Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development

(negative-strand RNA virus/paramyxovirus/reverse genetics/polymerase)

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ABSTRACT  Infectious human respiratory syncytial virus (RSV) was produced by the intracellular coexpression of five plasmid-borne cDNAs. One cDNA encoded a complete positive-sense version of the RSV genome (corresponding to the replicative intermediate RNA or antigenome), and each of the other four encoded a separate RSV protein, namely, the major nucleocapsid N protein, the nucleocapsid P phosphoprotein, the major polymerase L protein, or the protein from the 5' proximal open reading frame of the M2 mRNA [M2(ORF1)]. RSV was not produced if any of the five plasmids was omitted. The requirement for the M2(ORF1) protein is consistent with its recent identification as a transcription elongation factor and confirms its importance for RSV gene expression. It should thus be possible to introduce defined changes into infectious RSV. This should be useful for basic studies of RSV molecular biology and pathogenesis; in addition, there are immediate applications to the development of live attenuated vaccine strains bearing predetermined defined attenuating mutations.

Human respiratory syncytial virus (RSV) is the most important pediatric viral respiratory pathogen worldwide (1-3). This ubiquitous highly infectious agent emerges each year in seasonal epidemics and nearly everyone is infected at least once within the first 2 years of life. RSV disease is responsible for considerable morbidity and mortality and lacks an approved vaccine or highly effective antiviral therapy. Research on RSV is impeded by its poor growth in tissue culture, the instability of the virion, and the lack of a highly permissive experimental animal other than the chimpanzee.

Resistance to RSV reinfection induced by natural infection is incomplete but increases incrementally with repeated exposure. Thus, RSV can infect multiple times during childhood and later life, but serious disease usually is limited to the first and sometimes second infections of life. The minimum goal of RSV immuno prophylaxis is to induce sufficient resistance to prevent serious disease associated with the first or second infection.

RSV is a member of the pneumovirus genus of the paramyxovirus family (1, 4). Its genome is a single strand of negative-sense RNA of 15,222 nt. RNA synthesis appears to follow the general pattern of the nonsegmented negative-strand viruses. The genomic template is tightly encapsidated with the major nucleocapsid N protein and is associated with the P phosphoprotein and large L polymerase subunit protein. Transcription begins at the 3' extragenic leader region and proceeds along the entire length by a sequential stop--start mechanism guided by short template signals flanking the genes. This yields 10 major species of mRNA that encode 10 major proteins. RNA replication occurs by a switch to the synthesis of a full-length positive-sense antigenome that also is tightly encapsidated and serves as the template for the synthesis of progeny genome.

The development of methods for introducing designed changes into genomic RNA of nonsegmented negative-strand RNA viruses was impeded by the lack of homologous viral recombination and the lack of infectivity of naked genomic RNA. The supposition that the minimum unit of infectivity for this type of virus is a nucleocapsid competent for RNA synthesis suggested a different strategy to produce infectious virus from viral cDNA. This involved the intracellular coexpression, from separate transfected plasmids, of cDNA-encoded genomic or antigenomic RNA and those viral proteins necessary to generate a transcribing and replicating nucleocapsid. cDNA expression would be driven by T7 RNA polymerase supplied by a vaccinia recombinant virus. This approach was developed first by using short internally deleted analogs of genomic or antigenomic RNA ("minigenomes") that were shown to participate in transcription and replication when synthesized intracellularly in the presence of the appropriate viral proteins (5-10). This strategy was extended to its logical conclusion by the recent demonstrations (11, 12) with two rhabdoviruses (rabies and vesicular stomatitis viruses) that infectious virus could be produced by coexpression of a complete cDNA-encoded antigenomic RNA in the presence of the nucleocapsid (N), phosphoprotein (P), and large polymerase subunit (L) proteins.

The RSV N (391 aa), P (241 aa), and L (2165 aa) proteins were recently shown to be necessary and sufficient to direct RNA replication of cDNA-encoded RSV minigenomes (8, 9). However, fully processive sequential transcription required the coexpression of a fourth protein from the 5' proximal open reading frame of the M2 mRNA [M2(ORF1)] (194 aa), which was identified as a transcription elongation factor (9, 10). The M2(ORF1) protein (also called 22K or M2) is encoded by the first upstream ORF of the M2 mRNA (13). The M2 mRNA also contains a second downstream ORF [M2(ORF2)] (90 codons) that overlaps the first (13); expression of M2(ORF2) was associated with a strong inhibition of minigenomic RNA synthesis.

Abbreviations: RSV, respiratory syncytial virus; RT, reverse transcriptase or transcription; ORF, open reading frame; N, nucleocapsid; P, phosphoprotein; L, large polymerase subunit; M2(ORF1), 5' proximal ORF of the M2 mRNA; M2(ORF2), second ORF of the M2 mRNA.

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synthesis (10). We show coexpression of the N, P, L, and M2(ORFI) proteins in the presence of cDNA-encoded antigenic RNA is necessary and sufficient to produce infectious RSV.

MATERIALS AND METHODS

Transfection and Recovery of Recombinant RSV. Monolayer cultures of HEp-2 cells (in a six-well dish) were infected with 1 focus-forming unit per cell of strain MVA vaccinia virus recombinant expressing T7 RNA polymerase (MVA–T7) (14) and transfected with antigenome, N, and P plasmids (each at 0.4 µg per well) and L and M2(ORFI) plasmids (each at 0.1 µg per well) using LipofectACE (Life Technologies, Grand Island, NY) in an overnight transfection by the protocol of the supplier (9, 10). On day 3 clarified medium supernatants were passaged onto fresh HEp-2 cells and overlaid with methylcellulose (for subsequent antibody staining) or agarose (for plaque isolation). Antibody staining was with a mixture of three murine monoclonal antibodies to the RSV F protein followed by an anti-mouse antibody linked to horseradish peroxidase as described (15).

RESULTS

A cDNA clone encoding the antigenome of RSV strain A2 was constructed from cDNA segments that were synthesized by reverse transcription (RT)-coupled PCR (RT–PCR) from intracellular RSV mRNA or virion-derived genome RNA (Fig. 1). The leader end was constructed to abut the promoter for T7 RNA polymerase that included three transcribed guanosine residues for optimal activity; transcription would result in the donation of these three nonviral guanosines to the 5' end of the antigenome (9). To generate a nearly correct 3' end, the trailer end was constructed to be adjacent to a previously described hammerhead ribozyme followed by tandem terminators of T7 transcription (9). Ribozyme cleavage would donate a single 3'-phosphorylated uridine residue to the 3' end of the encoded RNA. The ribozyme sequence was followed by a tandem pair of terminators of T7 RNA polymerase. The addition of three 5' guanosine residues and one 3' uridine residue to a cDNA-encoded RSV minigenome containing the chloramphenicol acetyltransferase reporter gene had no effect on the expression of chloramphenicol acetyltransferase when complemented by RSV (data not shown).

Four restriction site markers (Fig. 2) were introduced into the antigenomic cDNA by incorporating the changes into oligonucleotide primers used in RT–PCR. This was done to facilitate assembly and provide a means to identify recombinant virus. Three sites were in intergenic regions and the fourth in a nontranslated gene region, and they involved a total of five nucleotide substitutions and a single nucleotide insertion. This increased the length of the encoded antigenome by 1 nt from that of wild type to a total of 15,223 nt.

The strategy for producing infectious RSV from cDNA-encoded antigenome involved its coexpression with those RSV proteins that are necessary and sufficient (i) to produce an antigenomic nucleocapsid capable of RNA replication and (ii) to render the progeny genomic nucleocapsid competent for both RNA replication and transcription. Transcription by the genomic nucleocapsid would provide all of the other RSV proteins and initiate a productive infection. The strategy of providing cDNA-encoded antigenome rather than genome was based on the premise (suggested to us by D. Kolakofsky, University of Geneva, personal communication) that this would avoid hybridization with positive-sense transcripts of the complementing plasmids. This same strategy subsequently was able to produce rabies and vesicular stomatitis viruses from cDNA (11, 12).

Previous experiments with RSV minigenomes indicated that the RSV proteins necessary and sufficient for RNA replication and transcription are N, P, L, and M2(ORFI) (9, 10). Plasmid-borne cDNA encoding the antigenome was transfected with plasmids encoding these four proteins into HEp-2 cells that had been infected with a recently described vaccinia virus MVA strain recombinant that expresses the T7 RNA polymerase (MVA–T7) (14). The MVA strain is a host-range mutant that grows permissively in avian cells, whereas in mammalian cells there is a block at a late stage in virion maturation that greatly reduces the production of infectious virus.

Three days after transfection, the culture supernatants were passaged onto fresh cells and incubated for 5 days under methylcellulose. The cells were fixed and stained by an indirect

![Fig. 1. Construction of RSV antigenic cDNA (not to scale) and structure of the encoded RNA. The diagram of the antigenome (at top) includes the following features: the 5'-terminal nonviral guanosine triplet contributed by the T7 promoter, the four sequence markers at positions 1099 (which adds 1 nt to the length), 1139, 5611, and 7559 (numbered according to the first nucleotide of the resulting restriction site), the ribozyme, tandem T7 terminators, and the single nonviral 3'-phosphorylated uridine residue contributed to the 3' end by ribozyme cleavage (the site of cleavage is indicated with an arrow) (9). Cloned cDNA segments (in the middle) representing in aggregate the complete antigenome were assembled in a version of pBR322 (at the bottom) in which the naturally occurring BamHI site had been ablated by mutagenesis and the Pst I–EcoRI fragment was replaced with a synthetic polylinker containing unique restriction sites designed to facilitate assembly. The box illustrates the removal of the BamHI site: the naturally occurring BamHI–Sal I fragment (the BamHI site is shown in the top line in positive sense and underlined) was replaced with a PCR-generated Bgl II–Sal I fragment [the Bgl II site is shown in the bottom line, underlined; its 4 nt sticky end (italic type) is compatible with that of BamHI]. This resulted in a single-nucleotide C → T change (underlined) that was silent at the amino acid level.](image-url)
The primer that each represented the intracellular rate restricted slightly strain the " Plaque-purified thrice-passaged NS2/GE F

FIG. 2. Sequence markers in the cDNA-encoded antigenomic RNA. Sequences are positive sense; identities between strains A2 and 18537 (16), representing subgroups A and B, respectively, are indicated with dots; sequences representing restriction sites in the cDNA are underlined; gene-start (GS) and gene-end (GE) transcription signals are boxed. Restriction site N GS is in italic type; and the sequence markers are shown underneath each sequence. (Top) A single cytidine residue was inserted at position 1099 to create an Afl II site in the NSII-N intergenic region, and the AG at positions 1139 and 1140 immediately upstream of the N ORF were replaced with CC to create a Neo I site. (Middle) Substitution of guanosine and uridine at positions 5612 and 5616, respectively, created a Stu I site in the G-F intergenic region. (Bottom) A cytidine replacement at position 7500 created a Sph I site in the F-M2 intergenic region.

horseradish peroxidase method using F-specific monoclonal antibodies as primary antibody (15). Numerous RSV-like plaques were detected against a background of cytopathogenicity that presumably was due to a low level of MVA-T7 recombinant virus (data not shown). The plaques contained an abundant amount of the RSV F protein, as indicated by brown-black coloration, and displayed cytopathic effects characteristic of RSV, notably syncytium formation.

The RSV-like plaques were picked from plates that had been prepared in parallel but incubated under agarose and stained with neutral red. The plaques were propagated and compared to a laboratory RSV strain, A2, by plaque assay and antibody staining. As shown in Fig. 3 Lower, the plaques derived from the transfected cultures closely resembled those of the laboratory strain (Fig. 3 Upper). One difference was that the plaques derived from the transfected cultures appeared to be slightly smaller than those from the laboratory strain, with centers that were less well-cleared. This raised the possibility that the putative recombinant virus differed phenotypically from this particular wild-type isolate, possibly being slightly more restricted in cell-to-cell spread and exhibiting a reduced rate of cell killing. Further work will be needed to resolve this point. With regard to the propagation of released virus, the yields of the recombinant vs. laboratory virus in HEP-2 cells were roughly identical at 32° or 37°C N ORF was in italic type: preliminary studies, the recombinant and laboratory viruses were indistinguishable with regard to the accumulation of intracellular RSV mRNAs and proteins (data not shown).

Plaque-purified thrice-passaged recombinant RSV was analyzed in parallel with laboratory virus by RT-PCR using three primer pairs flanking the four inserted markers. PCR products of the expected sizes were produced (Fig. 4 A-C). The production of each was dependent on the RT step, indicating that each was derived from RNA rather than contaminating cDNA. Restriction enzyme digestion (Fig. 4 D-F) showed that the PCR products representing the recombinant virus contained the expected restriction site markers while those representing the laboratory strain did not. Nucleotide sequence analysis of cloned PCR product confirmed the sequences spanning the restriction site markers (data not shown).

The efficiency of RSV production when complemented by N, P, L, and M2(OFR1) was relatively high, ranging in three experiments from an average of 9.9 to 94.8 plaques per 0.4 μg of input antigenomic cDNA and 1.5 x 10^6 cells. Since these plaques were derived from passage, it is not known how many infected cells were present in each well of the original transfection. But, nearly every transfected well (54 of 56 in Table 1) produced virus. Since the yield of released RSV per infected cell typically is very low (≈10 plaque-forming units) even under ideal conditions and since many wells yielded many times this amount (up to 169 plaques, see Table 1), it seems likely that several RSV-producing cells were present in many of the wells of transfected cells.

RSV was not recovered if any of the plasmids were omitted (Table 1 and data not shown). The requirement for M2(OFR1) also could be satisfied with the complete gene, M2(OFR1+2), provided the level of its input cDNA was low [0.016 μg per 1.5 x 10^6 cells (Table 1), which was the optimum for transcription determined with the minigenome system (10)]. At higher levels, the production of virus was greatly reduced (data not shown), suggesting that the inhibition of minigenomic RNA synthesis associated with M2(OFR2) (10) also operates on the complete genome during productive infection.

DISCUSSION

Challenges to the production of infectious RSV from cDNA included its poor growth in tissue culture, lengthy replication cycle, virion instability, negative-sense RNA genome strategy, and complexity of genome organization and gene products. The reliable and efficient method described here should be of considerable utility for introducing defined mutations into this important uncontrolled pathogen.

The RSV M2(OFR1) protein was recently identified as a transcription elongation factor that was required for processive sequential transcription (10). Consistent with this, the present
study showed that M2(ORF1) was required for the production of infectious RSV and, thus, is an obligatory component of functional nucleocapsids during productive infection. Expression of M2(ORF2) mRNA, which was not previously thought to be an RSV gene, was associated with inhibition of minigenome RNA synthesis (10). Consistent with this, higher levels of input M2(ORF1+2) plasmid reduced the production of infectious RSV (data not shown), presumably an effect of M2(ORF2).

Minigenomes of Sendai and measles viruses were shown to replicate efficiently only if their nucleotide length was a multiple of 6, which is thought to represent a requirement for a precise spacing of nucleotide residues relative to encapsidating NP protein (6, 19). In contrast, replication of an RSV–chloramphenicol acetyltransferase minigenome complemented by RSV was unaffected by incremental single-nucleotide changes in length, and sequence analysis confirmed that the lengths were maintained without compensatory changes (S. Samal and P.L.C., unpublished data). The length of the genome of wild-type RSV (15,222 nt) is a multiple of 6 but that predicted for the recombinant virus (15,223 nt) is not. The possibility exists that this single nucleotide addition was corrected by compensatory mutation elsewhere, although the relatively high efficiency of rescue makes this seem unlikely. Thus, rescue of infectious RSV did not appear to be constrained by a strict requirement of genome length. It should be interesting to determine whether the nonviral 5' guanosine triplet and 3'-terminal uridine are removed during replication.

The ability to introduce defined mutations into infectious RSV should have many applications in extending analyses of RSV molecular biology and pathogenesis. For example, the functions of the RSV proteins, especially the NS1, NS2, SH, M2(ORF1), and M2(ORF2) proteins, could be investigated by introducing mutations that ablate or reduce their level of expression or that yield mutant protein. As another example, each of the three glycoproteins could be modified to evaluate effects on growth in tissue culture and infection and pathogenesis in experimental animals. The roles of various genomic RNA structural features, such as promoters, intergenic regions, gene overlap, and transcription signals, could be evaluated. These studies should be performed in parallel by using the RSV minigenomes, whose helper-dependent status should be particularly useful for mutants that are too inhibitory to be recovered in replication-independent infectious virus.

A number of attenuated RSV strains as candidate vaccines for intranasal administration were developed recently by using the strategy of multiple rounds of chemical mutagenesis to introduce multiple mutations into a virus that had been attenuated during cold passage (18, 20). Preliminary nucleotide sequence analysis of some of these attenuated viruses indicates that each level of increased attenuation is associated with two or more additional nucleotides and amino acid substitutions (ref. 18 and unpublished data). It should thus be possible to distinguish between silent incidental mutations and those responsible for phenotype differences by introducing them, separately and in various combinations, into infectious wild-type RSV. This should directly identify mutations responsible for phenotypes such as attenuation, temperature sensitivity, cold adaptation, small plaque size, host range restriction, etc. Mutations from this menu can then be introduced in various combinations to “fine tune” vaccine virus. Importantly, it should also be possible to modify vaccine virus to accommodate antigenic drift in circulating virus.

Also, it should be possible to explore other methods of attenuation. One example would be to replace individual internal genes of human RSV with their bovine RSV counterparts, which might function with reduced efficiency in the human RSV background. The possibility of other types of
Table 1. Production of infectious RSV was dependent on expression of M2(ORFI)

<table>
<thead>
<tr>
<th>Complementing plasmids transfected (µg of cDNA)</th>
<th>Production of infectious RSV</th>
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<tbody>
<tr>
<td>N (0.4)/P (0.4)/L (0.1)</td>
<td>Exp. 1 Exp. 2 Exp. 3</td>
</tr>
<tr>
<td>N (0.4)/P (0.4)/L (0.1)/M2 [ORFI+2] (0.016)</td>
<td>0 x 24 0 x 12 0 x 12</td>
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<tr>
<td>N (0.4)/P (0.4)/L (0.1)</td>
<td>0 x 19 0 x 4 9 x 1</td>
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<td></td>
<td>1 x 2 3 x 1 10 x 1</td>
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<td></td>
<td>2 x 2 5 x 1 14 x 2</td>
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<td>3 x 1 6 x 1 22 x 1</td>
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<tr>
<td></td>
<td>9 x 1 28 x 1</td>
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<td>10 x 1 32 x 1</td>
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<td>13 x 1 49 x 1</td>
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<td></td>
<td>34 x 1 70 x 2</td>
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<td></td>
<td>51 x 1 166 x 1</td>
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<td></td>
<td>169 x 1</td>
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<tr>
<td>(av = 0.38)</td>
<td>(av = 10.9) (av = 48.6)</td>
</tr>
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| N (0.4)/P (0.4)/L (0.1/M2 [ORFI] (0.1)         | (av = 13.7) (av = 9.9) (av = 94.8) |

In addition to the plasmids, transfection mixtures also contained 1.5 x 10^6 cells and 0.4 µg of antigenomic cDNA. Supernatants from transfected cultures (10^6 cells per well) were passaged onto fresh HEp-2 cells, overlaid with methycellulose, and stained 5 days later with F-specific monoclonal antibodies to identify the RSV plaques. Data are reported as the number of wells containing the indicated number of plaques; for example, 0 plaques per well for 24 wells. av, Average value expressed as the number of plaques per well.

attenuating mutation is exemplified by the finding that a tissue-culture-adapted nonpathogenic strain of pneumonia virus of mice (the murine counterpart of RSV) lacked the cytoplasmic tail of the G protein (21). Other mutations that are candidates for evaluation in infectious virus include mutations in cis-acting signals identified during mutational analysis of RSV minigenomes.

An exciting possibility is that RSV might be engineered in ways that enhance its immunogenicity and induce a level of protection greater than that provided by natural infection. This would overcome the greatest obstacle to controlling RSV, namely, the incomplete nature of immunity induced by natural infection. It may be possible to insert foreign sequence into the RSV genome for coexpression. Genes of interest for evaluation include those encoding cytokines (e.g., interleukin 6), antagonists thereof, or proteins rich in T-helper-cell epitopes (the latter expressed as a separate protein or as a chimera engineered from a second copy of one of the RSV proteins such as SH). This offers the possibility of modifying and improving the immune response quantitatively and qualitatively. Coexpression of cytokine genes or antagonists thereof during RSV infection in experimental animals also might give insights into the contribution of immunity to viral pathogenesis. It also might be possible to identify and ablate possible epitopes associated with undesirable immunopathologic reactions. Inclusion of the G protein gene of RSV subgroup B would broaden the response to cover a wider spectrum of the relatively diverse subgroup A and B strains present in the human population, allowing a single virus to function as a bivalent vaccine. RSV can be evaluated as a possible vector for protective antigens of other respiratory tract pathogens. An RSV vector also might have utility in transient gene therapy of the respiratory tract.

We thank Drs. Linda Wyatt and Bernard Moss for the gift of the MVA-77 recombinant vaccinia virus and Dr. Daniel Kolakofsky for helpful discussion.