80 were precipitated efficiently from denatured samples by the IE2-specific antisera 1218 and 1219. As expected for a coprecipitated protein, p75 was recovered poorly or not at all from denatured extracts.

The recovery of p80 in immunoprecipitations of denatured cell extracts with IE2-specific antisera 1218 and 1219 provided evidence that p80 was an IE2 species. On the basis of its preferential labeling in short time periods (Fig. 3), p80 was a candidate for a short-lived precursor to p86. To examine the metabolic relationship of the proteins directly, infected cells were labeled for 10 min to maximize labeling of p80 relative to that of p86 and the label was chased for increasing times (Fig. 7A). No further increase in labeled total extract protein occurred after the initiation of the chase (data not shown). To avoid potential interference of labeled p75 with the analysis of the transfer of label from p80 to p86, cell extracts prepared for each time point were denatured prior to immunoprecipitation.

During the pulse, almost all of the labeled IE2 protein was p80, and the label chased completely into p86 (Fig. 7A). Early in the chase period, the combined incorporation of radioactivity into p80 and p86 increased slightly (data not shown); however, no further incorporation into IE2 species was observed, and the level actually declined after a 1-h chase, an observation that presumably reflects limited turnover of p86. Most importantly, there was a quantitative conversion of labeled p80 to p86 during intermediate chase times when total IE2 label was conserved (Fig. 7B). These data show directly that p80 is a precursor to p86.

Conditions for detection and probable origin of p75. Despite the abundance of labeled p75 in infected cells, it has not been reported previously. Although p75 would not have been detected in immunoblot studies of the major IE proteins, the protein was not revealed in immunoprecipitation experiments either. p75 could have eluded detection for several reasons. First, standard 10- to 15% polyacrylamide gels with short running times might have afforded insufficient resolution from p86. Second, most of the previous immunoprecipitation studies of the IE proteins used the Towne virus strain. If p75 is made in Towne-infected cells, it might be more difficult to resolve

FIG. 7. Precursor-product relationship of IE2 p80 and p86 polypeptides. (A) Pulse-chase analysis of p80 and p86. HEL cells were infected with HCMV for 48 h, labeled with [³⁵S]methionine for 10 min, and chased by incubation in nonradioactive medium supplemented with 5 mM L-methionine for the times indicated above the lanes. Cell extracts prepared for each time point were denatured and then processed for immunoprecipitation with IE2-specific antiserum 1218. The positions of p86 and p80 proteins are indicated on the right. The positions of molecular mass standards are indicated on the left. (B) Relative incorporation of [³⁵S]methionine into p80 and p86. Values plotted represent the fraction of label in each protein relative to total incorporation into p80 and p86 for each time point.

electrophoretically from Towne p86 than the proteins from AD169-infected cells. Finally, p75 might not be made in the conditions most commonly used to study p86, in cells infected in the presence of cycloheximide (CH) and later released from the protein synthesis block.

We first investigated whether p75 was made in Towne-infected cells. The major Towne IE1 and IE2 proteins migrated faster than the corresponding AD169 IE1 protein and p86 (Fig. 8). By using labeling conditions more suitable to the detection of AD169 p80, a similar Towne protein also was observed (data not shown). As expected, these IE1 and IE2 species also were immunoprecipitated from denatured extracts (data not shown). In addition, another abundant polypeptide, migrating faster than Towne p86, was precipitated from

FIG. 8. Comparison of IE proteins and p75 from cells infected with AD169 or Towne. HEL cells infected with AD169 or Towne were labeled with [³⁵S]methionine for 2 h at 48 hpi. The infected cells were harvested, and extracts were prepared for immunoprecipitation with the IE1- or IE2-specific antisera indicated above the lanes. The positions of p86, p75, and IE1 proteins are indicated on the right. The sizes of molecular mass standards (in kilodaltons [kD]) shown in the marker lane (M) are indicated on the left.

Towne-infected cells by antisera with IE2 specificity (1218) or shared specificity (8528; data not shown) but not by antisera against IE1 (8575). This protein was not precipitated from denatured extracts (data not shown). Thus, Towne-infected cells produced a protein resembling the p75 made in AD169-infected cells.

Unexpectedly, the electrophoretic mobility of the protein from Towne-infected cells was slower than that of the p75 made in AD169-infected cells (Fig. 8). This result was significant for several reasons. The mobility difference provided strong evidence against a trivial source for p75, such as a contaminating agent. Also, it showed that p75 was probably of viral origin, since it was unlikely that a cellular protein would be altered differently after infection with the different viral strains. Finally, it showed that the Towne proteins are more difficult to resolve electrophoretically than the AD169 proteins.

To determine whether p75 was made after reversal of a CH block, HEL cells were preincubated with CH and infected with HCMV (Towne) for 12 h in the presence of the inhibitor. The CH was washed out, and the cells were labeled and processed for immunoprecipitation. Inhibitors of RNA synthesis often are added during the labeling time to restrict protein synthesis to IE proteins. However, even with the less stringent protocol of omitting the RNA synthesis inhibitor following the CH reversal, p75 was not detected (Fig. 9). Therefore, p75 should not have been detected by others in conditions commonly used to amplify IE protein synthesis. Furthermore, on the basis of the time course results (Fig. 2) and the likely viral origin (Fig. 8), p75 is probably an early protein whose synthesis continues at late times.

DISCUSSION

We describe here the identification of two polypeptides in HCMV-infected cells after immunoprecipitation with specific antisera directed against the HCMV 86-kDa IE2 (p86) protein. On the basis of sequence relatedness and metabolic properties, the species designated p80 behaved as expected for a precursor polypeptide to p86. The p75 species was an unrelated protein of probably viral origin that formed a stable complex with p86 in infected cells.

FIG. 9. Effect of CH pretreatment on detection of p75. HEL cells were treated with 200 μ g of CH per ml 30 min prior to infection. After infection with HCMV (Towne) for 12 h, the CH was removed and cells were labeled for 3 h with [35 S]methionine. To provide standards for the proteins immunoprecipitated from CH-treated cells (CH⁺), untreated cells (CH⁻) infected for 48 h were labeled for 2 h. Infected (I) and mock-infected (M) cell lysates were processed for immunoprecipitation with antibody 9221. The positions of p86, p80, p75, and IE1 proteins are indicated on the right. The sizes of molecular mass standards (in kilodaltons [kD]) are indicated on the left.

The IE2 precursor polypeptide p80. Initially, p80 was resolved in gels run for extended time periods to improve separation of the abundant p86 and p75 polypeptides. Subsequently, better resolution was obtained by decreasing the concentration of bisacrylamide in the gels from 0.2 to 0.125%. However, even under the best resolving conditions, p80 often was difficult to detect when infected cells were labeled for more than 1 h, and p80 was not observed in the steady state by protein blotting. The kinetics of labeling of p80 (Fig. 3) showed rapid approach to steady state, a behavior characteristic of a low-abundance, short-lived precursor. The protein was recognized directly by IE2 antisera (Fig. 6), and labeled p80 chased quantitatively to p86 (Fig. 7). These results clearly identified p80 as a precursor to the more slowly migrating IE2 polypeptide.

An abundant polypeptide most likely corresponding to p86, on the basis of recognition by IE2 antisera, is phosphorylated in HCMV-infected cells (26). The 580-amino-acid IE2 protein encoded by HCMV strain AD169 has 64 serine residues, 48 threonine residues, and 6 tyrosine residues; four of the serine residues are not conserved in the 579-amino-acid Towne strain homolog. Differential phosphorylation is a common source of altered gel mobility among species of phosphoproteins. In the case of adenovirus E1a protein, some of the conformers are sufficiently different in electrophoretic mobility as to be resolved easily in one-dimensional gels (49). Although E1a protein is phosphorylated on multiple residues, a single phosphorylation event produces a major mobility shift (15, 51, 66). Accordingly, if the p80-p86 mobility shift is the result of phosphorylation, then perhaps only a single phosphorylation event is responsible. Additional heterogeneity in the phosphorylated forms of the protein also might be present; however, better separation of the IE2 species might be required to observe such variation.

As of yet, no other posttranslational modifications of IE2 have been noted. There are several potential N-linked glycosylation sites (56). Whatever the source of the conversion of p80 to p86, the modification did not result in any obvious differential reactivity of the two proteins with any of the IE2-specific antisera used in this study. The role of protein modification in IE2 function is completely unknown. Our

identification of p80 will facilitate studies regarding the role of posttranslational modifications in the function of IE2 or the regulation of its activity.

The protein p75 and its interaction with IE2 p86. The recovery of p75 by immunoprecipitation in the absence of its direct immunodetection (Fig. 4) provided the first evidence that p75 was unrelated in primary amino acid sequence to IE2 proteins. Two additional findings confirmed this interpretation: p75 was not precipitated from denatured samples (Fig. 6 and 7A), and its partial proteolytic fragments were distinct from those of p86 (Fig. 5). Together with the complex stability in vivo and in vitro, these properties are the signature of a protein-protein complex formed in the intact cell and define the first observation of a specific protein-protein interaction involving the IE2 polypeptide in the infected cell.

The discovery of different mobilities of the polypeptides coprecipitated from cells infected with two different strains of HCMV effectively excluded trivial explanations for the detection of p75. We note that modifications of the protocols used to propagate and plaque the virus, such as the preparation of whole-cell lysate virus stocks or the use of dexamethasone (see Materials and Methods), did not affect the detection of p75 (data not shown). Proof of the biological significance of the complex awaits identification of the p75 gene and the preparation of the appropriate genetic and biochemical reagents for analysis of the complex and its function.

It is not surprising that p75 has not been reported by others, since our data show that p75 was found through a special combination of circumstances. p75 certainly would not have appeared in the protein-blotting studies of IE proteins. p75 was not synthesized in cells treated with CH to enhance the synthesis of IE proteins, a protocol also used in the large majority of published studies of IE2. Also, we found it difficult to resolve p75 from p86 in the 10- to 15% polyacrylamide gels and under the running conditions most commonly used (50). This limitation applied particularly to the proteins from Towne-infected cells, since the Towne p86 migrates faster than the AD169 p86, and the Towne p75 migrates more slowly.

Recent reports indicate that a p86 fusion protein interacts with p86 and a number of cellular proteins in vitro (7, 17, 22). If p75 is viral, it cannot be one of the p86-interacting proteins in uninfected cell extracts (17). However, p75 was not detected even when infected cell extracts were assayed (17). We speculate that either p75 was not resolved from p86 electrophoretically or p75 did not associate with the p86 fusion protein, because p75 in the infected cell extracts is already bound to p86. Conversely, we found no evidence by immunoprecipitation of abundant coprecipitating species corresponding to the cellular p86-binding proteins identified by others in vitro. Perhaps p86 complexes containing the cellular proteins are present in very low concentrations and/or are disrupted by binding of p75.

There could be a direct link between the p75 association with an IE2 protein and the maturation of p80 to p86. In pulse-chase analysis of nondenatured samples, labeled p75 appears in the immunoprecipitates with the same kinetics as labeled p86 (50). The simplest, though not only, interpretation of this observation is that p75 preferentially interacts with the mature form (p86) and not the precursor form (p80) of the IE2 protein.

Our evidence suggests that p75 is an early viral gene product that becomes more abundant late in infection. Thus, p75 may belong to the class of early-late genes. The most obvious role for p75 would be as a partner in transcription regulation by p86. In this case, one might expect p75 to be a nuclear phosphoprotein, as is p86. However, p75 did not label with

phosphate under conditions in which p86 is labeled easily (50). Also, since both activation and repression of target genes by IE2 occur in transient expression assays, presumably in the absence of p75, a viral p75 cannot be required for transcription regulation by p86. However, a viral p75 could act as a modulator of IE2 activity during a lytic or nonlytic host-virus encounter. For example, p75 could augment or disrupt other protein-protein interactions of p86, including self-association.

Among the early HCMV gene products that have been identified are nonstructural DNA-binding proteins, phosphoproteins, glycoproteins, and enzymes, some of which presumably are involved in viral DNA replication (see reference 33 for a review). Allowing for differences in molecular masses reported from gel mobilities, early proteins with molecular masses within the 60- to 90-kDa range must be considered candidates for the protein we call p75. These candidates include the 65-kDa UL84 gene product (24), the 76-kDa product of the HWLF1 reading frame ICP22 (41), glycoproteins of 60 and 63 kDa (62), a 68-kDa protein kinase (52), and an 84-kDa phosphorylated nuclear protein (72). If p75 is not phosphorylated, then the latter two proteins are probably not p75. Identification of the p75 gene and its function will add important information to our understanding of the activities of IE2 in the life cycles of HCMV.

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REFERENCES

- Barry, P. A., E. Pratt-Lowe, B. M. Peterlin, and P. A. Luciw. 1990. Cytomegalovirus activates transcription directed by the long terminal repeat of human immunodeficiency virus type 1. *J. Virol.* **64**:2932-2940.
- Blanton, R. A., and M. J. Tevethia. 1981. Immunoprecipitation of virus-specific immediate-early polypeptides from cells lytically infected with human cytomegalovirus strain AD169. *Virology* **112**:262-273.
- Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner. 1985. A very strong enhancer is located upstream of an immediate-early gene of human cytomegalovirus. *Cell* **41**:521-530.
- Chang, C.-P., C. L. Malone, and M. F. Stinski. 1989. A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. *J. Virol.* **63**:281-290.
- Cherrington, J. M., E. L. Khoury, and E. S. Mocarski. 1991. Human cytomegalovirus *ie2* negatively regulates α gene expression via a short target sequence near the transcription start site. *J. Virol.* **65**:887-896.
- Cherrington, J. M., and E. S. Mocarski. 1989. Human cytomegalovirus *ie1* transactivates the α promoter-enhancer via an 18-base-pair repeat element. *J. Virol.* **63**:1435-1440.
- Chiou, C. J., J. Zong, I. Waheed, and G. S. Hayward. 1993. Identification and mapping of dimerization and DNA-binding domains in the C terminus of the IE2 regulatory protein of human cytomegalovirus. *J. Virol.* **67**:6201-6214.
- Cleveland, D. W. 1983. Peptide mapping in one dimension by limited proteolysis of sodium dodecyl sulfate-solubilized proteins. *Methods Enzymol.* **96**:222-223.
- Colberg-Poley, A. M., L. D. Santomenna, P. P. Harlow, P. A. Benfield, and D. J. Tenney. 1992. Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. *J. Virol.* **66**:95-105.
- Davis, M. G., S. C. Kenney, J. Kamine, J. S. Pagano, and E.-S. Huang. 1987. Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **84**:8642-8646.
- DeMarchi, J. M. 1981. Human cytomegalovirus DNA: restriction enzyme cleavage and map locations for immediate early, early and late RNAs. *Virology* **114**:23-28.
- DeMarchi, J. M., M. L. Blankship, G. D. Brown, and A. S. Kaplan. 1978. Size and complexity of human cytomegalovirus DNA. *Virology* **89**:643-646.
- DeMarchi, J. M., C. A. Schmidt, and A. S. Kaplan. 1980. Pattern of transcription of human cytomegalovirus in permissively infected cells. *J. Virol.* **35**:277-286.
- Depto, A. S., and R. M. Stenberg. 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. *J. Virol.* **63**:1232-1238.
- Dumont, D. J., M. L. Tremblay, and P. E. Branton. 1989. Phosphorylation at serine 89 induces a shift in gel mobility but has little effect on the function of adenovirus type 5 E1A proteins. *J. Virol.* **63**:987-991.
- Fleckenstein, B., I. Muller, and J. Collins. 1982. Cloning of the complete human cytomegalovirus genome in cosmid. *Gene* **18**:39-46.
- Furnari, B. A., E. Poma, T. F. Kowalik, S. M. Huong, and E. S. Huang. 1993. Human cytomegalovirus immediate-early gene 2 protein interacts with itself and with several novel cellular proteins. *J. Virol.* **67**:4981-4991.
- Geelen, J. L. M. C., C. Walig, P. Wertheim, and J. van der Noordaa. 1978. Human cytomegalovirus DNA. I. Molecular weight and infectivity. *J. Virol.* **26**:813-816.
- Ghazal, P., H. Lubon, and L. Hennighausen. 1988. Specific interactions between transcription factors and the promoter-regulatory region of the human cytomegalovirus major immediate-early gene. *J. Virol.* **62**:1076-1079.
- Ghazal, P., H. Lubon, and L. Hennighausen. 1988. Multiple sequence-specific transcription factors modulate cytomegalovirus enhancer activity in vitro. *Mol. Cell. Biol.* **8**:1809-1811.
- Ghazal, P. J., E. Young, C. Giulietti, C. DeMattei, J. Garcia, R. Gaynor, R. M. Stenberg, and J. A. Nelson. 1991. A discrete *cis* element in the human immunodeficiency virus long terminal repeat mediates synergistic *trans* activation by cytomegalovirus immediate-early proteins. *J. Virol.* **65**:6735-6742.
- Hagemeyer, C., S. Walker, R. Caswell, T. Kouzarides, and J. Sinclair. 1992. The human cytomegalovirus 80-kilodalton but not the 72-kilodalton immediate-early protein transactivates heterologous promoters in a TATA box-dependent mechanism and interacts directly with TFIID. *J. Virol.* **66**:4452-4456.
- Harlow, E., P. Whyte, B. R. Franza, Jr., and C. Schley. 1986. Association of early-region 1A proteins with cellular polypeptides. *Mol. Cell Biol.* **6**:1579-1589.
- He, Y. S., L. Xu, and E. S. Huang. 1992. Characterization of human cytomegalovirus UL84 early gene and identification of its putative protein product. *J. Virol.* **66**:1098-1108.
- Hermiston, T. W., C. L. Malone, and M. F. Stinski. 1990. Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. *J. Virol.* **64**:3532-3536.
- Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski. 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. *J. Virol.* **61**:3214-3221.
- Hinds, P. W., C. A. Finlay, A. B. Frey, and A. J. Levine. 1987. Immunological evidence for the association of p53 with a heat shock protein hsp70, in p53-plus-ras-transformed cell lines. *Mol. Cell Biol.* **7**:2863-2869.
- Jeang, K.-T., and W. Gibson. 1980. A cycloheximide-enhanced protein in cytomegalovirus infected cells. *Virology* **107**:362-374.
- Jupp, R., S. Hoffmann, A. Depto, R. M. Stenberg, P. Ghazal, and J. A. Nelson. 1993. Direct interaction of human cytomegalovirus IE86 protein with the *cis* repression signal does not preclude TBP

- from binding to the TATA box. *J. Virol.* **67**:5595–5604.
30. Klucher, K. M., M. Sommer, J. T. Kadonaga, and D. H. Spector. 1993. In vivo and in vitro analysis of transcriptional activation mediated by the human cytomegalovirus major immediate-early proteins. *Mol. Cell. Biol.* **13**:1238–1250.
 31. LaFemina, R. L., M. C. Pizzorno, J. D. Mosca, and G. S. Hayward. 1989. Expression of the acidic nuclear immediate-early protein (IE1) of human cytomegalovirus in stable cell lines and its preferential association with metaphase chromosomes. *Virology* **172**:584–600.
 32. Lakeman, A. D., and J. E. Osborn. 1979. Size of infectious DNA from human and murine cytomegaloviruses. *J. Virol.* **30**:414–416.
 33. Landini, M. P., and S. Michelson. 1988. Human cytomegalovirus proteins. *Prog. Med. Virol.* **35**:152–185.
 34. Lang, D., and T. Stamminger. 1993. The 86-kilodalton IE-2 protein of human cytomegalovirus is a sequence-specific DNA-binding protein that interacts directly with the negative autoregulatory response element located near the cap site of the IE1/2 enhancer-promoter. *J. Virol.* **67**:323–331.
 35. Liu, B., T. W. Hermiston, and M. F. Stinski. 1991. A *cis*-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. *J. Virol.* **65**:897–903.
 36. Macias, M. P., and M. F. Stinski. 1993. An in vitro system for human cytomegalovirus immediate early 2 protein (IE2)-mediated site-dependent repression of transcription and direct binding of IE2 to the major immediate early promoter. *Proc. Natl. Acad. Sci. USA* **90**:707–711.
 37. Malone, C. L., D. H. Vesole, and M. F. Stinski. 1990. Transactivation of human cytomegalovirus early promoters by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. *J. Virol.* **64**:1498–1506.
 38. Mazon, M. C., G. Jahn, and B. Plachter. 1992. Monoclonal antibody E-13 (M-810) to human cytomegalovirus recognizes an epitope encoded by exon 2 of the major immediate early gene. *J. Gen. Virol.* **73**:2699–2703.
 39. McDonough, S. H., and D. H. Spector. 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. *Virology* **125**:31–46.
 40. Michelson, S., F. Horodniceanu, M. Kress, and M. Tardy-Panit. 1979. Human cytomegalovirus-induced immediate early antigens: analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis after immunoprecipitation. *J. Virol.* **32**:259–267.
 41. Mocarski, E. S., L. Pereira, and A. L. McCormick. 1988. Human cytomegalovirus ICP22, the product of the HWLF1 reading frame, is an early nuclear protein that is released from cells. *J. Gen. Virol.* **69**:2613–2621.
 42. Nankervis, G. A., and M. L. Kumar. 1978. Diseases produced by cytomegaloviruses. *Med. Clin. North Am.* **62**:1021–1035.
 - 42a. Nelson, J. Personal communication.
 43. Pizzorno, M. C., and G. S. Hayward. 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. *J. Virol.* **64**:6154–6165.
 44. Pizzorno, M. C., M.-A. Mullen, Y.-N. Chang, and G. S. Hayward. 1991. The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. *J. Virol.* **65**:3839–3852.
 45. Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward. 1988. *trans*-Activation and autoregulation of gene expression by immediate-early 2 gene products of human cytomegalovirus. *J. Virol.* **62**:1167–1179.
 46. Plachter, B., W. Britt, R. Vornhagen, T. Stamminger, and G. Jahn. 1993. Analysis of proteins encoded by IE regions 1 and 2 of human cytomegalovirus using monoclonal antibodies generated against recombinant antigens. *Virology* **193**:642–652.
 47. Puchtler, E., and T. Stamminger. 1991. An inducible promoter mediates abundant expression from the immediate-early 2 gene region of human cytomegalovirus at late times after infection. *J. Virol.* **65**:6301–6306.
 48. Rando, R. F., A. Srinivasan, J. Feingold, E. Gonczol, and S. Plotkin. 1990. Characterization of multiple molecular interactions between human cytomegalovirus (HCMV) and human immunodeficiency virus type 1 (HIV-1). *Virology* **176**:87–97.
 49. Richter, J. D., J. M. Slavicek, J. F. Schneider, and N. C. Jones. 1988. Heterogeneity of adenovirus type 5 E1A proteins: multiple serine phosphorylations induce slow-migrating electrophoretic variants but do not affect E1A-induced transcriptional activation or transformation. *J. Virol.* **62**:1948–1955.
 50. Samaniego, L. A. Unpublished results.
 51. Smith, C. L., C. Debouck, M. Rosenberg, and J. S. Culp. 1989. Phosphorylation of serine residue 89 of human adenovirus E1A proteins is responsible for their characteristic electrophoretic mobility shifts, and its mutation affects biological function. *J. Virol.* **63**:1569–1577.
 52. Somogyi, T., S. Michelson, and M. J. O. Masse. 1990. Genomic location of a human cytomegalovirus protein with protein kinase activity (PK68). *Virology* **174**:276–285.
 53. Stamminger, T., E. Puchtler, and B. Fleckenstein. 1991. Discordant expression of the immediate-early 1 and 2 gene regions of human cytomegalovirus at early times after infection involves posttranscriptional processing events. *J. Virol.* **65**:2273–2282.
 54. Staprans, S. I., D. K. Rabert, and D. H. Spector. 1988. Identification of sequence requirements and *trans*-acting functions necessary for regulated expression of a human cytomegalovirus early gene. *J. Virol.* **62**:3463–3473.
 55. Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson. 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. *J. Virol.* **63**:2699–2708.
 56. Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal. 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early protein involves common and unique protein domains. *J. Virol.* **64**:1556–1565.
 57. Stenberg, R. M., D. R. Thomsen, and M. F. Stinski. 1984. Structural analysis of the major immediate-early gene of human cytomegalovirus. *J. Virol.* **49**:190–199.
 58. Stenberg, R. M., P. R. Witte, and M. F. Stinski. 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. *J. Virol.* **56**:665–675.
 59. Stinski, M. F. 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. *J. Virol.* **26**:686–701.
 60. Stinski, M. F., and T. J. Roehr. 1985. Activation of the major immediate-early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. *J. Virol.* **55**:431–441.
 61. Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein. 1983. Organization and expression of the immediate early genes of human cytomegalovirus. *J. Virol.* **46**:1–14.
 62. Sullivan-Tailleur, G., and H. M. Garnett. 1986. Plasma membrane proteins and glycoproteins induced by human cytomegalovirus infection of human embryonic fibroblasts. *J. Gen. Virol.* **67**:515–526.
 63. Tevethia, M. J., D. J. Spector, K. M. Leisure, and M. F. Stinski. 1987. Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. *Virology* **161**:276–285.
 64. Thompson, D. L., D. Kalderon, A. E. Smith, and M. J. Tevethia. 1990. Dissociation of Rb-binding and anchorage-independent growth from immortalization and tumorigenicity using SV40 mutants producing N-terminally truncated large T antigens. *Virology* **178**:15–34.
 65. Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski. 1984. Promoter regulatory region of the major immediate early gene of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **81**:659–663.
 66. Tremblay, M. L., D. J. Dumont, and P. E. Branton. 1989. Analysis of phosphorylation sites in the exon 1 region of E1A proteins of human adenovirus type 5. *Virology* **169**:397–407.
 67. Walker, S., C. Hagemeier, J. G. P. Sissons, and J. H. Sinclair. 1992. A 10-base-pair element of the human immunodeficiency virus type 1 long terminal repeat (LTR) is an absolute requirement

- for transactivation by the human cytomegalovirus 72-kilodalton IE1 protein but can be compensated for by other LTR regions in transactivation by the 80-kilodalton IE2 protein. *J. Virol.* **66**:1543–1550.
68. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate-early, early, and late times after infection. *J. Virol.* **41**:462–477.
69. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. *J. Virol.* **38**:446–451.
70. **Wentworth, B. B., and L. French.** 1970. Plaque assay of human cytomegalovirus strains of human origin (35031). *Proc. Soc. Exp. Biol. Med.* **135**:253–259.
71. **Weston, K.** 1988. An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. *Virology* **162**:406–416.
72. **Wright, D. A., S. I. Staprans, and D. H. Spector.** 1988. Four phosphoproteins with common amino termini are encoded by human cytomegalovirus AD169. *J. Virol.* **62**:331–340.