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The Carboxyl Terminus of the Murine MyD116 Gene Substitutes for the Corresponding Domain of the $\gamma_1$34.5 Gene of Herpes Simplex Virus To Preclude the Premature Shutoff of Total Protein Synthesis in Infected Human Cells

BIN HE,[1] JOANY CHOU,[1] DAN A. LIEBERMANN,[2] BARBARA HOFFMAN,[2] AND BERNARD ROIZMAN[1]*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637,[1] and Fels Institute for Cancer Research, Temple University School of Medicine, Philadelphia, Pennsylvania 19140[2]

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The herpes simplex virus 1 mutants from which both copies of the $\gamma_1$34.5 gene had been deleted trigger total shutoff of protein synthesis in human neuroblastoma cells and human foreskin fibroblasts but not in African green monkey (Vero) cells. The carboxyl-terminal 64 amino acids of $\gamma_1$34.5 are homologous to the corresponding domain of MyD116, a murine myeloid differentiation primary responsive gene. The carboxyl-terminal domain of $\gamma_1$34.5 is required to preclude the shutoff of protein synthesis (J. Chou and B. Roizman, Proc. Natl. Acad. Sci. USA 91:5247–5251, 1994). We report that in-frame substitution of the carboxyl terminus of $\gamma_1$34.5 with the corresponding domain of MyD116 in the context of the viral genome restored the ability of $\gamma_1$34.5 to preclude premature shutoff of protein synthesis in both neuroblastoma cells and in human foreskin fibroblasts. The results suggest that (i) in the course of its evolution, the virus "borrowed" a gene fragment to preclude a cell response to infection and (ii) the carboxyl terminus of MyD116 and its family of genes known as GADD34 may have a similar function(s) in cells stressed by growth arrest, DNA damage, and differentiation and in herpes simplex virus infection.

The herpes simplex virus 1 (HSV-1) genes are arranged in four clusters: i.e., in a unique long (U_L) sequence; in a sequence designated as *ab* and its inverted repeat, *b′a′*, which flank U_L; in a unique short (U_S) sequence; and in the sequences *a′c′* and *ca*, which flank U_S (19, 22). The *a* sequence varies in length (reviewed in reference 17). In the HSV-1 strain F [HSV-1(F)], this sequence is approximately 500 bp long and consists largely of reiterations of short sequences (15). The *a* sequence serves many functions involving the cleavage of mature, unit-length DNA from newly synthesized DNA concatemers for packaging into preformed capsids. The *a* sequence flanking the U_L sequence also contains a promoter for a gene designated $\gamma_1$34.5 (5). Because it is located within inverted repeats, the $\gamma_1$34.5 gene is present in two copies per genome. As its designation indicates, the expression of the gene is only partially induced by inhibitors of DNA synthesis. In human cells, but particularly human neuroblastoma cell lines and human foreskin fibroblast (HFF) cultures infected with mutants from which both copies had been deleted, all protein synthesis ceases within hours of the onset of viral DNA synthesis (4, 7). Not surprisingly, mutants lacking the $\gamma_1$34.5 gene are avirulent in experimental murine and guinea pig models (3, 24). The deletion mutant replicates as well as the wild-type virus in Vero cells. The function of the $\gamma_1$34.5 gene product appears to be to preclude the premature shutoff of total protein synthesis in selected human cells. Experiments detailed elsewhere (4) indicate that the amounts of $\gamma_1$34.5 protein required for this purpose can be very small and may be supplied in *trans* by cells carrying the viral $\gamma_1$34.5 gene. The $\gamma_1$34.5 protein, predicted to contain 263 amino acids, consists of three domains: i.e., a 159-amino-acid amino-terminal domain, 10 repeats of three amino acids (AlaThrPro), and a 74-amino-acid carboxyl-terminal domain (6). The number of the triplet repeats appears to vary among virus strains (6). A stretch of 64 amino acids at the carboxyl terminus of the $\gamma_1$34.5 gene is homologous to a corresponding stretch of amino acids of the carboxyl terminus of a murine protein known as MyD116 (13, 14) and a Chinese hamster protein known as GADD34 (10). The MyD116 protein is predicted to contain 657 amino acids and consists of a long amino-terminal domain, 4.5 repeats of a sequence of 38 amino acids, and a relatively short carboxyl-terminal domain. This protein was shown to be part of a subset of proteins induced during myeloid differentiation (13). GADD34, structurally closely related to MyD116, is also one of a subset of proteins induced after DNA damage or cell growth arrest (10, 25). It is thought that the function of GADD34 or MyD116 alone or in association with GADD45 is to suppress cell division during DNA repair and differentiation of lesions to preclude stress responses which could result in cell death (18, 25). The precise function of the MyD116 and of GADD34 genes is not known.

In an earlier report (8) from this laboratory, it was shown that the carboxyl terminus of $\gamma_1$34.5 (i.e., the domain homologous to the carboxyl terminus of MyD116 and GADD34) is necessary to preclude the premature shutoff of protein synthesis. In the studies reported in this article, we inserted into the $\gamma_1$34.5 deletion mutant either a $\gamma_1$34.5 gene from which the carboxyl terminus had been deleted or a chimeric gene consisting of the amino terminus and the 10 reiterations of the

* Corresponding author. Mailing address: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 E. 58th St., Chicago IL 60637. Phone: (312) 702-1898. Fax: (312) 702-1631.

AlaThrPro triplet fused to the carboxyl terminus of the MyD116 gene. We report that the carboxyl terminus of the MyD116 protein substitutes for the carboxyl terminus of the $\gamma_1$34.5 protein in precluding premature shutoff of protein synthesis in both human neuroblastoma cells and in HFFs.

MATERIALS AND METHODS

Cells. The Vero and human neuroblastoma SK-N-SH cell lines used were obtained from the American Type Culture Collection. The human 143TK mutant cells were obtained from Carlo Croce. The HFF cell strain used was obtained from George Kemble (Aviron, Inc., Burlingame, Calif.). The media for propagation of cells consisted of Dulbecco's modified Eagle's medium supplemented with 10% (SK-N-SH cells) or 5% (Vero cells) fetal bovine serum and 100 μg of bromodeoxyuridine per ml (143TK mutant cells).

Viruses. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (9). The construction of recombinant viruses R3617, R3616, and HSV-1(F)Δ305 was reported elsewhere (3, 16). As illustrated in Fig. 1, HSV-1(F)Δ305 lacks the 501-bp *Bgl*II-*Sac*I fragment from the domain of the thymidine kinase (*tk*) gene contained in the *Bam*HI Q fragment (16). R3617, derived from HSV-1(F)Δ305, lacks the 501-bp fragment from the domain of the *tk* gene and a 1-kb stretch from the coding sequences of both copies of the $\gamma_1$34.5 gene. The sequences deleted from the *tk* gene in R3617 were restored in the recombinant R3616 (3). Recombinants R8300 and R8301 were constructed by cotransfection of the intact viral DNA of R3616 with either plasmid pRB4871 or pRB4872 on rabbit skin cells originally obtained from J. McClaren. *tk* mutant recombinants were selected on 143TK mutant cells in medium consisting of mixture 199 supplemented with 100 μg of bromodeoxyuridine per ml and 2% fetal calf serum as described previously (16). Viral DNAs were extracted from cells and prepared as previously described (21). Preparation of viral stocks and titrations of infectivity were done with Vero cells.

Plasmids. The 2.3-kbp *Eco*RI fragment containing the full-length MyD116 cDNA (13) was cloned into the *Eco*RI site of pBluescript SK. Plasmid pRB4867 contained the *Bam*HI Q fragment of HSV-1(F) cloned into the *Bam*HI site of pUC9. Plasmid pRB3207 contained approximately 100 bp of the $\gamma_1$34.5 5′ noncoding sequence, the coding sequence of the $\gamma_1$34.5 gene, and the domain of the α0 gene contained in the *Bam*HI S fragment. To construct pRB4871, pRB3207 was cleaved with *Dra*III and *Bsp*EI, and then the 0.25-kbp DNA fragment containing the coding sequences of the carboxyl-terminal 58 codons of the $\gamma_1$34.5 gene was replaced with the double-stranded DNA oligomer linker (GTGCTG CAGGAATTCT and its complement, CCGGAGAATTCCTGCAGCACCAG) to yield plasmid pRB4868. A 474-bp *Pst*I-*Eco*RI fragment from murine MyD116 cDNA was inserted into the *Pst*I and *Eco*RI sites of pRB4868; the resulting plasmid was designated pRB4869. Plasmid pRB4870 was constructed by cloning an 887-bp *Hin*dIII-*Xba*I fragment containing the U_L 26.5 promoter from pRB4090 (11) into the *Hin*dIII and *Xba*I sites of pRB4869. Finally, a 2.7-kbp *Sph*I fragment containing the U_L26.5 promoter, the coding sequences of the $\gamma_1$34.5 gene for the amino-terminal portion of 205 amino acid residues, and the coding sequences of MyD116 for the carboxyl-terminal portion of 133 amino acid residues was cloned into the *Sph*I site of the *tk* gene in plasmid pRB4867. The resulting plasmid was designated pRB4871. In this plasmid, the expression of the $\gamma_1$34.5-MyD116 chimeric gene was driven by the U_L26.5 promoter.

To construct plasmid pRB4872, pRB4871 was cleaved with *Dra*III and *Stu*I to remove a 0.8-kbp DNA fragment containing the coding sequences of the MyD116 gene. The oligomer (GTGTGACTAGTCTAGAGG and its complement, CCTCTAGACTAGTCACACCAG) containing stop codons was ligated into the *Dra*III and *Stu*I sites. In this plasmid, the $\gamma_1$34.5 gene driven by the U_L26.5 promoter lacks the coding sequences for the carboxyl-terminal 58 amino acids.

Antibodies. The rabbit polyclonal antibody BR4 to the 10 repeats of the AlaThrPro triplet in the HSV-1(F) $\gamma_1$34.5 protein was described elsewhere (1). Rabbit polyclonal antibody to MyD116 was prepared as follows. An *Acc*I-*Eco*RI fragment encoding the carboxyl-terminal 174 amino acids of the MyD116 cDNA was inserted into the vector pGEX3X (Pharmacia) cleaved with *Bam*HI and *Eco*RI, and the *Bam*HI site was blunted with Klenow fragment before ligation. The resulting plasmid, pRB4873, was predicted to encode glutathione *S*-transferase (GST) fused in frame with the carboxyl-terminal portion of MyD116. The junction of GST and MyD116 was sequenced to verify that the fusion was in frame. Expression of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium with *Escherichia coli* BL21 cells transformed with pRB4873, followed by affinity purification of the fusion protein from bacterial lysates on agarose beads conjugated with glutathione. The fusion protein was used for immunization of rabbits for production of polyclonal antibody.

Analyses of viral DNAs. Digestion of viral DNAs with appropriate restriction enzymes, electrophoretic separation in agarose gels, transfer to nitrocellulose sheets, hybridization with radiolabeled probes, and autoradiography were performed as previously described (1, 3, 4, 12, 20).

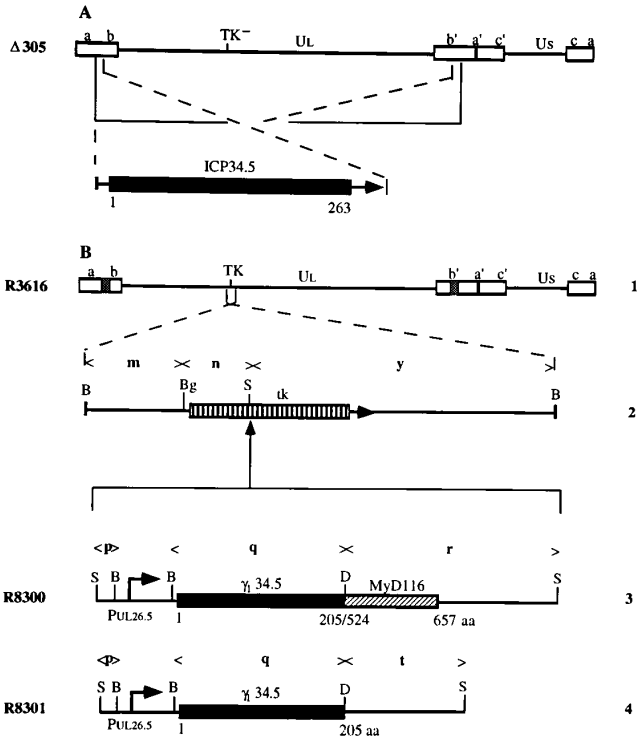

FIG. 1. (A) Schematic representation of the genome structure and sequence arrangements of HSV-1(F)Δ305 and the location of the $\gamma_1$34.5 gene. The two covalently linked components of HSV-1 DNA, L and S, each consist of unique sequences, U_L and U_S, respectively, flanked by inverted repeats (19, 22). The reiterated sequences flanking U_L, designated as *ab* and *b′a′*, are each 9 kbp in size, whereas the repeats flanking U_S, designated *a′c′* and *ca*, are 6.3 kbp in size (22). TK^- identifies the position of thymidine kinase (*tk*) gene and of the *Bgl*II-*Sac*I fragment deleted from HSV-1(F)Δ305 (16). The location of the $\gamma_1$34.5 gene is shown in the expanded portions of the inverted repeat sequences *b* and *b′*. The solid bar and the arrow indicate the coding region and the direction of transcription of $\gamma_1$34.5. Since the *b* sequence is repeated in an inverted orientation, there are two copies of the $\gamma_1$34.5 gene per genome. (B) Schematic representation of the sequence arrangements of the HSV-1 R3616 mutants and of the recombinant viruses derived from it. Line 1, sequence arrangement of R3616, a tk^+ virus. The recombinant R3617 is a *tk* mutant. Approximately 1,000 bp, represented as a filled box, had been deleted from both copies of the $\gamma_1$34.5 gene (3). Line 2, enlarged portion of the R3616 genome showing the *Bam*HI Q fragment of the HSV-1-containing *tk* gene. The hatched bar and arrow represent the coding region of the *tk* gene and the direction of transcription, respectively. Lines 3 and 4, sequence arrangements of the recombinant viruses R8300 and R8301, which were selected from among the progeny of transfection of intact R3616 DNA with either plasmid pRB4871 or pRB4872 as described in Materials and Methods. The solid bars indicate the amino-terminal portion of the $\gamma_1$34.5 gene encoding amino acids 1 to 205, whereas the hatched bar indicates the carboxyl terminus of the MyD116 gene encoding amino acids 524 to 657. The thin line on the left side (5′) of the $\gamma_1$34.5 gene represents the promoter sequences from U_L26.5 of HSV-1(F). The arrow indicates the site of transcription initiation and the direction of transcription. The thin line on the right side of MyD116 or the $\gamma_1$34.5 gene indicates the DNA sequences downstream of the $\gamma_1$34.5 coding region of the *Bam*HI S fragment. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; S, *Sph*I; D, *Dra*III. Abbreviations: m, 0.78-kbp *Bam*HI-*Bgl*II fragment; n, 0.44-kbp *Bgl*II-*Sph*I fragment; y, 2.37-kbp *Sph*I-*Bam*HI fragment; p, 0.15-kbp *Sph*I-*Bam*HI fragment; q, 0.71-kbp *Bam*HI-*Dra*III fragment; r, 1.0-kbp *Dra*III-*Sph*I fragment; t, 0.343-kbp *Dra*III-*Sph*I fragment.

Separation of nuclear and cytoplasmic fractions. Infected cells were harvested, rinsed with phosphate-buffered saline, and lysed in hypotonic buffer (2) consisting of 1.6 mM $MgCl_2$, 6 mM KCl, 10 mM Tris (pH 8.0), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 0.5% Nonidet P-40 and kept on ice for 5 min. After a brief vortexing, the nuclei were pelleted by centrifugation on a tabletop centrifuge for 10 min and rinsed with phosphate-buffered saline. The cytoplasmic and nuclear fractions were solubilized in disruption buffer (50 mM Tris-HCl [pH 7.2], 2% sodium dodecyl sulfate [SDS], 2.75% sucrose, 5% β-mercaptoethanol) and subjected to electrophoresis in denaturing gels as previously described (1, 3, 4, 12).

Immunoblots. Virus-infected cells were rinsed, harvested, solubilized, denatured by boiling, and subjected to electrophoresis in SDS–12%-polyacrylamide gels, electrically transferred to nitrocellulose, blocked with 5% nonfat milk, reacted with either the GST-MyD116 antiserum or $\gamma_1$34.5 antiserum, rinsed, and reacted with anti-rabbit immunoglobulin coupled to alkaline phosphatase as recommended by the manufacturer (Bio-Rad).

[^{35}S]methionine labeling of infected cells. Replicate cultures were either infected or mock infected and incubated at 37°C. At 14 h after infection, the cells were overlaid with 1 ml of medium lacking methionine but supplemented with 50 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) for 1 h. The cells were then harvested, solubilized, subjected to electrophoresis in denaturing polyacrylamide gels, and transferred to a nitrocellulose sheet and subjected to autoradiography as previously described (7).

RESULTS

Construction of recombinant viruses. The purpose of this series of experiments was to determine whether the carboxyl-terminal domain of MyD116 could substitute for the homologous domain of $\gamma_1$34.5 in the context of HSV-1-infected human cells. As described in Materials and Methods and shown schematically in Fig. 1B, in the chimeric gene, the codons encoding amino acids 206 to 263 of the $\gamma_1$34.5 [$\gamma_1$34.5(ΔC)] gene were replaced in frame with the MyD116 sequence encoding amino acids 524 to 657. In recombinant virus R8300, the coding sequence of the chimeric $\gamma_1$34.5(ΔC)-MyD116 gene linked to the promoter of the U_L26.5 gene was inserted into the *Sph*I site of *Bam*HI Q. The parent virus used in these constructions was R3616, which lacks approximately 1 kbp from the coding sequences of the $\gamma_1$34.5 gene. To construct the recombinant, rabbit skin cells were cotransfected with intact R3616 viral DNA and plasmid pRB4871 containing the *tk* gene, into which was inserted the coding sequence of the chimeric gene $\gamma_1$34.5-MyD116 driven by the U_L26.5 promoter. The progeny of the transfection was plated on 143TK mutant cells overlaid with medium containing bromodeoxyuridine as described in Materials and Methods. The *tk* mutant progeny viruses were plaque purified, and their DNAs were analyzed for the presence of the chimeric gene by hybridization to electrophoretically separated digests of viral DNA transferred to a nitrocellulose sheet with ^{32}P-labeled *Bam*HI Q DNA. The hybridization patterns of the *Bam*HI-*Dra*III digest (Fig. 2, lane 3) of the DNA of the isolate designated R8300 revealed the presence of fragment *mnp* (1.37 kbp), derived from *Bam*HI Q and the U_L26.5 promoter; and fragment *yr* (3.4 kbp), derived from the carboxyl terminus of MyD116 and the remaining portion of *Bam*HI Q as predicted from Fig. 1, lines 2 and 3. The *Bam*HI-*Bgl*II digest (Fig. 2, lane 4) revealed the presence of the predicted fragment *yqr* (4.2 kbp), consisting of a portion of *Bam*HI Q, MyD116, and $\gamma_1$34.5; and the fragments *m* (0.8 kbp) and *n* (0.44 kbp), derived from *Bam*HI Q. As expected, the parent virus with an intact *Bam*HI Q fragment yielded a single 3.6-kbp band on digestion with *Bam*HI (Fig. 2, lane 6) and the predicted two 2.8- and 0.8-kbp bands on digestion with *Bam*HI and *Bgl*II (Fig. 2, lane 5).

To construct the recombinant virus R8301 (Fig. 1, line 4), rabbit skin cells were cotransfected with intact R3616 viral DNA and the plasmid pRB4872. This plasmid contains the amino-terminal 205 codons of the $\gamma_1$34.5 [$\gamma_1$34.5(ΔC)] gene linked to the U_L26.5 promoter. The recombinant virus (R8301), obtained and analyzed as described above, was found to be as predicted in Fig. 1, lines 2 and 4. Specifically, hybridization of the electrophoretically separated *Bam*HI-*Dra*III digest with the ^{32}P-labeled *Bam*HI Q fragment revealed (Fig. 2, lane 1) fragment *yt* (2.7 kbp), derived in part from *Bam*HI Q and $\gamma_1$34.5 (*Bam*HI S), and fragment *mnp* (1.37 kbp), derived from *Bam*HI Q and the U_L26.5 promoter. Similarly, the *Bam*HI-*Bgl*II digest (Fig. 2, lane 2) revealed three bands: i.e., fragment *yqt* (3.43 kbp), consisting of part of *Bam*HI Q and $\gamma_1$34.5 (*Bam*HI S); and fragments *m* and *n* described above.

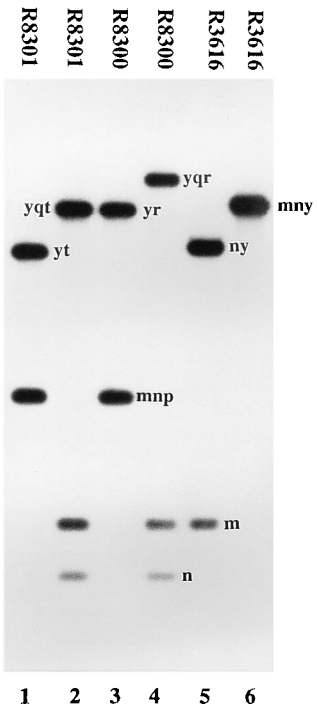

FIG. 2. Autoradiographic images of electrophoretically separated restriction endonuclease digests of the recombinant virus DNAs hybridized to the labeled *Bam*HI Q fragment. Viral DNAs were digested, electrophoretically separated on 0.8% agarose gels, transferred to a nitrocellulose sheet, hybridized to the ^{32}P-labeled *Bam*HI Q DNA fragment, and exposed to Kodak X-ray film. Lanes 1 and 3, *Bam*HI-*Dra*III digest of DNAs of recombinant viruses R8301 and R8300, respectively; lanes 2, 4, and 5, *Bam*HI-*Bgl*II digest of DNAs from recombinant viruses R8301, R8300, and R3616, respectively; lane 6, *Bam*HI digest of R3616 DNA. The lowercase letters identify the DNA sequences expected to be present in each band as predicted in Fig. 1. Recombinant virus R8301 contained codons 1 through 205 of the $\gamma_1$34.5 gene linked to the U_L26.5 promoter. The *Bam*HI-*Dra*III digest of R8301 yielded (lane 1) a 2.7-kbp band (yt) from a portion of the *Bam*HI Q fragment (y) and a portion of the *Bam*HI S fragment (t) and a 1.37-kbp band (mnp) containing a portion of the *Bam*HI Q fragment (m plus n) and a portion of the U_L26.5 promoter sequence (p). The *Bam*HI-*Bgl*II digest of R8301 yielded (lane 2) a 3.4-kb band (yqt) consisting of a portion of the *Bam*HI Q fragment (y) and a portion of the *Bam*HI S fragment (q plus t), a 0.7-kbp band (m), and a 0.4-kbp (n) band, both derived from *Bam*HI Q. Recombinant virus R8300 contained the chimeric $\gamma_1$34.5-MyD116 gene consisting of codons 1 through 205 of the $\gamma_1$34.5 gene fused in frame to codons 524 to 657 of the MyD116 gene and driven by the U_L26.5 promoter. This gene was inserted into the *Sph*I site of the *tk* gene. The *Bam*HI-*Dra*III digest of R8300 yielded (lane 3) a 3.4-kbp band (yr) consisting of a portion of the *Bam*HI Q fragment (y), a portion of the *Bam*HI S fragment and the MyD116 coding sequences (r), and a 1.37-kbp band (mnp) described above. The *Bam*HI-*Bgl*II digest of R8300 yielded (lane 4) a 4.2-kbp band (yqr) consisting of a portion of the *Bam*HI Q fragment (y) and a portion of the *Bam*HI S fragment (q plus r), a 0.8-kbp band (m), and a 0.44-kbp band (n), both derived from the *Bam*HI Q fragment. The R3616 DNA digest served as a control for the purity of the recombinant viruses constructed from it and as a marker. The *Bam*HI-*Bgl*II digest of R3616 (lane 5) yielded a 2.8-kbp band (ny) and a 0.8-kbp band (m), whereas the *Bam*HI digest (lane 6) yielded an intact *Bam*HI Q fragment (mny).

Reactivity of the $\gamma_1$34.5 proteins specified by the recombinant viruses in immunoblots. In this series of experiments, lysates of Vero cells mock infected or infected with R8301, R8300, HSV-1(F)Δ305, or R3617 were electrophoretically separated in denaturing gels, electrically transferred to nitrocellulose sheets, and reacted with either the rabbit polyclonal antibody BR4 (1) directed against the 10 repeats (Fig. 3B) or the polyclonal antibody to the carboxyl terminus of MyD116, prepared as described in Materials and Methods. The viruses

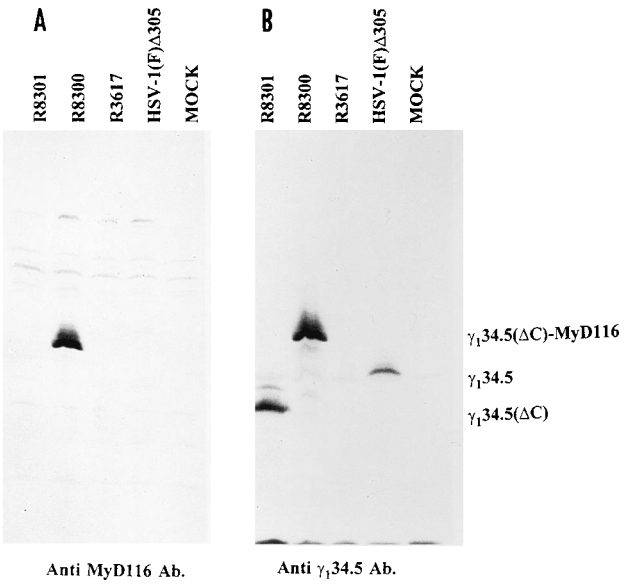

FIG. 3. Immunoblots of electrically separated lysates of infected Vero cells probed with either a GST-MyD116 antiserum or BR4 antiserum (1). Vero cells were infected with 5.0 PFU of the indicated viruses. At 15 h after infection, the cells were harvested, solubilized, subjected to electrophoresis in a denaturing polyacrylamide gel, electrically transferred to nitrocellulose sheets, and reacted with either the rabbit polyclonal antibody to the GST-MyD116 fusion protein (A) or with the rabbit R4 serum against a synthetic peptide consisting of the AlaThrPro trimer repeated 10 times (B). The positions of the bands containing the chimeric protein $\gamma_1$34.5(ΔC)-MyD116, the truncated protein $\gamma_1$34.5(ΔC), and the wild-type protein $\gamma_1$34.5 are shown. Ab., antibody.

used in this study were all *tk* mutants inasmuch as the chimeric $\gamma_1$34.5(ΔC)-MyD116 gene and the truncated $\gamma_1$34.5(ΔC) gene were inserted into the viral *tk* gene, disrupting it. Since all $\gamma_1$34.5 derivatives contained the AlaThrPro repeats, the BR4 antibody reacted with a relatively abundant band containing the $\gamma_1$34.5(ΔC)-MyD116 protein with an apparent M_r of 52,000 in the lane containing the lysates of R8300, the $\gamma_1$34.5 protein in the lane containing the lysate of cells infected with HSV-1(F)Δ305, and a fast-migrating protein with an apparent M_r of 36,000 expected to consist of the amino-terminal domain of $\gamma_1$34.5 [$\gamma_1$34.5(ΔC)] in lysates of cells infected with R8301. The serum did not react with the lysates of cells mock infected or infected with the R3617 virus. The results shown in Fig. 3A indicate that the serum against the carboxyl terminus of MyD116 reacted only with a protein with an apparent M_r of 52,000 in lysates of cells infected with R8300.

We conclude that the recombinant viruses R8300 and R8301 contain the appropriate $\gamma_1$34.5 gene derivatives described above. It is noteworthy that identical amounts of proteins were loaded in each lane. The results show that the chimeric $\gamma_1$34.5(ΔC)-MyD116 protein and the truncated $\gamma_1$34.5 protein were either overexpressed or were more stable than the authentic $\gamma_1$34.5 protein. A simple explanation of the results is that the $\gamma_1$34.5 protein was made from mRNA transcribed from its natural promoter, whereas the chimeric and truncated $\gamma_1$34.5 proteins were made from mRNA transcribed from the U_L26.5 promoter. In this and other studies, the U_L26.5 promoter appeared to be a much stronger promoter than that of the natural $\gamma_1$34.5 gene.

The effect of $\gamma_1$34.5 and its derivatives contained in parent and recombinant viruses on protein synthesis in Vero cells, human neuroblastoma SK-N-SH cells, and HFFs. In this series of experiments, replicate Vero, human neuroblastoma SK-N-SH, or HFF cultures were infected with either 10 (Fig. 4A, C, and E) or 50 (Fig. 4B, D, and F) PFU of HSV-1(F)Δ305, R3617, R8301, or R8300 per cell. At 14 h after infection, the cells were labeled for 1 h with [^{35}S]methionine and then harvested, solubilized in disruption buffer, and subjected to electrophoresis in denaturing polyacrylamide gels. The following results were obtained. (i) Labeled proteins were detected in Vero cells infected with all of the viruses tested (Fig. 4A and B). (ii) Premature shutoff of protein synthesis was observed in

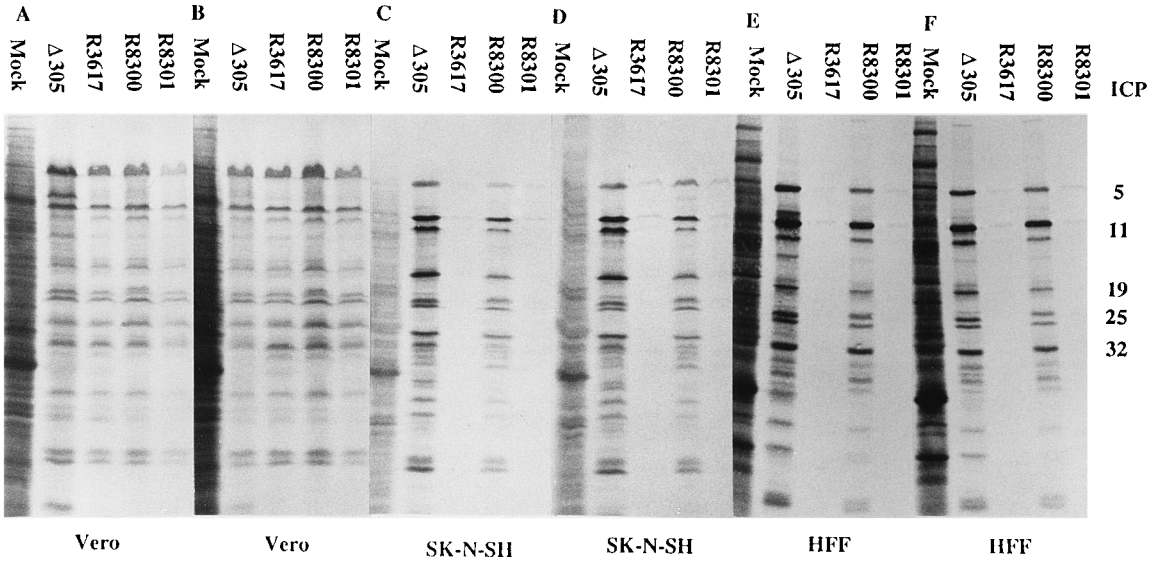

FIG. 4. Autoradiographic images of electrophoretically separated, [^{35}S]methionine-labeled proteins from lysates of cells infected with HSV-1(F)Δ305 containing a wild-type $\gamma_1$34.5 gene at its natural location or viruses containing mutated $\gamma_1$34.5 genes. All viruses were *tk* mutants because the mutated $\gamma_1$34.5 genes were inserted into the *tk* gene. The cells were infected with either 10 (A, C, and E) or 50 (B, D, and F) PFU per cell and labeled at 14 h postinfection for 1 h. The procedures for infection, [^{35}S]methionine labeling, preparation of cell extracts, separation of proteins by electrophoresis in denaturing polyacrylamide gels cross-linked with *N,N′*-diallytartardiamide, transfer of polypeptides to nitrocellulose sheets, and autoradiography were done as described previously (7).

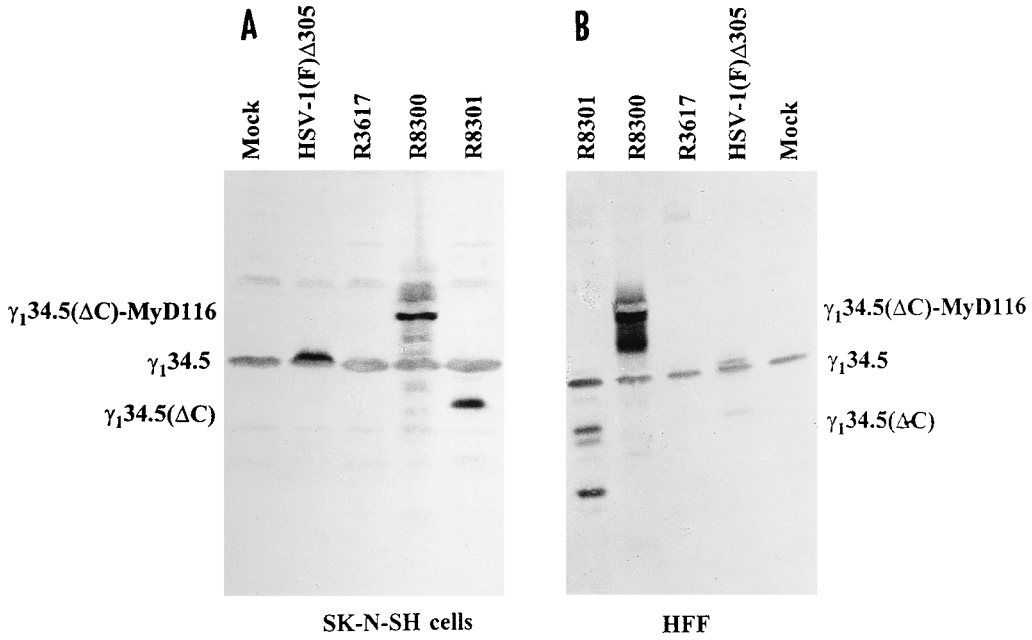

FIG. 5. Immunoblots of electrophoretically separated lysates of infected SK-N-SH cells or HFFs reacted with BR4 antiserum (1). Replicate cultures of human neuroblastoma SK-N-SH cells and HFFs were either mock infected or were infected with 10 PFU of the indicated viruses per cell. The cells were harvested 15 h after mock infection or infection, solubilized, subjected to electrophoresis on 12% denaturing polyacrylamide gels, transferred electrically to nitrocellulose sheets, and reacted with R4 antiserum. The position of bands containing the wild-type or mutated $\gamma_1$34.5 proteins are shown.

SK-N-SH cells and HFFs infected with R3617 or R8301 but not in cells infected with HSV-1(F)Δ305 or R8300 (Fig. 4C, D, E, and F), indicating that the carboxyl terminus of MyD116 protein substituted for the corresponding homologous domain of the $\gamma_1$34.5 protein.

To determine whether the chimeric $\gamma_1$34.5(ΔC)-MyD116 protein and the truncated $\gamma_1$34.5(ΔC) protein were expressed in the SK-N-SH cells and in the HFFs infected with the appropriate viruses, infected cell lysates were solubilized, electrophoretically separated in denaturing gels, transferred electrically to a nitrocellulose sheet, and reacted with the BR4 antiserum. The results shown in Fig. 5 indicate the presence of the predicted bands in both SK-N-SH cells and HFFs. We should note that in the experiment shown, the BR4 antibody reacted with a host band migrating slightly faster than the $\gamma_1$34.5 protein. The abundance of the $\gamma_1$34.5 protein detected by the antibody in SK-N-SH cells infected with HSV-1(F)Δ305 was greater than that present in HFFs infected with the same virus. The antibody reacted with bands corresponding to full-length and truncated $\gamma_1$34.5(ΔC) protein in HFFs and with a single band of the predicted full length in lysates of SK-N-SH cells. The chimeric $\gamma_1$34.5(ΔC)-MyD116 protein formed multiple bands, including one of the expected size in both cell lines infected with R8300 recombinant virus.

Compartmentalization of the $\gamma_1$34.5 protein and its derivatives in infected cells. In earlier studies (1), this laboratory reported that $\gamma_1$34.5 protein was present in both the nuclear and cytoplasmic fractions of infected cells. The purpose of these experiments was to determine whether the compartmentalization of the $\gamma_1$34.5(ΔC)-MyD116 and $\gamma_1$34.5(ΔC) proteins differed from that of the parent $\gamma_1$34.5 protein. In this series of experiments, the nuclear and cytoplasmic fractions were solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with the BR4 antibody. The results of a representative experiment are shown in Fig. 6. All three proteins, $\gamma_1$34.5, $\gamma_1$34.5(ΔC)-MyD116, and $\gamma_1$34.5(ΔC), accumulated in both the nucleus and cytoplasm, although the relative amounts varied from one experiment to another. In this and other experiments, we noted more extensive degradation of cytoplasmic forms of the chimeric protein $\gamma_1$34.5(ΔC)-MyD116 than in the total cell extracts shown in Fig. 5.

DISCUSSION

The salient features of the results reported here and their significance are as follows. (i) In order to detect the substitution of the carboxyl terminus of $\gamma_1$34.5 with that of MyD116, we had to make an antibody specific for the swapped domain of MyD116. We report that the antibody reacts with the chi-

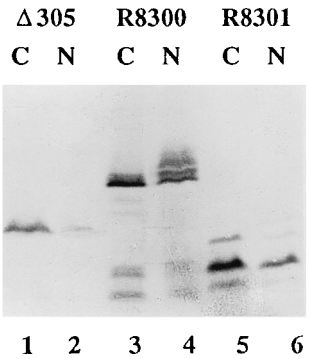

FIG. 6. Immunoblots of electrophoretically separated nuclear and cytoplasmic fractions of HEp-2 cells infected with 5 PFU of HSV-1(F)Δ305, R8300, or R8301 per cell. The cells were infected with the indicated viruses and harvested 15 h after infection. Cytoplasmic (C) and nuclear (N) fractions were prepared as described in Materials and Methods, subjected to electrophoresis on 12% polyacrylamide denaturing gels, transferred electrically to nitrocellulose sheets, and reacted with the BR4 antiserum. Lanes 1, 3, and 5, polypeptides from cytoplasmic fractions; lanes 2, 4, and 6, polypeptides from nuclear fractions. The positions of bands representing the wild-type or mutant $\gamma_1$34.5 proteins are shown.

meric protein but not with intact $\gamma_1$34.5 protein or with cellular proteins in Vero cells, HEp-2 cells (not shown), SK-N-SH cells, or HFFs of a size predicted for the MyD116/GADD34 series of proteins. The failure to detect such proteins suggests that they were either absent, present at a very low level of abundance, or did not react with the antibody to the murine protein.

(ii) In earlier studies (7), the $\gamma_1$34.5 protein was truncated by the insertion of a sequence containing stop codons in all six reading frames 11 codons after the 10 repeats of the AlaThr-Pro triplet. Whereas protein synthesis was shut off in both neuroblastoma cells and in HFFs infected with the 1-kbp deletion mutant R3616, protein synthesis was shut off in neuroblastoma cells but not in HFFs infected with viruses specifying a $\gamma_1$34.5 protein truncated by the insertion (4). These studies were interpreted to indicate that the stop codon is suppressed and that the small amount of full-length protein made under those conditions was sufficient to preclude shutoff in HFFs but not in neuroblastoma cells. In this study, we show that the $\gamma_1$34.5 protein from which the carboxyl terminus was deleted is unable to preclude the shutoff of protein synthesis in either neuroblastoma cells or HFFs.

(iii) The key observation is that the carboxyl terminus of the murine MyD116 protein functionally substituted for the carboxyl terminus of the $\gamma_1$34.5 protein in precluding the shutoff of protein synthesis. This observation has several implications. First, MyD116 is a much larger protein and most likely has more than one function. The successful swapping of domains suggests that the carboxyl terminus of MyD116 has a similar function under the conditions in which it is expressed. It is conceivable that the conditions which cause the expression of MyD116/GADD34 genes result in the shutoff of protein synthesis and that one of the functions of these proteins is to preclude this response. It would appear that the HSV "borrowed" a portion of the MyD116/GADD34 genes to prevent a similar cell response—but why not the whole gene? Part of the answer may be that the amino-terminal domains of the MyD116/GADD34 proteins perform a different function than the amino-terminal domain of $\gamma_1$34.5 protein. Another plausible possibility is that the $\gamma_1$34.5 amino-terminal domain brings together in one complex the proteins interactive with the carboxyl terminus and other viral proteins needed to preclude the shutoff of protein synthesis. This hypothesis remains to be tested. We should note that in preliminary experiments, we failed to overcome the premature shutoff of protein synthesis by insertion into the viral genome of the carboxyl terminus of MyD116 alone (data not shown). These results, however, were not readily interpretable, since the amounts of the MyD116 carboxyl-terminus peptide were very small and the protein may also have been very unstable.

The second observation of interest stems from the observation noted in this and other reports (4, 8) that $\gamma_1$34.5 mutant virus cannot be differentiated from wild-type virus in Vero cells. The effects of $\gamma_1$34.5 mutant viruses on cells of nonhuman derivation are generally less drastic than those seen in human cell lines. One possibility, therefore, was that the carboxyl terminus of the murine MyD116 protein would not functionally substitute for the corresponding domain of $\gamma_1$34.5 protein because of potential species differences. The observation that the carboxyl terminus of the murine MyD116 protein substituted for the corresponding terminus of $\gamma_1$34.5 suggests at least two possibilities. (i) The apparent species specificity does not reside within the carboxyl terminus of MyD116. (ii) The species specificity resides in the virus function which triggers the premature shutoff of protein synthesis and not in the function which blocks the response.

(iv) Attempts to determine the cellular compartmentalization of the $\gamma_1$34.5 protein have yielded mixed results. Fractionation of cells with nonionic detergents showed the presence of $\gamma_1$34.5 protein in both nuclear and cytoplasmic fractions. The distribution of the protein varied from one experiment to another. By immunofluorescence, the protein appeared to be primarily cytoplasmic, but we must conclude that it is available in both compartments. It is of interest that in most experiments, the chimeric $\gamma_1$34.5(ΔC)-MyD116 protein tended to be more heavily represented in the nucleus, whereas the carboxyl terminus of MyD116 expressed by itself was primarily nuclear, although the amounts were too small to exclude the possibility that some of the protein was also localized in the cytoplasm (data not shown). These observations do not exclude the possibility that one of the functions of the amino-terminal domain is to deliver the carboxyl terminus to the appropriate site for its function. That site remains to be determined.

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