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# Recombinant Respiratory Syncytial Virus from Which the Entire SH Gene Has Been Deleted Grows Efficiently in Cell Culture and Exhibits Site-Specific Attenuation in the Respiratory Tract of the Mouse

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**The small hydrophobic protein SH of human respiratory syncytial virus (RSV) is a short transmembrane surface protein of unknown function. A full-length cDNA of RSV strain A2 (subgroup A) antigenomic RNA was modified such that the entire SH gene, including the transcription signals and the complete mRNA-encoding sequence, was deleted and replaced by a synthetic intergenic region. This reduced the length of the antigenome by 398 nucleotides and ablated expression of 1 of the 10 RSV mRNAs. Recombinant virus containing this engineered deletion was recovered, and the absence of the SH gene was confirmed by reverse transcription in conjunction with PCR. Northern blot analysis of intracellular RNAs and gel electrophoresis of labeled intracellular proteins confirmed the lack of expression of the SH mRNA and protein. The absence of the SH gene did not noticeably affect RNA replication, but two effects on transcription were noted. First, synthesis of the G, F, and M2 mRNAs was increased, presumably due to their being one position closer to the promoter in the gene order. Second, transcription of genes downstream of the engineered site exhibited a steeper gradient of polarity. On monolayers of HEp-2 cells, the SH-minus virus produced syncytia which were at least equivalent in size to those of the wild type and produced plaques which were 70% larger. Furthermore, the SH-minus virus grew somewhat better (up to 12.6-fold) than wild-type recombinant RSV in certain cell lines. While the function of the SH protein remains to be determined, it seems to be completely dispensable for growth in tissue culture and fusion function. When inoculated intranasally into mice, the SH-minus virus resembled the wild-type recombinant virus in its efficiency of replication in the lungs, whereas it replicated 10-fold less efficiently in the upper respiratory tract. In mice, the SH-minus and wild-type recombinant viruses were similarly immunogenic and effective in inducing resistance to virus challenge.**

Human respiratory syncytial virus (RSV) is the most important viral agent of pediatric respiratory tract disease worldwide, causing bronchiolitis and pneumonia (reference 13 and references therein). It is a member of the family *Paramyxoviridae*, which, together with the families *Rhabdoviridae* and *Filoviridae*, constitutes the order *Mononegavirales*, comprising the nonsegmented negative-strand RNA viruses (32).

The genome of RSV is a single strand of negative-sense RNA of 15,222 nucleotides (nt). The RSV gene map (designated according to the proteins described below) is 3′-NS1-NS2-N-P-M-SH-G-F-M2-L-5′. The viral genome is tightly encapsidated by the nucleocapsid N protein, which together with the phosphoprotein P and large polymerase subunit L forms the minimum unit for RNA replication. RNA replication involves the synthesis of a positive-sense, exact-copy, encapsidated, replicative intermediate called the antigenome, which serves in turn as the template for progeny genome. In addition to the N, P, and L proteins, transcription requires the M2 open reading frame 1 (ORF1) protein which is a processivity factor that lacks a known counterpart in other nonsegmented negative-strand viruses examined to date (12). Transcription initiates at the 3′ end of the genome and proceeds by a sequential stop-start process which is mediated by the transcription gene-start (GS) and gene-end (GE) signals that flank each gene (26) and yields 10 major subgenomic mRNAs.

In addition to the four nucleocapsid-associated proteins described above, RSV encodes a matrix protein that is thought to mediate interaction between the nucleocapsid and envelope during virion morphogenesis. RSV also encodes several putative accessory proteins. There are two nonstructural proteins, NS1 and NS2, whose functions are unknown, although NS1 appears to be a negative regulatory factor for RNA synthesis (2). There also is evidence that a second, overlapping translational ORF in the M2 mRNA encodes an additional protein, called M2 ORF2, which also is a negative regulatory protein for RNA synthesis (12).

Finally, RSV encodes three surface envelope proteins that are components of the virion: the attachment protein G, the fusion protein F, and the small hydrophobic protein SH. Identification of the G protein as the viral attachment protein is based on the finding that G-specific antibodies inhibited the adsorption of the virion to cells (28). The heavily glycosylated G protein has little apparent similarity to the attachment proteins of other nonsegmented negative-strand RNA viruses. It is highly divergent between strains, but it contains a conserved domain which is thought to be involved in receptor binding (21). The fusion function of the F protein was originally identified by the finding that certain F-specific monoclonal antibodies inhibit syncytium formation (39). The RSV F protein is clearly related to the F proteins of other paramyxoviruses, although the amount of amino acid sequence identity is low

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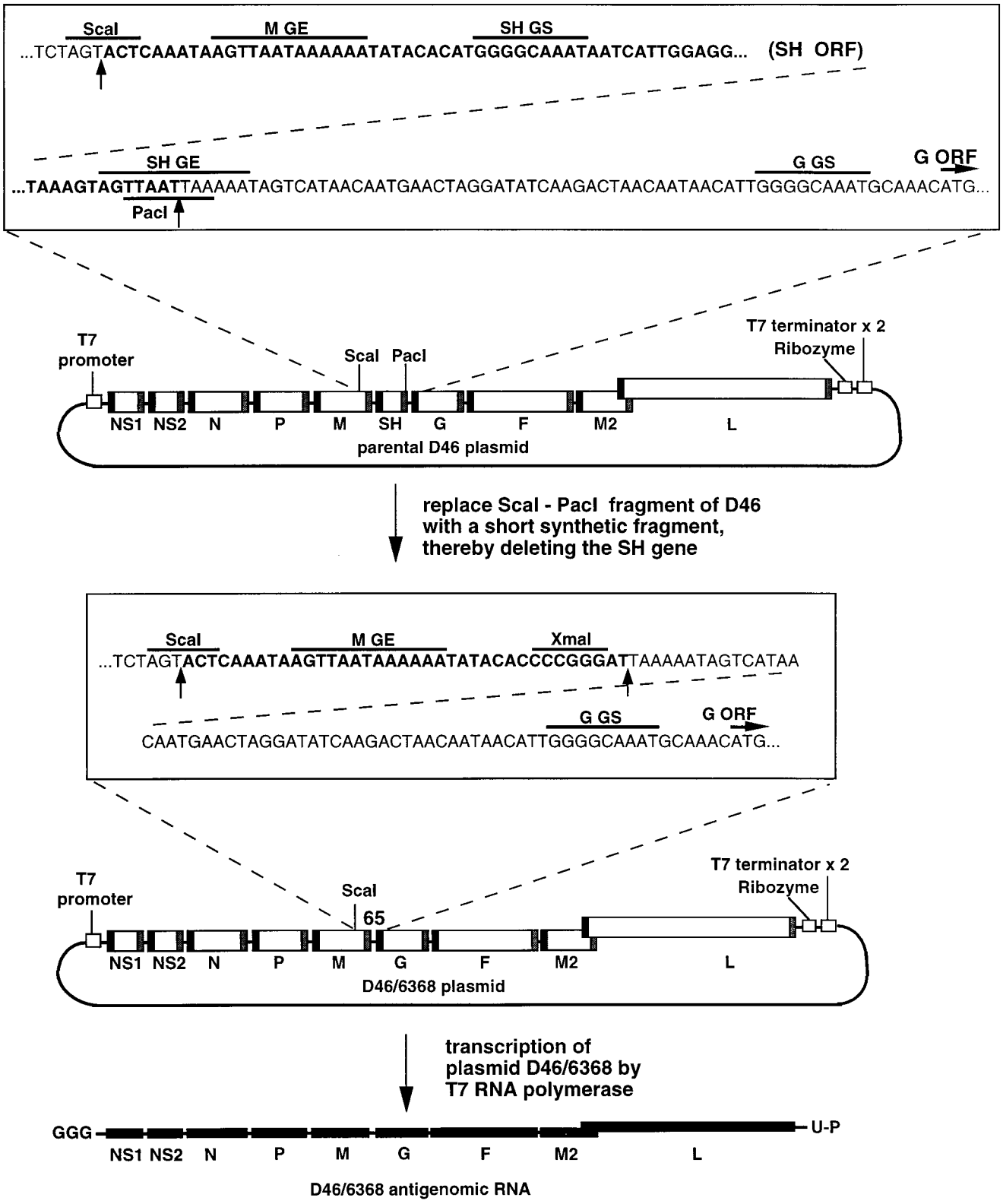


FIG. 1. Diagrams (not to scale) of the parental wild-type D46 plasmid encoding an RSV antigenome (top) and the D46/6368 derivative in which the SH gene has been deleted (bottom). The RSV genes are shown as open rectangles; the GS and GE transcription signals are shown as black and gray bars, respectively. The T7 phage promoter (left) and hammerhead ribozyme and T7 terminators used to generate the 3′ end of the RNA transcript (right) are shown as open boxes. The *Sca*I-*Pac*I fragment of D46 was replaced with a short synthetic fragment, resulting in D46/6368. The sequence flanking the SH gene in D46 and the sequence of the engineered region in D46/6368 are each shown in a box over the respective plasmid map. The sequence of the *Sca*I-*Pac*I fragment in D46 and its replacement in D46/6368 are shown in bold and demarcated with arrows facing upward. The M GE, SH GS, SH GE, and G GS sites are indicated with overlining. The new M-G intergenic region in D46/6368 is labeled 65 to indicate its nucleotide length. The positive-sense T7 transcript of the SH-minus D46/6368 construct is illustrated at the bottom; the three 5′-terminal nonviral G residues contributed by the T7 promoter and the 3′-terminal U residue are shown (11). These nonviral nucleotides are not included in length measurements.

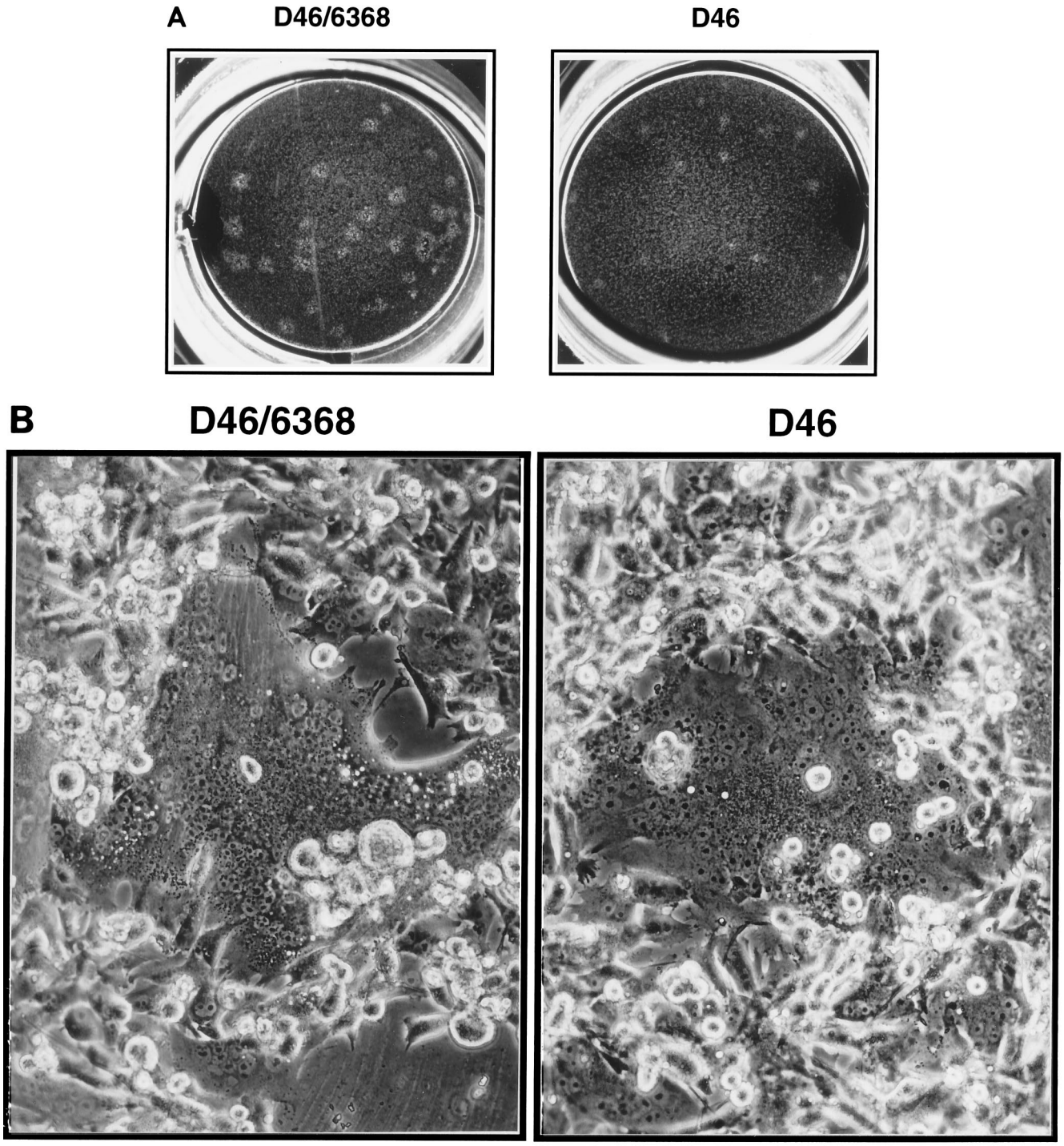


FIG. 2. Comparison of D46/6368 SH-minus and D46 wild-type recombinant RSV with regard to the ability to form plaques (A) and syncytia (B) in monolayers of HEp-2 cells. (A) Comparison of plaque size. Cells were infected, incubated for 6 days at 37°C under a methylcellulose overlay, fixed with methanol, and photographed. (B) Cells were infected, covered with methylcellulose, incubated for 3 days at 37°C, and photographed without further treatment.

(37). The F protein is synthesized as an $F_0$ precursor which is cleaved endoproteolytically into two disulfide-linked subunits, $F_1$ and $F_2$.

The third surface species, the SH protein, is small (64 amino acids in the case of strain A2) and contains a putative transmembrane domain at amino acid positions 14 to 41. It is oriented in the membrane with the C terminus exposed (10), and there are potential glycosylation sites in both the C-terminal and N-terminal domains (9). In infected cells, the SH protein of strain A2 accumulates in four forms (31): (i) SH0 ($M_r$, 7,500), the full-length, unglycosylated form that is the most abundant (8); (ii) SHg ($M_r$, 13,000 to 15,000), which is the

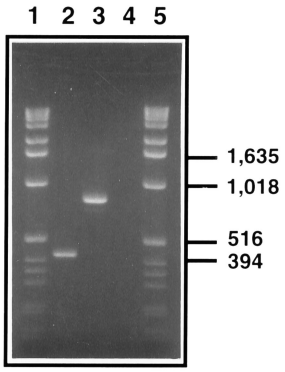


FIG. 3. RT-PCR analysis of total intracellular RNA from cells infected with the D46 wild-type or D46/6368 SH-minus virus to confirm the deletion in the SH locus. RT was performed with a positive-sense primer that anneals upstream of the SH gene, and the PCR used, in addition, a negative-sense primer that anneals downstream of the SH gene. Lanes: 1 and 5, markers consisting of a 1-kb DNA ladder (Life Technologies); 2, D46/6368 RNA subjected to RT-PCR; 3, D46 RNA subjected to RT-PCR; 4, D46/6368 RNA subjected to PCR alone. PCR products were electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. Nucleotides lengths of some marker DNA fragments are shown to the right.

full-length form containing a single N-linked carbohydrate chain; (iii) SHp ($M_r$, 21,000 to 40,000), a modified version of SHg in which the single N-linked carbohydrate chain is modified by the addition of polylactosaminoglycan (1); and (iv) SHt ($M_r$, 4,800), a truncated, unglycosylated form which is initiated from the second methionyl codon (position 23) and which alone among the different forms does not appear to be transported to the cell surface. The SH0 and SHp forms have been detected in purified virions, suggesting that there is a selectivity at the level of virion morphogenesis (10).

Among the paramyxoviruses, ostensibly similar SH proteins have been found in simian virus 5 (20), bovine RSV (34), mumps virus (16), and turkey rhinotracheitis virus (29). The small hydrophobic VP24 protein of filoviruses is thought to be a surface protein (3) and might also be a counterpart of the paramyxovirus SH protein. The function of the SH protein has not been clearly defined. In a fusion assay in cells expressing plasmid-encoded proteins, efficient fusion of CV-1 cells by RSV proteins required the coexpression of the F, G, and SH proteins (19). Several possible functions of the RSV SH protein can be imagined: (i) it might enhance viral attachment or penetration (19); (ii) it might be involved in virion morphogenesis; or (iii) it might have a "luxury" function distinct from a direct role in virus growth, such as interaction with components of the host immune system as recently described for the V protein of Sendai virus (23). Function i or ii might involve an activity that modifies membrane permeability, as has been suggested by others for some hydrophobic proteins of various viruses (6, 27, 36).

The recent development of reverse genetic systems for nonsegmented negative-strand RNA viruses and the successful recovery of the recombinant viruses (14) have provided new methods for analysis of genomes and for investigation of the functions of individual proteins. As a first step to investigate the function of the RSV SH protein, we engineered a full-length DNA copy of the RSV antigenome in which the SH gene was deleted in its entirety. Recombinant virus was recovered and analyzed in vitro and by infection of mice.

## MATERIALS AND METHODS

**Plasmid construction.** The previously described plasmid D46 encodes a complete antigenomic RNA of strain A2 and was used previously to recover recombinant RSV (11). This antigenome is 1 nt longer than the naturally occurring one and contains several restriction site markers. Plasmid D46 was modified in the present study so that the complete SH gene was deleted, yielding plasmid D46/6368. The construction of plasmid D46/6368 involved two parental subclones, D50, which contains a T7 promoter attached to the left-hand end of the genome encompassing the leader region to the L GS, and D39, which contains the downstream end of the M2 gene and the complete L gene attached at the downstream end to a hammerhead ribozyme and tandem T7 transcription terminators. Plasmid D50 was digested with *Sca*I (position 4189 in the complete 15,223-nt antigenome sequence) and *Pac*I (position 4623), and the resulting 435-bp fragment was replaced with a short DNA fragment constructed from two complementary oligonucleotides. The *Sca*I-*Pac*I fragment which was removed included the downstream end of the M gene, including its transcription GE signal, and the SH gene in its entirety except the last six nucleotides of the GE signal (Fig. 1). The short DNA fragment which was inserted in its place was made by annealing the following two partially complementary synthetic oligonucleotides, 5′-**ACT**CAAATAAGTTAATAAAAAATAT*CCCGGG***AT**-3′ (positive-sense strand; the M GE sequence is underlined, an inserted *Xma*I site is shown in italics, and the *Sca*I half-site and *Pac*I sticky end at the left and right, respectively, are shown in boldface) and 5′-*CCCGGG*ATATTTTTTATTAACTTATT TG**AGT**-3′ (negative-sense strand, annotated in the same way as the positive-sense oligonucleotide). The modified D50 plasmid containing the SH deletion is called D50/6368. Thereafter, the *Bam*HI-*Mlu*I fragment of D39 was cloned into the *Bam*HI-*Mlu*I window of D50/6368 to generate the full-length D46/6368 cDNA.

**Transfection, virus growth, passage, plaque purification, and antibody staining of viral plaques.** The procedures were performed as described earlier (4, 11), but with two modifications: (i) cytosine arabinoside, an inhibitor of vaccinia virus, was not used during transfection; and (ii) HEp-2 cells used for transfection were incubated at either 32 or 37°C, and all recovered viruses were propagated at 37°C. Recovered viruses were plaque purified twice before amplification and analysis.

**Total RNA and poly(A)$^+$ RNA isolation.** Cell monolayers from one or two wells of a six-well plate were scraped and resuspended in 100 μl of water, and total intracellular RNA was isolated by using Trizol reagent (Life Technologies) according to the manufacturer's recommendation except that after the isopropanol precipitation, the RNA was extracted twice with phenol-chloroform and then precipitated with ethanol. Poly(A)$^+$ RNA was isolated by using a Oligotex mRNA kit (Qiagen).

**RT-PCR.** The SH gene region was copied into cDNA and amplified. Total intracellular RNA was subjected to reverse transcription (RT) with Superscript II (Life Technologies), using as primer the positive-sense synthetic oligonucleotide 5′-GAAAGTATATATTATGTT-3′. This primer is complementary to nt 3958 to 3975 of the RSV genome, which are upstream of the SH gene. An aliquot of the cDNA product was used as template in PCR using as primer the above-mentioned oligonucleotide together with the negative-sense oligonucleotide 5′-TATATAAGCACGATGATATG-3′. This latter primer corresponds to nt 4763 to 4782 of the genome, which are downstream of the SH gene. An initial 2-min denaturation step was performed, during which the *Taq* DNA polymerase was added, and then 33 cycles were performed (denaturation, 1 min at 94°C; annealing, 1 min at 39°C; elongation, 2 min at 72°C). The products were analyzed on a 2.5% agarose gel.

**Northern blot hybridization.** By using previously described procedures, RNA was separated by electrophoresis on agarose gels in the presence of formaldehyde and blotted to nitrocellulose (18). The blots were hybridized with [$^{32}$P]dCTP-labeled DNA probes of the M, SH, G, F, M2, and L genes which were synthesized individually in vitro from cDNAs by Klenow polymerase with random priming using synthetic hexamers (Boehringer Mannheim). Hybridized radioactivity was quantitated with a Molecular Dynamics model 445 SI PhosphorImager.

**[$^{35}$S]methionine labeling, immunoprecipitation, and SDS-PAGE.** The procedures were performed as described previously (4). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), precast 4 to 20% Tris-glycine gels (Novex) were used.

**In vitro growth analysis.** HEp-2, 293, CV-1, Vero, MRC-5, African green monkey kidney (AGMK-21), bovine turbinate (BT), and MDBK cell monolayers were used for a single-step growth cycle analysis. For each type of cell, three 25-cm$^2$ culture flasks were infected with multiplicity of infection of 2 PFU of the D46/6368 (SH-minus) or D46 (wild-type recombinant) virus per cell. Opti-MEM (Life Technologies) with 2% fetal bovine serum (FBS; Summit) was used for HEp-2, Vero, 293, BT, MRC-5, and AGMK-21 cells; E-MEM (Life Technologies) with 1 or 2% FBS was used for MDBK or BT cells, respectively. After 3 h of adsorption at 37°C, cells were washed three times with 4 ml of medium, 4 ml of medium was added, and the cells were incubated at 37°C with 5% $CO_2$. Then, at various times after inoculation (see Results), 200-μl aliquots of supernatant were removed, adjusted to contain 100 mM magnesium sulfate and 50 mM HEPES buffer (pH 7.5), flash-frozen, and stored at −70°C until titration; each aliquot taken was replaced with an equal amount of fresh medium. For titration,

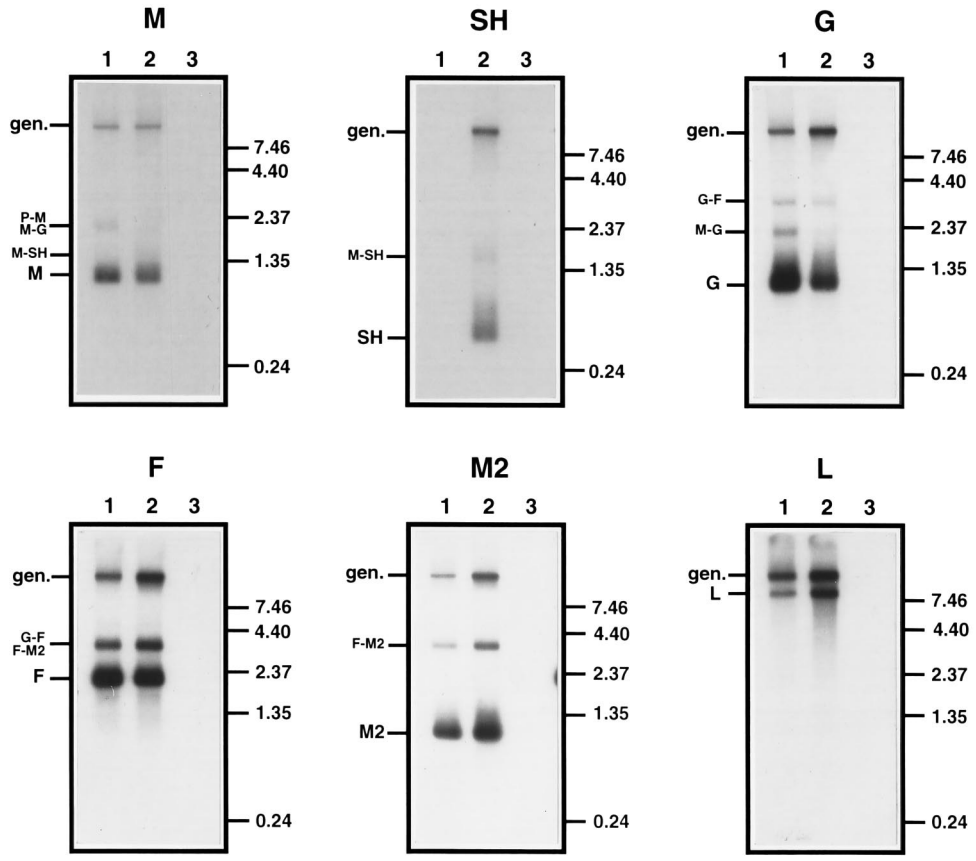


FIG. 4. Northern blot hybridization of RNAs encoded by the D46 wild-type and D46/6368 SH-minus viruses. Total intracellular RNA was isolated from infected cells and subjected to oligo(dT) chromatography without a prior denaturation step, conditions under which the selected RNA also includes genomic RNA due to sandwich hybridization. RNAs were electrophoresed on formaldehyde-agarose gels and blotted onto nitrocellulose membrane. Replicate blots were hybridized individually with $^{32}$P-labeled DNA probes of the M, SH, G, F, M2, or L gene, as indicated. Lanes: 1, D46/6368 RNA; 2, D46 RNA; 3, uninfected HEp-2 cell RNA. Positions of the genomic RNA (gen.), mRNAs (large letters), and readthrough transcripts (small letters) are shown on the left. The positions of readthrough transcripts P-M and M-G (M probe) coincide, as do the positions of G-F and F-M2 transcripts (F probe). The positions of the 0.24- to 9.5-kb RNA ladder molecular weight markers (Life Technologies), which were run in parallel and visualized by hybridization with $^{32}$P-labeled DNA of phage lambda, are shown on the right (with the exception of the 9.5-kb band).

HEp-2 cells (24-well plates) were infected with 10-fold dilutions of each virus and overlaid with Opti-MEM containing 2% FBS and 0.9% methylcellulose (MCB Reagents). After incubation for 7 days, the medium was removed and the cell monolayer was fixed with 80% methanol at 4°C. Plaques were immunostained with a mixture of three monoclonal antibodies specific for the RSV F protein, followed by goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (30).

**Evaluation of virus replication, immunogenicity, and protective efficacy in mice.** Respiratory-pathogen-free 13-week-old BALB/c mice in groups of 24 were inoculated intranasally under light methoxyflurane anesthesia on day 0 with $10^6$ PFU per animal in a 0.1-ml inoculum of wild-type recombinant D46 virus, SH-minus recombinant D46/6368 virus, or biologically derived cold-passaged (*cp*) temperature-sensitive (*ts*) virus *cpts*248/404 (reference 17 and references therein). On days 4, 5, 6, and 8 postinoculation, six mice from each group were sacrificed by $CO_2$ asphyxiation, nasal turbinates and lung tissue were obtained separately and homogenized, and virus titers were determined in plaque assay using the antibody staining procedure described above. To evaluate immunogenicity and protective efficacy, four additional groups of six mice were bled and then inoculated as described above with the wild-type recombinant, its SH-minus derivative, or the *cpts*248/404 virus or were mock infected. Four weeks later, the mice were anesthetized, serum samples were collected, and a challenge inoculation of $10^6$ PFU of biologically derived RSV strain A2 per animal was administered intranasally. Four days later, the animals were sacrificed, and nasal turbinates and lung tissues were harvested and assayed for infectious RSV as described above. Serum immunoglobulin G antibodies which bind to the RSV F protein were quantitated in an enzyme-linked immunosorbent assay (ELISA) using F glycoprotein which had been immunoaffinity purified from cells infected with RSV Long strain (30).

## RESULTS

**Recovered recombinant virus lacking the SH gene: plaque size and syncytium formation.** We previously described the recovery of infectious recombinant RSV strain A2 from plasmid D46, which encodes a full-length RSV antigenome that contains several restriction site markers and is 1 nt longer than the naturally occurring, 15,222-nt genome (11). The SH gene was replaced with a synthetic fragment which represents a copy of the SH-G intergenic sequence with the addition of an *Xma*I site (Materials and Methods; Fig. 1). The *Xma*I site was introduced as a marker and as a potential insertion site for future studies. The resulting plasmid, D46/6368 (Fig. 1), encodes an antigenome that is 14,825 nt, 398 nt (2.6%) shorter than the D46 antigenome (11).

In accordance with published procedures (11), plasmid D46/6368 was cotransfected into HEp-2 cells together with plasmids encoding the N, P, L, and M2 ORF1 proteins, and the cells were simultaneously infected with a recombinant of the MVA strain of vaccinia virus that expresses T7 RNA polymerase (41). Parallel cultures were transfected with the D46 wild-type cDNA under the same conditions. Supernatants were harvested 3 days posttransfection and passaged once, and recom-

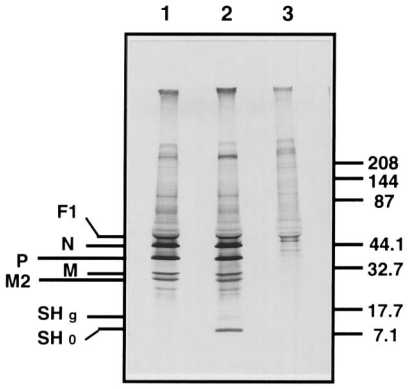


FIG. 5. SDS-PAGE of $^{35}$S-labeled proteins synthesized in uninfected HEp-2 cells (lane 3) and in cells infected with the D46/6368 SH-minus virus (lane 1) or D46 wild-type virus (lane 2). Proteins were subjected to immunoprecipitation with antiserum raised against purified RSV virions and analyzed by electrophoresis in a precast gradient 4 to 20% Tris-glycine gel (Novex). Positions of RSV proteins are indicated to the left; positions and molecular masses (in kilodaltons) of marker proteins (Kaleidoscope Prestained Standards; Bio-Rad), are shown to the right.

binant viruses were plaque purified twice and amplified. The D46 wild-type and D46/6368 SH-minus viruses were inoculated onto HEp-2 cells, incubated for 6 days under methylcellulose overlay, fixed with methanol, and photographed. This analysis revealed a rather striking difference in plaque size (Fig. 2A). Measurement of 30 plaques of each virus showed that the plaques of the D46/6368 virus were on average 70% larger than those of the D46 virus.

Additional HEp-2 cells were infected with the D46 wild-type or D46/6386 SH-minus virus, incubated for 3 days under methylcellulose, and examined by light microscopy. As shown in the photomicrographs in Fig. 2B, the most prominent cytopathic effect of both viruses involved the formation of large syncytia. The ability of the SH-minus virus to form syncytia was undiminished relative to the wild type.

**RT-PCR analysis of recombinant virus.** To confirm the absence of the SH gene in the genome of the recovered D46/6368 virus, cells were infected with the first passage of wild-type or SH-minus recombinant virus, and total intracellular RNA was recovered and analyzed by RT-PCR. RT was performed with a positive-sense primer that annealed upstream of the SH gene at genome positions 3958 to 3975. PCR was performed with the same primer together with a negative-sense primer representing nt 4763 to 4782, which lie downstream of the SH gene. As shown in Fig. 3, wild-type D46 virus yielded a single PCR product corresponding to the predicted 824-bp fragment between positions 3958 and 4782 (lane 3). In the case of the D46/6368 virus, the PCR product was shorter and corresponded to the predicted 426-bp fragment containing the deletion (lane 2). The generation of the PCR products was dependent on the RT step (lane 4), showing that they were derived from RNA rather than DNA, as expected. Thus, RT-PCR analysis demonstrates that the genome of D46/6368 virus contains the expected 398-nt deletion at the SH locus.

TABLE 1. Levels of replication of D46/6368 and D46 viruses in various cell lines

| Cell type | Host | Tissue origin | D46/6368 virus replication compared to that of D46 |
|---|---|---|---|
| HEp-2 | Human | Larynx | Increased |
| 293 | Human | Kidney | Increased |
| MRC-5 | Human | Lung | Similar |
| Vero | Monkey | Kidney | Similar |
| AGMK-21 | Monkey | Kidney | Increased |
| CV-1 | Monkey | Kidney | Similar |
| BT | Bovine | Turbinate | Similar |
| MDBK | Bovine | Kidney | Similar |

**Northern blot analysis of mRNAs.** To examine the transcription of genes located upstream and downstream of the SH gene, poly(A)$^+$ mRNA was isolated from cells infected with the D46 or D46/6368 virus and analyzed by Northern blot hybridization (Fig. 4). The intracellular RNA purposefully was not denatured prior to poly(A)$^+$ selection; thus, as shown below and described previously (7), the selected mRNA also contained genomic RNA due to sandwich hybridization to mRNA. This permitted simultaneous analysis of mRNA and genome, so that the abundance of each mRNA could be normalized to genomic RNA contained in the same gel lane. The blots were hybridized with $^{32}$P-labeled DNA probes which were synthesized from cDNA clones by random priming and thus contained probes of both polarities. The probes individually represented the M, SH, G, F, M2, and L genes.

As expected, the SH probe hybridized to both subgenomic SH mRNA and genomic RNA in the case of the wild-type D46 virus but not for the D46/6368 virus (Fig. 4). The probes specific for other RSV genes hybridized in each case to the genome and to the expected major monocistronic mRNA for both viruses. In addition, a number of previously described dicistronic readthrough mRNAs, such as the F-M2, P-M, and G-F mRNAs, were detected with both viruses. Also, the G-specific probe hybridized to an novel species specific to the D46/6368 virus which appeared to be a readthrough of the M and G genes. This combination was possible due to the deletion of the intervening SH gene. The same species, specific to D46/6368 but not D46, appeared to be hybridized by the M-specific probe, although this identification is somewhat obscured by its comigration with the P-M mRNA, which would be encoded by both D46 and D46/6368 viruses. We then quantitated the relative level of synthesis of each mRNA by D46 and D46/6368. For each pairwise comparison, the amount of mRNA in a given gel lane was normalized to the amount of genome in the same lane. This comparison showed that D46/6368 and D46 expressed the following mRNAs in the indicated ratios (D46/6368 to D46): M, 1.1; G, 3.9; F, 2.5; M2, 1.3; and L, 0.75.

**Protein analysis by radioimmunoprecipitation assay.** To compare the viral proteins synthesized by the D46 and D46/6368 viruses, HEp-2 cells were infected with a multiplicity of infection of 2 PFU per cell and labeled by incubation with [$^{35}$S]methionine from 16 to 20 h postinfection. Cell lysates were prepared and analyzed directly or following immunoprecipitation using a rabbit antiserum raised against purified RSV virions. Total and immunoprecipitated proteins were analyzed by SDS-PAGE on 4 to 20% gradient gels; the pattern of immunoprecipitated proteins is shown in Fig. 5. In the case of the D46 virus, the pattern of immunoprecipitated proteins included the unglycosylated form of SH protein, SH0, and the N-glycosylated form, SHg, whereas neither species was evident for the D46/6368 virus (Fig. 5). The SH0 protein also could be detected in the pattern of total infected-cell proteins in the case of D46 but not D46/6368 (data not shown). Otherwise, the patterns of proteins synthesized by D46 versus D46/6368 were essentially indistinguishable. PhosphorImager analysis of the N, P, M, $F_1$, and M2 proteins in the pattern of immunoprecipitated proteins showed that equivalent amounts were made by both viruses (not shown). It was somewhat surprising that

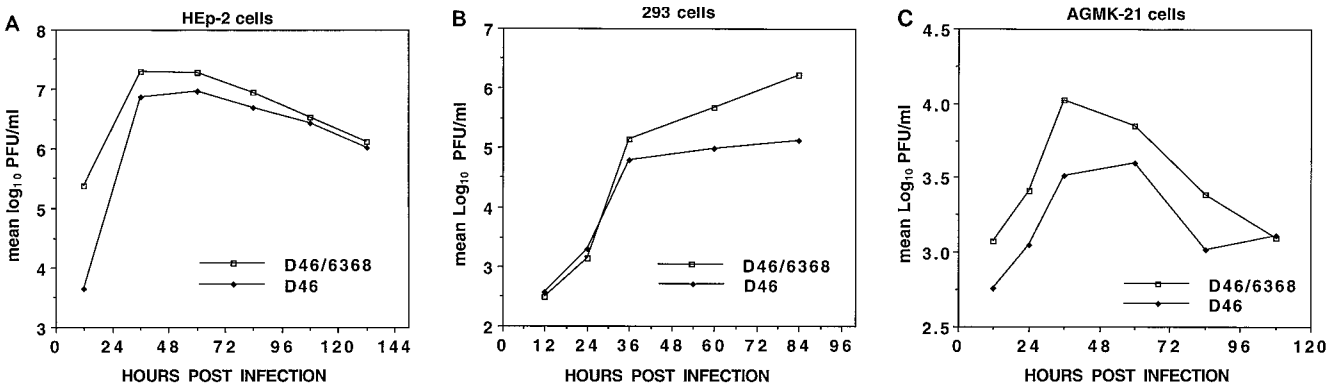


FIG. 6. Growth curves for D46 wild-type and D46/6368 SH-minus viruses in HEp-2 cells (A), 293 cells (B), and AGMK-21 cells (C). Triplicate cell monolayers in 25-cm$^2$ culture flasks were infected with 2 PFU per cell of either virus and incubated at 37°C. Aliquots were taken at indicated time points, stored at −70°C, and titrated in parallel by plaque assay with antibody staining. Each point shown is the mean titer of three supernatants from infected cell monolayers.

the higher relative levels of G and F mRNAs noted above were not associated with correspondingly higher levels of G and F proteins.

**Growth curve analysis in eight cell lines.** Eight different cell lines representing different species and different tissue origins were used to compare efficiencies of replication of the D46 and D46/6368 viruses (Table 1). Triplicate monolayers of each type of cell were infected with either virus, samples were taken at 12- or 24-h intervals, and virus was quantitated by plaque assay facilitated by antibody staining. Surprisingly, the D46/6368 virus grew to higher titer than the D46 wild-type virus in three cell lines, HEp-2, 293, and AGMK-21. In HEp-2 cells (Fig. 6A), the titer of progeny D46/6368 virus at 12 h postinfection was 53 times greater than for the D46 virus, and at 36 h the difference was 2.6-fold. In 293 cells (Fig. 6B), the yield of the D46/6368 virus was 2-fold greater than that of the D46 virus at 36 h postinfection, and this difference increased to 4.8- and 12.6-fold at 60 and 84 h postinfection, respectively. In AGMK-21 cells (Fig. 6C), the yield of D46/6368 virus was 3.2 times that of D46 virus at 36 h postinfection. In MRC-5, Vero, CV-1, MDBK, and BT cells, a significant difference in level of virus replication was not observed. Thus, within this collection of eight cell lines, a moderate host range effect in the growth of the SH-minus virus was observed in three cell lines.

**Replication, immunogenicity, and protective efficacy following intranasal administration to mice.** The D46 and D46/6368 viruses were compared for the ability to replicate in the upper and lower respiratory tracts of mice following intranasal inoculation of $10^6$ PFU. For comparison, the biologically derived *cpts*248/404 vaccine candidate was included (reference 17 and references therein). This virus has been extensively characterized in rodents, chimpanzees, and humans and is highly restricted in replication in the upper and lower respiratory tracts of the mouse. Animals were sacrificed at 4, 5, 6, and 8 days postinoculation, and the nasal turbinates and lung tissues were harvested, homogenized, and analyzed by plaque assay to determine the level of virus replication. In the upper respiratory tract (Fig. 7A), the SH-minus D46/6368 virus exhibited an attenuation phenotype. Specifically, its level of replication was 10-fold lower than that of the wild-type virus and was comparable to that of the *cpts*248/404 virus. In contrast, in the lower respiratory tract (Fig. 7B), the level of replication of the D46/6368 virus was very similar to that of the wild type, whereas the *cpts*248/404 virus was highly restricted, consistent with previous results.

The immunogenicities of the D46 wild-type, D46/6368 SH-minus, and *cpts*248/404 viruses were compared in mice (Table 2). Groups of mice were infected intranasally as described above. On day 0 and 4 weeks later, serum samples were taken and the mice were challenged by the intranasal instillation of $10^6$ PFU per animal of biologically derived wild-type RSV strain A2. Four days later, the mice were sacrificed and the nasal turbinates and lung tissues were harvested and assayed for virus (Table 2). The serum samples were assayed by ELISA against purified F glycoprotein. Mice which had been infected on day 0 with any of the three viruses developed high levels of F-specific serum antibodies and were highly resistant in both the upper and lower respiratory tracts to the replication of RSV challenge virus. Thus, the SH-minus mutant could not be distinguished from its wild-type parent on the basis of its ability to induce RSV-specific serum antibodies and protection in the mouse.

## DISCUSSION

In this study, we deleted the SH gene from a cDNA encoding the RSV antigenome, which was then used to recover infectious recombinant SH-minus RSV. The presence of the deletion in genomic RNA of the recombinant virus was confirmed by RT-PCR. In addition, the recombinant RSV was analyzed by plaque assay with antibody staining, Northern blot hybridization, SDS-PAGE of total and immunoprecipitated proteins, single-step growth curve assay in eight different cell lines, assay of virus replication in the upper and lower respiratory tracts of mice, and evaluation of the ability to induce RSV-specific serum antibodies and resistance to virus challenge. Interestingly, the deletion tended to improve rather than impair growth in tissue culture. Replication in mice was affected only for the upper respiratory tract, where it was reduced 10-fold, and the SH-minus virus was equivalent to its wild-type parent in the ability to induce serum antibodies and protection against virus challenge in the mouse.

It is worth noting that the recombinant virus encoded by the parental D46 cDNA has been shown to have a wild-type phenotype with regard to replication and virulence in immunologically naive chimpanzees, a fully permissive experimental animal (40). This finding indicates that the parental recombinant virus, assembled from a laboratory strain, contains all of the genes required for replication and virulence in a permissive host. This would seem to eliminate the trivial possibility that we might be deleting a gene whose loss is without effect because its encoded function had already been debilitated by mutation during tissue culture passage.

It might have been anticipated that deletion of the SH gene

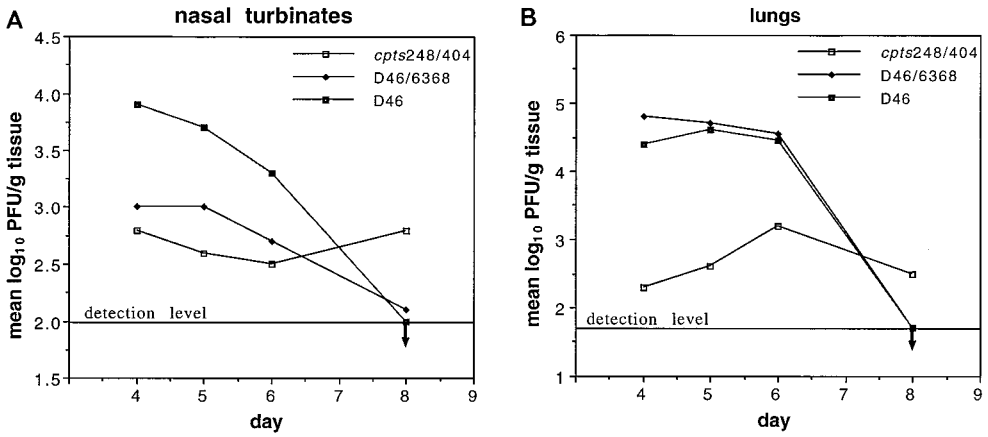


FIG. 7. Kinetics of virus replication in the upper (A) and lower (B) respiratory tracts of mice inoculated intranasally with the D46 wild-type virus, D46/6368 SH-minus virus, or biologically derived *cpts*248/404 virus. Mice in groups of 24 were inoculated intranasally with $10^6$ PFU of the indicated virus. Six mice from each group were sacrificed on the indicated day, the nasal turbinates and lung tissues were removed and homogenized, the level of infectious virus was determined by plaque assay on individual specimens, and mean $\log_{10}$ titers were determined.

would significantly impair RSV growth in tissue culture. For example, studies with cDNA-expressed RSV proteins in tissue culture suggested that the SH protein is important for efficient syncytium formation and, by inference, for virus penetration (19). However, the SH-minus D46/6368 virus was fully capable of forming syncytial plaques. Also, the growth characteristics of the SH-minus virus in tissue culture provided no evidence of a significant impairment in a critical viral function such as penetration, although it is possible that some defect will be uncovered by more detailed characterization such as a kinetic analysis of penetration by preadsorbed virus. Thus, the RSV SH protein is a dispensable accessory protein whose function remains to be identified. One possible model for the SH protein is the Vpu protein of human immunodeficiency virus type 1, which forms ion channels and facilitates virion maturation but is dispensable for virus growth (references 27 and 36 and references therein).

There is a naturally occurring case of a knockout mutation of the SH gene in mumps virus (38). In this case, tissue culture-adapted and egg-adapted isolates of the Enders strain of mumps virus were found to contain a point mutation in the GE signal of the F gene, which is immediately upstream of the SH gene. This results in the synthesis of an F-SH readthrough mRNA to the apparent exclusion of a monocistronic SH mRNA. However, the possibility cannot be excluded that small amounts of SH mRNA are made and that small amounts of SH protein are synthesized either from such an mRNA or from the F-SH dicistronic mRNA. In the present study, the possibility of trace levels of synthesis of the RSV SH protein was precluded by deletion of the entire gene. Also, since the manner in which the mumps virus knockout was acquired during adaptation and passage is unknown, it is possible that second-site mutation occurred to accommodate the loss or reduction of SH protein. In the present work, the efficiencies of recovery of the wild-type and SH-minus viruses were indistinguishable, and from the outset the latter grew better and formed larger plaques than the former. These observations, together with the low-passage history, make the possibility of second-site mutation seem unlikely.

TABLE 2. The RSV D46/6368 SH-minus virus is immunogenic and protects the upper and lower respiratory tract of mice against wild-type challenge

| Immunizing virus[a] | Serum antibody titer[b] (reciprocal of mean $\log_2$) | | RSV A2 replication after challenge[c] (mean $\log_{10}$ PFU/g of tissue) | |
|---|---|---|---|---|
| | Day 0 | Day 28 | Nasal turbinates | Lung |
| D46 | 7.3 | 15.0 | <2.0 | <1.7 |
| D46/6368 | 7.3 | 15.0 | 2.1 | <1.7 |
| *cpts*248/404 | 7.0 | 12.6 | 2.3 | <1.7 |
| None | 7.6 | 7.3 | 4.6 | 5.1 |

[a] Groups of six BALB/c mice were immunized intranasally with $10^6$ PFU of the indicated virus on day 0.

[b] Serum IgG antibody response was quantitated in an ELISA using immunopurified F glycoprotein from RSV subgroup A.

[c] Mice were intranasally administered $10^6$ PFU of RSV A2 on day 28 and sacrificed 4 days later.

Dispensable accessory proteins have been identified for several other nonsegmented negative-strand RNA viruses by recovering recombinant knockout viruses (15, 23, 24, 33, 35). These include the C protein of vesicular stomatitis virus (24) and measles virus (33) and the V protein of Sendai virus (15, 23) and measles virus (35). One difference is that these previous studies involved the insertion of translation termination codons into ORFs or disruption of the RNA editing site. Here, our approach of actually deleting the complete gene is a more reliable means of ensuring the complete absence of expression. These previous examples also differ from the SH gene in that they involve ORFs that overlap with that of P, and therefore their maintenance during in vitro passage is perhaps less surprising. In contrast, the RSV SH gene is encoded by a non-overlapping ORF that is expressed as a separate mRNA. That its ORF has been maintained in a seemingly intact state in various RSV strains during extensive tissue culture passage over four decades is somewhat surprising given that its deletion is without apparent effect in vitro or, in some cases, was associated with improved growth.

The D46 and D46/6368 viruses were similar with regard to the efficiency of RNA replication. The magnitudes of overall transcription were comparable, but Northern blot analysis revealed differences in the accumulation of individual mRNA species. Whereas transcription of the M gene, which is upstream of the SH gene, was essentially the same for the two viruses, transcription of its downstream neighbors, the G and F

genes, was increased in the SH-minus virus. We interpreted this finding in terms of the polar mechanism of gene transcription: in the D46 virus, the G and F genes are seventh and eighth, respectively, in the gene order. Deletion of the SH gene advances them to the sixth and seventh positions, a change which should increase their frequency of transcription. The increase in transcription associated with this change was similar to the 2.5- to 3-fold increase which would be anticipated based on previous studies (26). This observation shows that a change in gene order in a recombinant virus results in a change in the efficiency of transcription.

A more surprising finding was that there seemed to be a steeper gradient of transcriptional polarity for the genes after the G gene (F, M2, and L) in the D46/6368 than in the wild-type D46 virus. We note that in D46/6368, the engineered M-G intergenic region that was left following the deletion is 65 nt in length. In comparison, the longest naturally occurring intergenic regions in strain A2 are the 44-nt M-SH, 46-nt F-M2, and 52-nt G-F intergenic regions, and strain 18537 has an F-M2 intergenic region of 56 nt (22). Previously, we showed that the naturally occurring intergenic regions of strain A2, which range in size from 1 to 52 nt, did not differ significantly with regard to their effects on transcriptional readthrough and polarity in a dicistronic minigenome (25). However, we did not test regions longer than the 46-nt F-M2 region, and it might be that there is an upper limit after which the polymerase is affected more severely. Thus, it might be that the greater length of the intergenic region that was engineered between M and G is responsible for the increased gradient of polarity of transcription of the downstream genes. The idea that a long intergenic region might affect transcription in this way is being further evaluated.

Growth of the SH-minus mutant in tissue culture equaled or exceeded that of the wild-type recombinant virus, with the production of infectious virus being up to 12.6-fold better in 293 cells and the size of the plaques formed in HEp-2 cells under semisolid medium being 70% greater. It is possible that this modest enhancement of growth is a direct consequence of the absence of the SH protein. An alternative possibility is that it is due to the increased expression of the G and F genes, although it is curious that we did not observe an increase in the accumulation of cell-associated G and F proteins. Another possibility is the SH-minus virus grows better because its genome is slightly smaller. We noted previously that the addition of 762 nt of chloramphenicol acetyltransferase sequence resulted in a 20-fold decrease in virus yield and a slight (10%) reduction in plaque size (4). We subsequently have found that a further increase in the size of the insert has the effect of increasing the level of attenuation (5). It is possible that the converse is true; i.e., the small reduction in genome length due to the SH deletion had the effect of enhancing viral growth. With regard to vaccine development, mutations which (i) increase the expression of viral antigens or (ii) improve growth in vitro would be highly desirable.

When inoculated intranasally into mice, the SH-minus virus was indistinguishable from wild type with regard to replication in the lower respiratory tract but was restricted 10-fold in the upper respiratory tract. This result showed that, remarkably, the SH gene is dispensable for growth in vivo. It will be very interesting to determine whether this also is true in a fully permissive experimental animal such as the chimpanzee. The level of restriction in the upper respiratory tract of the mouse was comparable to that of the *cpts*248/404 vaccine candidate virus. It is not yet known whether the attenuation of the SH-minus mutant is due to the loss of the SH protein, the change in gene transcription, the increase in intergenic length, or the decrease in genome size. Experiments to distinguish between these possibilities are in progress. However, the SH-minus and *cpts*248/404 viruses resembled the recombinant wild-type virus in the ability to induce high levels of RSV-specific serum antibodies and protection in both the upper and respiratory tracts against challenge virus replication. Thus, the SH protein does not appear to contribute to the antigenic structure of the RSV F and G proteins, which are the major protective antigens.

The finding that the SH-minus virus exhibits site-specific attenuation in the upper respiratory tract has implications for development of a live attenuated recombinant vaccine virus (reference 17 and references therein). A vaccine virus that is restricted at that site might be particularly desirable in the case of young infants, because this vulnerable age group breathes predominantly through the nose. Also, the incidence of otitis media might be reduced. As a general principle, it is desirable to include various types of mutations in a recombinant vaccine virus, and an SH-minus deletion mutation will be a useful addition to the *ts* and *cp* host range mutations which exist in current RSV vaccine virus candidates (17). These different mutations could be readily placed together in a recombinant vaccine virus. Furthermore, the deletion of nearly 400 nt encoding an entire mRNA represents a type of mutation which should be highly refractory to reversion.

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