JOURNAL OF VIROLOGY, Oct. 1996, p. 6634–6641 Vol. 70, No. 10
0022-538X/96/$04.00+0

Recovery of Infectious Respiratory Syncytial Virus Expressing an Additional, Foreign Gene

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Received 22 March 1996/Accepted 17 June 1996

A previous report described the recovery from cDNA of infectious recombinant respiratory syncytial virus (RSV) strain A2 (P. L. Collins, M. G. Hill, E. Camargo, H. Grosfeld, R. M. Chanock, and B. R. Murphy, Proc. Natl. Acad. Sci. USA, 92:11563–11567, 1995). Here, the system was used to construct recombinant RSV containing an additional gene encoding chloramphenicol acetyltransferase (CAT). The CAT coding sequence was flanked by RSV-specific gene-start and gene-end motifs, the transcription signals for the viral RNA-dependent RNA polymerase. The RSV-CAT chimeric transcription cassette was inserted into the region between the G and F genes of the complete cDNA-encoded positive-sense RSV antigenome, and infectious CAT-expressing recombinant RSV was recovered. Transcription of the inserted gene into the predicted subgenomic polyadenylated mRNA was demonstrated by Northern (RNA) blot hybridization analysis, and the encoded protein was detected by enzyme assay and by radioimmunoprecipitation. Quantitation of intracellular CAT, SH, G, and F mRNAs showed that the CAT mRNA was efficiently expressed and that the levels of the G and F mRNAs (which represent the genes on either side of the inserted CAT gene) were comparable to those expressed by a wild-type recombinant RSV. Consistent with this finding, the CAT-containing and wild-type viruses were very similar with regard to the levels of synthesis of the major viral proteins. Each of 25 RSV isolates obtained by plaque purification following eight serial passages expressed CAT, showing that the foreign gene was faithfully maintained in functional form. Analysis by reverse transcription and PCR did not reveal evidence of deletion of the foreign sequence. This finding demonstrated that the RSV genome can accept and maintain an increase in length of 762 nucleotides of foreign sequence and can be engineered to encode an additional, 11th mRNA. The presence of the additional gene resulted in a 10% decrease in plaque diameter and was associated with delay in virus growth and 20-fold decrease in virus yield in vitro. Thus, introduction of an additional gene into the RSV genome might represent a method of attenuation. The ability to express foreign genes by recombinant RSV has implications for basic studies as well as for the development of live recombinant vaccines.

Human respiratory syncytial virus (RSV) is a prototype member of genus *Pneumovirus* of family *Paramyxoviridae*, order *Mononegavirales* (the nonsegmented negative-strand RNA viruses). RSV is the most important viral pediatric respiratory tract pathogen, causing serious bronchiolitis or pneumonia with the peak incidence between 2 and 7 months of age and with frequent reinfections. An effective vaccine against RSV is not yet available (reference 6 and references therein). Studies with RSV are impeded by its instability and poor growth in tissue culture.

Direct manipulation of the genome of an RNA virus through a DNA intermediate was first demonstrated by Taniguchi et al. in 1978 with the positive-sense RNA bacteriophage Qβ (35) and was shown by Racaniello and Baltimore in 1981 with the mammalian positive-sense RNA poliovirus (29). The flow diagram of these experiments was as follows: virion genomic RNA → full-length cDNA → genetic manipulations → cDNA-encoded genomic RNA → recombinant virus.

For negative-strand RNA viruses, this approach was precluded by the lack of infectivity of naked genomic RNA. Instead, the minimum unit of infectivity is a viral nucleocapsid competent to begin the cycle of RNA transcription and replication. Thus, cDNA-encoded genomic RNA would need to be associated with the virus-specific proteins of the nucleocapsid and the polymerase complex. This approach was developed first with systems based on analogs of genomic RNA, or minigenomes. These were made competent for transcription and RNA replication by complementation with the proteins of the nucleocapsid and polymerase complex. In the case of the segmented influenza virus, the complementing proteins were purified from standard virus, whereas in the case of nonsegmented negative-strand viruses, it was necessary to deliver the proteins by infection with standard virus or from transfected cDNAs (2, 7, 8, 15, 19, 23, 24, 33, 38). As the next step of this successful strategy, single cDNA-encoded gene segments have been introduced into infectious influenza virus (10, 12, 25), and complete infectious recombinant virus has been recovered from full-length cDNA for several nonsegmented negative-strand viruses, namely, rabies virus (32), vesicular stomatitis virus (18, 36), RSV (4), measles virus (30), and Sendai virus (13).

The genome of RSV is a single strand of negative-sense RNA of 15,222 nucleotides. RSV RNA replication is directed by the nucleocapsid N, phosphoprotein P, and polymerase L proteins; transcription requires in addition the M2 protein, which has been identified as a transcription elongation factor (5). As in other nonsegmented negative-strand RNA viruses, the RSV proteins are encoded by individual subgenomic mRNAs which are synthesized by sequential stop-start transcription guided by the gene-start (GS) and gene-end (GE)

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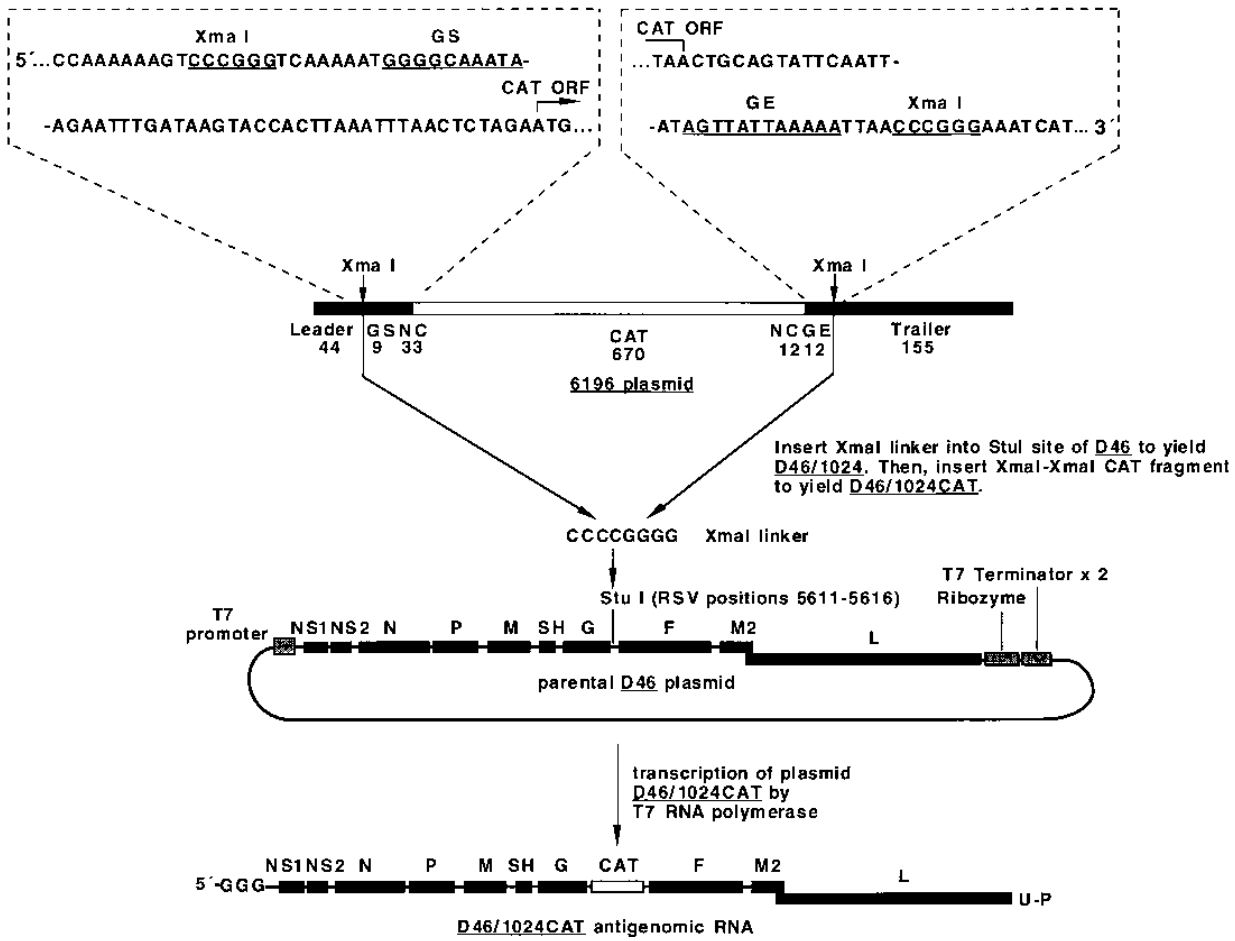

FIG. 1. Construction of D46/1024CAT cDNA encoding an RSV antigenome containing the CAT ORF flanked by RSV transcription signals (not to scale; RSV-specific segments are shown as filled boxes, and the CAT sequence is shown as an open box). The source of the CAT gene transcription cassette was RSV-CAT minigenome cDNA 6196 (diagram at the top). The RSV-CAT minigenome contains the leader region, GS, and GE signals, noncoding (NC) RSV gene sequences, and the CAT ORF, with *Xma*I restriction endonuclease sites preceding the GS signal and following the GE signal. The nucleotide lengths of these elements are indicated, and the sequences (positive sense) surrounding the *Xma*I sites are shown above the diagram. An eight-nucleotide *Xma*I linker was inserted into *Stu*I site of the parental plasmid D46 to construct the plasmid D46/1024. The *Xma*I-*Xma*I fragment of plasmid 6196 was inserted into plasmid D46/1024 to construct plasmid D46/1024CAT. The RNA encoded by the D46 cDNA, including the three 5′-terminal nonviral G residues contributed by the T7 promoter and the 3′-terminal phosphorylated U residue contributed by cleavage of the hammerhead ribozyme (4), is shown at the bottom; the nucleotide lengths given in this report do not include these nonviral nucleotides. The L gene is drawn offset to indicate the gene overlap.

motifs, the regulatory elements for viral RNA-dependent RNA polymerase (references 4 to 6 and references therein).

It was unknown whether the genome of a nonsegmented negative-strand RNA virus could be used to express an additional, foreign gene. A number of impediments were conceivable, including constraints on an increase in nucleotide length, possible deleterious effects from adding additional transcription signals encoding an additional mRNA, or rapid inactivation of the foreign gene by nucleotide deletion or substitution as a result of an error-prone polymerase. In the work described in this report, the translational open reading frame (ORF) encoding the chloramphenicol acetyltransferase (CAT) protein was engineered to be flanked by the RSV GS and GE signals. This transcription cassette was inserted into the G-F intergenic region of a cDNA encoding RSV antigenomic RNA. Recombinant RSV which expressed the CAT ORF as an additional subgenomic mRNA was recovered.

MATERIALS AND METHODS

Plasmid construction. A copy of the CAT ORF flanked by the RSV GS and GE transcription signals was derived from the previously described RSV-CAT H15 minigenome (7, 17). The H15 minigenome contains the CAT ORF bordered on the upstream end by the nonstructural NS1 protein gene GS and noncoding sequences and on the downstream end by L gene noncoding and GE sequences. This was modified as part of a mutational analysis to contain the insertion of an *Xma*I site four nucleotides downstream of the GE signal (14a). A second *Xma*I site was placed eight nucleotides upstream of the GS signal by inserting a short synthetic DNA fragment into a naturally occurring *Bst*XI site to yield plasmid 6196 (Fig. 1). The synthetic DNA was constructed by annealing two partially complementary synthetic oligonucleotides, 5′-GTCCCGGGTCAAAA -3′ (positive-sense strand; the *Xma*I site is underlined) and 5′-GACCCGGGA CTTTT-3′ (negative-sense strand). In the original construction of plasmid D46, which encodes the previously described 15,223-nucleotide antigenome, a new *Stu*I site was placed in the G-F intergenic region (4). This site was modified by the insertion of an *Xma*I linker constructed from a self-complementary oligonucleotide 5′-CCCCGGGG-3′. This resulted in plasmid D46/1024, which encodes an antigenome of 15,231 nucleotides. Finally, the 753-nucleotide *Xma*I-*Xma*I fragment of plasmid 6196, containing the CAT ORF flanked by the GS and GE signals, was inserted into *Xma*I site of plasmid D46/1024 to yield D46/1024CAT, encoding an antigenome of 15,984 nucleotides (Fig. 1). The insertions were confirmed by restriction mapping and/or DNA sequencing. Standard molecular biological methods were used (31).

Transfection, virus growth, and passages. Transfection experiments were carried out as described previously (4). Briefly, HEp-2 cells (six-well plates) were infected at 1 focus-forming unit per cell with strain MVA vaccinia virus expressing T7 RNA polymerase (37). A mixture of four plasmids containing the RSV N, P, L, or M2 (ORF1) ORF under the control of the T7 promoter (0.4, 0.4, 0.2, 0.2

μg per well, respectively) and a fifth plasmid encoding either the D46 wild-type or D46/1024CAT antigenome (0.4 μg) was transfected with LipofectACE (Life Technologies) as recommended by the supplier. Cells were incubated in a CO_2 incubator at 32°C. Twenty-four hours later, the medium was replaced with Opti-MEM medium (Life Technologies) containing 2% fetal bovine serum and 40 μg of cytosine arabinoside, per ml, added at 2 ml per well. After 48 h, medium supernatants were passaged to fresh HEp-2 cells. Six days later, when cytopathology was apparent, the cells were harvested and one-eighth of the medium supernatant was passaged to fresh cells in 25-cm^2 flasks. This was followed by a total of eight serial passages, at a passage interval of 5 to 6 days. The cell pellets were saved for CAT assay and RNA isolation. Cytosine arabinoside was maintained for the first six passages; the first six passages were performed at 32°C in 25-cm^2 flasks, and the last two passages were performed in 162-cm^2 flasks.

Virus was isolated by plaque purification using an overlay of Eagle minimal essential medium (Quality Biologicals, Inc.) containing 2% fetal bovine serum and 0.75% agarose. Plaque assays for visualization by antibody staining were performed with an overlay consisting of Opti-MEM medium supplemented with 2% fetal bovine serum and 0.9% methylcellulose (MCB Reagents). After incubation for 5 to 7 days at 32 or 37°C, the overlay was removed and the monolayer was fixed with 80% methanol (4°C). The plaques were incubated with a mixture of murine monoclonal antibodies 1129, 1243, and 1269, specific to the RSV F protein, and then with sheep anti-mouse immunoglobulin G labeled with horseradish peroxidase (21).

CAT assay and analysis of the stability of CAT expression. Cell pellets were used to prepare lysates, which were analyzed for the ability to acetylate [^{14}C]chloramphenicol as visualized by thin-layer chromatography (7, 14). To study the stability of CAT expression during passage of recombinant virus, 20 plaques were picked from the third passage and 25 were picked from the eighth passage. Each plaque was picked only when that particular well contained only one or a few well-separated plaques; the low yield of virus in vitro also minimizes cross-contamination. Each plaque was amplified in 25-cm^2 flasks for CAT assay, and as necessary virus growth was monitored by plaque assay.

Isolation of total RNA and poly(A)$^+$ RNA. Cell pellets were resuspended in 100 μl of water, and RNA was extracted with Trizol reagent (Life Technologies) as recommended by the manufacturer except that following the isopropanol precipitation, the RNA was extracted twice with phenol-chloroform and precipitated with ethanol. Poly(A)$^+$ mRNAs were isolated from the samples of total RNA by using an Oligotex mRNA kit (Qiagen).

RT in conjunction with PCR (RT-PCR). One microgram of total infected-cell RNA was used as the template for reverse transcription (RT) in a 50-μl reaction primed with the synthetic oligonucleotide 5′-ACATCATAACTACACTAC-3′, which is positive sense and represents positions 5412 to 5429 in the 5′-to-3′ wild-type antigenome sequence. Then, 15 μl of the reaction mixture was used for PCR in a 100-μl reaction mixture containing 1 μg of the above-mentioned oligonucleotide and 1 μg of a downstream, negative-sense oligonucleotide, 5′-AGCAAAACAAAATGTGACTG-3′, which represents nucleotides 5730 to 5711 in the 3′-to-5′ sequence of the wild-type genome. Thus, the two primers flank the *Stu*I insertion site located at positions 5611 to 5616. The PCR included a 4-min initial denaturation step (94°C) during which the polymerase was added, followed by 33 cycles (denaturation at 94°C for 1 min, annealing at 37°C for 1 min, and elongation at 72°C for 2 min).

Northern blot hybridization. The procedure for Northern (RNA) blot hybridization was described in detail elsewhere (15). Briefly, RNA samples were subjected to electrophoresis on 1.5% agarose gels containing 0.44 M formaldehyde (31), transferred to nitrocellulose (3), and used for hybridization with [^{32}P]-CTP-labeled riboprobes synthesized by runoff transcription of cloned cDNA in vitro. The negative-sense CAT probe was synthesized by in vitro transcription of linearized RSV-CAT C2 minigenome plasmid (15), and the SH, G, and F negative-sense probes were synthesized from linearized plasmids containing these genes under the control of T7 RNA polymerase. Quantitation of the amount of hybridized radioactivity was performed with a Molecular Dynamics PhosphorImager 445 SI.

Radiolabeling, immunoprecipitation, and polyacrylamide gel electrophoresis (PAGE). HEp-2 cell monolayers grown in six-well plates were infected with D46/1024CAT or D46 virus at a multiplicity of infection of 5 PFU per cell. At 20 h postinfection, the cells were washed three times with methionine-free RPMI medium 1640 (Life Technologies) and incubated for 4 h in the same medium containing 100 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham) per ml. The labeled cells were washed three times with cold phosphate-buffered saline and lysed in 0.5 ml of cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8.0], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1.0% Triton X-100) per well. The lysates were clarified by centrifugation at 7,000 × *g* for 5 min and frozen at −70°C until use. Aliquots (20 μl) of each lysate were combined with 30 μl of RIPA buffer and either (i) 5 μl of rabbit antiserum raised against gradient-purified RSV (5, 15), (ii) 5 μl of purified CAT-specific polyclonal rabbit antibody (5 Prime-3 Prime), or (iii) 5 μl each of F-specific murine monoclonal antibodies 1129, 1243, and 1269 (21) and then incubated at room temperature for 2 h. To precipitate immune complexes, 30 μl of *Staphylococcus aureus* protein A bound to agarose beads (Calbiochem) was added, and samples were shaken at 4°C for 1 h. The beads were collected by centrifugation for 20 s at 7,000 × *g*, the supernatants were saved for a second round of immunoprecipitation (below), and the beads were washed twice with cold RIPA buffer. Proteins were eluted by boiling in 35 μl of sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and analyzed by electrophoresis on precast 8 to 16% gradient polyacrylamide Tris-glycine minigels (Novex). Following electrophoresis, the gels were fixed for 30 min in 30% methanol–10% acetic acid, dried, and exposed to X-ray film or analyzed by phosphor imagery. When the supernatant remaining after the first precipitation of immune complexes with protein A beads was combined with additional antibody for a second round of immunoprecipitation, the amount of recovered protein was less than 2% of the amount from original precipitation. This result showed that the initial recovery of antigen had been essentially complete.

RESULTS

Construction of cDNA encoding RSV antigenomic RNA containing the CAT gene. We previously constructed cDNA D46, which encodes a complete, 15,223-nucleotide RSV antigenome (one nucleotide longer than that of wild-type RSV) and which was used to produce recombinant infectious RSV (4). During its construction, the antigenome cDNA had been modified to contain four new restriction sites as markers. One of these, a *Stu*I site placed in the region between the G and F genes (positions 5611 to 5616 in the 3′-5′ sequence of the wild-type genome), was chosen for evaluation here as an insertion site for the foreign CAT gene. A copy of the CAT ORF flanked on the upstream end by the RSV GS signal and on the downstream end by the RSV GE signal was derived from a previously described RSV-CAT minigenome (Materials and Methods). The insertion of this RSV-CAT transcription cassette into the *Stu*I site, to yield the D46/1024CAT cDNA, increased the length of the encoded antigenome to a total of 15,984 nucleotides. Also, whereas wild-type RSV encodes 10 major subgenomic mRNAs, the recombinant virus predicted from the D46/1024CAT antigenome would encode in addition the CAT gene as an 11th mRNA. The strategy of construction is shown in Fig. 1.

Recovery of infectious virus. The strategy for producing infectious RSV from cDNA-encoded antigenomic RNA was described in detail previously (4). It involved the coexpression in HEp-2 cells of the five cDNAs encoding the antigenomic RNA and the N, P, L and M2 (ORF1) proteins, which are necessary and sufficient for viral RNA replication and transcription. cDNA expression was driven by T7 RNA polymerase supplied by a vaccinia-T7 recombinant virus based on the MVA strain, which is a host range mutant that does not produce infectious virus efficiently in mammalian cells (37). Despite this host range restriction, the MVA-T7 recombinant virus produced an amount of infectious progeny that was sufficient to cause extensive cytopathogenicity upon passage, perhaps a consequence of the long incubation necessitated by the slow growth of RSV (not shown). Therefore, cytosine arabinoside, an inhibitor of vaccinia virus replication, was added 24 h following the transfection and maintained during the first six passages.

Two antigenome cDNAs were tested for the recovery of RSV: the previously described D46 cDNA and the D46/1024CAT cDNA. Each one yielded infectious recombinant RSV, and as described below, cells infected with the D46/1024CAT recombinant virus expressed abundant levels of CAT enzyme. For each virus, transfection supernatants were passaged to fresh cells, and a total of eight serial passages were performed at intervals of 5 to 6 days and multiplicities of infection of less than 0.1 PFU per cell.

Analysis of mRNAs by Northern blot hybridization. The CAT sequence in the D46/1024CAT genome was flanked by RSV GS and GE signals and thus should be expressed as an additional, separate, polyadenylated mRNA. The presence of this predicted mRNA was tested by Northern blot hybridiza-

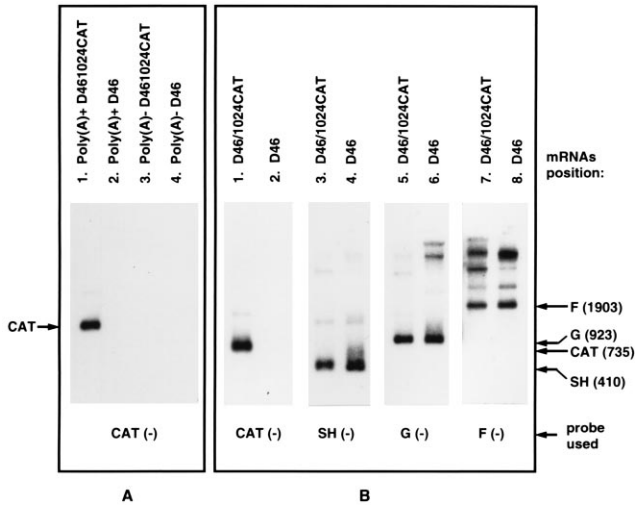

FIG. 2. Northern blot hybridization of intracellular mRNAs encoded by recombinant D46 or D46/1024CAT RSV. Total intracellular RNA was isolated and separated into poly(A)$^+$ and poly(A)$^-$ fractions by oligo(dT) chromatography. RNAs were electrophoresed on formaldehyde-agarose gels and analyzed by Northern blot hybridization with the indicated negative-sense riboprobe. (A) Hybridization with a CAT-specific probe of the poly(A)$^+$ and poly(A)$^-$ fractions encoded by the two viruses. Lane 1, poly(A)$^+$ RNA, D46/1024CAT virus; lane 2, poly(A)$^+$ RNA, D46 virus; lane 3, poly(A)$^-$ RNA, D46/1024CAT virus; lane 4, poly(A)$^-$ RNA, D46 virus. (B) Hybridization of poly(A)$^+$ mRNA encoded by the D46/1024CAT virus (lanes 1, 3, 5, and 7) or D46 virus (lanes 2, 4, 6, and 8) with riboprobe of the following specificities: lanes 1 and 2, CAT; lanes 3 and 4, SH; lanes 5 and 6, G; lanes 7 and 8, F. The predicted nucleotide length of each mRNA excluding poly(A) is indicated.

tion of RNA from cells infected with D46/1024CAT virus or D46 virus, each at passage 8 (Fig. 2).

Hybridization with a negative-sense CAT-specific riboprobe detected a major band which was of the appropriate size to be the predicted CAT mRNA, which would contain 735 nucleotides not including poly(A). This species was completely retained by oligo(dT) latex particles, showing that it was polyadenylated (Fig. 2A). In some cases, a minor larger CAT-specific species which was of the appropriate size to be a G-CAT readthrough mRNA was detected (Fig. 2B, lane 1). The D46/1024CAT virus had been subjected to eight passages at low multiplicities of infection prior to the infection used for preparing the intracellular RNA shown in Fig. 2. It is noteworthy that there was no evidence of shorter forms of the CAT mRNA, as might have arisen if the CAT gene was subject to deletion.

Replicate blots were hybridized with negative-sense riboprobe specific to the CAT, SH, G, or F gene (Fig. 2B). The latter two genes were of particular interest because they flanked the inserted CAT gene. This analysis showed that the levels of expression of the subgenomic SH, G, and F mRNAs appeared to be similar for the two viruses. To analyze this further, phosphor imagery was used to compare the amounts of hybridized radioactivity in the three RSV mRNA bands for D46/1024CAT and D46. This strategy could not be used to determine the relative molar amounts of the various mRNAs because a different probe was used for each. However, the ratio of radioactivity between D46/1024CAT and D46 was determined for each mRNA: SH, 0.77; G, 0.87; and F, 0.78. The deviation from unity probably indicates that slightly less RNA was loaded for D46/1024CAT than for D46, although it also is possible that the overall level of mRNA accumulation was slightly less for D46/1024CAT RSV. More importantly, the demonstration that the three ratios were similar confirms that the levels of expression of each of these mRNAs were approximately the same for D46/1024CAT and D46. Thus, the insertion of the CAT gene between the G and F genes did not, for example, drastically affect the level of transcription of either gene.

Analysis of proteins by radioimmunoprecipitation assay. To characterize viral protein synthesis, infected HEp-2 cells were labeled with [^{35}S]methionine, and cell lysates were analyzed by PAGE either directly or following immunoprecipitation under conditions (see Materials and Methods) in which the recovery of labeled antigen was essentially complete (Fig. 3). Precipitation with a rabbit antiserum raised against purified RSV showed that the D46/1024CAT and D46 viruses expressed similar amounts of the major viral proteins F_1, N, P, M, and M2 (Fig. 3A). That similar levels of M2 protein were recovered for both viruses was noteworthy because the M2 gene is downstream of the inserted CAT gene. Accumulation of the F protein, which is encoded by the gene located immediately downstream of the insertion, also was examined by immunoprecipitation with a mixture of three anti-F monoclonal antibodies. Similar levels of the F_1 subunit were recovered for both viruses (Fig. 3A). Phosphor imagery analysis of the major viral proteins mentioned above was performed for several independent experiments (not shown). This analysis showed that there was sample-to-sample variability, but overall the two viruses could not be distinguished on the basis of the level of recovered proteins. Precipitation with anti-CAT antibodies recovered a single species for the D46/1024CAT virus but not for the D46 virus (Fig. 3B). Analysis of the total labeled protein showed that the N, P, and M proteins could be detected without immunoprecipitation (although detection of the latter was complicated by its comigration with a cellular species) and confirmed that the two viruses yielded similar patterns. The position corresponding to that of the CAT protein contained more radioactivity in the D46/1024CAT pattern compared with that of D46, as was confirmed by PhosphorImager analysis of independent experiments. This finding suggested that the CAT protein could be detected among the total labeled proteins without precipitation, although this demonstration was complicated by the presence of a comigrating background band in the uninfected and D46-infected patterns (Fig. 3B).

Analysis of the CAT gene insert by RT-PCR of the genome of recovered virus. RT-PCR was used to confirm the presence

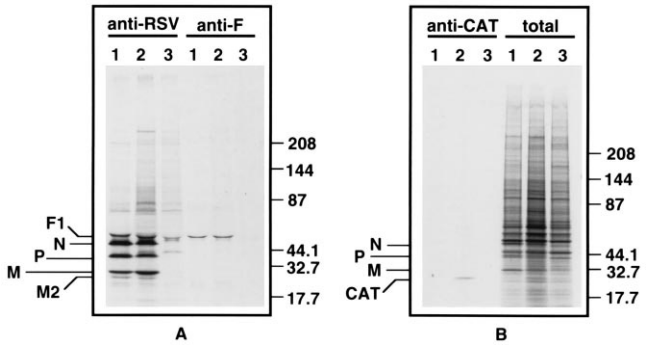

FIG. 3. Analysis of [^{35}S]methionine-labeled viral proteins expressed by recombinant D46 or D46/1024CAT RSV. Lane 1, D46 virus; lane 2, D46/1024CAT virus; lane 3, noninfected cells. Proteins were precipitated as indicated with anti-RSV rabbit serum (A), anti-F monoclonal antibodies (A), or anti-CAT polyclonal antibodies (B). Also, aliquots of total protein were analyzed in parallel (B). Positions of viral proteins are indicated at the left; molecular masses of marker proteins (in kilodaltons) are indicated at the right.

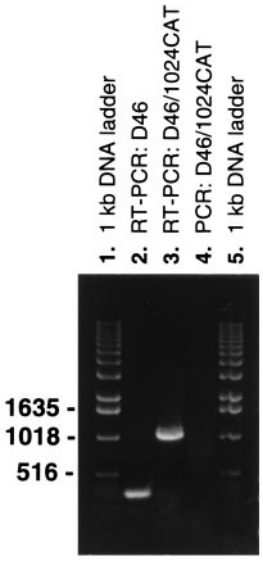

FIG. 4. Confirmation of the location of the CAT gene insert in the recombinant RSV genome. Intracellular RNA was purified from cells infected with the eighth passage of the D46 or D46/1024CAT virus and analyzed by RT-PCR using primers which flanked the insertion site in the G-F intergenic region. The PCR products were analyzed on a neutral 1% agarose gel and stained with ethidium bromide. Lanes: 1 and 5, commercially prepared DNA ladder (1 kb ladder; Life Technologies) (several of the bands are identified at the side by length in base pairs); lane 2, RT-PCR products of D46 RSV; lane 3, RT-PCR products of D46/1024CAT RSV; lane 4, PCR of D46/1024CAT RSV without the RT stage.

of the CAT gene in the predicted location of the genome of recombinant RSV. Total intracellular RNA was isolated from the cell pellet of passage 8 of both D46/1024CAT and D46 RSV. Two primers that flank the site of insertion, the *Stu*I restriction endonuclease site at RSV positions 5611 to 5616, were chosen; the upstream positive-sense primer corresponding to positions 5412 to 5429, and the downstream negative-sense primer corresponded to positions 5730 to 5711. The positive-sense primer was used for the RT step, and both primers were included in the PCR.

As shown in Fig. 4, RT-PCR of the D46 virus (lane 2) yielded a single product that corresponded to the predicted fragment of 318 nucleotides, which would represent the G/F gene junction without additional foreign sequence. Analysis of D46/1024CAT viral RNA yielded a single product whose electrophoretic mobility corresponded well with that of the predicted 1,079-nucleotide fragment, which would represent the G/F gene junction containing the inserted CAT transcription cassette. It is noteworthy that the latter PCR yielded a single major band; the absence of detectable smaller products indicated that the population of recombinant genomes did not contain a large number of molecules with a deletion in this region. When PCR analysis was performed on D46/1024CAT virus RNA without the RT step, no band was seen (lane 4), confirming that the analysis was specific to RNA. Thus, the RT-PCR analysis confirmed the presence of an insert of the predicted length in the predicted location in the genomic RNA of the D46/1024CAT recombinant virus.

Stability of the CAT gene measured by enzyme expression. Cell pellets from all of the passages beginning with the third were tested for CAT expression. For the D46/1024CAT virus, all of these assays displayed conversion of ^{14}C-labeled chloramphenicol into acetylated forms (Fig. 5). To investigate stability of CAT expression, virus from 20 or 25 individual plaques from passage 3 to 8, respectively, was analyzed for CAT expression. All samples were positive. Importantly, the levels of expression of CAT were similar for all of the 25 isolates from passage 8, as judged by assay of equivalent aliquots of cell lysate (not shown). This finding indicated that the activity of the CAT protein encoded by each isolate remained unimpaired by mutation.

Antibody staining of plaques. Beginning with the second passage, one-eighth of the medium supernatant (i.e., 0.5 ml) harvested from each passage stage was used to infect fresh HEp-2 cells in six-well plates that were incubated under methylcellulose overlay for 5 to 7 days. Then, the cells were fixed and stained by incubation with monoclonal antibodies against RSV F protein followed by a second antibody linked to horseradish peroxidase.

Recombinant RSV produced from cDNA D46 in previous work was indistinguishable from a naturally occurring wild-type RSV isolate with regard to efficiency of plaque formation over a range of temperatures in vitro and to the ability to replicate and cause disease when inoculated into the respiratory tracts of previously uninfected chimpanzees (36a). For these reasons, the D46 recombinant RSV was considered to be a virulent wild-type strain. The plaques produced by the D46 and D46/1024CAT recombinant viruses were compared by antibody staining. Photomicrographs of typical antibody-stained plaques of RSV recovered from the D46/1024CAT or D46 plasmid are presented in Fig. 6. Plaque morphologies were very similar for the two viruses, although the average diameter of the CAT-containing recombinant plaques was 90% of that of the D46 virus, as determined by measurement of 30 randomly-selected plaques for each virus. The effect of foreign sequence on plaque size will be examined in greater detail in future work with different and larger inserts.

Growth of the RSV-CAT recombinant virus in vitro. The efficiencies of replication in tissue culture of the D46 and D46/1024CAT viruses were compared in a single-step growth cycle. Triplicate monolayers of cells were infected with either virus, and samples were taken at 12-h intervals and quantitated by plaque assay (Fig. 7). This analysis showed that the produc-

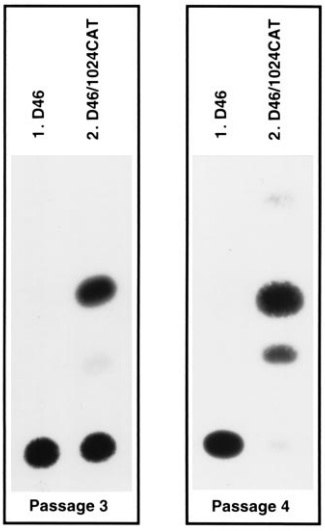

FIG. 5. CAT expression by recombinant D46 (lane 1) or D46/1024CAT (lane 2) RSV during passage 3 or passage 4. Equal cell equivalents of lysate were analyzed for acetylation of [^{14}C]chloramphenicol as visualized by thin-layer chromatography.

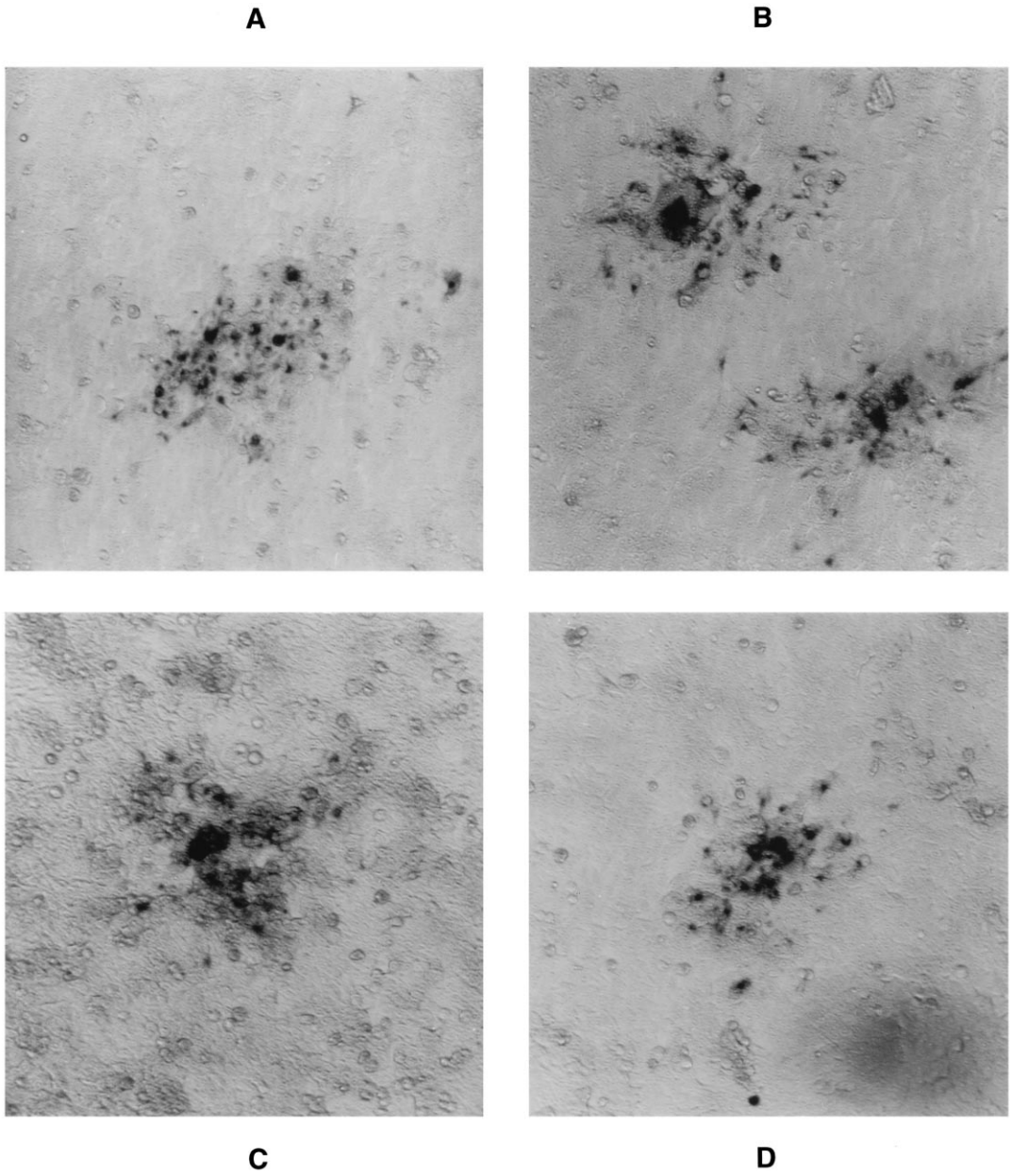

FIG. 6. Photomicrographs of plaques of D46 (A and B) and D46/1024CAT (C and D) viruses. Medium supernatants were harvested following the second passage, and aliquots were used to infect HEp-2 cells, which were overlaid with methylcellulose, incubated for 5 days at 32°C, fixed, and stained with a mixture of three monoclonal antibodies against the F protein of RSV.

tion of D46/1024CAT virus relative to that of D46 virus was delayed and achieved a maximum titer which was 20-fold lower.

DISCUSSION

This report shows that it is possible to construct recombinant, helper-independent RSV expressing a foreign gene, in this instance the CAT gene. The CAT gene was flanked by the RSV GS and GE transcription signals. This transcription cassette was inserted into the antigenomic cDNA such that the genomic RNA of recovered virus would contain CAT as an additional gene situated in the region between the G and F genes. Recombinant RSV was recovered by the intracellular synthesis of the antigenomic RNA together with the N, P, M2, and L proteins, each encoded by separate transfected plasmids whose expression was directed by bacteriophage T7 RNA polymerase supplied by a vaccinia virus recombinant (4). The recombinant RSV directed expression of the predicted polyadenylated subgenomic mRNA that encoded CAT protein that was detected both by enzyme assay and by radioimmunoprecipitation. Expression of CAT gene was shown to be stable through passage 8 (the highest passage level tested).

The presence of the additional gene was associated with a lag in the appearance of virus and a 20-fold decrease in virus production during a single-step growth curve. We now have recovered RSV recombinants with the luciferase gene inserted at the same site or with the CAT or luciferase gene inserted

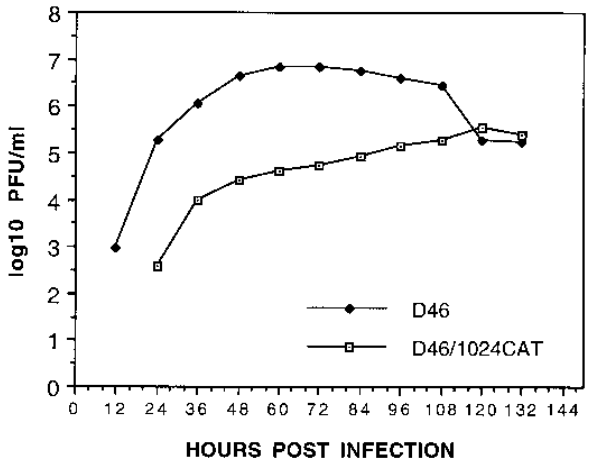

FIG. 7. Growth curves for D46 and D46/1024CAT viruses in HEp-2 cells. Cell monolayers in 25-cm^2 culture flasks were infected with 2 PFU of either virus per cell (three replicate flasks per virus). Samples were taken at the indicated time points, stored at −70°C, and titrated in parallel by plaque assay. Each single-step growth curve is based on the average of the virus titers of the three infected cell monolayers. For D46/1024CAT, no virus was detected 12 h after inoculation (in a 0.25-ml sample).

between the SH and G genes (unpublished data). These viruses also exhibit reduced growth, whereas the numerous wild-type recombinant viruses recovered to date exhibit undiminished growth (unpublished data). This finding indicates that the reduced growth indeed is associated with the inserted gene rather than due to chance mutation elsewhere in the genome. The reduced growth of the RSV-CAT recombinant is reminiscent of results with influenza virus, in which the substitution of a single cDNA-derived, neuraminidase-encoding gene segment containing an additional 917 nucleotides was associated with a 10-fold reduction in virus production in vitro (12). The finding that insertion of a foreign gene into recombinant RSV reduced its level of replication and was stable during passage in vitro suggests that this might be a strategy for effecting attenuation. It will be interesting to evaluate the effects of the insertion of the CAT gene and of other, larger genes on the growth and immunogenicity of recombinant RSV in experimental animals.

Several RNA viruses have been described as vectors for the expression of foreign peptides or proteins. Positive-sense RNA viruses such as poliovirus, Sindbis virus, Semliki Forest virus, or dengue virus have been used to express foreign peptides or complete proteins, either as helper-dependent replicons or as replication-independent, infectious recombinant virus (references 1, 20, 26, 27, and 28 and references therein; 34). For at least some positive-sense RNA virus vectors, the foreign insert often was unstable (20, 28). Among the negative-strand viruses, influenza A virus has been used to encode foreign peptides incorporated into viral proteins. Also, complete foreign proteins have been produced by fusing a foreign ORF to an influenza virus ORF, with the foreign protein engineered to be released by autoproteolysis. Alternatively, complete foreign proteins can be expressed from a second ORF in a bicistronic gene segment (10, 11, 25). The results described in this report show that a nonsegmented negative-strand RNA virus also can stably express a complete foreign protein. Indeed, this work illustrates an advantage of the strategy of gene expression of the nonsegmented negative-strand viruses, namely, that the foreign coding sequences can be introduced as a separate transcription cassette that is expressed as a separate mRNA. It will be interesting to see if the maintenance of foreign inserts with a high degree of stability is a general characteristic of the nonsegmented negative-strand RNA viruses.

This study showed that RSV can tolerate an increase of genome length of 762 nucleotides to a total of 15,984 nucleotides (1.05 times that of wild-type RSV). The Marburg and Ebola filoviruses, which also are members of order *Mononegavirales*, have genome lengths of about 19,000 nucleotides. This fact suggests that for paramyxoviruses like RSV whose genomes are shorter, the negative-strand strategy per se does not preclude an increase in genome size. As is the case with other paramyxoviruses, RSV particles produced in tissue culture can be highly variable in size and include filaments of up to 10 μm in length (6, 22). Since 99% of the infectivity of bovine RSV produced in vitro was trapped by a 0.45-μm-pore-size filter (22), the nature and size of the infectious particle is unclear. These observations suggest that RSV might not have a strict packaging limit and might tolerate a further increase in nucleocapsid length.

As with other nonsegmented negative-strand viruses, the relative abundance of each RSV mRNA decreases with the distance of its gene from the promoter, presumably as a result of polymerase fall-off during sequential transcription (9). This finding had suggested the possibility that the insertion of an additional transcription cassette into the RSV genome might increase transcriptional polarity and reduce the transcription of downstream genes. Surprisingly, insertion of the relatively small CAT cassette into the G-F intergenic region did not appear to alter transcription of the SH, G, and F genes. We are investigating this further by using different gene inserts and different insertion sites in order to gain a more complete picture of the effects of gene insertion on transcription, RNA replication, and virus growth.

The viral RNA-dependent RNA polymerases are known to have an error-prone nature due to the absence of proofreading and repair mechanisms. In RNA virus genomes, the frequency of mutation is estimated to be as high as 10^{-4} to 10^{-5} per site on average (reference 16 and references therein). In the case of the recombinant D46/1024CAT RSV produced here, correct expression of the foreign gene would be irrelevant for virus replication and would be free to accumulate mutations. The passages described here involved multiplicities of infection less than 0.1 PFU per cell, and the duration of each passage level indicated that multiple rounds of infection were involved. Yields of infectious virus from RSV-infected tissue culture cells typically are low, on the order of 10 to 100 PFU per cell. But intracellular macromolecular synthesis is robust, and the poor yields of infectious virus seems to represent an inefficient step in packaging rather than low levels of RNA replication. Thus, the maintenance of CAT through eight serial passages involved many rounds of RNA replication. It is remarkable that the nonessential CAT gene remained intact and capable of encoding fully functional protein in each of the 25 isolates tested at passage 8. Also, RT-PCR analysis of RNA isolated from passage 8 did not detect deletions within the CAT gene. Monitoring the fate of a nonessential gene should prove to be a useful tool for studying the fidelity of the RNA-dependent RNA polymerase.

An important application of this work will be in the development of live vaccines against respiratory diseases. Because most of the antigenic difference between the two RSV antigenic subgroups resides in the G glycoprotein, it will be interesting to determine whether recombinant RSV can express the G protein of the heterologous subgroup as an additional gene to yield a divalent vaccine. Envelope protein genes of some other respiratory viruses, such as human parainfluenza 3 virus,

also will be tested for expression by recombinant RSV. Another important line of experimentation will be to test whether coexpression of immune modulators such as interleukin 6 can enhance the immunogenicity of infectious RSV. This might abrogate the most significant obstacle to successful RSV immunoprophylaxis, the relatively poor immunogenicity of the virus in the very young.

ACKNOWLEDGMENTS

We thank Myron Hill for technical assistance and Mark Peeples, Brian Murphy, and Robert Chanock for helpful discussion and for reviewing the manuscript.

ADDENDUM

While this report was under review, Schnell et al. (31a) reported the recovery of infectious recombinant vesicular stomatitis virus that expressed CAT as an additional gene located between the G and L genes.

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