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The UL45 Gene Product Is Required for Herpes Simplex Virus Type 1 Glycoprotein B-Induced Fusion

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Herpes simplex virus type 1 (HSV-1) syncytial (*syn*) mutants cause formation of giant polykaryocytes and have been utilized to identify genes promoting or suppressing cell fusion. We previously described an HSV-1 recombinant, F1 (J. L. Goodman, M. L. Cook, F. Sederati, K. Izumi, and J. G. Stevens, J. Virol. 63:1153–1161, 1989), which has unique virulence properties and a *syn* mutation in the carboxy terminus of glycoprotein B (gB). We attempted to replace this single-base-pair *syn* mutation through cotransfection with a 379-bp PCR-generated fragment of wild-type gB. The nonsyncytial viruses isolated were shown by DNA sequencing not to have acquired the expected wild-type gB sequence. Instead, they had lost their cell-cell fusion properties because of alterations mapping to the UL45 gene. The mutant UL45 gene in one nonsyncytial derivative of F1, A4B, was found to have a deletion of a C at UL45 nucleotide 230, resulting in a predicted frame shift and termination at 92 rather than 172 amino acids. Northern (RNA) analysis showed that the mutant UL45 gene was normally transcribed. However, Western immunoblotting showed no detectable UL45 gene product from A4B or from another similarly isolated nonsyncytial F1 derivative, A61B, while another such virus, 1ACSS, expressed reduced amounts of UL45. When A4B was cotransfected with the wild-type UL45 gene, restoration of UL45 expression correlated with restoration of syncytium formation. Conversely, cloned DNA fragments containing the mutant A4B UL45 gene transferred the loss of cell-cell fusion to other gB *syn* mutants, rendering them UL45 negative and nonsyncytial. We conclude that normal UL45 expression is required to allow cell fusion induced by gB *syn* mutants and that the nonessential UL45 protein may play an important role as a mediator of fusion events during HSV-1 infection.

One of the critical steps in the infection of cells with herpes simplex virus type 1 (HSV-1) is fusion between the viral envelope and the cell membrane, allowing penetration of the viral nucleocapsid into the cell. The mechanisms by which HSV-1 promotes membrane fusion are not well understood because of complex interactions of multiple viral and cellular proteins. Although HSV-1 lesions in vivo may show evidence of cell-cell fusion and polykaryocyte formation (43), clinical isolates of HSV-1 normally do not maximally fuse cells in tissue culture. However, mutant viruses which have a greatly enhanced ability to fuse cells in vitro (syncytial or *syn* mutants) have been isolated and used to help identify the viral proteins involved in fusion in vivo. *syn* mutations have been mapped to at least the following four HSV-1 genes: UL27, which encodes glycoprotein B (gB) (4, 40); UL53, which encodes glycoprotein K (gK) (3, 21, 37); UL20, which encodes a membrane protein (2, 29); and UL24 (24, 41). An additional *syn* locus has been mapped to the HSV-1 long repeat region (39).

The majority of mutations thus far characterized permit syncytium formation to occur, suggesting that expression of the respective wild-type proteins functions to modulate cell fusion (43). The normal regulatory roles of two of these proteins, gB and gK, are further supported by the fact that fusion induced by mutant gB or gK is inhibited by cells expressing the corresponding wild-type proteins (22). In contrast, there are few reports of HSV-1 proteins that function as mediators of cell fusion, that is, cases in which the wild-type protein is required to allow expression of the syncytial phenotype to occur (43). gB (also a modulator) and glycoprotein D are two HSV-1 proteins which have been suggested to serve as mediators of cell fusion (5, 28, 36), and by inference, these proteins could be critical to the virion-cell fusion process in vivo. Indeed, gB and glycoprotein D are both required for viral penetration and infectivity (5, 28) and gB is important in the spread of virus from one cell or tissue type to another (8, 14, 35). In addition, variations in these proteins among clinical and laboratory isolates can profoundly affect viral pathogenicity and invasiveness (10, 14, 23, 48).

In studies of HSV invasiveness which utilized inoculation of the chorioallantoic membrane (CAM) of fertilized chicken eggs (16, 34, 38), we found that HSV-2 strains form large pocks, invade the CAM mesoderm, and kill embryos while HSV-1 strains normally form smaller pocks and do not kill (15). We also reported that unlike other HSV-1 strains, syncytial strain ANG is virulent for chicken embryos and behaves like HSV-2 upon CAM inoculation (13). We found that a syncytial recombinant virus, F1, isolated from the progeny of the cotransfection of the cloned ANG *Eco*RI F fragment into wild-type virus 17*syn*$^+$ had a millionfold increase in invasiveness in the CAM model and a thousandfold increase in neuroinvasiveness following footpad inoculation of mice (13). To identify the specific gene or genes responsible for the invasiveness phenotype of F1, we are studying phenotypes shared by F1 and ANG but different from those of 17*syn*$^+$ as possible contributors to invasiveness both by selectively deleting them from F1 and transferring them, stepwise, to 17*syn*$^+$.

We first sought to determine whether the syncytial phenotype shared by ANG and F1 contributes to CAM invasiveness. F1 and ANG both carry a single-base-pair *syn* mutation in the

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TABLE 1. Viruses isolated in this study

Virus	Phenotype[a]	Parent virus	Donor fragment	Comment(s)
A4B	WT	F1	gB PCR fragment from 17*syn*$^+$	Retained gB *syn* mutation, acquired frame shift mutation in UL45, no detectable UL45 product
A61B	WT	F1	gB PCR fragment from 17*syn*$^+$	Retained gB *syn* mutation, no detectable UL45 product
1ACSS	WT	F1	gB PCR fragment from 17*syn*$^+$	Retained gB *syn* mutation, reduced UL45 product
C4A	Syn	17*syn*$^+$	gB PCR fragment from F1	Acquired gB *syn* mutation
T1	Syn	A4B	*Hin*dIII-*Sph*I UL45 fragment[b] from 17*syn*$^+$	Wild-type levels of UL45 product
T9	Syn	A4B	*Hin*dIII-*Sph*I UL45 fragment[b] from F1	Wild-type levels of UL45 product

[a] WT, normal nonsyncytial HSV-1 plaques; Syn, syncytial plaques.
[b] 797-bp *Hin*dIII-*Sph*I fragments subcloned from the respective HSV-1 *Eco*RI I fragments (see Fig. 3).

3′ end of the gB gene that alters a single amino acid in the C-terminal cytoplasmic domain of the protein (12, 14, 19). This gB mutation, by itself, does not cause the CAM invasiveness phenotype, since we have isolated several recombinants containing this mutation which are not CAM invasive (20). However, this same mutation, when introduced into 17*syn*$^+$, dramatically alters disease pathogenesis and viral spread in mice (10, 14). We therefore set out to evaluate whether this mutation at least contributes to the CAM invasiveness of F1 by rescuing it through cotransfection with sequences from the wild-type gB gene differing only with respect to the syncytial mutation.

As reported here, we isolated nonsyncytial derivatives of F1 from these initial experiments, but unexpectedly, all still carried the gB *syn* mutation present in F1. We found that this phenotypic suppression of the gB *syn* mutation was due to a frameshift mutation mapping to the HSV-1 UL45 gene which has recently been characterized as a membrane protein by Visalli and Brandt (44). We report the nucleotide sequence change involved and the ability of the mutated UL45 gene to be transferred to and cause loss of fusion induced by other HSV-1 strains with the same gB *syn* mutation. Conversely, restoration of wild-type UL45 gene expression to the UL45 mutants allowed full expression of the syncytial phenotype. From these studies, we conclude that the UL45 gene product is critical for full expression of the cell-cell fusion induced by HSV-1 gB syncytial mutants. We suggest that the UL45 gene product, the function(s) of which has not previously been described, is a mediator of cell fusion and may play an important role in cell-cell fusion processes.

MATERIALS AND METHODS

Cells and virus. Virus stocks were produced in rabbit skin (RS) cells maintained in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum as previously described (13, 15). The stock virus strains used included reference strains 17*syn*$^+$ and ANG (25). The origin of 17*syn*$^+$-ANG recombinant virus F1 has been previously described (13). Other viruses isolated during this study are described in Results and listed in Tables 1 and 2. Virus titers were determined by plating serial 10-fold dilutions of virus onto cells under medium supplemented with 0.3% human serum immune globulin. Plaque purifications were performed by three successive limiting dilutions onto RS cell monolayers in 96-well plates.

DNA isolation, molecular cloning, restriction, and hybridization analysis. Viral DNA was isolated either on sodium iodide gradients (46) or by a rapid miniprep method (10). Plasmid DNAs were isolated either by cesium chloride density gradients as previously described (18) or by using Qiagen columns (Qiagen, Chatsworth, Calif.). Single-stranded M13 templates were purified on Qiagen columns. Restriction and modification enzymes were purchased from New England Biolabs (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or Gibco/BRL (Gaithersburg, Md.) and used in accordance with manufacturer specifications. Viral restriction fragments were either shotgun cloned or first purified from agarose gels by using the Qiaex glass matrix system (Qiagen) and then ligated into plasmids pUC18 and pUC19 or into M13mp18 and mp19 by standard methods (30, 32). Plasmid constructs were transformed into *Escherichia coli* DH5α, and M13 clones were transformed into strain JM101 (30). PCR fragments were cloned into the pCRII vector by using the TA cloning kit (Invitrogen, San Diego, Calif.). These fragments were then excised from the plasmid vector and directionally cloned into mp18 and mp19 for sequencing. For DNA hybridization, restriction fragments separated on agarose gels were transferred to membranes (Magnagraph; Micron Separations, Westboro, Mass.) by capillary action. DNA probes were labelled by random priming with digoxigenin-11-dUTP by using the Genius labelling kit (Boehringer Mannheim Biochemicals). Hybridization (high stringency) and detection of the labelled DNA were performed with the Genius detection kit (Boehringer Mannheim Biochemicals).

PCR. HSV-1 DNA was subjected to PCR (33) with synthetic oligonucleotide 918 (CGTGG GTCTG TTGGT CCT) as the forward primer and synthetic oligonucleotide 1265 (TCACA GGTCG TCCTC GTC) as the reverse primer (Fig. 1). The reaction was performed in a buffer consisting of KCl (50 mM), Tris-HCl (pH 8.3) (10 mM), $MgCl_2$ (2.5 mM), and gelatin (0.01%, wt/vol), to which the four deoxynucleotide triphos-

TABLE 2. Suppression of syncytium formation in other gB *syn* mutants

Unit length viral DNA	A4B *Eco*RI I donor fragment	Approximate rescue frequency[a]	Representative nonsyncytial virus(es)
F1	Yes	4/64	F1-1A, F1-1B
F1	No	0/154	None
C4A	Yes	20/106	C4A-1B, C4A-1C, C4A-2D
C4A	No	10/300	C4A-alone A
ANG	Yes	57/497	ANG-1B2, ANG-2B
ANG	No	2/524	ANG-alone A, B

[a] Dilutions of the transfections were plated for single plaques, and all resulting plaques were scored as syncytial or nonsyncytial. Results are expressed as number of nonsyncytial plaques/total number of plaques (see Materials and Methods).

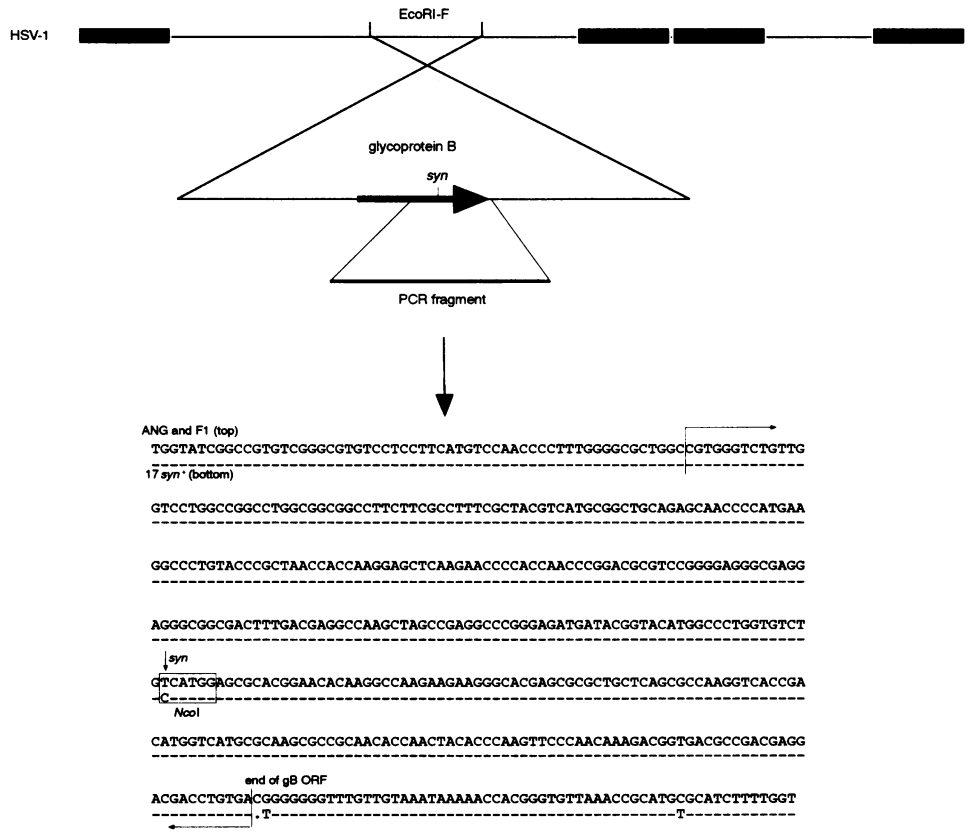

FIG. 1. Generation of PCR fragments used to transfer or rescue the *syn* mutation from HSV-1 recombinant F1. At the top is a diagrammatic depiction of the HSV-1 genome showing the position of the *Eco*RI F fragment and that of the gB gene within this fragment. The portion amplified by PCR is shown. At the bottom is the nucleotide sequence of the region of the gB gene amplified. The top line is the sequence from ANG and F1, and the bottom line is the corresponding sequence from 17*syn*$^+$. The dashes indicate identical bases, and the dot indicates a gap introduced to align the sequences. The positions of the forward, 918, and reverse, 1265, primers are shown by arrows. The nucleotide substitution responsible for the ANG and F1 *syn* mutation and the associated *Nco*I restriction site in the 17*syn*$^+$ sequence are boxed. ORF, open reading frame.

phates (25 μM each), the two primers (44 pM each), and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) were added. Target DNAs were added to a final volume of 100 μl. The PCR consisted of an initial 4-min denaturation step at 94°C, followed by 39 cycles of denaturation (94°C, 1 min), annealing (50°C, 1.5 min), and extension (72°C, 1.5 min). The 40th cycle concluded with a 6-min extension step.

DNA cotransfections, marker transfer and rescue, and screening for recombinant viruses. Marker rescues and transfers were performed by cotransfection of RS cells with unit length HSV-1 DNA and the DNA fragments of interest by the method of Graham and Van der Eb (17), as modified by Cai et al. (6). Briefly, 0.5 to 1.0 μg of HSV-1 DNA and 10 μg salmon sperm carrier DNA (Sigma, St. Louis, Mo.) were allowed to precipitate with 0.1 to 1.0 μg of linearized cloned DNA or, where mentioned, uncloned HSV-1 fragments isolated from agarose gels. This mixture was then added to 60-mm-diameter petri dishes seeded 24 h earlier with RS cells such that the cells were just confluent at the time of transfection. Following 4 h of incubation at 37°C, the cells were shocked with 15% glycerol in Tricene-buffered salts for 2 min at room temperature and washed with phosphate-buffered saline (PBS). Finally, 5 ml of growth medium was added and the cells were incubated for 2 to 4 days, until a complete cytopathic effect was evident. Transfections were screened by inoculating appropriate dilutions of the transfections onto RS cell monolayers in six-well plates and then incubating them under an overlay medium containing 1% low-melting-point agarose (Seaplaque; FMC, Rockland, Maine). Frequencies of syncytial and nonsyncytial plaques were determined by visually screening the resultant viral plaques from each transfection. The frequencies presented should be considered approximate, since the methodology does not account for possible sibling viruses. Representative plaques were transferred to individual wells of a 24-well plate with sterile toothpicks. These viruses were then grown to a 100% cytopathic effect and then plaque purified by three rounds of limiting dilution.

Nucleotide sequencing. Nucleotide sequencing was done by the dideoxy-chain termination method (42), with [α-^{35}S]dATP (New England Nuclear, Boston, Mass.) and the Sequenase Kit (U.S. Biochemical, Cleveland, Ohio) in accordance with the manufacturer-recommended protocol, except that termination reactions were performed at 50°C to facilitate sequencing of GC-rich herpesvirus DNA. All sequencing was done on single-stranded M13 templates. To verify the sequences of cloned PCR products, at least three different clones of each PCR product were sequenced. Either polyacrylamide or Long Ranger (J. T. Baker Chemical Co., Phillipsburg, N.J.) gels were used for sequencing. Sequences were analyzed by using MBIR software on a SUN microsystems computer workstation. Since sequencing was used only to detect minor differences from parent strain 17*syn*$^+$ (31), we did not submit new sequences to

the GenBank database. However, copies of the sequences in computer-readable form are available from us upon request.

RNA isolation and analysis. To prepare RNA, RS cells were infected with HSV at a multiplicity of infection of 5 to 10 PFU per cell. After a 1-h adsorption period at 37°C, medium was added to the cells. Six hours later, the infected cells were harvested and total RNA was isolated by using RNAzol B (Tel-Test, Inc., Friendswood, Tex.) in accordance with the manufacturer's protocol. Ten micrograms of denatured RNA was run in each lane on formaldehyde gels by standard methods (1) and transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) by capillary transfer. DNA probes were labelled with [^{32}P]dCTP by using random hexamers (Prime-it kit; Stratagene, La Jolla, Calif.) and hybridized to the RNA blots for 18 h at 42°C (1). Successive washes were done in 2×, 1×, and 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 1% sodium dodecyl sulfate at 55°C.

Protein analysis. Viral proteins were prepared by infecting RS cells in 75-cm^2 flasks at a multiplicity of infection of 2 PFU per cell. Twenty-four hours later, the infected monolayers were scraped into the growth medium and the cells were recovered by centrifugation at 500 × *g* for 10 min at 4°C. The cell pellets were washed in cold PBS and then resuspended in 50 μl of PBS. A virion-enriched preparation was obtained by ultracentrifugation of the supernatant from the above-described preparation at 60,000 × *g* for 1.5 h and resuspension of the pellet in 50 μl of PBS. Samples were denatured by addition of 0.5 volume of 6% SDS–6.2 M Tris/HCl (pH 6.8) and boiling for 2 min; this was followed by addition of 0.5 volume of 8 M urea–40 μg of bromphenol blue per ml–20% 2-mercaptoethanol and an additional 1 min of boiling. The denatured proteins were then separated by discontinuous denaturing electrophoresis (26) in 11% acrylamide gels at 20 mA of constant current for 5 h. The separated proteins were transferred to nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell) with a Transblot (Bio-Rad, Hercules, Calif.) apparatus at 30 V for 18 h followed by 60 V for 2 h. The membranes were incubated for 1 h with a 1:1,000 dilution of monospecific polyclonal rabbit anti-UL45 serum produced in *E. coli* (44; kindly provided by Curtis Brandt, University of Wisconsin) and then reacted for 1 h with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The blots were then developed with a 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) –nitroblue tetrazolium substrate (Kirkegaard & Perry).

Viral replication kinetics. Test viruses were inoculated onto monolayers in 24-well plates in a volume of 0.1 ml at a multiplicity of infection of 0.1 or 10 PFU per cell and incubated for 1 h at 37°C. The inoculum was removed, and the wells were washed twice with 1 ml of PBS prior to addition of 1 ml of normal growth medium. At each time point studied, duplicate wells for each virus were freeze-thawed and individually assayed on RS cell monolayers by serial dilution under immune globulin as described above. Duplicate titrations of the duplicate wells were averaged to calculate the final titer.

RESULTS

Isolation of nonsyncytial derivatives of recombinant F1. Strain 17*syn*$^+$ recombinant strain F1 contains, at amino acid 825 near the 3′ end of the gB gene, the syncytial mutation transferred from strain ANG (19). We set out to specifically rescue this mutation in F1 with wild-type 17*syn*$^+$ DNA, and as a control, we also sought to transfer the F1 *syn* mutation to the wild-type parent, 17*syn*$^+$. To accomplish these objectives, we PCR amplified from both 17*syn*$^+$ and F1 a 379-bp portion of the gB gene that contained the nucleotide residue altered in F1 (Fig. 1). This fragment flanked the area in which the single-base-pair *syn* mutation occurred in ANG and F1 but contained no other nucleotide differences between the strains (19). The amplified product containing the wild-type gB-coding sequence from 17*syn*$^+$ was cotransfected into RS cells with unit length DNA from F1, and viral progeny were screened for wild-type (nonsyncytial) plaques. In simultaneous control transfections, the PCR product from F1, containing the C-to-T nucleotide change responsible for the *syn* phenotype, was cotransfected with unit length 17*syn*$^+$ DNA and the progeny were screened for syncytial plaques.

We scored multiple transfections as to whether any progeny of the opposite phenotype were generated. From the 17*syn*$^+$ PCR fragment cotransfected with F1 DNA, we isolated nonsyncytial plaques at a very low frequency from three of seven separate transfections (A4, A6, and 1A series). From the F1 PCR fragment cotransfected with 17*syn*$^+$ DNA, we isolated syncytial plaques from one of six transfections (C4 series). Finally, in sets of three control transfections, each with the F1 PCR fragment cotransfected into F1 and the 17*syn*$^+$ PCR fragment cotransfected into 17*syn*$^+$, we did not detect any viral plaques of the opposite phenotype.

We plaque purified progeny viruses of the opposite phenotype from each of the transfections in which they were present. These progeny viruses and their respective derivations are listed in Table 1.

Characterization of progeny viruses. The single-base-pair *syn* mutation in the ANG gB gene disrupts an *Nco*I restriction site (14), allowing convenient screening for the gB *syn* mutation (Fig. 1). To determine whether this mutation was acquired in the newly syncytial viruses (C4 series) derived from cotransfection of the F1 *syn* mutation into 17*syn*$^+$ or, conversely, lost in the nonsyncytial viruses (A4, A6, and 1A series) derived from cotransfection of the corresponding wild-type 17*syn*$^+$ gB fragment into F1, viral DNAs from the parent strains and the transfection progeny were cut with *Nco*I and hybridized to the same 379-bp PCR product described above (Fig. 2). The syncytial C4 viruses all lost the *Nco*I site, as would be predicted if they had acquired the gB *syn* mutation from F1. In contrast, despite being nonsyncytial, A4B and A61B (Fig. 2), as well as 1ACSS (data not shown), did not acquire the expected *Nco*I restriction site from the wild-type gB sequence used in the cotransfections. To confirm these results, we sequenced the PCR products amplified from both nonsyncytial viruses A4B and A61B, as well as from syncytial virus C4A. As expected, the C4A virus contained the single-base F1 *syn* mutation incorporated into its gB sequence. The sequences from the nonsyncytial A4B and A61B viruses, however, showed no differences from syncytial parent strain F1. We therefore concluded that these nonsyncytial isolates were derivatives of F1 but had become nonsyncytial because of a spontaneous or transfection-induced (7, 27) mutation(s) elsewhere which suppressed the syncytial phenotype conferred by the gB *syn* mutation.

Rescue and genetic mapping of the A4B *syn* suppressor mutation. To genetically map the *syn* suppressor (SS) mutation in virus A4B, we cloned a library of the *Eco*RI fragments from strain F1 and utilized these clones as donor fragments in cotransfections performed with unit length DNA from nonsyncytial SS mutant A4B. When we screened the transfection progeny, we found *syn* plaques (at 20%, a frequency well above the background [<0.1%]) only among progeny from cotransfections utilizing the cloned F1 *Eco*RI I fragment (13.3 kb, 0.635 to 0.723 map unit). This fragment contains seven com-

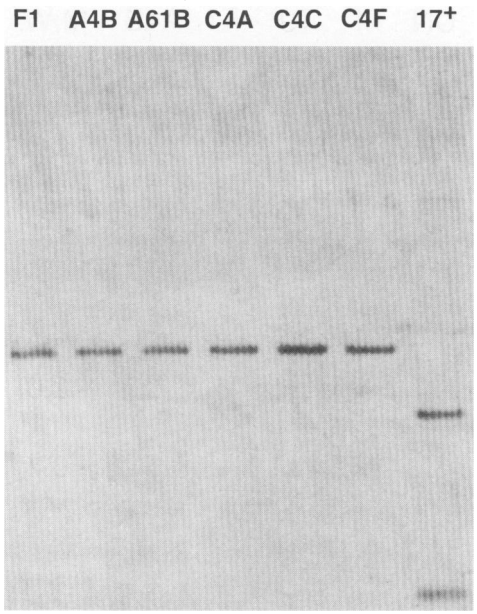

FIG. 2. Southern blot of *Nco*I-digested viral DNAs hybridized with a digoxigenin-labelled PCR fragment amplified from the gB gene of virus F1. Only 17*syn*$^+$ has retained the wild-type gB *Nco*I restriction site; all of the other viruses contain the *syn* mutation.

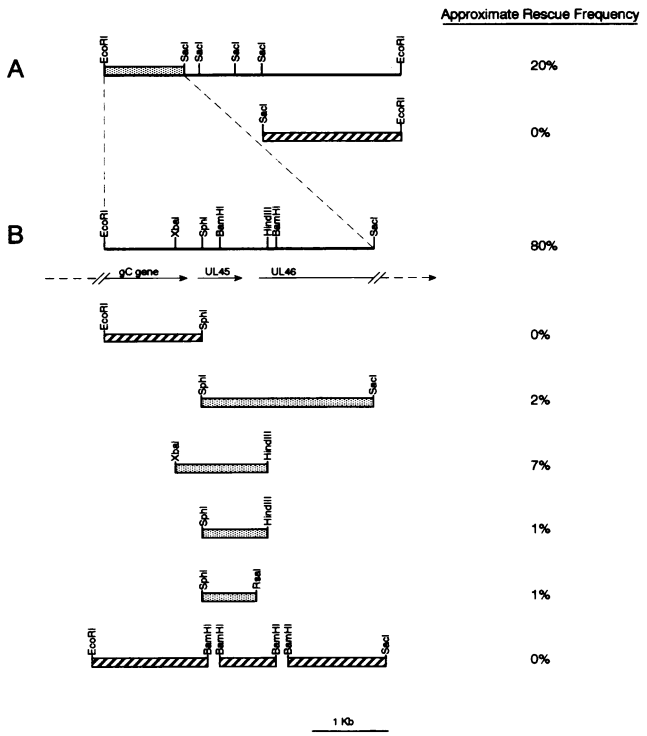

FIG. 3. Rescue of the *syn* suppressor mutation in virus A4B. The cloned subfragments that rescue the mutation are shown as stippled boxes, and the subfragments that did not rescue are shown as diagonally striped boxes. The approximate rescue frequencies are shown on the right. (A) Map of the HSV-1 *Eco*RI I fragment. (B) Map of the 3.7-kb *Eco*RI-*Sac*I subfragment of *Eco*RI-I. The three open reading frames contained or partially contained in this fragment are shown below the restriction map. The various cloned subfragments used in marker rescue experiments are shown below the open reading frames. The scale for the map in panel B is shown at the bottom.

plete open reading frames (UL45 to UL51) and portions of UL44 and UL52 (31).

We next performed a series of cotransfections with subfragments of the F1 *Eco*RI I fragment. The ability to restore the syncytial phenotype (see frequencies of rescue next to fragments in Fig. 3) was mapped first to a 3.5-kb *Eco*RI-*Sac*I fragment at the left-hand side of *Eco*RI-I, narrowed to a 1,158-bp *Xba*I-*Hin*dIII fragment, and then mapped to a cloned 797-bp *Sph*I-*Hin*dIII fragment containing the UL45 open reading frame and 31 bp of UL46. Finally, we were able to rescue *syn* suppression by using a cloned 528-bp *Sph*I-*Rsa*I fragment of F1 that contains only UL45. As a control, no syncytial progeny were obtained from cotransfections of A4B unit length DNA with the corresponding cloned *Sph*I-*Rsa*I fragment from A4B itself. Despite several attempts, we were unable to rescue the *syn* suppression with either the 1,507-bp *Eco*RI-*Bam*HI fragment or the 721-bp *Bam*HI fragment. These fragments overlap the region where other fragments did rescue the *syn* suppression (Fig. 3), a finding which suggested that the *syn* suppressor mutation might map near the *Bam*HI site within the UL45 gene.

If A4B contained a mutation in its UL45 gene which suppressed the expression of the F1 syncytial phenotype, we hypothesized that the transfer of wild-type UL45 genes to A4B should restore its syncytial phenotype. To determine if this was the case, we used the 797-bp *Hin*dIII-*Sph*I fragments containing UL45 cloned from F1, from 17*syn*$^+$ (the parent strain of F1), and as a control, from A4B itself in cotransfections with unit length A4B DNA and screened the progeny for syncytial plaques. Both the F1 and 17*syn*$^+$ fragments, but not the corresponding fragment from A4B, restored the syncytial phenotype. We concluded, then, that the wild-type UL45 gene was capable of restoring the syncytial phenotype to A4B and that the A4B UL45 gene carried the mutation which caused suppression of the syncytial phenotype. Representative syncytial viruses were plaque purified from these backcross transfections rescued with either the 17*syn*$^+$ fragment (T1 series) or the F1 fragment (T9 series). These viruses and their derivations are listed in Table 1.

Determination of the nucleotide sequence of the mutant UL45 gene of A4B. To precisely define the SS mutation in A4B, we cloned the 797-bp *Sph*I-*Hin*dIII fragments from A4B, F1, ANG (the donor parent strain of F1), and 17*syn*$^+$ into M13mp18 and mp19 and determined their DNA sequences (Fig. 4A). The F1, 17*syn*$^+$, and ANG sequences were identical within the UL45 open reading frame, except for a single-nucleotide change in ANG that did not affect the deduced amino acid sequence. We found that A4B, on the other hand, had a single-base (C) deletion at nucleotide 230 of the UL45 open reading frame, which would be predicted to result in a frameshift. We believe that the close proximity (3 bp) of this deletion to the *Bam*HI site in UL45 was, as theorized, the reason we were unable to rescue the suppressor mutation with the F1 *Bam*HI fragments. The deduced amino acid sequence of the mutant UL45 protein (Fig. 4B) terminated 49 bp downstream from the frameshift mutation, encoding a potential translation product of 92 amino acids (approximately 9 kDa) rather than the 172 amino acids (18 kDa) of the wild-type UL45 gene.

Transcriptional analysis of A4B. We next investigated whether the frameshift mutation in the A4B UL45 gene affected the transcription of either UL45 or, since the mRNAs

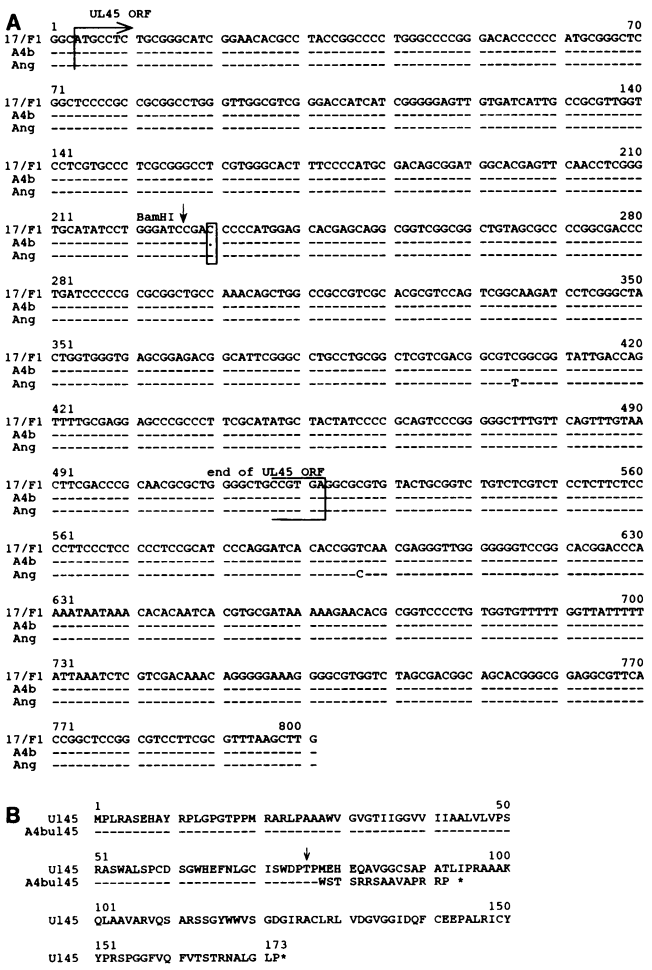

FIG. 4. (A) Nucleotide sequences of the UL45 genes of 17*syn*$^+$, F1, A4B, and ANG. The sequences of 17*syn*$^+$ and F1, being identical, are shown on the same line. Identical bases in the lower aligned sequences are depicted as dashes, and the single-base deletion at nucleotide 230 in A4B is depicted as a dot and is boxed. The UL45 open reading frame (ORF) and the *Bam*HI site within it are marked. (B) Comparison of the deduced UL45 amino acid sequences from 17*syn*$^+$, F1, ANG (top line), and A4B (bottom line). Identical amino acid residues are depicted as dashes in the lower sequence, and the respective termination codons are marked with asterisks. The point of the frameshift mutation in A4B is marked with an arrow.

for glycoprotein C (gC) and UL45 are 3′ coterminal (11), gC. Equivalent amounts of total RNAs from A4B and F1, as well as the T1 and T9 backcross recombinants, were subjected to electrophoresis and Northern (RNA) analysis with the 797-bp *Sph*I-*Hin*dIII fragment containing UL45 as a probe. As shown in Fig. 5, we noted two major known RNA transcripts, the 2.7-kb gC message and the 0.73-kb UL45 message, as described by others (9, 11, 45). The UL45 and gC transcripts in A4B were comparable in size and were expressed at levels similar to that of the parent virus, F1. We also observed hybridization of the probe to an unmapped message of approximately 5 kb which was present in all of the viral RNAs.

Immunodetection of the UL45 protein. We subjected both infected-cell lysates and virion-enriched preparations of A4B, F1, T1, and T9 to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western immunoblotting with

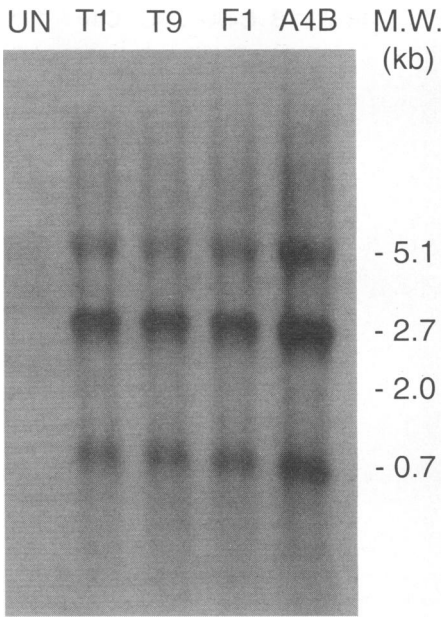

FIG. 5. Northern blot of total HSV-1-infected cell RNAs hybridized with the 797-bp *Sph*I-*Hin*dIII subclone of HSV-1 *Eco*RI-I (Fig. 3). Approximate molecular sizes (M.W.) are shown at the right. The UL45 mRNA migrates at approximately 0.73 kb. UN, uninfected cells.

polyclonal rabbit antiserum raised against the UL45 gene product. As shown in Fig. 6, we detected the expected 18-kDa UL45 protein in syncytial parent virus F1 and in backcross syncytial recombinants T1 and T9 (Table 1). These results were obtained by using either infected-cell lysates or virion-enriched preparations. Despite using several approaches, including overloaded gels, different percentages of polyacrylamide, and a

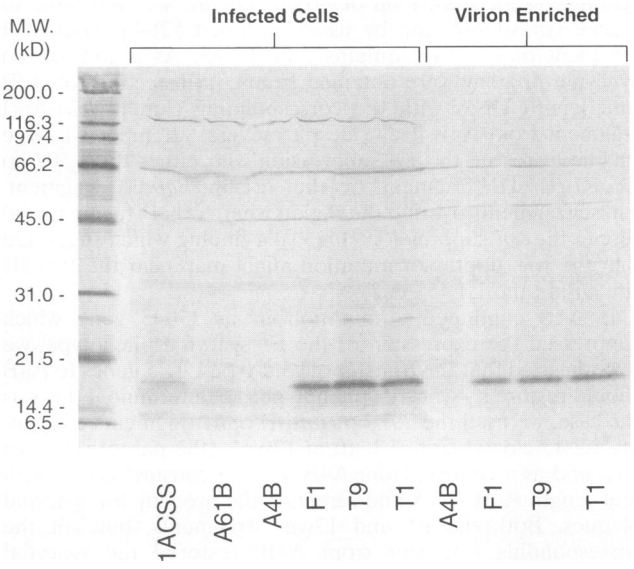

FIG. 6. Western blot showing expression of the 18-kDa UL45 protein. Viral proteins from infected cells or from crude virion preparations were transferred to nitrocellulose and reacted with monospecific polyclonal antiserum against UL45. Molecular size standards are shown to the left. kD, kilodaltons.

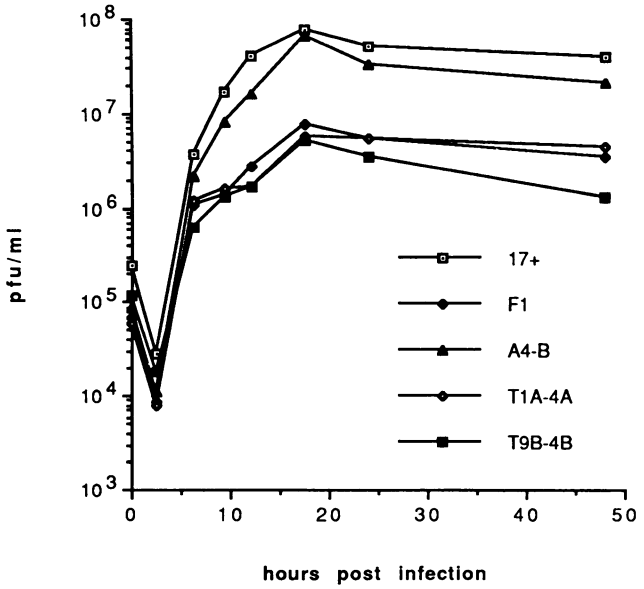

FIG. 7. One-step growth kinetics of the various virus strains on RS cells.

variety of transfer membranes and buffers, we were unable to detect any immunoreactive protein bands of 18 kDa or smaller in either fraction from cells infected with A4B.

We also used the Western blot to screen the other independently isolated mutants in which the *syn* mutation of F1 was suppressed (Fig. 6). Virus A61B, like A4B, showed no protein immunoreactive with the UL45 antiserum. Virus 1ACSS, on the other hand, produced an immunoreactive protein of the correct size, but the intensity of the protein band detected was reproducibly lower than that of the band obtained with F1. These results suggested that A61B and 1ACSS also sustained mutations in UL45 resulting, respectively, in either absent or altered production of the gene product and in suppression of the syncytial phenotype.

Replication of UL45-deficient mutants. Visalli and Brandt recently reported that deletion of the UL45 gene from HSV-1 strain KOS (nonsyncytial) retarded viral replication by approximately 20-fold (44). To determine whether the mutation in the A4B UL45 gene and the absence of an immunoreactive UL45 gene product affected its replication, we compared the growth kinetics of the relevant viruses in RS cells under one-step conditions (Fig. 7). All of the viruses tested demonstrated similar eclipse and exponential-growth kinetics. The extent of exponential growth and the peak titer of A4B closely resembled those of 17*syn*$^+$ and were, in fact, more than 10-fold higher than those of F1. This result was reproduced in several experiments, with both RS cells and chicken embryo fibroblasts, and similar results were seen with the other UL45-altered viruses, A61B and 1ACSS, and at the lower multiplicity of infection of 0.1 PFU per cell (data not shown). The earlier leveling off and lower peak titer of F1 were consistent with previous results obtained with that virus (13) and were reproduced in several experiments. The T1 and T9 backcross *syn* rescues of A4B, which expressed UL45 normally, replicated with kinetics resembling those of parent virus F1. Therefore, the UL45 mutation and/or the resultant suppression of the syncytial phenotype in A4B in fact resulted in enhanced growth of the virus in tissue culture, such that viral growth was similar to that of wild-type, nonsyncytial viruses.

The UL45 mutation in A4B also suppressed syncytium formation in other gB *syn* mutants. We next investigated whether the UL45 frameshift mutation in A4B would suppress syncytium formation by gB *syn* mutant viruses other than F1. We introduced the UL45 SS mutation into gB *syn* mutants ANG and C4A by cotransfection with the cloned A4B *Eco*RI I fragment (for higher recombination efficiencies due to its larger size). The various cotransfections and controls and the frequencies of nonsyncytial progeny are presented in Table 2. Multiple cotransfections with the A4B *Eco*RI I fragment into unit length DNAs from F1, C4A, and ANG all produced typical nonsyncytial plaques at frequencies above the background, although the frequencies were not as high as in the converse experiments in which we rescued the UL45 *syn* suppressor mutation in A4B (Fig. 3). In the control transfections with the various unit length viral DNAs alone, we observed 10 nonsyncytial plaques in the C4A transfection, 2 nonsyncytial plaques in the ANG transfection, and no nonsyncytial plaques in the F1 transfection (Table 2). Representative nonsyncytial plaques were picked and plaque purified from the A4B *Eco*RI I cotransfections along with three of the presumably spontaneously arising nonsyncytial plaques noted among the progeny of control transfections of ANG or C4A DNA alone.

We prepared infected-cell lysates from these nonsyncytial isolates and screened them by Western immunoblotting for expression of UL45 (see Fig. 9). As expected, consistent with the transfer of the A4B UL45 SS mutation, all of the nonsyncytial viruses (F1-1A, F1-1B, C4A-1B, C4A-1C, C4A-2D, ANG-1B2, and ANG-2B) isolated from cotransfections of parental *syn* mutant viral DNAs (from F1, C4A, and ANG, respectively) with the A4B *Eco*RI I fragment were UL45 negative by Western blot (see Fig. 9). Photographs of plaques formed by the parental viruses and the progeny of representative cotransfections from this and previously described experiments are shown in Fig. 8.

We also characterized UL45 expression in the three spontaneous nonsyncytial revertants which were isolated from the control transfections of ANG or C4A unit length DNA alone. Alterations were noted in the expression of UL45 by both nonsyncytial isolates from ANG DNA transfected alone (ANG-alone A and B) (Fig. 9). ANG-alone A (lane 7) underproduced UL45, and that protein which was present appeared to migrate more slowly. ANG-alone B (lane 6) produced no detectable UL45. In contrast, the nonsyncytial virus isolated from a transfection of C4A DNA alone (lane 12) apparently expressed UL45 normally. To rule out the possibility that spontaneous reversions of the gB *syn* mutation were responsible for the nonsyncytial phenotypes observed in these three viruses, all were screened for the presence of the wild-type *Nco*I site in gB by restriction digests of the PCR product surrounding the *syn* mutation (data not shown). None had reverted to the wild-type sequence, demonstrating that the wild-type phenotype was in no case due to reversion of the original gB mutation. These results suggest that the viruses ANG-alone A and B developed transfection-induced or spontaneous mutations in the UL45 gene resulting in the nonsyncytial phenotype. Whether or not C4A-alone A sustained an undetected mutation in UL45 remains to be determined.

DISCUSSION

We isolated and characterized an HSV-1 virus, A4B, with a frameshift mutation in its UL45 gene. This mutation resulted in the loss of an immunodetectable UL45 gene product and the loss of cell fusion induced by a *syn* mutation in the carboxyl

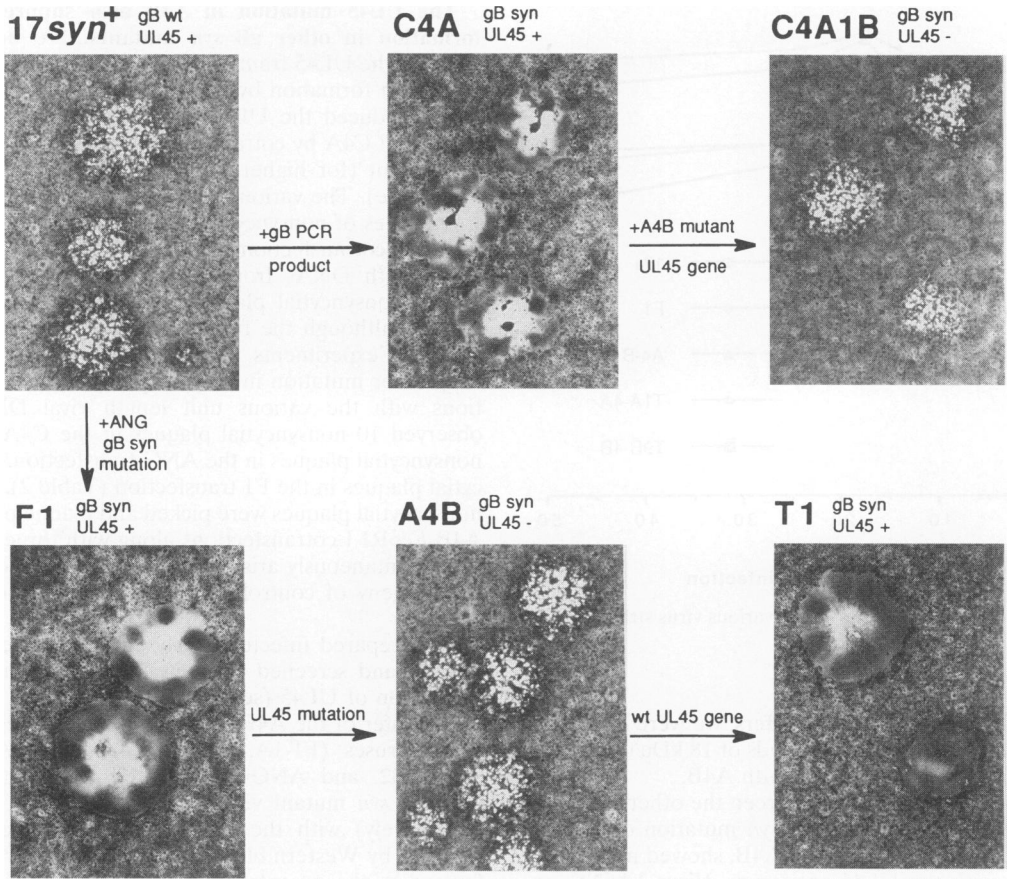

FIG. 8. Photographs of representative plaques of viruses on RS cells. Their derivations, the nature of their gB genes, and whether or not they express UL45 are indicated. wt, wild type.

cytoplasmic domain of gB. Furthermore, we isolated several other UL45 mutant viruses, both spontaneously occurring and from cotransfections with the mutant UL45 gene from A4B, and in all of these the reduction of UL45 expression was associated with suppression of syncytium formation.

These results demonstrate that UL45 plays a role in facilitating the fusion of HSV-infected cells, the first function ascribed to the UL45 gene product. Since no UL45 gene product could be detected in A4B or A61B, we hypothesize that the wild-type UL45 gene product normally interacts with other proteins involved in the fusion process and that this interaction promotes cell-cell fusion in gB syncytial mutants.

At least one of our *syn* suppressor mutants, 1ACSS, expressed, albeit at lower levels, a UL45 gene product, and we cannot completely exclude the possibility that a truncated UL45 gene product is expressed by A4B or A61B. A truncated gene product either might be more susceptible to degradation or might not contain immunologic epitopes required to react with the polyvalent UL45 antiserum. It is possible that an altered UL45 gene product, if present, does not interact normally with putative target proteins to promote cell-cell fusion, giving the same results as expected if UL45 were not expressed at all. On the other hand, rather than UL45 being an active promoter of cell fusion as we hypothesize, an undetected but altered UL45 protein could conceivably function as an inhibitor of syncytium formation. Complementation analysis of UL45 null mutants in cell lines expressing wild-type and

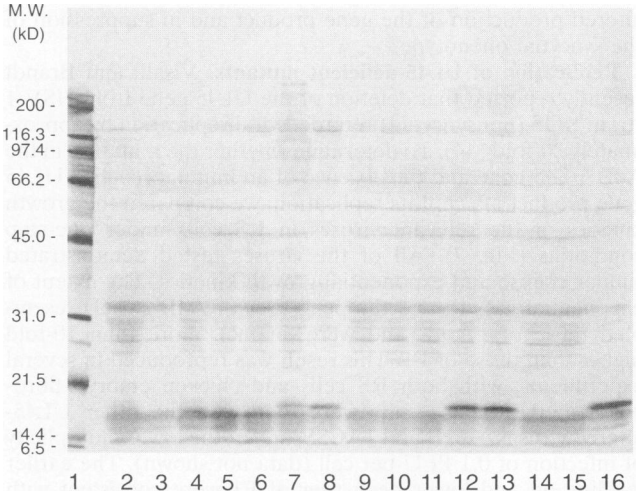

FIG. 9. Western blot of viral proteins extracted from nonsyncytial viruses isolated from cotransfections of ANG and C4A unit length DNAs with a cloned fragment containing the mutant UL45 gene from A4B. Nonsyncytial plaques picked from control transfections without the A4B UL45 gene (ANG-alone and C4A-alone series) are also shown (see Table 2). Lanes: 1, molecular size (M.W.) standards (kD, kilodaltons); 2, mock-infected cells; 3, A4B; 4, ANG-2B; 5, ANG-1B2; 6, ANG-alone B; 7, ANG-alone A; 8, ANG; 9, C4A-2D; 10, C4A-1C; 11, C4A-1B; 12, C4A-alone A; 13, C4A; 14, F1-1A; 15, F1-1B; 16, F1.

mutant UL45 genes would be helpful to further define the role of the UL45 protein in cell fusion.

We were initially surprised that we could not isolate nonsyncytial recombinant derivatives of F1 whose gB sequences had been rescued directly through cotransfections with the PCR product containing 379 bp of the wild-type gB gene of 17*syn*$^+$. Several possibilities might explain this result. One is that the use of a small PCR fragment to rescue the *syn* mutation in F1 yielded a recombination frequency below a detectable level. This is the most likely hypothesis, since we have recently recovered nonsyncytial derivatives of F1 at a significant frequency by using the much larger *Eco*RI F fragment of 17*syn*$^+$ (data not shown). Nonetheless, in three separate cotransfection experiments with the wild-type PCR fragment, all of the nonsyncytial variants of F1 we recovered had alterations in the UL45 gene and not in gB, even though we easily recovered syncytial derivatives of 17*syn*$^+$ carrying the gB *syn* mutation in converse experiments with the analogous PCR fragment from F1. The single nonsyncytial virus studied which was isolated from a transfection of C4A DNA alone (C4A-alone A) retained the original gB *syn* mutation but also expressed wild-type levels of the UL45 gene product. This virus may well have an undetected mutation that affects its UL45 protein. However, given the complexity of HSV fusion processes, it is also possible that a *syn* suppressor mutation exists at yet another genetic locus.

Throughout all of the cotransfections we performed, regardless of donor fragment size, the frequency of conferral of the syncytial phenotype to nonsyncytial viruses was always higher than the frequency of restoration of the nonsyncytial phenotype to syncytial viruses. Weise et al. reported obtaining similar results while trying to rescue the gB syncytial mutation in strain ANG (the donor parent of F1) with fragments from strain KOS (47). Thus, it is possible that the wild-type gB gene, for unknown reasons, exerts adverse effects when expressed in certain viruses, such as ANG and F1, and therefore is selected against in cotransfections.

The UL45 gene product has been characterized as an 18-kDa protein which is synthesized as a true late gene product and is associated with virions, most likely in the viral envelope (45). These results support our hypothesis that the UL45 gene product is involved in fusion processes, in that the protein is localized such that it could interact with virion glycoproteins involved in fusion processes. Visalli and Brandt reported that their UL45 null mutant, UL45Δ, while viable in tissue culture, had reduced replication (44). This result differs from our finding that UL45 mutant A4B replicated better than its UL45-positive parent and with kinetics similar to those of wild-type HSV. Several possibilities could explain the replicative differences between these two UL45-deficient viruses. First, it is possible that the 4-kB *lacZ* insertion in UL45Δ resulted in diminished replicative capabilities through mechanisms unrelated to its deficiency in UL45. These investigators did show that the insertion significantly reduced expression of the gene just upstream, i.e., that for gC. Second, as stated previously, we cannot absolutely rule out the possibility that our UL45 mutant, A4B, makes a truncated UL45 protein which can still carry out some function missing in the UL45 null mutant described by Visalli and Brandt. Third, the UL45 null mutant described by Visalli and Brandt was derived from KOS, a nonsyncytial strain of HSV-1. It is possible that the syncytial mutation still present in gB of F1 somehow compensates for growth and/or fusion deficiencies due to the lack of UL45, thus resulting in wild-type growth of the virus. Finally, HSV *syn* mutants generally yield reduced viral progeny compared with wild-type viruses and it is possible that elimination of gB-induced cell fusion resulting from the UL45 mutation in A4B removes such a generic impediment to viral replication.

In summary, we have demonstrated that a frameshift mutation in the UL45 gene suppresses the fusogenic properties, but not the in vitro replication, of HSV-1 gB syncytial mutants and that the UL45 gene product can function as an important player in mediating virus-induced cell-cell fusion processes. The UL45 protein may be important in the fusion events that occur normally during infection of cells by HSV-1.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. Greene/Wiley Interscience, New York.
2. **Baines, J. D., P. L. Ward, G. Campadelli-Fiume, and B. Roizman.** 1991. The U_L20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J. Virol. **65:**6414–6424.
3. **Bond, V. C., and S. Person.** 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology **132:**368–376.
4. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137:**185–190.
5. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in entry and cell fusion. J. Virol. **62:**2596–2604.
6. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61:**714–721.
7. **Calos, M. P., J. S. Lebkosky, and M. R. Botchan.** 1983. High mutation frequency in DNA transfected into mammalian cells. Proc. Natl. Acad. Sci. USA **80:**3015–3019.
8. **DeLuca, N., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology **122:**411–423.
9. **Draper, K. G., R. H. Costa, G. T.-Y. Lee, P. G. Spear, and E. K. Wagner.** 1984. Molecular basis of the glycoprotein-C-negative phenotype of herpes simplex virus type 1 macroplaque strain. J. Virol. **51:**578–585.
10. **Engel, J. P., E. P. Boyer, and J. L. Goodman.** 1993. Two novel single amino acid syncytial mutations in the carboxy terminus of glycoprotein B of herpes simplex virus type 1 confer a unique pathogenic phenotype. Virology **192:**112–120.
11. **Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner.** 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. **45:**634–647.
12. **Gage, P. J., M. Levine, and J. C. Glorioso.** 1993. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. J. Virol. **67:**2191–2201.
13. **Goodman, J. L., M. L. Cook, F. Sederati, K. Izumi, and J. G. Stevens.** 1989. Identification, transfer, and characterization of cloned herpes simplex virus invasiveness regions. J. Virol. **63:** 1153–1161.
14. **Goodman, J. L., and J. P. Engel.** 1990. Altered pathogenesis in herpes simplex virus type 1 infection due to a syncytial mutation mapping to the carboxy terminus of glycoprotein B. J. Virol. **65:**1770–1778.
15. **Goodman, J. L., and J. G. Stevens.** 1986. Passage of herpes simplex

virus type 1 on chick embryo fibroblasts confers virulence for chick embryos. Virus Res. **5:**191–200.
16. **Goodpasture, E. W., A. M. Woodruff, and G. J. Buddingh.** 1931. The cultivation of vaccine and other viruses in the chorioallantoic membrane of chick embryos. Science **74:**371–372.
17. **Graham, R. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
18. **Haanes, E. J., and P. P. Cleary.** 1989. Identification of a divergent M protein gene and an M protein-related gene family in *Streptococcus pyogenes* serotype 49. J. Bacteriol. **171:**6397–6408.
19. **Haanes, E. J., and J. L. Goodman.** Unpublished data.
20. **Haanes, E. J., and J. L. Goodman.** Unpublished data.
21. **Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson.** 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66:**5603–5609.
22. **Hutchinson, L., F. L. Graham, W. Cai, C. Debroy, S. Person, and D. C. Johnson.** 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology **196:**514–531.
23. **Izumi, K. M., and J. G. Stevens.** 1990. Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. J. Exp. Med. **172:**487–496.
24. **Jacobson, J. G., S. L. Martin, and D. M. Coen.** 1989. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. **63:**1839–1843.
25. **Kaerner, H. C., C. H. Schroder, A. Ott-Hartmann, G. Kumel, and H. Kirchner.** 1983. Genetic variability of herpes simplex virus: development of a pathogenic variant during passaging of a nonpathogenic herpes simplex virus type 1 virus strain in mouse brain. J. Virol. **46:**83–93.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lebkowski, J. S., R. B. DuBridge, E. A. Antell, K. S. Greisen, and M. P. Calos.** 1984. Transfected DNA is mutated in monkey, mouse, and human cells. Mol. Cell. Biol. **4:**1951–1960.
28. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62:**1486–1494.
29. **Maclean, C. A., S. Efstathiou, M. L. Elliot, F. E. Jamieson, and D. J. McGeoch.** 1991. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. J. Gen. Virol. **72:**897–906.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
32. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
33. **Mullis, K. B., and F. A. Faloona.** 1978. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. **155:**335–350.
34. **Nahmias, A. J., W. R. Dowdle, Z. M. Maib, A. Highsmith, R. W. Harwell, and W. E. Josey.** 1968. Relation of pock size on the chorioallantoic membrane to antigenic type of herpesvirus hominis. Proc. Soc. Exp. Biol. Med. **127:**1022–1028.
35. **Navarro, D., P. Paz, and L. Pereira.** 1992. Domains of herpes simplex virus 1 glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology **186:**99–112.
36. **Noble, A. G., G. T.-Y. Lee, and P. G. Spear.** 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology **129:**218–224.
37. **Pogue-Geile, K. L., G. T.-Y. Lee, S. K. Shapira, and P. G. Spear.** 1984. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. Virology **136:**100–109.
38. **Rodgers, F. G.** 1973. Infection, haemorrhage and death of chick embryos after inoculation of herpes simplex virus type 2 on the chorioallantoic membrane. J. Gen. Virol. **21:**187–191.
39. **Romanelli, M. G., E. M. Cattozzo, L. Faggioli, and M. Tognon.** 1991. Fine mapping and characterization of the *syn* 6 locus in the herpes simplex virus type 1 genome. J. Gen. Virol. **72:**1991–1995.
40. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29:**677–697.
41. **Sanders, P. G., N. M. Wilkie, and A. J. Davison.** 1982. Thymidine kinase deletion mutants of herpes simplex virus type 1. J. Gen. Virol. **63:**277–287.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
43. **Spear, P.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Inc., Boca Raton, Fla.
44. **Visalli, R. J., and C. R. Brandt.** 1991. The HSV-1 UL45 gene product is not required for growth in Vero cells. Virology **185:**419–423.
45. **Visalli, R. J., and C. R. Brandt.** 1993. The HSV-1 UL45 18kDa gene product is a true late protein and a component of the virion. Virus Res. **29:**167–178.
46. **Walboomers, J. M., and J. ter Schegget.** 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology **74:**256–258.
47. **Weise, K., H. C. Kaerner, J. Glorioso, and C. H. Schroder.** 1987. Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes of plaque morphology and neuropathogenicity. J. Gen. Virol. **68:**1909–1919.
48. **Yuhasz, S. A., and J. G. Stevens.** 1993. Glycoprotein B is a specific determinant of herpes simplex virus type 1 neuroinvasiveness. J. Virol. **67:**5948–5954.