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# Herpes simplex virus 1 $\gamma_1$34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage

**(programed cell death/protein synthesis/neuroblastoma cells)**

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ABSTRACT **The $\gamma_1$34.5 gene of herpes simplex virus is dispensable in some cell lines (e.g., Vero). In others (e.g., human neuroblastoma cell line SK-N-SH), the $\gamma_1$34.5$^-$ deletion mutant triggers a premature total shutoff of all protein synthesis, thereby rendering the cell nonviable and reducing drastically viral yields. The inability to prevent the cellular stress response that causes the infected cell to die may be responsible for the inability of the deletion mutant to multiply and cause pathology in the central nervous system of mice. The $\gamma_1$34.5 gene consists of an amino-terminal domain, a variable linker sequence consisting of 3 amino acids repeated 5–10 times, and a carboxyl-terminal domain homologous to the corresponding domain of *MyD116*, a gene expressed in myeloid leukemia cells induced to differentiate by interleukin 6, and growth arrest and DNA damage gene 34 (GADD34), a gene induced by growth arrest and DNA damage. We have constructed several viral mutants from which various domains of the $\gamma_1$34.5 gene had been deleted or rendered mute by the insertion of a stop codon. Studies on those mutants show that the domain of the $\gamma_1$34.5 gene necessary to preclude the total shutoff of protein synthesis corresponds to the carboxyl-terminal domain of the $\gamma_1$34.5 gene homologous to the corresponding coding domain of the *MyD116* and GADD34 genes.**

The $\gamma_1$34.5 gene of herpes simplex virus 1 (HSV-1) maps in the inverted repeats flanking the long unique sequence, and therefore, it is present in two copies per genome (1–4). The gene is dispensable inasmuch as mutants lacking both copies of the gene multiply nearly as well as wild-type virus in Vero cells (5). A remarkable property of the deletion mutants is that they fail to multiply and cause encephalitis after intracerebral inoculation of mice (5–8). Further studies of the deletion mutants in cells in culture revealed that in neuroblastoma cells (for example, the human neuroblastoma cell line SK-N-SH), the deletion mutant failed to prevent cell death resulting from total cessation of protein synthesis relatively early in infection, whereas the wild-type parent virus precluded the shutoff of protein synthesis and enabled the virus to multiply (9). These and other observations on the properties of the deletion mutant lead us to conclude that the onset of viral DNA synthesis or a product made thereafter triggers a stress response that results in total shutoff of protein synthesis and cell death. The function of the $\gamma_1$34.5 gene appears to be to preclude this response (9).

The $\gamma_1$34.5 gene of HSV-1 strain F [HSV-1(F)] is predicted to encode a protein of 263 amino acids consisting of a large amino-terminal domain, a linker or swivel region of 3 amino acids repeated 10 times, and a carboxyl-terminal domain (3). The 3-amino acid repeats (Ala-Thr-Pro, ATP codons) are a constant feature of all strains, but the number of repeats varies from strain to strain (3). The carboxyl-terminal domain is partially homologous to the corresponding domains of protein encoded by *MyD116* gene and growth arrest and DNA damage gene 34 (GADD34) (Fig. 1) (10, 12, 13). The *MyD116* gene was cloned from murine myeloid leukemia cells induced to differentiate by interleukin (IL) 6 (10). Like the $\gamma_1$34.5 protein, the MyD116 protein is predicted to consist of a large amino-terminal domain, a 38-amino acid sequence repeated 4.5 times, and a carboxyl terminus containing sequences partially homologous to an 82-amino acid stretch in $\gamma_1$34.5 protein (Fig. 1) (10). The hamster GADD34 gene induced by growth arrest and DNA damage (13) predicts a similar protein (Fig. 1).

IL-6 is a multicellular pleiotropic cytokine that induces cell-type-dependent differentiation and an acute-phase injury response in many cell types including neuronal tissues by viruses, bacteria, and other infectious agents (14). *MyD116* is one of several genes cloned as cDNA from myeloid leukemia cells induced to differentiate by IL-6 and is an immediate-response gene; its expression peaks 1 hr after exposure of cells to IL-6. Steady-state levels of MyD116 mRNA have been detected in the terminally differentiated cells. The precise function of the *MyD116* gene has not been established. One of the functions of the genes expressed after differentiation of the myeloid leukemic cells is to preclude the terminally differentiated cells from undergoing apoptosis. The GADD34 protein appears to be closely related and may have a similar function (Fig. 1) (13). The predicted amino acid sequence is similar to that of MyD116; its carboxyl terminus is even more homologous to $\gamma_1$34.5 than MyD116. In this report we show that the $\gamma_1$34.5 sequence, which precludes the total shutoff of protein synthesis and premature death of the cell, maps in the carboxyl terminus of $\gamma_1$34.5, i.e., in the region shared with GADD34 and MyD116 protein.

## MATERIALS AND METHODS

**Cells.** The Vero cell line and the human neuroblastoma cell line SK-N-SH (American Type Culture Collection) were propagated in Dulbecco's modified Eagle's medium supplemented with 5% and 10% fetal bovine serum, respectively.

**Viruses.** HSV-1(F) is the prototype HSV-1 strain used in this laboratory (15). The procedure for transfection and selection of viral recombinants carrying desired mutations has been described (16). In R3616 virus, 1 kbp was deleted in both copies of the $\gamma_1$34.5 gene between the *Bst*EII and *Stu* I sites (Fig. 2) (5). In R4002 virus (B virus, Fig. 2), a DNA fragment containing the HSV-1 thymidine kinase gene driven by the promoter of the $\alpha$27 gene ($\alpha$27–*tk*) and first codon of the glycoprotein H gene was inserted into both copies of the $\gamma_1$34.5 gene in such a manner that the promoter and first

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Abbreviations: HSV-1, herpes simplex virus 1; IL, interleukin; GADD34, growth arrest and DNA damage gene 34.

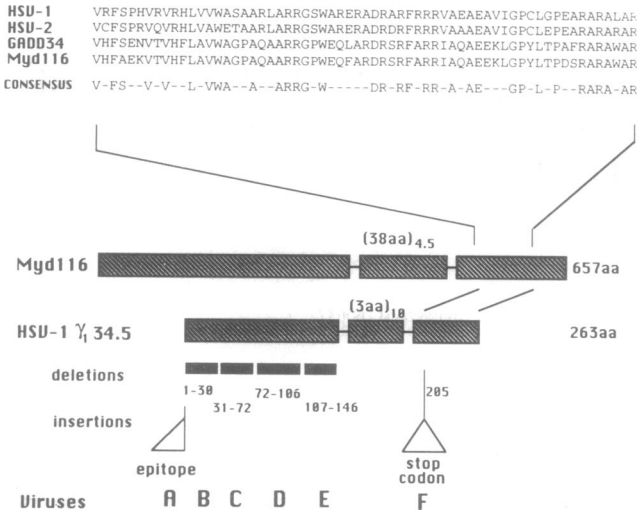


FIG. 1. Schematic representations of the MyD116 and $\gamma_1$34.5 proteins and of the mutations introduced into the HSV-1(F) $\gamma_1$34.5 gene. The predicted amino acid sequences of the carboxyl-terminal domains of the HSV-1 (3) and HSV-2 (11) $\gamma_1$34.5 proteins, GADD34 and MyD116 (10) proteins, and the conserved (identical) amino acids of the aligned carboxyl-terminus domains of $\gamma_1$34.5 proteins to GADD34 or MyD116 (consensus) are shown. In MyD116, 38 amino acids repeated 4.5 times separate a large highly charged amino-terminal domain from the carboxyl terminus. In $\gamma_1$34.5, 3 amino acids (ATP codons) repeated 10 times also separate a highly charged amino terminus from the carboxyl terminus. The solid bars underneath the $\gamma_1$34.5 gene represent the location and the extent of deletions within the mutant viruses, B–E, used in this study. The numbers represent the first and last codons deleted from the gene. The A virus contains a 17-amino acid epitope inserted at codon 1 (5). The F virus contains a six-frame stop codon inserted at codon 205, that is, 11 codons after the last repeat of the ATP codons. The sequence of the GADD34 protein was obtained from the GenPept Updates (February 15, 1994; accession no. L28147).

codon of the glycoprotein H was fused to codon 28 of the $\gamma_1$34.5 genes. The virus contains a 500-bp deletion in the thymidine kinase gene at its natural location (5). R4003 virus (A virus, Fig. 2) containing a 17-amino acid insertion encoding an $\alpha$4 epitope at the first amino acid of $\gamma_1$34.5 has been described (5).

**Plasmids.** Plasmids containing desired mutations were cloned using appropriate restriction enzymes and ligase supplied by New England Biolabs. The oligonucleotides shown in boxes of Fig. 2 were synthesized on a model 380B DNA synthesizer, Applied Biosystems, in this laboratory. After deprotection, the complementary oligonucleotides were annealed at 80°C, gradually cooled to room temperature in water bath, and used directly in cloning.

## RESULTS

**Construction of Viral Mutants.** The nucleotide sequence arrangements of mutants used in these studies are shown in Fig. 1. Viral mutants A (R4003) and B (R4002) are described above. The construction of all other mutants is described in Fig. 2. In mutants C, D, and E, fragments encoding codons 39, 41, and 34, respectively, were replaced by linker sequences designed to maintain the remainder of the gene in-frame and to provide a diagnostic restriction endonuclease cleavage site. The linker sequence and the diagnostic restriction sites are shown in Fig. 2. The electrophoretic profiles of the restriction enzyme digests hybridized with a $\gamma_1$34.5-specific probe show that the diagnostic sites were indeed present in both copies of the gene in the recombinant viruses (Fig. 3). To verify that the $\gamma_1$34.5 protein actually reflects the changes in the nucleotide sequence, lysates of Vero cells

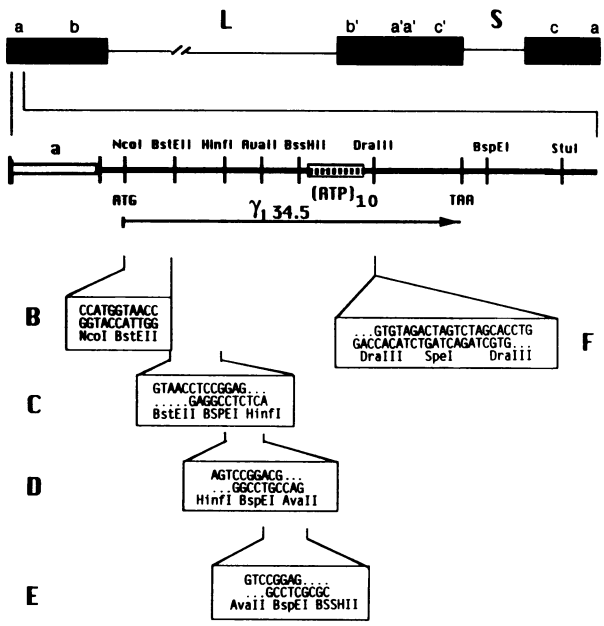


FIG. 2. Schematic representations of $\gamma_1$34.5 gene and construction of deletion mutants. The top line represents the sequence arrangement of the HSV-1 genome. The thin line represents unique sequences flanked by inverted repeats (solid rectangles) of the L component (ab and b'a') and of the S component (a'c' and ca) (17). The location and restriction map of $\gamma_1$34.5 gene at the terminus of the viral genome are shown; a second copy of the gene is located in the b sequence of the internal inverted repeats (1). The promoter of this gene is mapped to the 500-bp a sequence shown as an open rectangle to the left of the genome (1). The location of the ATP codon repeat is shown as a hatched rectangle. The coding sequence domain of $\gamma_1$34.5 is also shown. ATG and TAA represents the first and termination codon in the gene. To construct all other viruses, the plasmid containing the $\gamma_1$34.5 gene was cleaved with the appropriate restriction endonucleases and religated with a linker sequence containing a diagnostic restriction endonuclease cleavage sites as shown in the individual boxes. For the F virus, the plasmid was cleaved with *Dra* III and ligated with a linker that contains a six-frame stop codon and a diagnostic *Spe* I restriction site. Viruses D and E were generated by cotransfection of R3616 with plasmid D and E, respectively. Plaques were harvested and screened for expression of $\gamma_1$34.5 proteins migrating faster than the wild-type protein in polyacrylamide gels. Viruses C and F were generated by cotransfection of R4002 virus (B virus) DNA with plasmids C and F and the mixtures were selected on 143TK$^-$ cells in the presence of bromodeoxyuridine (100 $\mu$g/ml of medium). Candidate viruses were then screened for $\gamma_1$34.5-restored sequences and the presence of truncated proteins that reflect the deletions or insertions in the coding domain of its gene. The plaque-purified progenies selected for further studies were then restored to their natural *tk* locus by cotransfection with *Bam*HI Q fragment (data not shown).

infected with the various mutants were subjected to electrophoresis in denaturing gels, electrically transferred to a nitrocellulose sheet, and incubated with rabbit polyclonal antibody made against an oligopeptide consisting of 10 repeats of the Ala-Thr-Pro triplet as described (2). As shown in Fig. 4, the electrophoretic mobility of the band reactive with the polyclonal antibody increased for mutants B–F or decreased, as would be expected for mutant A whose $\gamma_1$34.5 protein increased in size by 17 amino acids.

**Phenotype of the $\gamma_1$34.5 Mutants in Vero and SK-N-SH Cell Lines.** The purpose of these studies was to map the domain of the $\gamma_1$34.5 protein that precludes the total shutoff and premature death of the infected cell. To attain this objective, the parental wild type, the deletion mutant described in the earlier publication (5), and the mutant viruses made for these studies and described above were used to infect replicate cultures of SK-N-SH and Vero cells. The cultures were

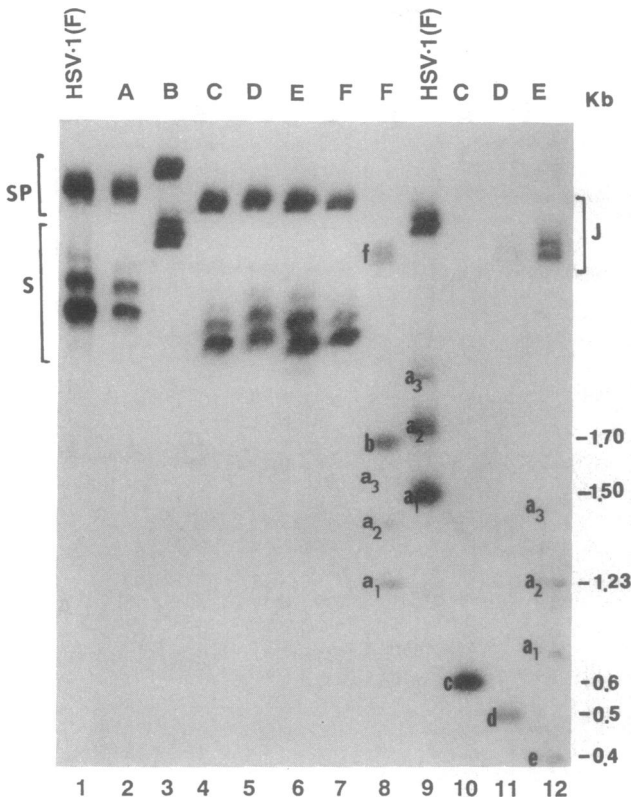


FIG. 3. Autoradiographic images of electrophoretically separated restriction endonuclease DNA fragments transferred to a nitrocellulose sheet and hybridized to $^{32}$P-labeled probes. Lanes: 1–7, *Bam*HI digests of DNAs of HSV-1(F) and recombinant viruses A–F, respectively; 8, *Bam*HI and *Spe* I digest of F recombinant; 9–12, *Bsp*EI digests of HSV-1(F) and C, D, and E recombinant DNAs, respectively. The digests were subjected to electrophoresis on a 1.2% agarose gel at 50 V in 50 mM Tris phosphate (pH 7.5), transferred to a nitrocellulose sheet, and probed with a 600-bp *Bst*EII–*Sac* I DNA fragment derived from the $\gamma_1$34.5 gene (Fig. 2). In lanes 1–7, the probe reacted with both the L–S junction fragment *Bam*HI SP (SP) and the terminal fragment *Bam*HI S (S). Both *Bam*HI SP and S bands show multiple ladder bands whose sizes differ by 500 bp due to variability in the number of a sequences in these fragments. The B virus in lane 3 contains the $\alpha$27–*tk* chimeric gene inserted in both *Bam*HI SP and S fragments, resulting in slower migrating bands as shown (5). All viruses show correct-size *Bam*HI SP and S fragments. Virus F contains a six-frame translation stop codon and a diagnostic *Spe* I restriction site in the stop codon sequence inserted in the *Dra* III site. To verify the presence of stop codons, F virus was digested with *Bam*HI (lane 7) or with *Bam*HI and *Spe* I (lane 8). As shown in lane 8, *Bam*HI S fragment is cleaved by *Spe* I into smaller size fragments of 1.7 kb (designated as b) and a ladder of terminal fragments containing sequences marked as $a_1$, $a_2$, and $a_3$. SP fragment of F virus is also cleaved by *Spe* I restriction endonuclease; however, only band f (lane 8) reacts with the probe. Further analyses of the deletions in viruses C–E by digestion with *Bsp*EI are shown in lanes 9–12. In lane 9, the $\gamma_1$34.5 sequence probe reacted with a set of *Bsp*EI junction fragments of wild-type HSV-1(F) DNA, marked collectively as J, and terminal fragments, marked as $a_1$, $a_2$, and $a_3$, with sizes starting at 1.5 kbp and increments thereon (lane 9). A unique *Bsp*EI site was present at the end of the $\gamma_1$34.5 gene in all viruses and an additional *Bsp*EI site was present in the linker sequence replacing the deleted sequence of viruses C–E (see Fig. 2). Cleavage of viruses C–E with *Bsp*EI restriction endonuclease yielded fragments c, d, and e of 0.6, 0.5, and 0.4 kb, respectively (lane 10, 11, and 12). The sizes of these fragments match the expected lengths of fragments when digested with *Bsp*EI. The probe, a 600-bp *Bst*EII–*Sac* I fragment derived from $\gamma_1$34.5, reacted fully with band c generated from both junction and terminal fragments in virus C (lane 10), partially with band d and J fragments in virus D (lane 11), and less with band e, terminal fragments $a_1$, $a_2$, and $a_3$, and bands J in virus E (lane 12). The sizes of all fragments (kb) are shown to the right of the figure.

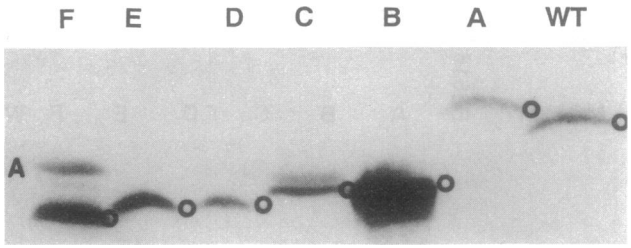


FIG. 4. Photographic images of immunoblots of ICP34.5 present in extracts of cells infected with HSV-1(F) or with mutants A–F. Replicate Vero cell cultures were each infected with HSV-1(F) (WT) or with mutant viruses A–F at 5–10 plaque-forming units per cell. The cells were harvested 24 hr after infection, solubilized, subjected to electrophoresis on denaturing 12% polyacrylamide gels, transferred electrically to nitrocellulose sheets, and reacted with the R4 rabbit polyclonal antibody to the Ala-Thr-Pro repeat in ICP34.5 as described (2). o, Location and presence of wild type and of ICP34.5 modified by mutagenesis of the gene. The letter A denotes actin, which was often nonspecifically stained by this polyclonal antibody.

labeled for 1 hr at 13 hr after infection with [$^{35}$S]methionine. As shown in Fig. 5, 13 hr after infection protein synthesis was shutoff in cells infected with the deletion mutant R3616 from which 1 kbp of coding sequences of the $\gamma_1$34.5 gene had been deleted and in cells infected with the mutant containing a stop codon in the carboxyl-terminal domain of the $\gamma_1$34.5 gene. Protein synthesis took place in cells infected with all other deletion or insertion mutants at levels comparable to those observed in wild-type-infected cells.

## DISCUSSION

Central to the results reported in this communication are the following features:

(*i*) The striking phenotype of $\gamma_1$34.5$^-$ virus is a complete loss of the capacity of the mutant virus to replicate and cause pathology in central nervous system of mice (5–8). In cells in culture, the $\gamma_1$34.5$^-$ virus multiplies in a fashion similar to that of wild-type virus in some cell lines (e.g., Vero cells). In these cells both the wild-type and mutant virus produce infectious progeny and, ultimately, destroy the infected cells. Although the rate of protein synthesis decreases gradually, it continues for many hours past the point of leveling of the synthesis of infectious progeny. In others (e.g., the human neuroblastoma cell line SK-N-SH), a decrease in protein synthesis was noted quite early in infection and all protein synthesis ceased several hours before the normal completion of the reproductive cycle. The shutoff of all cellular protein synthesis is triggered by the initiation viral DNA synthesis inasmuch as a drug that specifically blocks viral DNA synthesis (phosphonoacetate) precludes the shutoff. In earlier studies we concluded that the onset of viral DNA synthesis triggers a host response that results in the shutoff of protein synthesis and cell death and that the function of the $\gamma_1$34.5 gene is to preclude this response to enable the virus to multiply (9). We suspect that this stress response is the basis for the loss of the capacity of $\gamma_1$34.5$^-$ viruses to multiply in the central nervous system of mice, although the definitive experiments have not been completed as yet.

(*ii*) Acquisition and conservation of cellular genes by viruses for specific tasks is well recognized [e.g., DNA polymerase (19) and IL-10 (20)]. For $\gamma_1$34.5, the cellular homologs are MyD116 and GADD34 (10, 13). It is noteworthy that not all herpes viruses have conserved this gene. To date, it has been detected in HSV-2 (11) but not in other members of the $\alpha$-herpesvirinae subfamily of these viruses to which HSV-1 belongs. A homolog of MyD116, designated lmw23-NL (21), has been reported in the genome of African Swine Fever virus, an unrelated virus. As noted in the

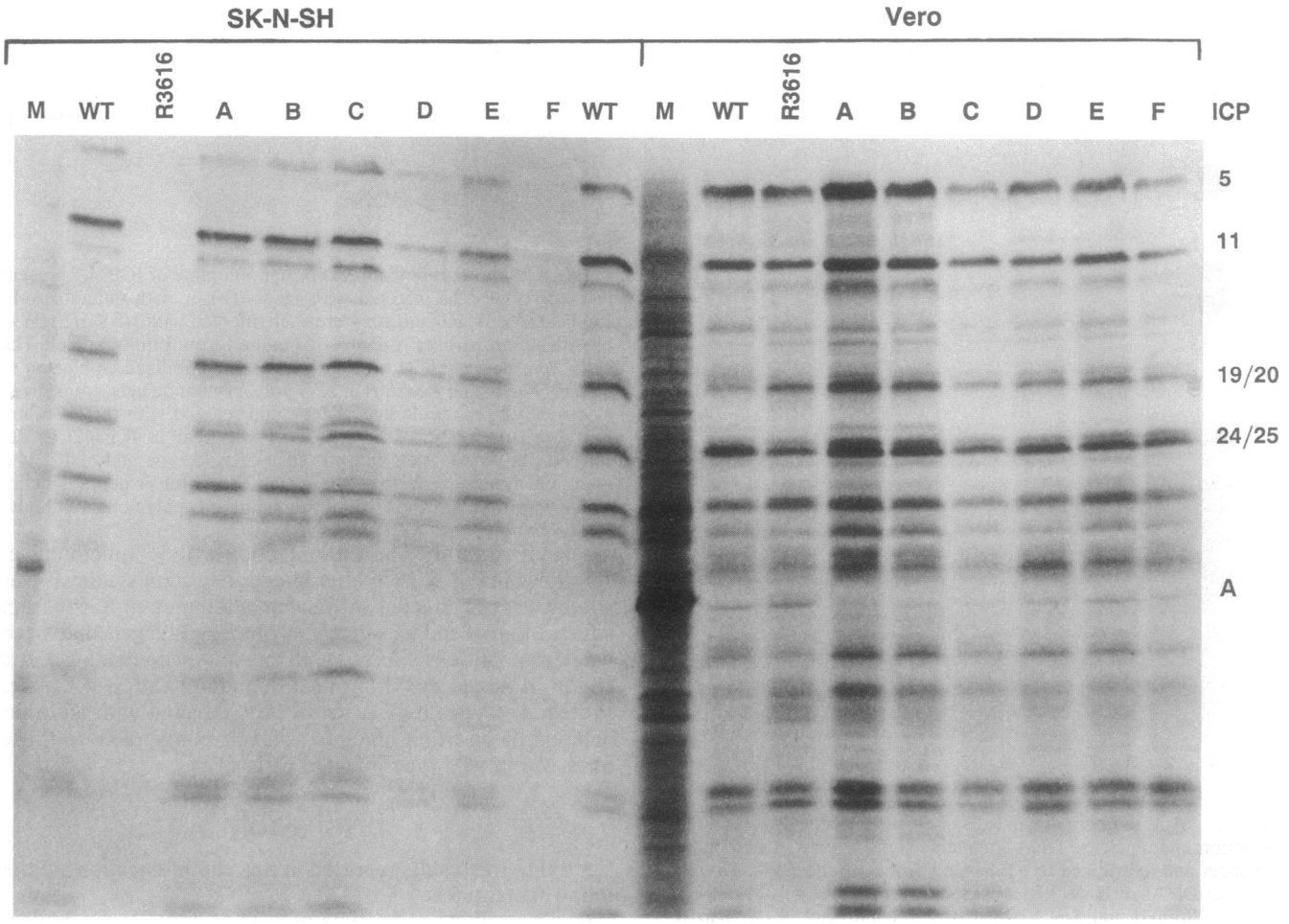


FIG. 5. Autoradiographic images of SK-N-SH and of Vero cells mock-infected (M) or infected with HSV-1(F) (WT) or $\gamma_1$34.5 mutant viruses A–F (10–50 plaque-forming units per cell) in 199V medium supplemented with 1% calf serum and incubated at 37°C. At 13 hr after infection, the cells were overlaid with 1 ml of 199V medium lacking methionine but supplemented with 50 $\mu$Ci of [$^{35}$S]methionine (specific activity, >1000 Ci/mmol; 1 Ci = 37 GBq), incubated for 1 hr, and then harvested. Cells were then solubilized, subjected to electrophoresis in denaturing gels, transferred to a nitrocellulose sheet, and subjected to autoradiography. The infected cell polypeptides (ICP) were labeled according to Morse *et al.* (18).

introduction, MyD116 protein is predicted to have a tripartite structure similar to that of $\gamma_1$34.5. However, the homologous domains are confined to the carboxyl terminus of the proteins. In this report we have shown that the domain of the $\gamma_1$34.5 necessary to preclude the total shutoff of protein synthesis is the domain of the protein that is homologous to the corresponding carboxyl-terminal domain of MyD116 and GADD34 proteins. The role of the amino-terminal domain of $\gamma_1$34.5 in this process is not known: our results show that at least in cells in culture, consecutive portions of the amino-terminal domain covering nearly the entire sequence are dispensable.

(*iii*) The scientific literature of recent years has revealed a growing interest and realization that disruption of the regulatory cascade in cells can result in apoptosis and that there are genes that both initiate and block this event. The pathway by which $\gamma_1$34.5 may act or the stress it precludes appears to be superficially, at least, different from that described to date and exemplified by the involvement of *bcl2*. *bcl2* has been shown to protect cells from apoptosis cell in stress (e.g., some viral infections) or during development (22, 23). *bcl2*, however, does not preclude apoptosis resulting from deprivation of IL-6 or the ciliary neurotrophic factor (24–26). Several studies have indicated that IL-6, ciliary neurotrophic factor, and leukemia inhibiting factor share common receptors and signal transduction pathways (26–28). On the basis of the results obtained with $\gamma_1$34.5 gene reported here, we predict that MyD116 and GADD34 proteins, particularly their carboxyl termini, may play a role in blocking apoptosis in terminally differentiated cells and that HSV appropriated the carboxyl-terminal domain of these genes for its own use.

It is noteworthy that many of the HSV proteins studied in detail to date have numerous functions (29). It is conceivable, therefore, that while the carboxyl-terminal domain of $\gamma_1$34.5 functions to block a cell response to the stress of infection in a manner similar to that of GADD34 and MyD116 protein, the amino-terminal domain has a different, potentially unrelated, function.

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