JOURNAL OF VIROLOGY, Apr. 1995, p. 2412–2419 Vol. 69, No. 4
0022-538X/95/$04.00+0

Functional cDNA Clones of the Human Respiratory Syncytial (RS) Virus N, P, and L Proteins Support Replication of RS Virus Genomic RNA Analogs and Define Minimal *trans*-Acting Requirements for RNA Replication

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Received 11 November 1994/Accepted 10 January 1995

The RNA-dependent RNA polymerase of human respiratory syncytial (RS) virus was expressed in a functional form from a cDNA clone. Coexpression of the viral polymerase (L) protein, phosphoprotein (P), and nucleocapsid (N) protein allowed us to develop a system for expression and recovery of replicable RS virus RNA entirely from cDNA clones. cDNA clones of the N, P, and L genes were constructed in pGEM-based expression plasmids and shown to direct expression of the appropriate polypeptides. Two types of RS virus genomic RNA analogs were expressed from an intracellular transcription plasmid that directed the synthesis of RNAs with defined 5′ and 3′ ends. One analog included the authentic 5′ and 3′ termini of the genome, and the second contained the authentic 5′ terminus and its complement at the 3′ terminus as found in copyback defective interfering RNAs of other negative-strand RNA viruses. Both types of genomic analogs were encapsidated and replicated in cells expressing the RS virus N, P, and L proteins. Omission of any of the three viral proteins abrogated replication, thereby defining the N, P, and L proteins as the minimal *trans*-acting proteins required for RNA replication. This system has the advantages that expression occurs at a level sufficient to allow direct biochemical analysis of the products of RNA replication and that neither the use of reporter genes nor wild-type RS helper virus is required. These features allow analysis of both *cis*- and *trans*-acting factors involved in the control of replication of RS virus RNA.

Human respiratory syncytial (RS) virus, the leading viral cause of severe lower respiratory tract disease in infants and young children, is the prototype of the genus *Pneumovirus* in the *Paramyxoviridae* family, possessing a single-stranded nonsegmented negative-sense RNA genome of 15,221 nucleotides (nt) (4). The genome of RS virus encodes 10 mRNAs (5, 8) and has a 44-nt leader sequence at the 3′ end and a 155-nt noncoding trailer sequence at the 5′ end (22). The gene order is 3′ (leader)-NS1-NS2-N-P-M-SH-G-F-22K-L-(trailer) 5′ (4). The genomic RNA of the virus is encapsidated with the N protein and associated with the P and the L proteins to form the ribonucleoprotein complex (16) which is believed to be responsible for RNA transcription (1, 17) and probably for replication as well, but this has yet to be demonstrated. A comparison of RS virus with members of the *Paramyxovirus* and *Morbillivirus* genera reveals that RS virus possesses several distinctive features. (i) It has several additional genes, i.e., NS1, NS2, and 22K, and the roles of these gene products in virus replication and pathogenesis are not clear; (ii) most of the virus proteins possess low amino acid homology with their counterparts; and (iii) the gene order differs from that of the other two genera (4). The relationship between these distinctive molecular features of RS virus and the pathogenesis of the disease remains unknown. These questions have not been addressed previously because it has not been possible to engineer site-specific alterations into the genome of the virus and to recover full-length infectious genomes from cDNA clones.

It is difficult to engineer changes into the genome of negative-sense RNA viruses at the cDNA level and recover replicable RNA because, in contrast to the situation with the positive-stranded RNA viruses, naked negative-strand genomic RNA is not infectious. Therefore, RNA of either polarity transcribed from full-length cDNA clones of the genomes of negative-strand RNA viruses is not by itself competent to initiate infection. Not only is the RNA-dependent RNA polymerase required for infectivity, but the RNA itself must be encapsidated with the viral nucleocapsid protein in order to be a functional template for the polymerase (12). Furthermore, constant nucleocapsid protein synthesis is required for RNA replication (18, 26). These facts have presented a formidable hurdle to the analysis of *cis*- and *trans*-acting factors involved in RNA transcription and replication of these viruses. However, the development of approaches to recover replicable RNA from a cDNA clone for the segmented negative-sense RNA virus, influenza virus (13, 21), stimulated the application of these techniques for nonsegmented negative-sense RNA viruses. Synthetic genomic RNA analogs of Sendai virus (23), parainfluenza 3 virus (10, 11), and RS virus (6, 7) containing a reporter gene(s) have been transfected into the corresponding wild-type virus-infected cells and rescued such that they are amplified, expressed, and in some cases packaged into infectious virus particles, as shown by the continued expression of the reporter gene(s) during sequential virus passages. However, all these systems rely on the presence of infectious, homologous wild-type helper virus to support the replication of the synthetic RNAs, and they work at low efficiency so that a reporter gene is required to detect expression from a virus containing the rescued RNA. Further, these systems cannot be used to assay the *trans*-acting protein requirements for RNA transcription and replication of the viruses because of the necessity for homologous wild-type helper virus.

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In order to circumvent these drawbacks, our laboratory (24) developed a system for recovery of infectious defective interfering (DI) particles of vesicular stomatitis virus (VSV), a nonsegmented negative-stranded RNA virus, entirely from cDNA clones. With this system, the *trans*-acting protein requirements for VSV RNA replication, nucleocapsid assembly, and budding of infectious particles were identified. Furthermore, the *cis*-acting sequence elements involved in control of RNA transcription and replication of VSV have been addressed (32). A similar approach has also been used to rescue DI RNA of Sendai virus (2) and synthetic RNA analogs of rabies virus (9) and to recover infectious rabies virus from a cDNA clone (28).

In this study we have prepared a functional cDNA clone of the RS virus RNA-dependent RNA polymerase which, in conjunction with functional cDNA clones of the RS virus N and P proteins, has allowed us to develop a system for expression and recovery of replicable RS virus RNA entirely from cDNA clones in cells. Advantages of this system are that expression is at a level sufficient to allow direct biochemical analysis of the products of RNA replication and that neither the use of reporter genes nor wild-type RS helper virus is required. These features allowed us to (i) recover replicable RS virus RNA analogs, (ii) demonstrate the cloning and expression of a functional RS virus RNA replicase, and (iii) assay the *trans*-acting protein requirements for RS virus RNA replication.

MATERIALS AND METHODS

Construction of full-length cDNAs encoding the RS virus N, P, and L proteins. All procedures and reaction conditions for plasmid constructions were carried out according to standard methods (27). The plasmid constructs were verified by DNA sequence determination of the relevant regions by the dideoxy chain termination method, using denatured plasmid DNA as templates (15).

In order to express RS virus proteins in the vaccinia virus-T7-based expression system, cDNAs of the RS virus N, P, and L genes were cloned into pGEM3 vectors downstream of the T7 RNA polymerase promoter. The clones were designated pRSV-N, pRSV-P, and pRSV-L, respectively. Briefly, pRSV-N was prepared by transferring a *Bam*HI-*Pst*I fragment containing the entire N gene from pAQ330 (19) into a pGEM3 vector. cDNA encoding the P protein was generated by reverse transcription of RS virus genomic RNA, followed by PCR amplification (RT-PCR). The cDNA was then cloned between the *Kpn*I and *Bam*HI sites of pGEM3. Because of the size of the L gene, (6,578 nt [30]) the full-length L gene clone was constructed through several steps of subcloning and finally assembled from four segments in a pGEM3 vector between the *Kpn*I and *Pst*I sites. Three of the four segments were generated by RT-PCR of RS virus genomic RNA, and the fourth segment was derived from an existing clone, pRSV-L-35, which was prepared by oligo(dT)-primed cDNA synthesis (8). This segment had an error at nt 4762 of the L gene, which differed from the consensus sequence of the L gene by the deletion of an adenosine residue (30). This mutation was repaired by site-directed mutagenesis.

Generation of cDNA clones encoding RS virus genomic analogs. cDNA clones that transcribe two types of RS virus genomic analogs were constructed. The first type (wild type) contained the authentic 3′ and 5′ termini of the genome, but deleted the majority of the internal genes, and the second type (panhandle type) contained complementary termini, derived from the 5′ terminus of the genome, surrounding a partial L gene (Fig. 1).

The wild-type analog plasmid (pWT1) was prepared as follows. cDNA containing the 3′ leader, NS1, NS2, N, and part of the P genes was synthesized by RT-PCR of RS virus genomic RNA with a pair of oligonucleotide primers corresponding to nt 1 to 29 and 2378 to 2360 of the genome. A 2.3-kb PCR product was cloned between the *Kpn*I and *Sal*I sites of pGEM3, and the resulting plasmid was digested with *Kpn*I and *Mun*I to release a 0.4-kb fragment containing the 3′ 44-nt leader and nt 1 to 375 of the NS1 gene. Consequently, this *Kpn*I-*Mun*I fragment was ligated with a *Mun*I-*Pst*I fragment, containing the 1,031-nt L gene 5′ end (positions 5547 to 6578 of the L gene) and the 155-nt trailer, and cloned into pGEM3. The resulting clone was digested with *Bsi*WI, and the termini of the released 1.6-kb *Bsi*WI fragment were repaired by partial filling with dGTP, dTTP, and the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories), followed by digestion with mung bean nuclease. This generated a blunt-ended DNA fragment whose terminal sequences precisely matched the authentic termini of the RS virus genome. This fragment was then inserted between the *Sma*I and *Stu*I sites of a transcription plasmid between the T7 promoter and the antigenomic strand of the hepatitis delta virus autolytic ribozyme followed by T7 terminator sequences (24). In this sequence context, the wild-type genomic RNA analog synthesized by the T7 RNA polymerase was predicted to contain two non-RS virus G residues at the 5′ end preceding the trailer and, after autolytic cleavage, a terminus that corresponded exactly to the authentic genome 3′ end.

The panhandle-type analog (pPH3) was derived from the trailer region and the L gene end sequences. A 1.2-kb *Mun*I-*Pst*I fragment containing the L gene 5′ end and the trailer region was isolated from pRSV-L, fused with an *Apo*I-*Pst*I fragment comprising 75 nt of the extreme 5′ end of the trailer, and subsequently cloned into the *Pst*I site of pGEM3. The resulting clone was treated as described above for the wild-type analog construction and inserted between the *Sma*I and *Stu*I sites of the intracellular transcription plasmid. Therefore, the T7 transcripts from the panhandle-type analog plasmid were predicted to contain two non-RS virus G residues at the 5′ end preceding the trailer and, after autolytic cleavage, a 3′ end whose sequence was complementary to the first 75-nt of the trailer, surrounding the 1,031-nt L gene 5′ end.

Virus infections and DNA transfections. 293 cells were grown in Dulbecco's modified eagle medium (D-MEM; GIBCO Laboratories) containing 10% heat-inactivated fetal bovine serum (FBS) in 60-mm-diameter plates. A subconfluent cell monolayer (about 3×10^6 cells per plate) was infected with recombinant vaccinia virus vTF7-3 (10 PFU per cell) that expresses T7 RNA polymerase. After 45 min of virus adsorption, the cells were washed once with D-MEM (without FBS) and then transfected with appropriate plasmid DNAs, using lipofectin according to the manufacturer's (Bethesda Research Laboratories) instructions. For protein expression, the cells were transfected with 5 μg of pRSV-N, pRSV-P, or pRSV-L, whereas for the RNA replication assay, the cells were transfected with 5 μg of pPH3 or pWT1 and a combination of 5 μg of pRSV-N, 2 μg of pRSV-P, and 0.25 to 2.0 μg of pRSV-L. The transfected cells were then incubated in D-MEM (without FBS) at 37°C for 12 to 16 h before being labeled with radioisotopes.

Immunoprecipitation and electrophoretic analysis of proteins. For radiolabeling of expressed proteins, at 12 h posttransfection the cells were incubated in methionine-free medium (GIBCO) for 45 min and then exposed to [^{35}S]methionine (20 μCi/ml; Du Pont/NEN) for 3 h. Cytoplasmic extracts of cells were prepared and virus-specific proteins were immunoprecipitated as described previously (25), except that goat polyclonal antiserum raised against RS virus was used (Chemicon International). For detecting expression of the L protein, rabbit antisera were raised against L protein-specific peptides. Three peptides corresponding to amino acid positions 1696 to 1713, 1721 to 1733, and 2094 to 2110, respectively (30), were synthesized by the UAB Protein Synthesis Core Facility and conjugated to keyhole limpet hemacyanin, and antisera were raised in rabbits by Lampire Biological Laboratories Inc. A combination of the anti-L-peptide sera was used for immunoprecipitation of the L protein. Immunoprecipitated proteins were analyzed by electrophoresis in 10% polyacrylamide gels and detected by fluorography as described previously (3, 20).

Analysis of RNA replication. To analyze RNA replication, cells were exposed to [^{3}H]uridine (25 μCi/ml; Du Pont/NEN) between 16 and 24 h posttransfection in the presence, where indicated, of actinomycin D (10 μg/ml, water-soluble mannitol complex; Sigma Chemical Co.). Cells were harvested, and cytoplasmic extracts were prepared as described previously (25). Either total RNA or N protein-encapsidated RNA selected by immunoprecipitation with the goat anti-RS virus antiserum was extracted and analyzed by electrophoresis in 1.75% agarose-urea gels and detected by fluorography as described by Wertz and Davis (31).

RNase protection assay of replication products. An RNase protection assay was used to detect strand-specific RNA synthesis by using an RPA II kit according to the manufacturer's instructions (Ambion). Briefly, N protein-encapsidated RNAs from one 60-mm-diameter plate of cells transfected with pPH3 and the N, P, or L gene plasmid as described above (without radiolabeling and actinomycin D treatment) were selectively enriched by immunoprecipitation and used in an RPA for each reaction. A strand-specific RNA probe was generated by T7 RNA polymerase in vitro transcription of a pGEM3 plasmid with incorporation of ^{35}S-UTP (Du Pont/NEN) according to the T7 RNA polymerase manufacturer's instructions (New England Biolabs). The pGEM3 plasmid containing a *Bcl*I-*Bgl*II fragment of the L gene end (positions 5655 to 6518) was linearized by digestion with *Ssp*I (position 6158), so that T7 polymerase transcription produced a 391-nt negative-sense RNA probe. The RNA probe was purified by polyacrylamide gel electrophoresis and 6×10^3 cpm of probe was used in each reaction of the assay. The protected RNA was analyzed by electrophoresis in 4.5% denaturing polyacrylamide gels and detected by fluorography.

RESULTS

Expression of RS virus proteins. To establish a helper virus-free system that could be used to engineer changes into the genome of RS virus at the cDNA level and recover replicable RNA, it was first necessary to prepare cDNA clones capable of expressing the RS virus proteins involved in RNA replication. By analogy with other negative-stranded RNA viruses, these would most likely be the N, P, and L proteins, although at the outset it was not known if the nonstructural proteins NS1 and

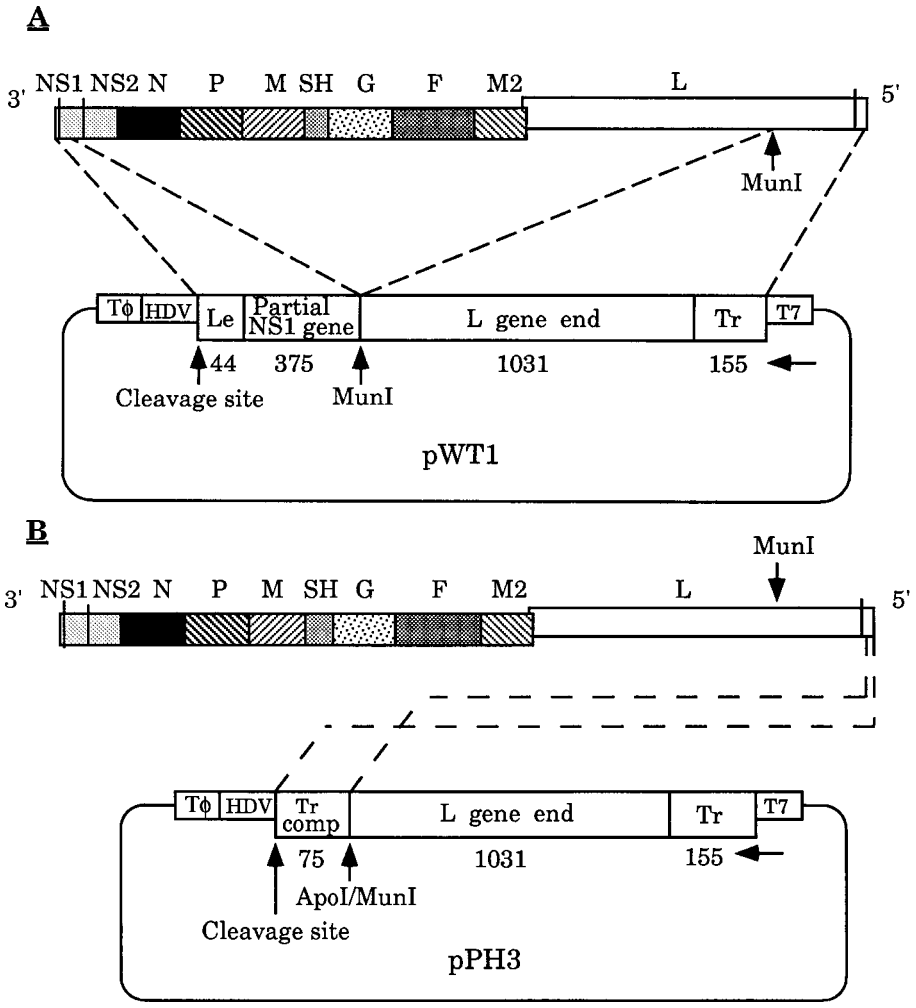

FIG. 1. Diagrams of RS virus genomic analogs. (A) Relevant regions of the wild-type genomic analog pWT1, showing the terminal regions of the RS virus genome that were included and the positions of the T7 polymerase promoter (T7), hepatitis delta virus ribozyme (HDV), leader region (Le), and T7 polymerase terminator (Tϕ). (B) Regions of the RS virus genome included in the RS virus panhandle-type analog pPH3. Tr, trailer region; Tr comp, trailer complement. The number of nucleotides from each region of the RS virus genome is indicated.

NS2 might be required. Full-length cDNA clones of the N, P, and L genes were prepared as described in Materials and Methods. To detect whether these cDNA clones expressed N, P, and L proteins, the recombinant vaccinia virus-T7 RNA polymerase expression system was used (14). 293 cells were infected with vTF7-3, which expresses T7 RNA polymerase in the cytoplasm of cells, and transfected with plasmid pRSV-N, pRSV-P, or pRSV-L. At 12 h posttransfection the cells were labeled with [^{35}S]methionine for 3 h. Cytoplasmic extracts were prepared, and proteins were immunoprecipitated with anti-RS virus antibody in the case of the N and P proteins or anti-L-peptide antisera in the case of the L protein and analyzed by electrophoresis. As shown in Fig. 2A, vTF7-3-infected cells transfected with pRSV-N expressed a protein (Fig. 2A, lane 5) which comigrated with the authentic N protein synthesized in RS virus-infected cells (Fig. 2A, lane 4). Similarly, vTF7-3-infected cells transfected with pRSV-P also expressed a protein (Fig. 2A, lane 6) which comigrated with the authentic P protein (Fig. 2A, lane 4). Neither untransfected nor uninfected cells produced these proteins (Fig. 2A, lanes 2 and 3) although two proteins can be seen in the control as well as transfected extracts that were nonspecifically precipitated by the anti-serum. These data suggested that pRSV-N and pRSV-P expressed the appropriate viral proteins. Anti-L-peptide sera were prepared in rabbits and used to immunoprecipitate the expression products of the L gene cDNA clone. A polypeptide was specifically immunoprecipitated from cells transfected with the L gene clone (Fig. 2B, lane 5), which comigrated with the L protein immunoprecipitated from RS virus-infected cells (Fig. 2B, lane 4). A few faint bands migrating faster than the L protein were also observed; these may represent products derived from premature termination or downstream initiations of translation, or they could be degradation products of the L protein. This experiment demonstrated that the full-length clone of the L gene was capable of directing synthesis of a protein having the same electrophoretic mobility as the authentic RS virus L protein. Consequently, this cDNA clone was used in the following RNA replication experiments to test whether the expressed L protein was a functional polymerase.

Expression of genomic RNA analogs. The second step to develop a helper virus-free reverse genetic analysis system for RS virus was to construct cDNA clones that could transcribe RS virus genomic RNA analogs in cells. The details of these constructs are described in Materials and Methods. Two types

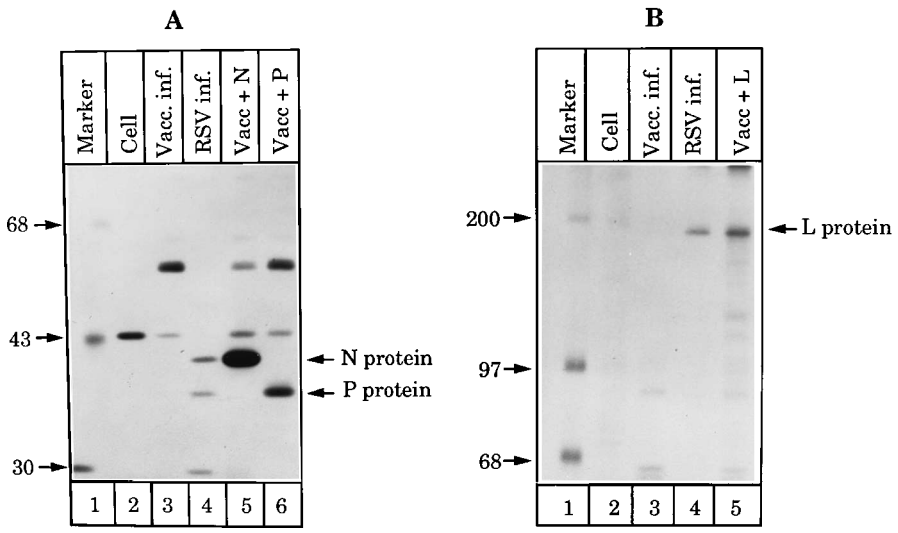

FIG. 2. Analysis of the expression of the RS virus N, P, and L proteins. (A) Cells were infected with vTF7-3 and then transfected with plasmid pRSV-N (lane 5) or pRSV-P (lane 6). The cells were labeled with [^{35}S]methionine for 3 h at 12 h posttransfection. Cytoplasmic extracts were prepared, and the proteins were immunoprecipitated with anti-RS virus antibodies. The [^{35}S]methionine-labeled proteins from uninfected (lane 2), vTF7-3 infected (lane 3), or RS virus-infected (lane 4) cells were also immunoprecipitated with the anti-RS virus antibodies. All immunoprecipitates were analyzed by electrophoresis in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel followed by fluorography. (B) [^{35}S]methionine-labeled proteins from vTF7-3-infected cells transfected with the L gene plasmid (lane 5) or from uninfected (lane 2), vTF7-3-infected (lane 3), or RS virus-infected (lane 4) cells were immunoprecipitated with anti-L-peptide antisera. The immunoprecipitates were analyzed by electrophoresis in an SDS–10% polyacrylamide gel. Lanes 1, ^{14}C-labeled molecular size markers; the molecular sizes (in kilodaltons) of three of the markers are shown for each gel. The positions of the RS virus N, P, and L proteins are indicated.

of RS virus genomic analogs were prepared as shown in Fig. 1. The wild-type cDNA clone, pWT1, encoded an analog of RS virus genomic RNA in which the majority of the internal genes were deleted (Fig. 1A). Transcription of pWT1 by T7 RNA polymerase would yield a 1,607-nt negative-sense RNA with the authentic 3′ terminus of the RS virus genome, created by autolytic cleavage by the ribozyme of the primary transcript. This RNA would have the following structural features (listed in 3′ to 5′ order): (i) 44-nt leader region; (ii) 375-nt of the 3′ end of the NS1 gene; (iii) 1,031-nt of the L gene 5′ end; (iv) 155-nt trailer region; and (v) two non-RS virus G residues encoded by the vector. Similar to pWT1, the panhandle-type cDNA clone, pPH3, encoded an RS virus genomic analog in which most of the internal genes had been deleted. However in contrast to pWT1, pPH3 contained DI-like termini as found in other negative-stranded RNA viruses, i.e., complementary termini surrounding a partial L gene (Fig. 1B). As with pWT1, the panhandle-type genomic analog sequences were placed in the transcription plasmid behind a T7 promoter and followed by the hepatitis delta virus ribozyme and T7 terminator. T7 RNA polymerase transcription of pPH3 would produce a 1,263-nt negative-sense RNA consisting of two non-RS virus G residues and the 155-nt trailer at the 5′ end, a complement of the first 75-nt of the trailer at the 3′ end, and 1,031 nt from the 5′ end of the L gene between the complementary termini. After autolytic cleavage, the 3′ end of the panhandle-type RNA analog should be exactly complementary to the authentic 5′ end of the genome.

To examine the ability of these two constructs to generate transcripts of the appropriate sizes in 293 cells, pWT1 and pPH3 were transfected separately into vTF7-3-infected cells and cells were exposed to [^{3}H]uridine for 8 h at 16 h posttransfection. The total cytoplasmic RNA synthesized during this period was analyzed by electrophoresis in an agarose-urea gel. The major species of RNA labeled in the pWT1-transfected sample was an RNA of 1.6 kb and one of 1.2 kb in the pPH3-transfected sample (Fig. 3, lanes 3 and 4). These RNAs had the correct predicted sizes and were not seen in untransfected, vTF7-3-infected cells (Fig. 3, lane 2). The minor bands migrating slightly slower than the major negative-sense RNA transcripts were RNAs that had not undergone autolytic cleavage by the ribozyme at the time of analysis. The identity of these cleaved and uncleaved transcripts was confirmed by comparison with the cleaved and uncleaved transcripts from the same

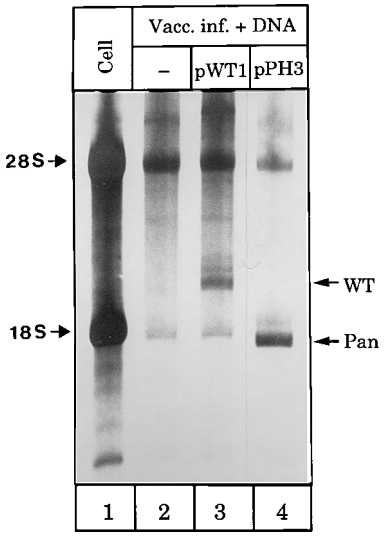

FIG. 3. Intracellular transcription of RS virus genomic RNA analogs. Cells were infected with vTF7-3, transfected with pWT1 (lane 3) or pPH3 (lane 4), and exposed to [^{3}H]uridine (25 μCi/ml) between 16 and 24 h posttransfection. Total cytoplasmic RNA species were analyzed by electrophoresis in a 1.75% agarose-urea gel and detected by fluorography. The [^{3}H]uridine-labeled RNA species in uninfected (lane 1) or vTF7-3-infected but nontransfected cells (lane 2) are also shown. Major RNA transcripts from the genomic analogs are indicated (WT or Pan).

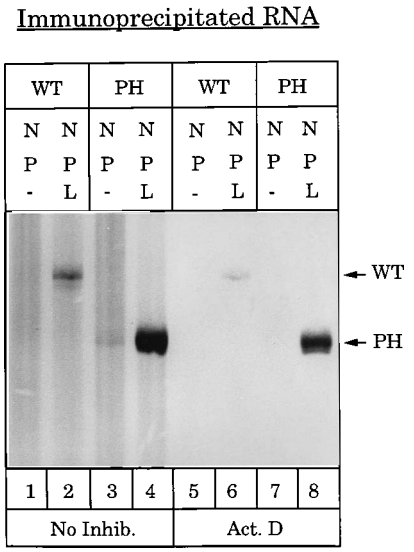

FIG. 4. Analysis of encapsidation and replication of RS virus genomic RNA analogs. Cells were infected with vTF7-3 and then transfected with pWT1 (lanes 1 and 2 and 5 and 6) or pPH3 (lanes 3 and 4 and 7 and 8) and the indicated combinations of plasmids encoding the N, P, and L proteins. The absence of a plasmid is indicated (−). Cells were exposed to [^{3}H]uridine (25 μCi/ml) between 16 and 22 h posttransfection in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of actinomycin D (10 μg/ml), after which they were harvested and cytoplasmic extracts were prepared. N protein-encapsidated RNA was isolated by immunoprecipitation, phenol extraction, and analysis by electrophoresis in a 1.75% agarose-urea gel.

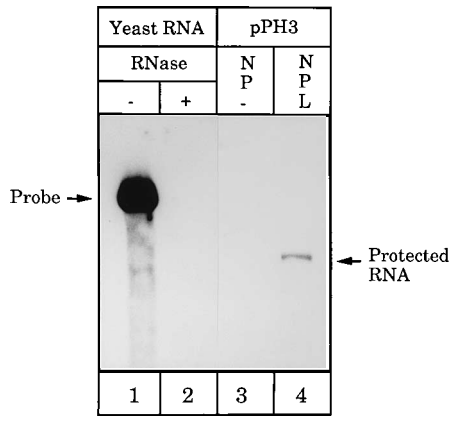

FIG. 5. Analysis of positive-stand RNA replication. Synthesis of positive-strand RNA of the panhandle-type genomic analog during RNA replication was identified by an RNase protection assay. N protein-encapsidated RNAs from cells transfected with pPH3 and the N and P (lane 3) or the N, P, and L (lane 4) plasmids were selectively enriched by immunoprecipitation. A ^{35}S-labeled negative-sense RNA probe was annealed to the RNA samples and then digested with RNase. The protected RNA was analyzed by electrophoresis in a 4.5% denaturing polyacrylamide gel and detected by fluorography. Lanes 1 and 2 show the probe hybridized with yeast RNA and then treated with (lane 2) or without (lane 1) RNase. Lanes 3 and 4 were exposed to the film 10-fold longer than lanes 1 and 2.

plasmids generated by in vitro transcription (data not shown). The majority of the RNA synthesized during the labeling period was cleaved by the ribozyme, releasing a 200-base RNA that contained the ribozyme and terminator sequences and that migrated near the bottom of the gel (data not shown).

Encapsidation and replication of genomic RNA analogs when coexpressed with the N, P, and L proteins. The active template for RNA synthesis by negative-strand RNA viruses is the RNA in the form of a ribonucleocapsid. To determine whether the RNAs transcribed in cells by T7 polymerase could be encapsidated with the nucleocapsid protein and replicated, vTF7-3-infected cells were transfected with pWT1 or pPH3 and with combinations of plasmids encoding the N, P, and L proteins. At 16 h posttransfection, the cells were exposed to [^{3}H]uridine for 6 h. We focused on the process of replication in these studies by selecting for encapsidated, replicated RNAs by immunoprecipitation followed by analysis of the extracted RNAs in an agarose-urea gel.

Immunoprecipitation of ^{3}H-labeled RNA by anti-RS virus polyclonal serum demonstrated that wild-type and panhandle-type RNA analogs were encapsidated when pRSV-N, pRSV-P, and pRSV-L were cotransfected (Fig. 4, lanes 2 and 4). However, in the absence of pRSV-L, encapsidated RNA was barely detected (Fig. 4, lanes 1 and 3). These results suggested that only a small percentage of the original T7 negative-sense RNA transcripts was encapsidated and that the majority of the encapsidated RNA arose from replication of the original transcripts by the RS virus polymerase. To test whether the labeled and encapsidated RNA was replicated by the RS virus RNA-dependent RNA polymerase, the effect of actinomycin D on synthesis and encapsidation of RNA was analyzed. Actinomycin D inhibits DNA-dependent RNA synthesis but not RNA-dependent RNA synthesis. As shown in Fig. 4, in the presence of actinomycin D, no incorporation of [^{3}H]uridine into the RS virus genomic analog occurred when only pRSV-N and pRSV-P were present in the cotransfection (lanes 5 and 7). However, when pRSV-L was included in the cotransfection, synthesis of the genomic analog was readily detected (lanes 6 and 8). These results demonstrated that the labeled RNAs were the products of replication by the RS virus RNA-dependent RNA polymerase. Comparisons of lane 2 with 6 and of lane 4 with 8 of Fig. 4 demonstrated that the majority of the labeled, encapsidated RNAs represent RNAs replicated by the RS virus polymerase and that only a small percentage of the original T7 transcripts was encapsidated. The amount of RNA replicated from the wild-type genomic analog was less than that from the panhandle-type construct, although a similar molar ratio of plasmids was used in the transfection. This is similar to the finding with VSV for which it has been shown that RNAs having copyback DI-like termini replicate better than the wild-type RNA (32). Due to its higher RNA replication efficiency, the panhandle-type analog, pPH3, was used in the following assays for strand-specific RNA synthesis and to determine the *trans*-acting protein requirements for RNA replication.

Analysis of positive-strand RNA synthesis. During RNA replication of negative-stranded RNA viruses, the encapsidated negative-sense genome must first replicate a positive-sense RNA antigenome which in turn would be encapsidated and serve as a template for the synthesis of progeny negative-sense RNA. Therefore, the synthesis of a positive-strand RNA intermediate is critical evidence for establishing that replication of the original negative-strand RNA has occurred. To test for RS virus positive-strand RNA synthesis, an RNase protection assay was carried out with a strand-specific probe. Encapsidated RNA was selected by immunoprecipitation from cells cotransfected with pPH3 and combinations of the N, P, and L gene plasmids. A 391-nt ^{35}S-labeled RNA probe was used, of which 360 nt were transcribed from the L gene sequence and complementary to the positive-sense RNA and the other 31 nt corresponded to the polylinker region of the vector. Hybridization of the probe with the positive-strand RNA should pro-

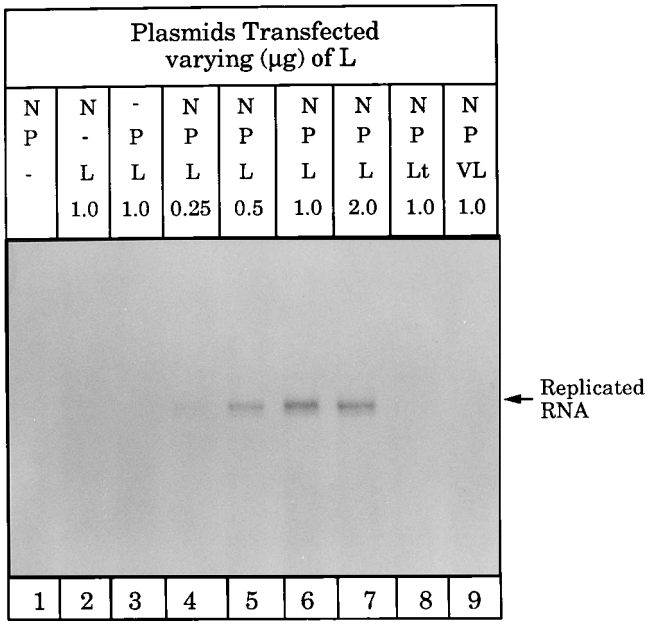

FIG. 6. Analysis of the *trans*-acting proteins required for RNA replication. Cells were infected with vTF7-3 (all lanes), transfected with 5 μg of pPH3 and combinations of 5 μg of pRSV-N, 2 μg of pRSV-P, and various amounts of pRSV-L as indicated. −, no plasmid. Cells were exposed to [^{3}H]uridine (25 μCi/ml) between 16 and 22 h posttransfection in the presence of actinomycin D (10 μg/ml). The RNAs extracted from the cell lysates were analyzed by electrophoresis in a 1.75% agarose-urea gel and detected by fluorography. In lanes 8 and 9, plasmid pRSV-L, was replaced with a truncated form of the RS virus L gene plasmid (Lt, lane 8) or the VSV L gene plasmid (VL, lane 9) in the cotransfection.

duce a double-strand RNA hybrid which, after nuclease digestion to remove the overhanging nucleotides, would be 360 bp long. Indeed, electrophoretic analysis of the protected RNA products demonstrated that the positive-strand RNA was synthesized when all three viral gene plasmids (N, P, and L) were cotransfected (Fig. 5, lane 4) but not when the L gene plasmid was omitted from the transfection (Fig. 5, lane 3). This protected RNA migrated at the predicted size (360 nt). The undigested probe (391 nt) shown in lane 1 is the probe hybridized with yeast RNA; lane 2 illustrates the complete degradation of this probe after treatment with RNase, thus indicating that the nuclease digestion was complete and that the RNA in lane 4 represented the specifically protected product. These data demonstrated that positive-strand RNA was replicated from the initial negative-sense RNA transcribed in cells and confirmed that RNA replication occurred only when all three viral proteins, N, P, and L, were provided by cotransfection. The presence of positive-sense RNA was also confirmed by primer extension analysis with a negative-sense oligonucleotide primer (data not shown).

The N, P, and L proteins are the minimal *trans*-acting proteins required for RNA replication. To determine the minimal *trans*-acting protein requirements for RS virus genomic RNA replication and to optimize the conditions of RNA replication, pPH3-transfected cells were cotransfected with various combinations of plasmids encoding the N, P, and L proteins. At 16 h posttransfection, the cells were labeled with [^{3}H]uridine for 6 h in the presence of actinomycin D. The RNAs extracted from cell lysates were analyzed by electrophoresis in an agarose-urea gel. The results clearly showed that any combination of two of the three plasmids in the cotransfection did not support RNA replication (Fig. 6, lanes 1 to 3). Only when all three plasmids were present in the cotransfection did replication of the RNA analog occur (Fig. 6, lanes 4 to 7). This result defined the N, P, and L proteins as the minimal *trans*-acting viral proteins required for RNA replication of the RS virus genomic analog. As the amount of pRSV-L was increased from 0.25 to 1 μg in the cotransfection, the yield of replicated RNA products also increased (Fig. 6, lanes 4 to 6). However, when 2 μg of pRSV-L was cotransfected the efficiency of replication no longer increased (Fig. 6, lane 7). Maximum RNA replication occurred when the molar ratio of transfected N, P, and L genes was 12:5:1 (Fig. 6, lane 6).

To test the specificity of the requirement for the viral RNA-dependent RNA polymerase for RS virus RNA replication, a VSV L gene plasmid that had been shown to support VSV RNA replication in a similar system (24, 32) and a truncated form of RS virus L gene plasmid that expressed a carboxy-terminally truncated form of L (predicted size, 170 kDa) were used to replace pRSV-L in the cotransfection. As shown in Fig. 6, lanes 8 and 9, neither the heterologous VSV RNA polymerase nor the truncated RS virus polymerase supported RNA replication. These data demonstrate that the RNA replication of the genomic analog requires the RS virus-specific, functional polymerase. Further work will be required to see if homologous mixtures of the VSV proteins will encapsidate and replicate the RS virus genomic analogs.

DISCUSSION

The ability to manipulate nonsegmented negative-strand RNA virus genomes at the cDNA level and to recover replicable RNA is important for analyses of the role of *cis*- and *trans*-acting elements in the control of RNA replication and transcription and for the recovery of infectious virus from a cDNA clone in order to study the roles of individual gene products in viral replication and pathogenesis.

We previously achieved the recovery of infectious DI particles of VSV entirely from cDNA clones (24). The DI genome was expressed intracellularly by transfection of a specialized T7 transcription plasmid. Simultaneously, plasmids were transfected that expressed the viral proteins necessary for encapsidation and replication, such that the genomic RNA analog was expressed in the cytoplasm in the presence of the viral N, P, and L proteins. This optimized the chance for encapsidation of the genomic RNA with the nucleocapsid protein to form functional ribonucleocapsids. This approach required functional cDNA clones for these viral genes. Major hurdles for establishing a similar system for RS virus were the lack of a complete cDNA clone of the polymerase gene and the lack of an assay by which such a clone could be identified as functional. For RS virus, unlike VSV, reconstitution of in vitro transcription activity from purified proteins has not been accomplished and therefore cannot be used to screen for a functional polymerase. Moreover, no temperature-sensitive mutants have been defined for the RS virus polymerase gene, which obviates the approach of complementation to identify a functional polymerase as was done successfully for VSV by Schubert et al. (29).

In the work described here, we report the construction of a complete cDNA clone for the RS virus RNA-dependent RNA polymerase. This cDNA clone expressed a polypeptide that comigrated with the authentic RS virus L protein. The expressed L protein was shown to be functional by testing its ability to support replication of RS virus RNAs when coexpressed in cells with the RS virus N and P proteins. Replication was shown to be due to an RNA-dependent RNA polymerase

by its insensitivity to actinomycin D, and the level of replication of the viral RNA depended on the level of expression of the polymerase. There was an optimum molar ratio of the transfected N, P, and L genes for supporting RNA replication. When the molar ratio altered, such as with under- or overexpression of the polymerase, the level of RNA replication decreased, which is in agreement with the observation of Schubert et al. (29) that overexpression of the VSV polymerase inhibited complementation.

Previous work reporting the rescue of RS virus genomic analogs from cDNA clones used the approach of transfecting RNA transcripts of genomic analogs into cells infected with wild-type RS virus as helper to supply the proteins required for replication (6, 7). The efficiency of recovery of replicated RNA using this approach was low and required the use of sensitive reporter genes for detection. In contrast, in the work reported here, replication occurred at levels that were measurable by direct biochemical means without the use of reporter genes. Most importantly, wild-type helper virus was not needed to provide the *trans*-acting viral proteins necessary for RNA replication. Instead, we established an RNA recovery system in which the *trans*-acting protein factors required for replication were supplied entirely from cDNA clones. This differentiates this work from previous approaches in that it circumvented the need to use wild-type RS virus as helper and thereby allowed analysis of the individual *trans*-acting protein requirements for RNA replication.

The data presented here establish that the N, P, and L proteins are the minimal *trans*-acting proteins required for RS virus RNA replication. The two nonstructural proteins of RS virus, NS1 and NS2, whose genes are located at the 3′ terminus of the genome following the leader gene and prior to the N gene, have been postulated to play a role in RNA replication or transcription solely because of the location of their genes at the 3′ terminus of the genome. However, the work presented here shows that these nonstructural proteins are not essential for RNA replication. The replicon having wild-type termini (pWT1) is expected to carry out transcription as well as replication. The studies presented here have focused on analysis of RNA replication, and further work is in progress to determine whether these nonstructural proteins play a role in modulating RNA replication and transcription. The system described here will allow these questions to be addressed.

The recovery of replicable RNA directly from cDNA clones and its measurement by direct metabolic labeling also make it possible to assay the *cis*-acting elements as well as the *trans*-acting factors that are important in controlling RNA replication. The data presented here, comparing the replication efficiencies of the wild-type RS virus genomic analog with that of the copyback DI or panhandle-type construct, showed that the panhandle-type construct replicated significantly better than the construct containing the wild-type 3′ leader and 5′ trailer region. This finding agrees with the observation that naturally occurring copyback-type DI RNAs of other negative-strand viruses outreplicate the corresponding wild-type RNAs and with the recent observation of Wertz et al. (32), who showed that the extent of terminal complementarity of VSV genomic RNA correlates with the efficiency of replication and controls the balance between replication and transcription. These data differ from the previous report of Collins et al. (7) in which no difference was noted in recovery of chloramphenicol acetyltransferase (CAT) reporter gene activity from a wild-type or a panhandle-type RS virus genomic analog. However, the work reported here used assays in which the process of RNA replication could be measured by direct analysis of RNA synthesis. The studies with VSV showed that changes in the termini of the VSV negative-sense RNA genome that affected the extent of terminal complementarity had dramatic effects on both transcription of mRNA and replication of the genome, and it seems likely that these effects account for the discrepancy with the CAT expression studies of Collins et al. (7).

In summary, the preparation of a cDNA clone of the functional RS virus RNA-dependent RNA polymerase and the establishment of a system to recover replicable RS virus genomic RNA analogs from cDNA clones enabled us to study RS virus RNA replication directly, without the use of either reporter genes or wild-type helper virus. This RS virus RNA replication system provided a means for identifying a functional clone of the RS virus RNA-dependent RNA polymerase and for detecting the minimal *trans*-acting protein requirements for RNA replication. Further development of this system should allow us to study the *cis*-acting sequences required for encapsidation, RNA replication, and transcription and to identify the gene products required for RS virus assembly and budding to yield infectious particles. Moreover, the clone for a functional RS virus RNA-dependent RNA polymerase has potential application in development of antiviral drugs and analysis of their mechanism of action and for developing genetically attenuated or recombinant vaccines.

ACKNOWLEDGMENTS

We thank Alison LeGrone, Fenglan Li, and Patrick Dean for excellent technical assistance and Andrew Ball and the members of the Wertz laboratory for advice and constructive comments.

The work was supported by Public Health Service grants AI12464 and AI20181 from NIAID to G.W.W.

REFERENCES

1. **Barik, S.** 1992. Transcription of human respiratory syncytial virus genome RNA in vitro: requirement of cellular factor(s). J. Virol. **66:**6813–6818.
2. **Calain, P., D. Curran, D. Kolakofsky, and L. Roux.** 1992. Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. Virology **191:**62–71.
3. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water-soluble fluoro, sodium salicylate. Anal. Biochem. **98:**132–135.
4. **Collins, P.** 1991. The molecular biology of human respiratory syncytial virus (RSV) of the genus Pneumovirus, p. 103–162. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
5. **Collins, P. L., Y. T. Huang, and G. W. Wertz.** 1984. Identification of a tenth mRNA of respiratory syncytial virus and assignment of polypeptides to the 10 viral genes. J. Virol. **49:**572–578.
6. **Collins, P. L., M. A. Mink, M. G. Hill III, E. Camargo, H. Grosfeld, and D. S. Stec.** 1993. Rescue of a 7502-nucleotide (49.3% of full-length) synthetic analog of respiratory syncytial virus genomic RNA. Virology **195:**252–256.
7. **Collins, P. L., M. A. Mink, and D. S. Stec.** 1991. Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proc. Natl. Acad. Sci. USA **88:**9663–9667.
8. **Collins, P. L., and G. W. Wertz.** 1983. cDNA cloning and transcriptional mapping of the nine polyadenylated RNAs encoded by the genome of human respiratory syncytial virus. Proc. Natl. Acad. Sci. USA **80:**3208–3212.
9. **Conzelmann, K.-K., and M. Schnell.** 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. J. Virol. **68:**713–719.
10. **De, B. P., and A. K. Banerjee.** 1993. Rescue of synthetic analogs of genome RNA of human parainfluenza virus type 3. Virology **196:**344–348.
11. **Dimock, K., and P. L. Collins.** 1993. Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3. J. Virol. **67:**2772–2778.
12. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. **10:**297–309.
13. **Enami, M., W. Luytjes, M. Krystal, and P. Palese.** 1990. Introduction of site-specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA **87:**3802–3805.
14. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.

15. **Haltiner, M., T. Kemp, and R. Tjian.** 1985. A novel strategy for constructing clustered point mutations. Nucleic Acids Res. **13:**1015–1028.
16. **Huang, Y. T., P. L. Collins, and G. W. Wertz.** 1985. Characterization of the 10 proteins of human respiratory syncytial virus: identification of a fourth envelope-associated protein. Virus Res. **2:**157–173.
17. **Huang, Y. T., R. R. Romito, B. P. De, and A. K. Banerjee.** 1993. Characterization of the in vitro system for the synthesis of mRNA from human respiratory syncytial virus. Virology **193:**862–867.
18. **Huang, Y. T., and G. W. Wertz.** 1982. The genome of respiratory syncytial virus is a negative-stranded RNA that codes for at least seven mRNA species. J. Virol. **43:**150–157.
19. **King, A. M. Q., E. J. Stott, S. J. Langer, K. K.-Y. Young, L. A. Ball, and G. W. Wertz.** 1987. Recombinant vaccinia viruses carrying the N gene of human respiratory syncytial virus: studies of gene expression in cell culture and immune response in mice. J. Virol. **61:**2885–2890.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell **59:**1107–1113.
22. **Mink, M. A., D. S. Stec, and P. L. Collins.** 1991. Nucleotide sequences of the 3′ leader and 5′ trailer regions of human respiratory syncytial virus genomic RNA. Virology **185:**615–624.
23. **Park, K. H., T. Huang, F. F. Correia, and M. Krystal.** 1991. Rescue of a foreign gene by Sendai virus. Proc. Natl. Acad. Sci. USA **88:**5537–5541.
24. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
25. **Pattnaik, A. K., and G. W. Wertz.** 1990. Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. J. Virol. **64:**2948–2957.
26. **Patton, J. T., N. L. Davis, and G. W. Wertz.** 1984. N protein alone satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. J. Virol. **49:**303–309.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Schnell, M. J., T. Mebatsion, and K.-K. Conzelmann.** 1994. Infectious rabies viruses from cloned cDNA. EMBO J. **13:**4195–4203.
29. **Schubert, M., G. G. Harmison, C. D. Richardson, and E. Meier.** 1985. Expression of a cDNA encoding a functional 241-kilodalton vesicular stomatitis virus RNA polymerase. Proc. Natl. Acad. Sci. USA **82:**7984–7988.
30. **Stec, D. S., M. G. Hill III, and P. L. Collins.** 1991. Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. Virology **183:**273–287.
31. **Wertz, G. W., and N. Davis.** 1981. Characterization of mapping of RNase III cleavage sites in VSV genome RNA. Nucleic Acids Res. **9:**6487–6503.
32. **Wertz, G. W., S. Whelan, A. LeGrone, and L. A. Ball.** 1994. Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. Proc. Natl. Acad. Sci. USA **91:**8587–8591.