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RNA Replication by Respiratory Syncytial Virus (RSV) Is Directed by the N, P, and L Proteins; Transcription Also Occurs under These Conditions but Requires RSV Superinfection for Efficient Synthesis of Full-Length mRNA

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Previously, a cDNA was constructed so that transcription by T7 RNA polymerase yielded a ~1-kb negative-sense analog of genomic RNA of human respiratory syncytial virus (RSV) containing the gene for chloramphenicol acetyltransferase (CAT) under the control of putative RSV transcription motifs and flanked by the RSV genomic termini. When transfected into RSV-infected cells, this minigenome was "rescued," as evidenced by high levels of CAT expression and the production of transmissible particles which propagated and expressed high levels of CAT expression during serial passage (P. L. Collins, M. A. Mink, and D. S. Stec, Proc. Natl. Acad. Sci. USA, 88:9663–9667, 1991). Here, this cDNA, together with a second one designed to yield an exact-copy positive-sense RSV-CAT RNA antigenome, were each modified to contain a self-cleaving hammerhead ribozyme for the generation of a nearly exact 3′ end. Each cDNA was transfected into cells infected with a vaccinia virus recombinant expressing T7 RNA polymerase, together with plasmids encoding the RSV N, P, and L proteins, each under the control of a T7 promoter. When the plasmid-supplied template was the mini-antigenome, the minigenome was produced. When the plasmid-supplied template was the minigenome, the products were mini-antigenome, subgenomic polyadenylated mRNA and progeny minigenome. Identification of progeny minigenome made from the plasmid-supplied minigenome template indicates that the full RSV RNA replication cycle occurred. RNA synthesis required all three RSV proteins, N, P, and L, and was ablated completely by the substitution of Asn for Asp at position 989 in the L protein. Thus, the N, P, and L proteins were sufficient for the synthesis of correct minigenome and antigenome, but this was not the case for subgenomic mRNA, indicating that the requirements for RNA replication and transcription are not identical. Complementation with N, P, and L alone yielded an mRNA pattern containing a large fraction of molecules of incomplete, heterogeneous size. In contrast, complementation with RSV (supplying all of the RSV gene products) yielded a single discrete mRNA band. Superinfection with RSV of cells staging N/P/L-based RNA synthesis yielded the single discrete mRNA species. Some additional factor supplied by RSV superinfection appeared to be involved in transcription, the most obvious possibility being one or more additional RSV gene products.

Human respiratory syncytial virus (RSV) is an important agent of pediatric respiratory tract disease and is the prototype member of the pneumovirus subfamily of the paramyxoviruses. The genome of RSV is a single nonsegmented negative RNA strand of 15,222 nucleotides (nt) (4, 28, 38).

Recently, there has been considerable progress in developing methods for direct genetic manipulation of the RNAs and proteins involved in the replication, transcription, and packaging of negative-strand RNA viruses, both segmented and nonsegmented. For the segmented negative-strand influenza virus, it was shown that cDNA-encoded versions of single gene segments could be introduced into influenza virus-infected cells and transcribed, replicated, and incorporated stably into infectious virus (19, 25). For the nonsegmented negative-strand virus rabies, it was recently shown that cDNA-encoded antigenome RNA expressed intracellularly in the presence of plasmid-encoded viral proteins could be replicated, transcribed, and assembled into infectious virus (37). The ability to produce virus from cDNA provides the means to engineer new virus strains. Development of this capability for other negative-strand RNA viruses is in progress.

In addition, information on negative-strand viral RNA synthesis and protein function has been obtained by using cDNA-encoded, helper-dependent "minigenomes" which are introduced intracellularly and "rescued" by coexpression of viral proteins. These studies have been performed with influenza virus and, more recently, with several nonsegmented negative strand viruses, including vesicular stomatitis virus, rabies virus, Sendai virus, RSV, and parainfluenza type 3 virus (2, 6–9, 13–15, 19, 24–26, 29–31, 40). Rescue means that the minigenome participates in one or more of the major features of the intracellular viral replicative cycle, including RNA replication, transcription, and production of transmissible particles. Studies to date have employed minigenomes such as a cDNA-encoded copy of naturally occurring defective interfering RNA (2, 13, 31, 40) and an engineered version of genome RNA containing a large internal deletion and the insertion of a marker gene, usually that for bacterial chloramphenicol acetyltransferase (CAT), under the control of putative transcription signals (6–9, 14, 15, 19, 24–26, 29, 30).

The minigenome cDNAs, typically engineered to be under the control of a promoter for bacteriophage RNA polymerase,

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can be transcribed in vitro, followed by RNA transfection, or can be transfected as a plasmid and transcribed intracellularly by RNA polymerase supplied by a recombinant vaccinia virus. The viral proteins needed to drive rescue of the minigenome can be supplied by infection with standard homologous virus (virus complemented) or by plasmids encoding viral proteins under the control of a bacteriophage promoter (plasmid complemented). These systems have provided the basis for beginning the analysis of *cis*-acting sequences in the minigenome and *trans*-acting plasmid-encoded viral proteins. For the nonsegmented negative-strand viruses, studies to date have supported the model that the sequences critical to RNA replication lie at the genomic termini, that the 3′ terminus together with sequences flanking the viral genes is important for RNA transcription, and that the N, P, and L proteins are necessary and sufficient for RNA replication and transcription. However, analysis of nonsegmented negative-strand RNA viruses by these methods is still at an early stage.

RSV, representing the pneumoviruses, is somewhat distinct from the other paramyxoviruses in having several more mRNAs, several different proteins, differences in genome organization and expression, such as a gene overlap and lack of "RNA editing" (39 and references therein), and a general absence of sequence relatedness except for low levels of overall relatedness in the amino acid sequences of the fusion (F) and polymerase (L) proteins (4, 23, 28, 38).

Nonetheless, RSV resembles other paramyxoviruses, and indeed all nonsegmented negative-strand viruses, in many fundamental features. Pertinent to RNA synthesis, RSV encodes a major nucleocapsid (N) protein, a nucleocapsid-associated phosphoprotein (P), and an L protein, the three proteins which appeared to be required and sufficient for RNA replication and transcription in the few other viruses examined to date. The general features of RNA structure and synthesis also have important similarities: the genome is a single negative strand, it is transcribed in a sequential stop-start manner into mostly nonoverlapping mRNAs, its genes are bordered by consensus gene-start (GS) and gene-end (GE) motifs which presumably function in transcription, it appears to be replicated through synthesis of an intermediary (antigenome) which is an exact complementary copy of the genome, and the 3′ and 5′ genome termini have a high degree of complementarity for the first 24 to 26 nt, reflecting either a conservation of promoter sequence at the 3′ ends of genome and antigenome or a role for base-pairing between the two termini during the replicative cycle (4, 23).

Previous work showed that an RSV-CAT minigenome (6–8) and a complementary mini-antigenome (23a) synthesized in vitro and transfected in RSV-infected cells directed the synthesis of large amounts of CAT. More recently, we have been able to directly demonstrate that, under these conditions of "RSV-complemented" rescue, the input minigenomic RNA is replicated into mini-antigenome and progeny minigenome and is transcribed into mRNA (24; unpublished data).

Here, we have modified this system so that the minigenome or mini-antigenome is transcribed intracellularly from a transfected plasmid in the presence of RSV proteins supplied from cotransfected plasmids, driven by T7 RNA polymerase supplied by the recombinant vaccinia virus vTF7-3. The first goal of these studies was to identify the viral proteins required for RSV RNA replication and transcription.

MATERIALS AND METHODS

cDNAs. cDNA C2 was constructed to encode a 931-nt negative-sense RSV-CAT RNA, or minigenome, containing the CAT gene flanked by GS and GE signals and by the genome termini. cDNA C4 was constructed to encode its exact complement, a positive-sense RSV-CAT mini-antigenome (Fig. 1A).

The C2-encoded minigenome (Fig. 1A and B) is very similar to the previously described (6) 934-nt prototype. There are four differences. (i) Three G residues were introduced between the T7 promoter and the 5′ end of the transcript to increase the efficiency of T7 transcription. Sequence analysis confirmed that these three G's are retained in RNA transcripts made from this promoter in vitro (34a), making a 3-nt 5′ extension (heterologous terminal nucleotides are not included in the base numbering of the minigenome). (ii) A hammerhead-type ribozyme sequence and T7 transcription terminator were added to the 3′ end of the encoded transcript (Fig. 1B and C). The ribozyme, modified from a published two-molecule system (35), was designed to fold back, hybridize with the 3′-terminal RSV-specific sequence on RSV-CAT RNA, and execute self-cleavage to leave a single additional, non-RSV-specific 3′-phosphorylated U residue on the 3′ terminus. Following cleavage, the base-paired ribozyme presumably would dissociate from RSV-CAT, perhaps facilitated by displacement by N protein. (iii) The RSV-CAT insert was modified to contain a C in place of G at leader position 4 (Fig. 1B), a change which was associated with a 7- to 20-fold increase in transcription and replication in the RSV infection/RNA transfection system (8, 24; unpublished data) (see Discussion). (iv) Last, we (unpublished data) and others (34) found that transfection of CAT cDNA into vaccinia virus-infected cells resulted in the expression of CAT in a fashion which was independent of attached promoters and apparently resulted from promiscuous transcription by vaccinia virus polymerase. We successfully eliminated this background by the insertion into the RSV-CAT cDNA, at three different sites, of the vaccinia virus early RNA polymerase transcription termination motif (5′-TTTTTNT [33, 41]). One double terminator [5′$(T)_8$] was placed in the positive strand between the T7 terminator and the ribozyme. A second [5′$(T)_8$] was placed in the negative strand on the outside of the T7 promoter. The third, single terminator (5′-TTTTTAT) was inserted in the positive strand into the NS1 nontranslated region as follows. The sequence 5′-AAATTTAACTCTAGA (which is immediately upstream of the *Xba*I site [underlined] that joins the NS1 nontranslated sequence to the CAT translational open reading frame (ORF) [6] [also see Fig. 1A]), was changed to 5′-TTTTTATCTAGA (also reducing the length by 3 nt). All mutations were produced by PCR with synthetic oligonucleotides containing the desired changes, followed by restriction site replacement with conveniently placed restriction sites at the junctions of the RSV, CAT, and plasmid sequences in the prototype construct. Modified cDNA sequences were confirmed in their entirety.

For construction of cDNA C4, encoding the mini-antigenome, the positions of the T7 promoter and ribozyme/T7 terminator were exchanged to change the polarity of the encoded RNA from negative to positive. Also, because the 3′ ends of RSV genome and antigenome RNA have a few sequence differences, the hybridizing sequence of the ribozyme was modified accordingly, so that an exact match would be made.

cDNAs containing the ORFs of the N, P, and L proteins were placed into the *Nco*I-*Bam*HI (N and P) or *Nco*I-*Pst*I (L) window of plasmid pTM-1 (the generous gift of Bernard Moss). In this configuration, the insert cDNA is under control of the promoter for T7 RNA polymerase and is preceded by the internal ribosome entry site of encephalomyocarditis virus, so that the ATG of this entry site (contained within the *Nco*I site 5′-CCATGG) serves as the ATG for the RSV cDNA (16). None of the three RSV cDNAs contained a naturally occurring *Nco*I site flanking the translation start site. For the N gene, this was introduced by PCR mutagenesis with reverse-transcribed intracellular mRNA as the template, and the cDNA sequence was confirmed in its entirety to encode the same amino acid sequence as reported previously (5). The P gene contains an internal *Nco*I site. Therefore, PCR mutagenesis was used to place a *Bsa*I site upstream of the P ATG, so that cleavage would generate an *Nco*I-compatible sticky end. The sequence of the P cDNA was confirmed in its entirety; its encoded amino acid sequence was identical to the published one (36). The L cDNA contains a *Bam*HI site immediately downstream of the translational start site, and ligation of cleaved and filled-in *Bam*HI and *Nco*I sites correctly fused the L ORF to the pTM1 translation start site, as confirmed by sequence analysis. The complete L cDNA was resequenced in its entirety and found to contain a single change at the amino acid level (Asp to Asn at position 989) relative to the published consensus sequence (38). As shown below, this L protein, designated ΔL, was completely inactive in rescue. This single change lay near a unique *Eco*NI site and was restored to the consensus sequence by PCR mutagenesis and restriction site replacement. As shown below, it was found to encode a functional L protein.

Transfections. Confluent monolayers of 293 cells in six-well dishes (each 10-cm^2 well contained 1.4×10^6 to 1.7×10^6 cells) were infected with 10 PFU of the vaccinia virus vTF7-3, expressing the T7 RNA polymerase (18), per cell. At 45 min postinfection, the inoculum was replaced with transfection medium, consisting of Opti-MEM (Life Technologies) containing the indicated (see figure legends) amount of each plasmid and 10 μl of Lipofectamine (Life Technologies) per ml, mixed according to the supplier's protocols. Twelve hours later, the transfection medium was replaced with minimal essential medium (MEM) containing 10% fetal bovine serum. For comparison, as shown in Fig. 8 and 9, rescue was also performed by the previously described method of RNA transfection and RSV complementation. Briefly, cDNA C2 or C4 was used to direct transcription in vitro, followed by RNA transfection into RSV-infected cells (6). Cells were harvested at 24 or 48 h postinfection as indicated in the figure legends.

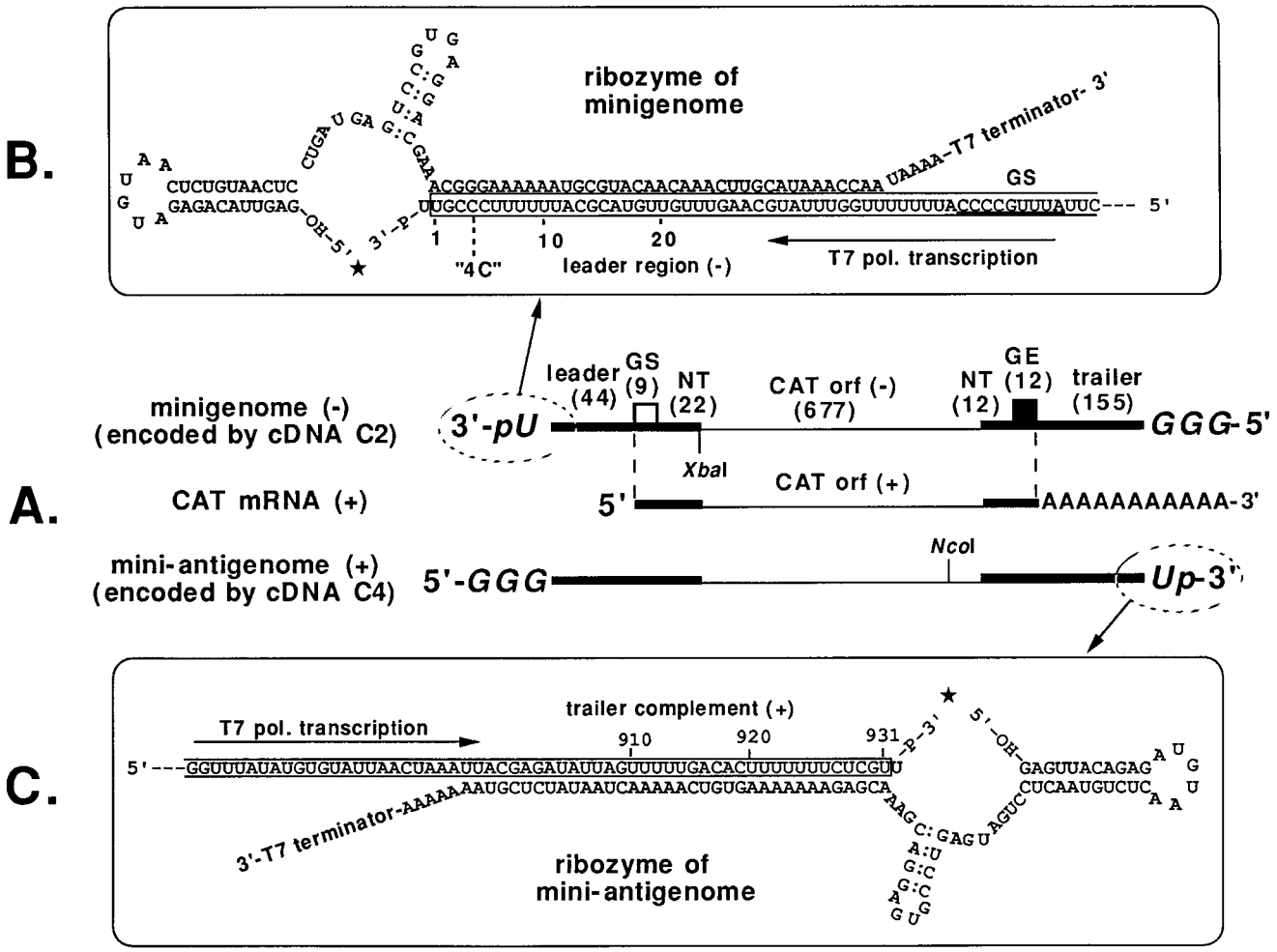

FIG. 1. cDNA-encoded RSV-CAT negative-sense minigenome and positive-sense mini-antigenome, and the structure of ribozymes used to generate nearly exact 3′ ends. (A) Line diagrams of the minigenome (encoded by cDNA C2) and mini-antigenome (cDNA C4) as they would appear following synthesis by T7 RNA polymerase and ribozyme self-cleavage and dissociation: these plasmid-templated forms contain non-viral nucleotides (bold italics) at the 5′ (a G triplet) and 3′ (a 3′-phosphorylated U) ends. Apart from these heterologous bases, the minigenome and mini-antigenome are exact complementary copies of 931 nt. The RSV-specific regions (thick lines) are the 3′-terminal 75 nt of the authentic genome (including the leader region, the GS signal, and 22 nt of the NS1 nontranslated [NT] region) and the 5′-terminal 179 nt (including 12 nt from the L NT region, the GE signal, and the trailer region). Between these viral segments are 677 nt containing the complement of the 660-nt CAT ORF flanked by restriction sites. Also shown is the predicted 732-nt [exclusive of poly(A)] positive-sense polyadenylated subgenomic mRNA. The *Nco*I and *Xba*I sites shown were used to linearize plasmid for in vitro run-off transcription for the preparation of positive- and negative-sense RNA probes, respectively (Materials and Methods). (B and C) The 3′ end and flanking ribozyme of the (B) minigenome and (C) mini-antigenome as it would appear with the ribozyme in folded form and self-cleavage having just occurred (the cleavage site is indicated with a star; following cleavage, the ribozyme presumably dissociates from RSV-CAT). The RSV-specific sequence is boxed and numbered according to the 3′ and 5′ minigenome sequence; the minigenome sequence also shows the C residue in place of G (negative sense) at position 4. pol, polymerase.

Oligo(dT) chromatography. From 30 to 100 μg of RNA was dissolved in 0.5 ml of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS) (elution buffer) in an Eppendorf tube, heated for 5 min in a bath of boiling water, quickly cooled, and adjusted to contain 0.5 M NaCl (binding buffer). Then, 150 μl of a 1:1 slurry of oligo(dT)-cellulose (Collaborative Research type 3) in binding buffer was added, and the mixture was rocked at room temperature for 5 min. Following very brief centrifugation at one-quarter speed, the supernatant (unbound fraction) was removed, clarified by centrifugation for 1 min, and precipitated with ethanol. The cellulose pellet was washed by three rounds of resuspension and pelleting in binding buffer. Bound RNA was recovered by four rounds of washing and pelleting in a total of 500 μl of 65°C elution buffer. The eluted fraction was clarified by centrifugation, adjusted to contain 0.2 M NaCl and 40 μg of tRNA per ml, and precipitated with ethanol. These conditions were determined to minimize artifactual binding through "sandwich" hybridization of genome to mRNAs, and each experiment contained controls consisting of intracellular RSV-CAT RNAs produced by RNA transfection/RSV complementation (not shown) to confirm that the binding of polyadenylated mRNA was complete and that artifactual binding of the minigenome and mini-antigenome was minimal.

Northern (RNA blot) hybridization with strand-specific probes. Total intracellular RNA was extracted by dissolving cell pellets in Trizol reagent (Life Technologies) according to the supplier's protocol, except that the RNAs were extracted twice with phenol-chloroform following the isopropanol precipitation. Samples (each representing one-fifth of one well of cells [approximately 15 μg in the case of total RNA]) were analyzed by electrophoresis in small 1.5% agarose gels (each 10-well gel was 10 cm wide and 7 cm long) containing 0.44 M formaldehyde, run at 90 V for 2 h. Equal cell equivalents of samples were loaded except as noted in the figure legends. Gels were transferred to 0.2-μm-pore-size nitrocellulose with a Turbo-Blot apparatus (Schleicher and Schuell) with a transfer buffer of 3 M NaCl and 8 mM NaOH (3). RNAs were fixed by UV cross-linking (Stratagene). With the use of radiolabeled RNA probes (see Fig. 3B, 4, and 6 through 9), prehybridization was performed for 6 h at 65°C in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS, 5× Denhardt's solution, and 0.5 mg of sheared DNA per ml. Hybridization was performed overnight under the same conditions with the addition of approximately 2×10^6 dpm of [^{32}P]CTP-labeled negative- or positive-sense RSV-CAT RNA. This was synthesized in vitro from *Xba*I-digested C2 cDNA (for the negative-sense probe) or *Nco*I-digested C4 cDNA (for the positive-sense probe) (see Fig. 1A for the positions of restriction sites), followed by extraction with phenol and column chromatography on Sephadex G-25. Washing was done in 2×

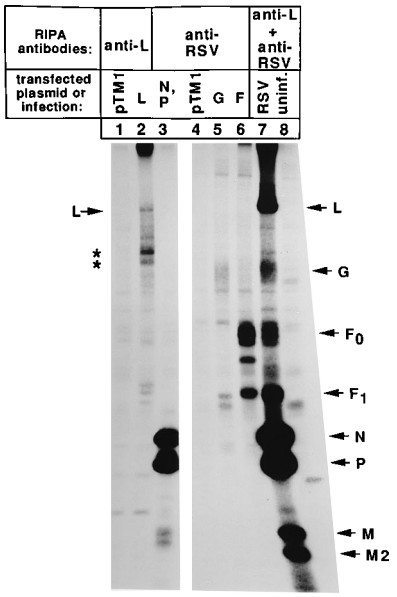

FIG. 2. Comparison by SDS–10% PAGE of [^{35}S]methionine-labeled, immunoprecipitated proteins synthesized in response to RSV or transfected plasmids. Lanes 1 to 6: cells were infected with vTF7-3 and transfected with 0.5 μg of plasmid pTM1 lacking insert (lanes 1 and 4) or encoding the L (lane 2), G (lane 5), or F (lane 6) protein. Cells in lane 3 received a mixture of 0.4 μg each of plasmids encoding N and P. Transfected cells were labeled at 10 to 12 h postinfection and harvested. Lanes 7 and 8: for comparison, cells were infected with RSV (lane 7) or mock infected (lane 8), labeled at 16 to 20 h postinfection, and harvested. The labeling periods differed because of the difference in kinetics of protein expression with the vaccinia virus-based system versus RSV infection. Immunoprecipitation was performed under conditions of antibody excess with rabbit antiserum raised against amino acids 132 to 186 of the L protein expressed in *Escherichia coli* as an N-terminal fusion protein with β-galactosidase (lanes 1 and 2), gradient-purified, disrupted RSV (lanes 3 to 6), or an equal mixture of the two antisera (lanes 7 and 8). Asterisks indicate two of the more abundant early quitters of plasmid-encoded L protein. Lanes 1 to 3 and 4 to 8 are separate parts from the same gel.

SSC–0.1% SDS at room temperature, followed by 2 h at 65°C. Subsequent washing in 0.1× SSC at 65°C had little effect and was omitted. For oligonucleotide hybridization, prehybridization was done for 6 h at 52°C in 6× SSC–0.1% SDS–6× Denhardt's solution, followed by overnight hybridization under the same conditions with the addition of 10^7 dpm per blot of oligodeoxynucleotide (see figure legend for description) which had been labeled with [γ-^{32}P]ATP and polynucleotide kinase, followed by phenol extraction and Sephadex G25 chromatography. Washes (15 min each) were done in 6× SSC, twice at room temperature and twice at 52°C. Blots were exposed to X-ray film for 0.5 to 24 h. In preliminary experiments (not shown), treatment of RNA samples prior to electrophoresis with sufficient DNase to fully degrade 1 μg of added plasmid had no effect on the bands described here as RNA. The strand specificity of negative- and positive-sense RNA probes was confirmed periodically against in vitro-synthesized negative- and positive-sense full-length RSV-CAT RNA.

RESULTS

Expression of the N, P, and L proteins. Plasmids containing the N, P, and L genes under the control of the promoter for T7 RNA polymerase were transfected into 293 cells which had been infected with vaccinia virus vTF7-3 expressing that polymerase. Expression was examined by Western (immunoblot) analysis (not shown) or by metabolic labeling with [^{35}S]methionine and SDS-PAGE directly (not shown) or following radioimmunoprecipitation (RIP) with rabbit antiserum against gradient-purified, disrupted RSV or against a bacterially expressed fusion protein containing amino acids 132 to 186 of the L protein (Fig. 2).

The levels of N and P detected by RIP in plasmid-transfected cells were somewhat less than in those infected with RSV (Fig. 2, compare lanes 3 and 7). N and P labeled under these conditions in response to RSV or the transfected plasmids could also be seen as major bands detectable without immunoprecipitation (not shown). Western blot analysis of side-by-side infections and transfections harvested at 24 h postinfection showed that the total accumulation of N and P in cells in response to plasmids versus RSV was essentially identical (not shown). In contrast, the level of L protein expressed by plasmid was much lower than that observed in RSV-infected cells and included shorter forms (Fig. 2, lane 2, asterisks), which might be the result of early cessation of T7 transcription, translation, or both. Comigration of the plasmid-expressed and RSV-expressed RSV proteins was confirmed by electrophoresis of mixed samples (not shown).

Synthesis of intracellular RSV RNA-CAT RNAs directed by N, P, and L. The plasmid-expressed proteins were tested for the ability to support rescue of the RSV-CAT minigenome or mini-antigenome expressed from a cotransfected plasmid (Fig. 3). Coexpression of the minigenome with the N, P, and L proteins resulted in the abundant expression of CAT. Expression was completely dependent on the expression of N and P (Fig. 3A, lanes 1 and 7) and was completely ablated if the L plasmid was replaced by one encoding an L protein (ΔL) which differed only in having Asn instead of Asp at position 989 (lane 13). These results indicated that positive-sense CAT-encoding RNA, perhaps the predicted subgenomic mRNA (Fig. 1A), was being synthesized from the plasmid-supplied minigenome and that synthesis was dependent on the three RSV proteins. Titration of the levels of N and P plasmid showed that activity was greatest when the two plasmids were present in equal amounts (approximately equimolar, given the similar size of the N and P ORFs) at 0.5 or 1.0 μg of each per well, conditions under which the two proteins were expressed in relative amounts that resembled those in RSV-infected cells (Fig. 2, compare lanes 3 and 7).

Northern blot hybridization of total intracellular RNA with strand-specific RNA probes was used to examine the RSV-CAT RNAs synthesized intracellularly in response to plasmid-encoded RSV-CAT minigenome or mini-antigenome and the N, P, and L proteins (Fig. 3B). In each blot, the probe was chosen to be of the same polarity as the RSV-CAT made intracellularly from plasmid, so that products of the supplied RNA template would be detected.

When the plasmid-supplied RNA was the mini-antigenome (Fig. 3B, top panel), a single major band, presumptively designated the minigenome, was detected (lanes 4, 5, 7, 8, and 11 to 14). When the plasmid-supplied RNA was the minigenome (Fig. 3B, bottom panel), the products included a species identified presumptively as the mini-antigenome and a smear of putative subgenomic mRNA (lanes 4, 5, 7, 8, and 11 to 14). Evidence to support these identifications is described below.

Within the levels tested, variation in the levels of input plasmid had primarily a quantitative effect on RNA synthesis, with the negative and positive templates generally exhibiting similar responses. As was indicated in the CAT assay in Fig. 3A, RNA synthesis was maximal when N and P were present in equal amounts (Fig. 3B, lanes 4 and 8). Significant levels of RNA synthesis occurred when there was less P than N (lanes 5 and 7), but synthesis was greatly reduced when there was less N than P (lanes 3 and 9). RNA synthesis was robust at the highest tested level of N (lane 5), whereas the comparable level of P was inhibitory (lane 9). Varying the level of input L plasmid over an eightfold range gave a relatively broad optimum (lanes 11 to 14).

Identification of RSV-CAT genome, antigenome, and subgenomic mRNA. Mixing experiments (Fig. 4) showed that the

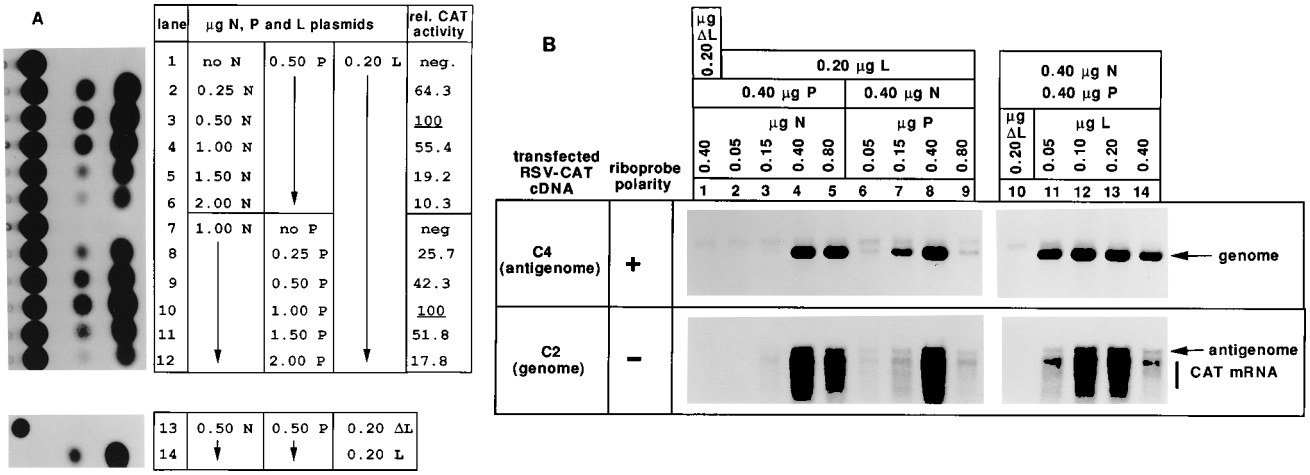

FIG. 3. Transcription and replication of plasmid-supplied RSV-CAT minigenome or mini-antigenome in response to plasmid-encoded RSV N, P, and L proteins, and optimization of the input amounts of N, P, and L plasmids. (A) Thin-layer plates showing levels of CAT expressed intracellularly in response to plasmid-supplied minigenome with different amounts of input N or P plasmids or in the presence of a defective L protein as a negative control. Cells (single wells of six-well plates) were infected with vaccinia virus vTF7-3 and transfected with (per well) 0.2 μg of cDNA C2 (encoding minigenome), 0.2 μg of plasmid encoding functional (lanes 1 to 12 and 14) or nonfunctional (lane 13) L protein (the latter designated ΔL), and the indicated amounts of N and P plasmids. Cells were harvested at 48 h posttransfection and 1/150 (lanes 1 to 12) or 1/10 (lanes 13 and 14) of the cells were processed for CAT assay (6, 21) and quantitated by liquid scintillation counting of excised spots. The results for lanes 1 to 6 were adjusted according to percent acetylation, with that in lane 3 being set at 100%, and the same was done for lanes 7 to 12 with lane 10 set at 100% (the relative conversion in lane 10 versus lane 3 was 1.56-fold). neg., negligible. (B) Analysis by Northern blot hybridization with strand-specific probes of RSV-CAT RNAs synthesized intracellularly when the amount of input N, P, or L plasmid was varied. Single wells of cells were infected with vTF7-3 and transfected with 0.2 μg of cDNA encoding the mini-antigenome (top panel) or minigenome (bottom panel), 0.4 μg each of the N and P plasmids or as indicated, and 0.2 μg of L plasmid or as indicated. As negative controls, the cells in lanes 1 and 10 received 0.2 μg of plasmid encoding nonfunctional L (ΔL) in place of that for functional L protein. Total intracellular RNA was extracted at 48 h posttransfection and analyzed by Northern blotting with ^{32}P-RSV-CAT RNA probe of the same polarity as that encoded by the transfected plasmid: positive-sense probe in the top panel, and negative-sense probe in the bottom panel.

putative minigenome synthesized intracellularly in response to the N, P, and L proteins (lane 2) comigrated with minigenome synthesized in vitro from plasmid C2 (lane 7), as demonstrated by mixing experiments (lanes 3 to 6). Similarly, mini-antigenome synthesized intracellularly comigrated with that synthesized in vitro (not shown). In vitro transcription of cDNA C2 (Fig. 4, lane 7) or C4 (not shown) yielded two bands. That the lower one was the appropriate size to be the minigenome (or mini-antigenome) was confirmed by its comigration with the 935-nt prototype RSV-CAT RNA transcribed from *Hga*I-digested cDNA (not shown). The upper band probably contained uncleaved ribozyme; self-cleavage in vitro was somewhat variable, being approximately 50% in the experiment shown in Fig. 4 (lane 7), whereas in others, cleavage was essentially complete (not shown).

The intracellular mini-antigenome and subgenomic mRNA were examined by Northern blot hybridization with two ^{32}P-labeled negative-sense oligonucleotides, one specific to the leader region and the other to the trailer (Fig. 5), after which

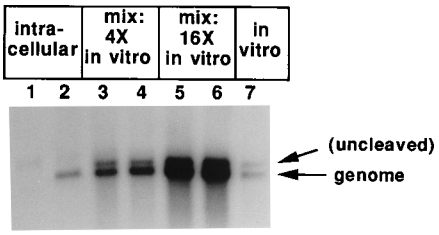

FIG. 4. Northern blot, probed with the positive-sense RSV-CAT RNA probe, showing the comigration of minigenome synthesized intracellularly in response to plasmid-expressed N, P, and L with minigenome synthesized in vitro by T7 polymerase. Lanes 1 and 2, RNA from cells which had been infected with vTF7-3 and transfected with plasmid C4 (encoding mini-antigenome) together with plasmids encoding the N, P, and nonfunctional (lane 1) or functional (lane 2) L protein. Lane 7, approximately 5 ng of minigenome transcribed from plasmid C2 in vitro by T7 RNA polymerase. The remaining lanes were mixing experiments: lanes 3 and 4, replicates of lanes 1 and 2, respectively, except that the samples were mixed prior to electrophoresis with RNA from lane 7 (in a fourfold-greater amount than shown in lane 7). Lanes 5 and 6, replicates of lanes 1 and 2, respectively, with a 16-fold-greater amount of the RNA represented in lane 7. The position of the minigenome is indicated, as is that of a second, larger band of in vitro-synthesized RNA which presumably represents non-self-cleaved ribozyme.

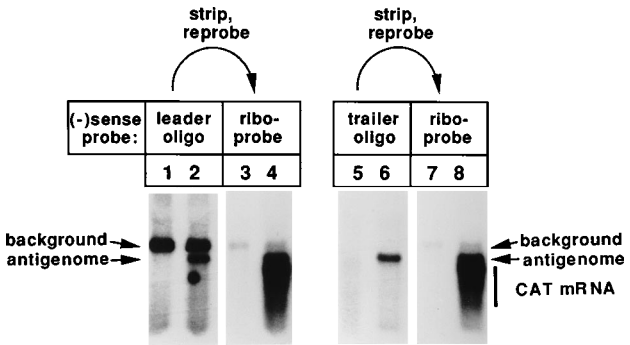

FIG. 5. Northern blot hybridization of intracellular mini-antigenome and mRNA with negative-sense oligonucleotide probes. Cells were infected with vTF7-3 and transfected with plasmid C2 (encoding the minigenome) together with plasmids encoding N, P, and either nonfunctional (lanes 1, 3, 5, and 7) or functional (lanes 2, 4, 6, and 8) L protein. Lanes 1 and 2 were probed with a negative-sense oligonucleotide representing nt 3 to 43 from the 3′ end of the minigenome (contained entirely within the 44-nt leader region). Lanes 5 and 6 were probed with a negative-sense oligonucleotide representing nt 82 to 122 from the 5′ end of the minigenome (contained entirely within the 155-nt trailer region). Following processing and exposure, the two blots were stripped and hybridized with the negative-sense RSV-CAT RNA probe: lanes 3 and 4 are reprobed lanes 1 and 2, respectively, and lanes 7 and 8 are reprobed lanes 5 and 6, respectively.

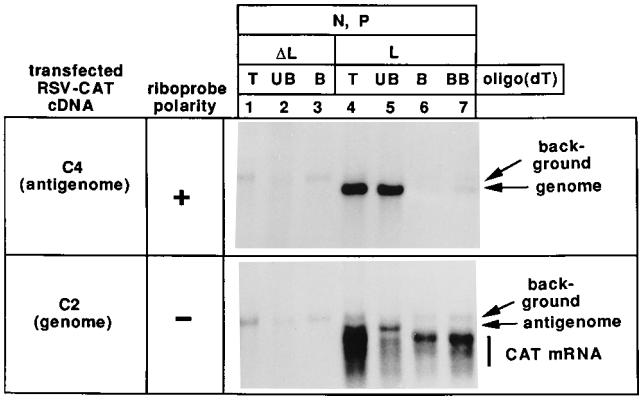

FIG. 6. Northern blots showing oligo(dT)-cellulose chromatography of RSV-CAT RNAs synthesized intracellularly in response to the N, P, and L proteins. Cells were infected with vTF7-3 and transfected with plasmid C4 (encoding the mini-antigenome) (top panel) or C2 (encoding the minigenome) (bottom panel) together with plasmids encoding the N and P proteins and nonfunctional (lanes 1 to 3) or functional (lanes 4 to 7) L protein. Lanes: 1 and 4, unfractionated (T, total) intracellular RNA; 2 and 5, RNA which did not bind to oligo(dT)-cellulose (UB, unbound); 3 and 6, RNA which bound to and was eluted from oligo(dT)-cellulose (B, bound); and 7, RNA from lane 6 which was denatured and subjected to a second round of binding to and elution from oligo(dT)-cellulose (BB, twice bound). Blots were hybridized with an RSV-CAT RNA probe of the same polarity as synthesized intracellularly by the transfected plasmid: positive-sense probe in the top panel, and negative-sense probe in the bottom panel. The positions of the major species are indicated.

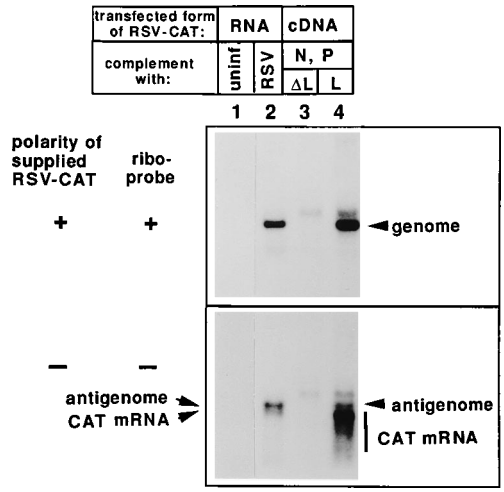

FIG. 7. Comparison, by Northern blot hybridization with strand-specific probes, of RSV-CAT RNAs synthesized during RSV-complemented rescue (lanes 1 and 2) versus N/P/L-complemented rescue (lanes 3 and 4). Lanes 1 and 2: cells were mock infected (lane 1) or infected with RSV (lane 2) and transfected with in vitro-synthesized mini-antigenome from plasmid C4 as the template (top panel) or minigenome from plasmid C2 as the template (bottom panel). Lanes 3 and 4: cells were infected with vTF7-3 and transfected with plasmid C4 (encoding the mini-antigenome) (top panel) or C2 (encoding the minigenome) (bottom panel) together with plasmids encoding N, P, and nonfunctional (lane 1) or functional (lane 2) L protein. Cells were harvested at 24 h (lanes 1 and 2) or 48 h (lanes 3 and 4) postinfection. Blots were hybridized with an RSV-CAT RNA probe of the same polarity as the plasmid-encoded template RNA: (top panel) positive sense; (bottom panel) negative sense.

the probes were removed by denaturation and the blots were hybridized with the negative-sense RSV-CAT riboprobe to confirm identifications. Each oligonucleotide probe hybridized to mini-antigenome and not to subgenomic mRNA, as would be expected. The leader-specific oligonucleotide also bound strongly to a background band which migrated slightly more slowly than the minigenome and whose synthesis was not dependent on the presence of L (lanes 1 and 2). This background species did not bind significantly with the trailer-specific probe (lanes 5 and 6), indicating that it did not contain the complete RSV-CAT sequence. In most of the Northern blots in this study, a similarly sized background band can be faintly detected with either C2 or C4 as the transfected plasmid, although it is not known whether the background band has the same species under each condition.

Oligo(dT) chromatography and Northern blot hybridization with strand-specific probes were used to characterize the intracellular RNAs synthesized in response to N, P, and L (Fig. 6). Neither the minigenome nor the mini-antigenome bound to oligo(dT) (upper and lower panels, respectively; compare lanes 5 and 6), consistent with expectations. The putative subgenome RSV-CAT mRNA was divided between the unbound and bound fractions (lower panel, compare lanes 5 and 6) under conditions in which a second cycle of chromatography of both the unbound and bound samples showed that the initial separation had been complete (lane 7 contains one sample from such rebinding experiments; the rest are not shown). The bound fraction was enriched for the larger species and included the largest and most distinct subgenomic species (located in Fig. 6 at the upper extremity of the vertical bar denoting mRNA), which was the expected size of the predicted full-length mRNA. This supported its identification. It was somewhat surprising to find that the smaller species of this smear of subgenomic mRNA were retained by oligo(dT), since only complete transcripts would be expected to be polyadenylated by the RSV polymerase. A second round of oligo(dT) chromatography (lane 7) confirmed this binding. These smaller species among the bound fraction might be the 3′-terminal ends of nucleolytic breakdown products of complete mRNAs or might be early quitters, polyadenylated either by an aberrant activity of the RSV polymerase or posttranscriptionally by the soluble vaccinia virus poly(A) polymerase (20).

Comparison with RNAs synthesized from transfected RSV-CAT RNA complemented by RSV infection. It was of interest to compare the spectrum of RSV-CAT RNAs produced when complementation was by RSV infection versus plasmid-expressed N, P, and L. It would have been desirable to have supplied the cDNA-encoded RSV-CAT template by the same method (either in vitro or intracellular transcription) for each type of complementation, but preliminary experiments indicated that, although RSV infection could be used to rescue RSV-CAT template synthesized intracellularly from transfected plasmid (not shown), the efficiency was low and insufficient for RNA analysis. This might be a consequence of the long lag time (16 to 20 h) before the peak of RSV protein synthesis and the substantial inhibition of RSV protein synthesis effected by coinfection with vTF7-3 (not shown; see below). And, although plasmid-expressed N, P, and L proteins were able to rescue transfected RSV-CAT RNA (not shown), the efficiency also was insufficient for RNA analysis. Thus, by necessity, the comparison involved RNA transfection/RSV complementation versus DNA transfection/plasmid complementation (Fig. 7).

The two systems were comparable in ability to synthesize the minigenome from the mini-antigenome template (Fig. 7, top panel, lanes 2 and 4) but differed with respect to the synthesis of positive-sense RNA from the minigenome template (Fig. 7, bottom panel, lanes 2 and 4). As described above, the plasmid-directed system synthesized a discrete band of mini-antigenome and a diffuse smear of subgenomic mRNA of heterogeneous size (bottom panel, lane 4). In contrast, the

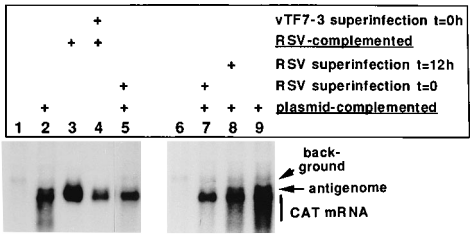

FIG. 8. Northern blot of mini-antigenome and mRNA synthesized from supplied minigenome template in response to N, P, and L (plasmid complemented), RSV (RSV complemented), or N, P, and L supplemented with RSV superinfection. Samples: lanes 1, 2, 6, and 9, cells infected with vTF7-3 and transfected with plasmid C2 (encoding the minigenome), together with plasmids encoding N, P, and nonfunctional (lanes 1 and 6) or functional (lanes 2 and 9) L; lane 3, cells infected with RSV and transfected with in vitro-synthesized minigenome; lane 4, same as lane 3 except that vTF7-3 was added simultaneously with RSV; lane 5, same as lane 2 except that RSV was added simultaneously with vTF7-3; lanes 7 and 8, same as lane 9 except that RSV was added simultaneously with vTF7-3 (lane 7) or 12 h later (lane 8). Cells were harvested at 48 h. Lanes 1 to 5 are from one blot, and lanes 6 to 9 are from another. The blot was hybridized with negative-sense RSV-CAT RNA.

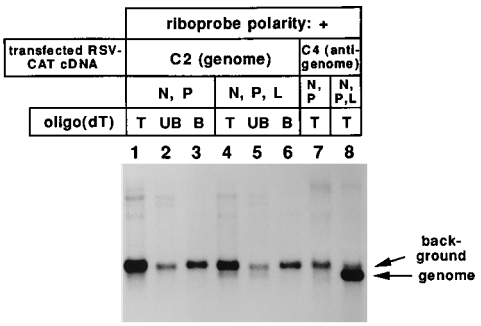

FIG. 9. Northern blot of RSV-CAT RNAs synthesized in cells transfected with plasmid C2 (encoding the minigenome) (lanes 1 to 6) or C4 (encoding the mini-antigenome) (lanes 7 and 8) together with plasmids encoding N and P (lanes 1 to 3 and 7) or N, P, and L (lanes 4 to 6 and 8). RNA was extracted at 48 h and subjected to chromatography on oligo(dT)-cellulose (T, UB, and B, total, unbound, and bound fractions, respectively). The samples were analyzed by Northern blot hybridization with the positive-sense RSV-CAT RNA probe (which would hybridize to template-sense RNA in lanes 1 to 6 and template-complementary RNA in lanes 7 and 8). Lanes 7 and 8 received nine times more cell equivalents of RNA than did lanes 1 to 6.

positive-sense RSV-CAT RNAs made by complementation with RSV migrated as a doublet of discrete bands. The larger component was identified as the mini-antigenome by its electrophoretic mobility and lack of binding to oligo(dT) (not shown). The lower, slightly diffuse component was bound by oligo(dT) (not shown), identifying it as subgenomic mRNA. The RNAs from the RNA transfection/RSV complementation system in this particular experiment had been harvested at 24 h, whereas those from the plasmid-complemented system were harvested at 48 h. This did not have much bearing on the comparison; when the RNA transfection/RNA complementation system samples were harvested at 48 h, the relative amount of mRNA was increased but the pattern was otherwise the same (see Fig. 8, lane 3).

An interesting possibility was that the mRNA patterns observed with the two systems differed because one involved only N, P, and L versus the full array of RSV gene products. If this difference was due to the lack of one or more RSV gene products in the N/P/L-based system, this might be remedied by superinfection with RSV. Indeed, this proved to be the case (Fig. 8). Coinfection of the plasmid-complemented system with RSV beginning at 0 h resulted in the production of a discrete mRNA species (Fig. 8, lane 5) similar to that observed with RNA transfection/RSV complementation (lanes 3 and 4). When superinfection of the plasmid-based system with RSV was delayed for 12 h, the pattern of subgenomic mRNA (lane 8) was intermediate between the RSV-complemented (lane 3) and plasmid-complemented (lane 9) patterns. As a control, coinfection of the RSV-complemented system with vTF7-3 reduced the quantity of positive-sense RNA produced but did not alter the discrete nature of the mRNA band (compare lanes 3 and 4), indicating that vaccinia virus was not responsible for the differences between the two systems. This control (lane 4) also shows that although vTF7-3 coinfection strongly inhibited protein synthesis by RSV (not shown), sufficient amounts were produced for RSV-CAT RNA replication and transcription. This supported the interpretation that RSV superinfection in the context of a vaccinia virus infection (Fig. 8, lane 5) supplied sufficient levels of RSV gene expression to remediate N/P/L-based transcription.

Examination of template-sense intracellular RNAs. In each of the previous Northern blot experiments, the probe had been chosen to be of the same polarity as the plasmid-supplied RSV-CAT RNA template in order to visualize RNAs copied off the plasmid-supplied template rather than the template itself. Out of interest, we also used Northern blot hybridization to examine intracellular RNA of the same polarity as the plasmid-expressed template. Figure 9 (lanes 1 to 6) shows intracellular RNAs where the transfected plasmid was C2 (encoding minigenome template) and the probe was positive-sense RSV-CAT RNA and thus would hybridize to the plasmid-encoded RNA as well as to any replicated minigenome. As a marker on the same blot with the same probe, RNAs (in ninefold excess) from cells in which the transfected plasmid was C4 (encoding the mini-antigenome) (lanes 7 and 8) are shown; in this case, the positive-sense probe would hybridize to the minigenome copied from the plasmid-supplied template but not to the template itself.

We had assumed that the cells expressing plasmid-encoded template would contain a very large accumulation of that RNA, much of which might be excess that never became involved in N/P/L-directed replication and transcription. Surprisingly, in the cells transfected with C2 plasmid (encoding the minigenome), the most abundant species of template-sense intracellular RNA was somewhat larger than the minigenome RNA (Fig. 9, compare lanes 1 and 4 with lane 8), and similar results were observed when the template was the mini-antigenome and the probe was negative-sense RSV-CAT RNA (not shown). The accumulation of this larger band was independent of the expression of L (compare lanes 1 and 4) and thus was a background band unrelated to N/P/L-specific RNA synthesis. Unexpectedly, oligo(dT) chromatography showed that more than half of this background band was polyadenylated (compare lane 2 with 3 and lane 5 with 6).

Interestingly, the minigenome could be detected as a minor band, but only in cells expressing the N, P, and L proteins (Fig. 9, lane 4); expression of N and P without L was insufficient (lane 1). Whereas the presence of the minigenome in lane 4 and its absence in lane 1 was somewhat obscured by the abundant trailing background band, oligo(dT) chromatography aided in the comparison by segregating some of the background material into the bound fraction. This helped confirm the presence of the minigenome in the unbound RNA fraction from cells expressing N, P, and L (lane 5) and its absence in the unbound fraction from cells expressing only N and P (lane 2).

The general absence of intracellular plasmid-encoded minigenome (Fig. 9, lane 1) or mini-antigenome (not shown) in

cells not competent for N/P/L-directed replication could be accounted for if naked RSV-CAT RNA were highly unstable. This would not be surprising, since it contains neither a 5′ cap nor 3′ poly(A). Encapsidation by N protein might have been expected to confer stability, but apparently this did not occur in amounts sufficient for detection, given that the analysis involved total cellular RNA rather than concentrated nucleocapsids (Fig. 9, lane 1). That the small amount of minigenome detected in lanes 4 and 5 depended on the presence of L suggests that it was the product of the RSV polymerase. This implies that a complete cycle of replication occurred; specifically, that the plasmid-supplied minigenome template was copied into the mini-antigenome, which in turn was copied into progeny minigenome. It is remarkable that this process was sufficiently efficient that its products were detectable among total cellular RNA and against the background of T7 transcription.

The nature of the background band remains unclear (although not critical for interpretation of the RNA synthesis directed by the RSV proteins). As shown in Fig. 9, cells accumulated a background band of the same polarity as the plasmid-encoded RSV-CAT RNA (lanes 1 to 6) as well as a background band of the opposite polarity (lanes 7 and 8), although the latter was more than ninefold less abundant, as judged from the ninefold-greater amount of RNA loaded in lanes 7 and 8. A likely explanation for the presence of transcripts from both strands of the input plasmid is promiscuous transcription by vaccinia virus. The background bands of different polarities probably have different origins; much of the template-sense background band presumably was derived from the RSV-CAT T7 transcript, whereas transcription from the opposing strand lacking the T7 promoter would be due to vaccinia virus. That the background bands could be detected under conditions in which naked plasmid-encoded minigenome or mini-antigenome was not suggests that they were stabilized by one or more factors. These could include the observed polyadenylation, presumably mediated by soluble vaccinia virus poly(A) polymerase (20), as well as vaccinia virus-mediated 5′ capping and methylation. Some of the template-sense background band might also be RSV-CAT on which the ribozyme failed to self-cleave and remained on the 3′ end as a snapback structure with the adjoining T7 terminator. Perhaps this structure conferred stability, although the stem-loop structure of the T7 terminator at the 3′ end alone did not do so in a previous study (17). Whatever the nature of this background RNA, these results indicate that plasmid-supplied minigenome or mini-antigenome was not abundant intracellularly except as the product of N/P/L-directed replication. While a substantial population of background plasmid-derived RNA accumulated independently of N/P/L-specific RNA synthesis, the products of N/P/L-directed replication could be readily detected against this background without purification or selective labeling.

DISCUSSION

Intracellular coexpression of the mini-antigenome template and the N, P, and L proteins resulted in the synthesis of minigenome (Fig. 3B). When the plasmid-supplied template was the minigenome, the products were mini-antigenome, subgenomic mRNA (Fig. 3B), and a smaller amount of progeny minigenome (Fig. 9). The synthesis of these species was completely dependent on the N, P, and L proteins. RNA synthesis was enhanced when the N plasmid was present in the same amount as or in excess to the P plasmid, but changes in input L plasmid levels over an eightfold range had modest effects (Fig. 3B).

The minigenome and mini-antigenome did not bind to oligo (dT), and the latter hybridized to leader- or trailer-specific negative-sense oligonucleotide. In contrast, much of the subgenomic mRNA bound to oligo(dT) and none of it hybridized to leader- or trailer-specific oligonucleotide. This was consistent with these RNAs having the general structures shown in Fig. 1A.

This functional assay for the RSV N, P, and L proteins confirmed that the published sequences encode functional proteins. These functions can now be probed by cDNA mutagenesis. This also provides a method for evaluating mutations in N, P, and L identified by sequence analysis of existing virus strains. For example, a series of temperature-sensitive attenuated RSVs generated by chemical mutagenesis are being characterized and evaluated as candidate vaccine strains (10, 11). Sequence analysis to date (unpublished data) has identified mutations within the N and L proteins which can now be introduced individually into the N and L cDNAs for functional evaluation as described here.

A single-amino-acid substitution in the L protein (Asp to Asn at position 989, representing a deviation from the published consensus sequence [38]) ablated activity so that even the sensitive CAT assay was negative (Fig. 3A). This particular segment had been made by reverse transcription coupled with PCR, and it is not known whether this mutation was present in the original molecule of mRNA or whether it was introduced during copying, amplification, and cloning. This change falls at the end of that region of the L protein which is the most highly conserved among the nonsegmented negative-strand RNA viruses (32), but whether this particular single change directly affects a catalytic domain or exerts its effect conformationally is unknown.

The RSV-CAT minigenome was constructed to contain a mutation, G to C in the negative sense, at leader position 4, and the mini-antigenome contained the complementary change. This mutation was previously shown to increase the level of CAT expression by the minigenome 7- to 20-fold in the RNA transfection/RSV complementation system (8, 24; unpublished data). In work to be presented elsewhere, this "up-mutation" in either the minigenome or mini-antigenome afforded corresponding increases in the levels of minigenome, mini-antigenome, and mRNA. While the full effects of this up-mutation have not been analyzed, and while we recognize the formal possibility that it might influence results in unanticipated ways, it should be noted that this same mutation was recently found in a cold-adapted subgroup B virus (12). The occurrence of this base assignment in nature justifies its use here. Under these conditions, all of the RSV-CAT species were readily detected from total cellular RNA without the need to concentrate nucleocapsids by immunoprecipitation or banding in CsCl and without the use of actinomycin D to reduce background transcription by vaccinia virus and T7 polymerase.

The plasmid-encoded forms of the minigenome and mini-antigenome would each have four nonviral terminal nucleotides, namely, three G residues at the 5′ end and one 3′-phosphorylated U residue at the 3′ end (Fig. 1). We have not yet investigated whether exactly correct termini were generated during replication; this work is in progress. Similar heterologous 5′-terminal extensions have been shown to be removed from cDNA-encoded defective interfering RNA of vesicular stomatitis virus during replication driven by viral proteins (31). In preliminary experiments with the RNA transfection/RSV complementation system, the presence of these few additional residues had no effect on rescue efficiency (not shown), although longer extensions (such as uncleaved ribozyme) would be predicted to ablate rescue (6).

Thus, the plasmid-supplied N, P, and L proteins appear to be fully sufficient to support RNA replication, a finding which has also been made with two rhabdoviruses, vesicular stomatitis virus and rabies virus (9, 31, 37), and the paramyxovirus Sendai virus (2, 13). RSV is sufficiently dissimilar that this confirmation is useful.

The RSV N, P, and L proteins alone also directed transcription, but the products contained a large proportion of short transcripts unless RSV superinfection was provided beginning at the start of the experiment. Thus, the minimum requirements for transcription and replication were not identical. Specifically, CAT mRNA produced by N, P, and L alone included a broad band of smaller, heterogeneously sized molecules, in contrast to the single discrete, full-sized mRNA observed with RSV complementation or superinfection. This difference was independent of vaccinia virus. Delayed addition of RSV resulted in a pattern intermediate between the discrete and disperse ones, suggesting that the remedial effect was at the level of synthesis (or stability) rather than repair of already formed defective transcripts. RSV superinfection also appeared to have the effect of reducing the accumulation of antigenome (Fig. 8 and data not shown). Thus, more than one activity appears to be involved, and further characterization is in progress.

We note that RSV nucleocapsids isolated from infected cells were active for highly authentic transcription in vitro (1, 22) and were shown to contain primarily N and P proteins with trace amounts of L protein (1). To reconcile this observation with our suggestion that an additional RSV-specific (or induced) protein or other factor is needed for the synthesis of complete transcripts, it must be postulated that small amounts of this factor (such that it was not detected previously [1]) are sufficient.

One trivial explanation for the remediation of transcription by RSV superinfection was that one or more of the plasmid-encoded proteins was defective. This cannot be absolutely ruled out. However, we note that these proteins contain consensus amino acid sequences determined from sequencing multiple independent cDNAs, reducing the likelihood of this type of artifact. Also, if a defect is involved, it would be subtle indeed (and of considerable interest), since it appeared to spare RNA replication. Superinfection with RSV did not increase the total levels of synthesis of positive-sense RSV-CAT RNA (Fig. 8) or minigenome (not shown). Indeed, RNA synthesis appeared to reduced somewhat by RSV superinfection in the experiment shown in Fig. 8 and was reduced to a greater extent in other experiments (not shown). This lack of stimulation of RNA synthesis by RSV superinfection would be consistent with the plasmid-encoded proteins' being fully functional.

Another possibility is that remediation by RSV superinfection boosted the intracellular levels of one or more proteins from a suboptimal, subfunctional level to one which was fully functional. The levels of plasmid-expressed N and P proteins were not greatly different from those expressed by RSV, but the lower levels of L expressed from the plasmid give this possibility some credence (Fig. 2). Variations in the levels of input plasmid, including L, did not have dramatic effects on the quality of transcription or replication (Fig. 3B), although admittedly these experiments might need to encompass a wider range and be accompanied by direct protein quantitation.

A third possibility, which we favor, is that the remediation by RSV of transcription by N, P, and L occurred through the expression of another essential gene product(s), this being most simply (but not necessarily) a protein. It is worth noting that RSV encodes three proteins, NS1, NS2, and M2, which lack counterparts in the other paramyxoviruses, as well as the transmembrane small hydrophobic (SH) protein, which has a counterpart only in simian virus 5 and mumps virus. The functions of these proteins are unknown. All of these, as well as the matrix (M) protein, are candidates to participate in RNA synthetic or regulatory activities. Since the sequences and sizes of the RSV N and P proteins are quite different from those of their counterparts in other paramyxoviruses, it would not be completely unexpected to find differences in the constitution and activities of the RNA polymerase between RSV and model systems such as vesicular stomatitis virus and Sendai virus.

Our confidence that the effects of RSV superinfection on N/P/L-directed transcription do not have a trivial basis is based on our recent finding that coexpression of the M2 cDNA (which has two ORFs) was sufficient to yield the single band of discrete, full-size mRNA (unpublished data). Examination of the full catalog of effects of this additional cDNA and assignment of the functions to the appropriate ORF(s) is in progress.

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ADDENDUM

While the manuscript was under review, Yu et al. reported that the RSV N, P, and L proteins are necessary and sufficient to support RNA replication by RSV minigenomes (40a). Regarding RNA replication, the results are in agreement. The present study extends this work by confirming the sequence of functional L protein and by examining transcription.

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