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Autoregulation of the Human Cytomegalovirus Major Immediate-Early Gene

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The gene coding for the human cytomegalovirus major immediate-early 72-kilodalton protein was cloned into simian virus origin of DNA replication plasmid pSVOd. Transfection of this plasmid (pSVCC2) into cells constitutively expressing the simian virus 40 T-antigen resulted in readily detectable levels of immediate-early region 1-specific RNA and protein. Partial restriction enzyme digestion of pSVCC2 was used to generate specific amino acid deletions within the 72-kilodalton protein. Mutant ΔS12, which contained a deletion of 145 amino acids at the carboxy terminus of the protein, accumulated at least 10 times more immediate-early region 1 RNA than wild-type pSVCC2 did. In contrast, normal levels of ΔS12-specific RNA were detected in cells cotransfected with wild-type pSVCC2. Therefore, the wild-type gene was capable of suppressing transcription of the mutant gene. Our results suggest that the wild-type major immediate-early protein of cytomegalovirus autoregulates transcription of immediate-early region 1 and that one of the regulatory domains is within the carboxy-terminal 145 amino acids of the viral protein.

Infection of permissive cells with either herpes simplex virus (HSV) or human cytomegalovirus (CMV) leads to sequential expression of the viral genes. These genes have been divided into the following three classes: immediate early (IE), early, and late (9–11, 16, 17, 24, 26, 32, 46, 67). The first genes expressed after infection are the IE or alpha genes. Expression of the IE genes does not require de novo virus-specific protein synthesis (9–11, 16, 17, 24, 26, 32, 46, 47). However, expression of herpesvirus IE genes has the unusual property of being activated in *trans* by a virion component (1, 4, 15, 20, 22, 23, 27, 28, 30, 39). Campbell et al. (4) have demonstrated that the HSV major tegument protein has a role in this *trans* activation. However, in stably transformed cells *trans* activation is virus specific; i.e., HSV can activate only the HSV IE promoter (28), while human CMV can activate only the CMV IE promoter (39). The IE genes for HSV and CMV are also considered to be responsible for the positive regulation of early and late gene expression (9–11, 16, 17, 24, 26, 32, 46, 47). In the case of HSV, mutants in the ICP 4 gene have been used to demonstrate the requirement of this viral protein for positive regulation (12, 29, 31, 32).

It was originally proposed that, in the sequential pattern of HSV gene expression, IE or alpha genes were negatively regulated by early or beta genes (16). However, the results of studies in which mutants with mutations in the ICP 4 gene were used suggested that the negative regulation might be a function of the IE gene themselves (8, 12, 29, 31, 32). Recently, Deluca et al. (8) demonstrated that a class of ICP 4 temperature-sensitive mutants overproduced IE proteins, while the normal levels of early proteins were synthesized. The data of these authors indicated that ICP 4 was autoregulating.

The expression of human CMV IE region 1, the gene that codes for the major IE 72-kilodalton(kDa) protein, reaches a maximum at about 4 to 5 h after infection, and then its expression decreases dramatically (38, 40). In contrast to the studies with HSV ICP 4, phenotypic mutants of human CMV have not been isolated and characterized. Therefore, we used plasmid pSVOd (25) and the COS-1 cell system (13) as a eucaryotic expression vector system to investigate the regulatory functions associated with the 72-kDa major IE protein. In this report we describe the capacity of the 72-kDa major IE protein to regulate its own expression in COS-1 cells. The carboxy-terminal domain of the 72-kDa protein plays a critical role in autoregulation. We also discuss the structure of the protein and its role in regulating transcription from the two adjacent transcription units, IE regions 1 and 2. Transcription in IE region 1 has been described previously (35), and transcription in IE region 2 is described in the accompanying paper (36).

MATERIALS AND METHODS

Cell culture and virus propagation. The growth of human foreskin fibroblasts (HF) and the propagation of CMV strain Towne have been described previously (38). COS-1 cells were generally passaged 1:4 and grown in Eagle minimal essential medium supplemented with 5% fetal bovine serum.

Enzymes. Enzymes were obtained from Bethesda Research Laboratories, Inc., Bethesda, Md, New England BioLabs, Inc., Beverly, Mass., and Boehringer Mannheim Biochemicals, Indianapolis, Ind., and were used as recommended by the suppliers.

Plasmid DNAs. Bacterial plasmid pSVOd (25) was provided by M. Wathen, National Animal Disease Laboratory. Plasmid DNAs were grown and amplified as previously described (44). Plasmid pSVCC2 was construced as described in the legend to Fig. 1. Briefly, the 3.6-kilobase *Cla*I restriction enzyme fragment which spans IE region 1 was chosen because it contains the entire gene plus the promoter-regulatory region (35, 43). The ends of the fragment were filled in with Klenow polymerase and deoxynucleotide triphosphates. *Hin*dIII linkers were ligated onto the blunt ends and digested with *Hin*dIII, and the fragment was inserted into the *Hin*dIII site of pSVOd. Figure 1 shows the final construction (pSVCC2), including the relative locations of the four exons of the RNA and the direction of transcription (arrow). Deletions of pSVCC2 were constructed by partial

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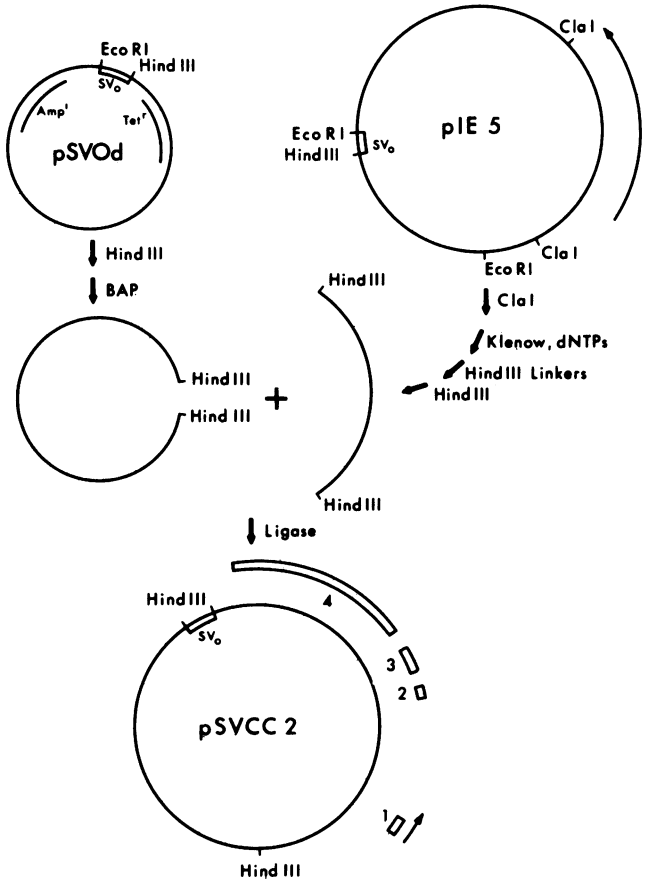

FIG. 1. Construction of pSVCC2. Recombinant plasmid pIE5, which contains *Eco*RI fragment I of CMV DNA, was digested to completion with *Cla*I. The 3.6-kilobase *Cla*I fragment containing CMV IE region 1 was reacted with a solution containing 0.5 mM dCTP, 0.5 mM dGTP, and 10 U of Klenow polymerase for 30 min at room temperature. The fragment was ethanol precipitated and ligated to excess *Hin*dIII linkers at 15°C overnight. The fragment was acrylamide gel purified, digested with *Hin*dIII, and ligated to pSVOd which had been digested with *Hin*dIII and dephosphorylated with bacterial alkaline phosphatase (BAP). Ligation was at 15°C overnight. Amp^r, Ampicillin resistance; Tet^r, tetracycline resistance; SV_o, simian virus 40 origin of DNA replication; dNTPs, deoxynucleoside triphosphates. The open boxes at the bottom (pSVCC2) indicate the exons of the major IE mRNA (35) (see Fig. 2). The arrow indicates the direction of transcription.

restriction enzyme digestion of pSVCC2 in such a way as to dissect out specific domains of the 72-kDa protein. Mutant ΔE24 was generated by partially digested pSVCC2 with *Eco*RV, selecting the appropriate band out of an agarose gel, and circularizing the molecule by blunt end ligation. Mutant ΔE24 contains a deletion of a 429-nucleotide *Eco*RV fragment. This removes exactly 143 amino acids from the middle of the protein, and therefore the reading frame is not altered (see Fig. 4). As a result, the amino- and carboxy-terminal ends of the 72-kDa protein remain intact.

Mutant ΔS12 was constructed by digesting pSVCC2 at the unique *Bgl*II site and partially digesting the resulting linear molecule with *Sau*3A I. The molecules were circularized and digested with *Bgl*III, and the appropriate plasmid was selected. Mutant ΔS12 contains a 40-nucleotide deletion downstream from the *Bgl*II site (see Fig. 4). This mutation causes a frameshift which contains a 145-amino acid deletion that removes the carboxy-terminal one-third of the protein.

DNA transfection. COS-1 cells were passaged 1:4 onto 100-mm plates 2 days before transfection. The cells were generally at 50 to 75% confluence at the time of transfection. The modification of the DEAE-dextran technique of McCutchan and Pagano (23) described by L. Villarreal (personal communication) was used. Cells were washed once in TSM (30 mM Tris hydrochloride, 150 mM NaCl, 1.5 mM $MgCl_2$) and transfected with (per plate) 1 ml of TSM containing 500 μg of DEAE-dextran (molecular weight, 10^6) per ml and 5 μg of the appropriate plasmid DNA. The quantity of DNA was verified by visualization on ethidium bromide-stained agarose gels. The cells were incubated at 37°C for 30 min and overlaid with 10 ml of Eagle minimal essential medium supplemented with 5% fetal bovine serum and 100 mM chloroquine phosphate (Sigma Chemical Co., St. Louis, Mo.). The cells were incubated for 3 h at 37°C, then the cells were washed once with TSM, overlaid with growth medium, and incubated for 44 to 48 h at 37°C.

Preparation of RNA. Cytoplasmic RNA was purified from Nonidet P-40–deoxycholate lysates of transfected cells by using previously described methods (5, 34). Whole-cell RNA was prepared by the guanidinium hydrochloride lysis method essentially as described by Strohman et al. (42). CMV IE polysome-associated RNA was purified as described by Stinski et al. (41).

RNA analysis. Preparation of radioactive DNA probes and analysis of RNA by the nuclease mapping technique of Berk and Sharp (2) have been described previously (35). The amount of virus-specific RNA was determined by scanning appropriately exposed autoradiograms with a Beckman model DU-8 spectrophotometer, using a model DU-8 gel scanning compuset module.

Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins was performed as described by Stinski (37, 38). Proteins were transferred to nitrocellulose by the method of Towbin et al. (45). The 72-kDa major IE protein was detected by using a 1/500 dilution of monoclonal antibody E-3 (14) (obtained from L. Goldstein), followed by the addition of 2×10^6 cpm of ^{125}I-labeled *Staphylococcus aureus* protein A (Amersham Corp., Arlington Heights, Ill.). A truncated major IE protein was detected as described above by using convalescent serum M-19 (obtained from S. Michelson).

Protein structure determination. Two-dimensional protein structures were determined by the method of Chou and Fasman (6) as previously described by Cohen et al. (7).

RESULTS

Expression of IE region 1 in CMV-infected cells. To examine the relative level of expression of IE region 1 throughout the replicative cycle, human fibroblasts were infected with CMV, and whole-cell RNA was isolated at different times after infection. The RNAs from equal numbers of infected cells were hybridized to a probe labeled at the 3′ end of the *Bgl*II site (Fig. 2) and were digested with mung bean nuclease, and the protected hybrids were subjected to alkaline agarose gel electrophoresis as described in Materials and Methods. The expected 550-nucleotide band representing the DNA-RNA hybrid was detected as early as 2 h after infection (Fig. 2). IE region 1 expression reached a maximum level at 5 h after infection, and then the relative level of expression declined sharply (Fig. 2). The initial decrease in the level of expression between 5 and 10 h postinfection was approximately 10-fold. This decrease in level of expression

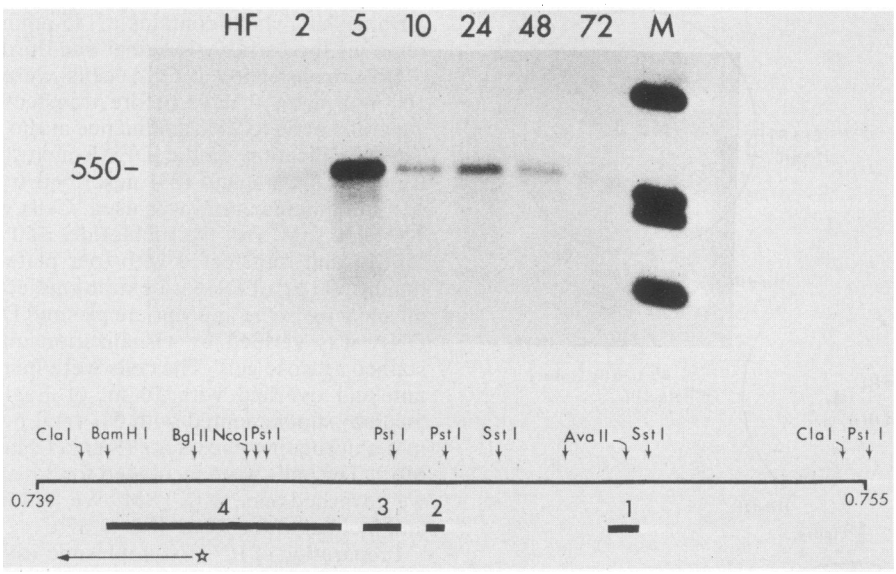

FIG. 2. Synthesis of IE region 1 RNA at different times after infection. Whole-cell RNA was isolated from uninfected cells (HF) or infected cells at different times (2, 5, 10, 24, 48, and 72 h; indicated at the top) and hybridized to a 3′ *Bgl*II probe. The star indicates the end-labeled probe. The map shows the location of the probe, and the thick numbered lines represent the exons of the 1.95-kilobase major IE mRNA. Relevant restriction enzyme sites and map units are shown. The hybrids were digested with mung bean nuclease and subjected to alkaline agarose gel electrophoresis as described in Materials and Methods. The position of the protected probe (550 nucleotides) is indicated. M, Markers.

of the major IE gene was consistent with our previous findings (40).

Expression of CMV IE region 1 on COS-1 cells. To test the level of expression of IE region 1, pSVCC2 was transfected into COS-1 cells, and cytoplasmic RNA was isolated as described in Materials and Methods. The RNA was analyzed as described above. We used the same *Bgl*II site labeled at the 3′ end. This probe was hybridized to CMV IE polysome-associated RNA isolated from infected human fibroblast cells and to cytoplasmic RNA isolated from pSVCC2-

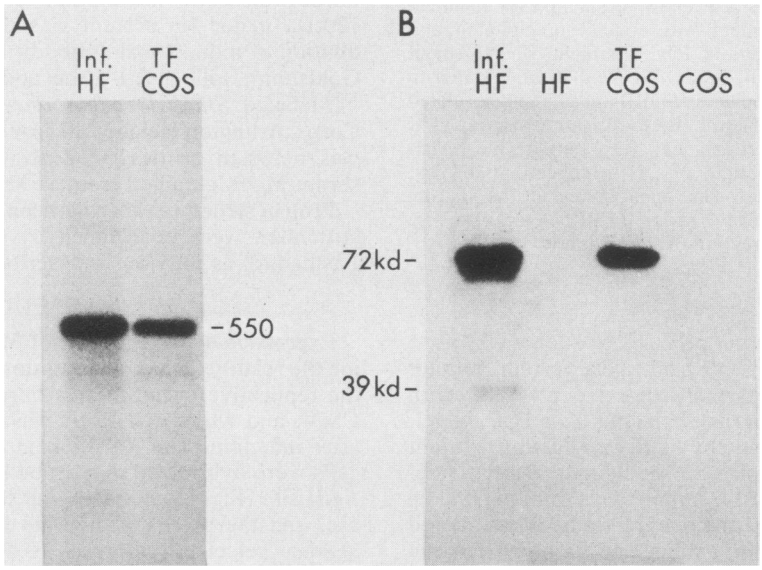

FIG. 3. Expression of pSVCC2 in COS-1 cells. COS-1 cells were transfected with pSVCC2 as described in Materials and Methods. (A) IE polysome-associated RNA (5 μg) (Inf. HF) and cytoplasmic RNA (25 μg) (TF COS) from transfected cells and analyzed as described in the legend to Fig. 1. The position of the protected probe (550 nucleotides) is indicated on the right. (B) Cell lysates from uninfected HF cells (HF), CMV-infected HF cells (Inf. HF), untransfected COS-1 cells (COS), and pSVCC2-transfected COS-1 cells (TF COS) were denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (37, 38). The proteins were transferred to nitrocellulose and probed with monoclonal antibody E-3 as described in Materials and Methods. The positions of the 72- and 39-kDa proteins are indicated.

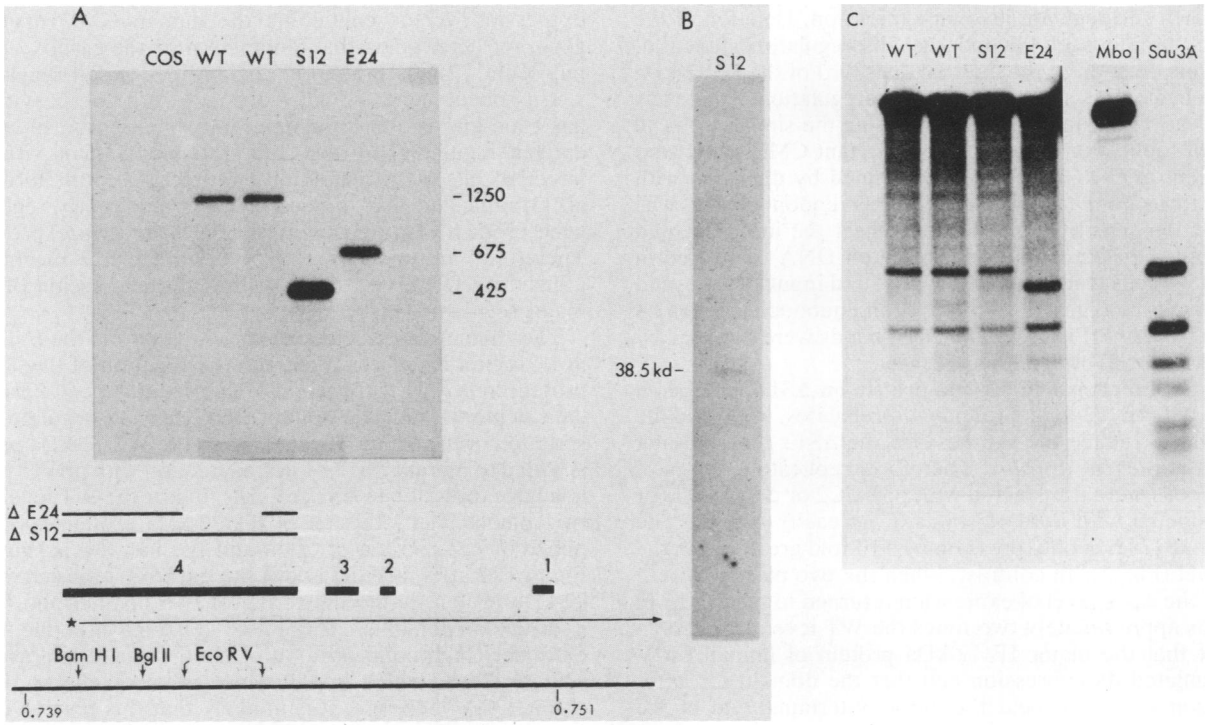

FIG. 4. Analysis of deletion mutants of pSVCC2. Plasmids were transfected into COS-1 cells as described in Materials and Methods. (A) Mutants ΔS12 and ΔE24 were constructed as described in Materials and Methods, and the deleted sequences are shown. The relative locations of the exons of the 1.95-kilobase IE mRNA (thick, numbered lines) and the 5'-end-labeled *Bam*HI probe used in the hybridization studies are shown at the bottom. The star indicates the end-labeled probe. Relevant restriction enzyme sites and map units are shown. Cytoplasmic RNAs from transfected and untransfected cells (COS) were analyzed as described in the legend to Fig. 3A. S12, pΔS12; E24 pΔE24. The positions of the protected probes (1,250, 675, and 426 nucleotides) are indicated. (B) Cell lysates from COS-1 cells transfected with pΔS12 were denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose and probed with convalescent serum M-19 as described in Materials and Methods. The position of the detected 38.5-kDa viral protein is indicated. (C) Soluble DNA extracted from cells transfected with WT, ΔS12, or ΔE24 was digested with restriction endonuclease *Mbo*I (methylation sensitive). The WT recombinant plasmid which replicated only in bacteria was not digested by *Mbo*I but was digested by restriction endonuclease *Sau*3A.

transfected COS-1 cells. Figure 3A shows that both RNAs protected a band at approximately 550 nucleotides. These data demonstrate that the major IE mRNA was expressed at readily detectable levels in COS-1 cells and contained the same 3'end as the RNA in CMV-infected cells. We also analyzed the 5' end of the major IE mRNA produced in COS-1 cells and found that it was identical to the 5' end of the mRNA produced by wild-type (WT) infection of HF cells (data not shown).

The viral protein produced in the transfected COS-1 cells was analyzed by Western blot analysis. Proteins from CMV-infected and mock-infected HF cells and from transfected and mock-transfected COS-1 cells were subjected to sodium-dodecyl sulfate-polyacrylamide gels electrophoresis. The proteins were transferred to nitrocellulose and the replicas were incubated with monoclonal antibody E-3 and then with ^{125}I-labeled protein A. A major 72-kDa protein and two less abundant bands which migrated slightly faster than the 72-kDa protein were detected in CMV-infected cells (Fig. 3B). In addition, 39-kDa minor band was present. This is the typical pattern that occurs in HF cells infected with CMV strain Towne and is consistent with our previous findings (38, 41). In COS-1 cells, a single species that comigrated with the major IE 72-kDa protein was detected (Fig. 3B). Therefore, the transfected COS-1 cells synthesized readily detectable amounts of the CMV major IE protein.

Analysis of deletion mutants with mutations in the 72-kDa protein. To determine whether the major IE 72-kDa protein has an autoregulatory function, we constructed deletions in the coding sequences of IE region 1 as described in Materials and Methods. The locations of the deletions in the two mutants used in this study are shown in Fig. 4. The mutant and WT plasmids were transfected into COS-1 cells, and the amount of IE region 1 RNA was quantitated as described above. Because the *Bgl*II site was deleted in mutant ΔS12, the RNA was hybridized to a 5'-end-labeled *Bam*HI probe (Fig. 4). The levels of WT expression, detectable as a 1,250-nucleotide band in two separate WT-transfected cultures, were identical, indicating that variation did not occur from sample to sample (Fig. 4). The level of expression with mutant ΔE24, detectable as a 675-nucleotide band, was approximately 50% greater than the level of WT expression. This variation could have been due to a perturbation in the structure of the viral protein, but is was considered to be within the range of experimental error. The level of expression with mutant ΔS12, detectable as a 425-nucleotide band, was 10-fold greater than that with the WT plasmid (Fig. 4A). This 10-fold increase was consistent from experiment to experiment. A truncated ΔS12 protein of approximately 40 kDa was predicted from the amino acid sequence. A viral protein of this approximate size was detected by using Western blot analysis and human convalescent serum (Fig. 4B). There was no reaction with nontransfected COS-1 cells. The data indicated that the human CMV 72-kDa protein has

the capacity to regulate its own expression. Deletion of the carboxy terminus eliminated the autoregulatory function. However, deletion of the internal one-third of the IE 72-kDa protein had only a slight effect on autoregulation. The levels of replication of the plasmids containing the simian virus 40 origin of replication and the WT or mutant CMV gene were equivalent in COS-1 cells, as determined by digestion with restriction endonuclease *Mbo*I. This endonuclease was methylation sensitive and did not digest the input plasmid DNA but did digest the nonmethylated DNA replicated in the COS-1 cells (Fig. 4C). The methylated input WT plasmid DNA could be digested by restriction endonuclease *Sau*3A but not by *Mbo*I (Fig. 4C). The DNA bands were detected by Southern blot hybridization.

Effect of functional IE 72-kDa protein on ΔS12 expression. If the WT IE 72-kDa protein autoregulates, it would be expected to reduce the synthesis of the ΔS12 gene product when it is present in *trans*. Therefore, replicate cultures of COS-1 cells were transfected with pSVCC2 or ΔS12 alone or cotransfected with both plasmids (5 μg each) (Fig. 5). The mutant ΔS12 level of expression was 10-fold greater than the WT level (Fig. 5). In contrast, when the two plasmids were mixed, the ΔS12 level of expression returned to a lower level that was approximately two times the WT level. These data suggest that the major IE 72-kDa protein of human CMV autoregulated its expression and that the domain for autoregulation was at or near the carboxy-terminal end of the protein.

DISCUSSION

In this report we describe the capacity of a herpesvirus IE protein to regulate its own expression. Using an amplifiable expression vector containing the human CMV major IE gene, we determined that a domain near the carboxy terminus of the 72-kDa protein is responsible for self-regulation.

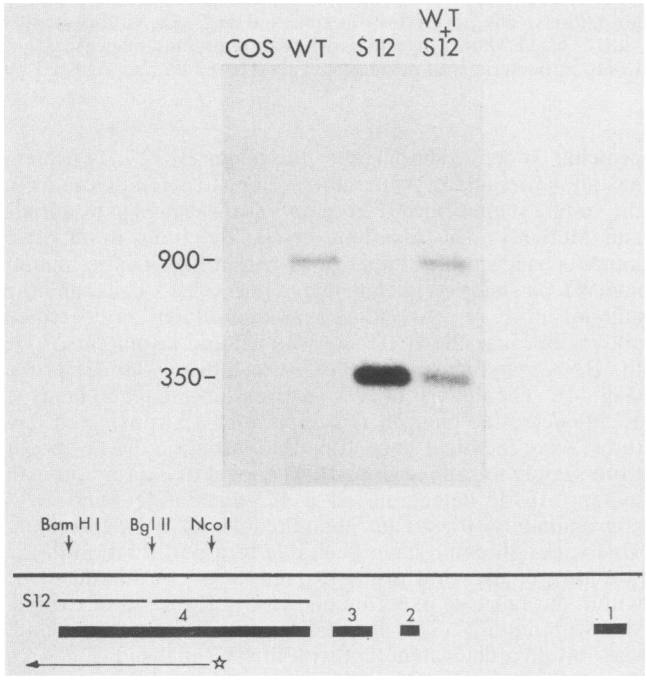

FIG. 5. Effect of functional IE 72-kDa protein on pΔS12 expression. COS-1 cells were transfected with plasmids and analyzed as described in Materials and Methods and the legends to Fig. 3A and 4A. The probe used in the study was 3′ end labeled at an *Nco*I site (shown at the bottom). The star indicates the end-labeled probe. The positions of the protected probes (900 and 350 nucleotides) are indicated.

This phenomenon is not restricted to the herpesvirus. It has been known for some time that simian virus 40 large-T antigen regulates its own expression in simian virus 40-infected cells (33). In addition, in a recent report Borrelli et al. (3) indicated that the adenovirus type 2 early region 1a gene product also negatively regulates its own expression. Therefore, autoregulation may be a general mechanism utilized by DNA viruses to control the expression of their major regulatory proteins.

The 40-nucleotide deletion downstream from the *Bgl*II site in IE region 1 leads to premature termination of the 72-kDa protein, resulting in a protein of approximately 40 kDa. Our data demonstrate that mutant ΔS12 fails to regulate transcription of the major IE gene. When the WT 72-kDa protein is added to mutant ΔS12 by cotransfection with pSVCC2, the level of expression in ΔS12 returns to near the WT level. The two-dimensional structure of the 72-kDa protein, as determined by the method of Chou and Fasman (6), is shown in Fig. 6. What is striking about the carboxy terminus of the 72-kDa protein is the clustering of two distinct and highly hydrophilic domains. These two domains are due to an extremely high concentration of glutamic acid and aspartic acid residues, which impart a net negative charge to this region (35). Therefore, it is unlikely that this portion of the protein functions to autoregulate by binding to the viral DNA. In fact, studies in our laboratory have demonstrated that the 72-kDa protein is not a DNA-binding protein when calf thymus DNA is used as a substrate (M. P. Landini and M. F. Stinski, unpublished data). Therefore, we propose that the major IE 72-kDa protein associates with another host or viral protein to regulate expression of the major IE gene or the 72-kDa protein reacts specifically with a defined viral DNA sequence.

It is possible that the ΔS12 truncated protein may not be transported to the nucleus. Recent studies with simian virus 40 large-T antigen have demonstrated that a region near the amino-terminal one-third of the protein has a role in transport of T antigen into the nucleus (18, 19). There is clustering of positvely charged amino acids at the extreme carboxy terminus of the human CMV major IE protein. Thus, failure of the 72-kDa protein to be transported into the nucleus would also result in an inability of the protein to autoregulate.

Additional studies in our laboratory have demonstrated that IE regions 1 and 2 are actually linked through a complicated series of splicing events (36). The region 2 mRNAs are transcribed from the region 1 promoter, with region 1 exons being spliced onto region 2. Therefore, the 72-kDa protein should also negatively regulate the expression of IE region 2. Although we have not directly addressed this question, the results of experiments described in the accompanying paper suggest a relationship between autoregulation of IE region 1 and the kind of RNA transcribed from IE region 2 (36). For example, under IE conditions (i.e., in the presence of an inhibitor of protein synthesis), IE region 2 RNAs are linked to the IE region 1 leader exons. Therefore, the synthesis of these RNAs is influenced by the IE region 1 promoter-regulatory region. After synthesis of the major IE 72-kDa protein, unspliced viral RNA is transcribed from IE region 2 with the 5′ end of the RNA adjacent to the IE region 2 promoter (26). These results suggest that after autoregulation of the IE region 1 promoter, the IE region 2 RNAs no longer have the IE region 1 leader exons and that

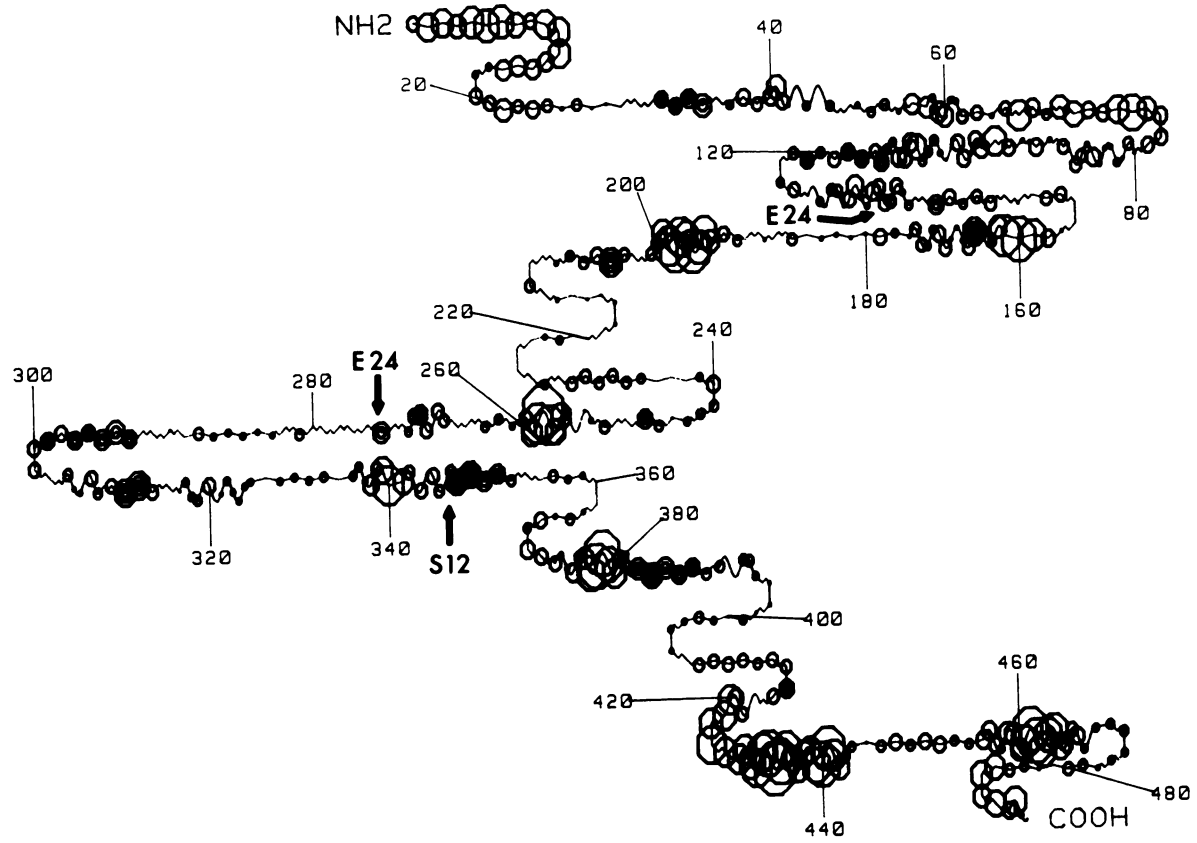

FIG. 6. Two-dimensional structure of the IE region 1 72-kDa protein. The structure was predicted by using the method of Chou and Fasman (6) as previously described by Cohen et al. (7). The locations of the amino (NH_2) and carboxy (COOH) termini are indicated. The open octagons indicate hydrophilic regions, and the concentric octagons indicate hydrophobic regions. The sizes of the octagons indicate the degree of hydrophilicity or hydrophobicity. The amino acids are numbered every 20 residues. The arrows indicate the locations of the ΔS12 and ΔE24 deletions. The amino acid sequence of the IE region 1 72-kDa protein has been described previously (35).

the IE region 2 promoter is preferentially used. Our studies have shown that the amount of IE region 1 RNA in the cells decreases 10-fold between 5 and 10 h after infection. This is equivalent to the change in the relative levels of expression between WT pSVCC2 and mutant ΔS12. Since whole-cell RNA is affected in this manner, it is unlikely that the increased amount of cytoplasmic RNA in ΔS12-transfected cells is due to the lack of some posttranscriptional control mechanism. Therefore, we propose that the major IE protein regulates its own expression.

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