VIROLOGY 236, 188–201 (1997)
ARTICLE NO. VY978734

Increased Expression of the N Protein of Respiratory Syncytial Virus Stimulates

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Received May 2, 1997; returned to author for revision June 9, 1997; accepted July 14, 1997

A popular model for RNA synthesis by nonsegmented negative-strand RNA viruses is that transcription and RNA replication are executed by the same polymerase complex and that there is a dynamic balance between the two processes that is mediated by the nucleocapsid N protein. According to this model, transcription occurs until sufficient soluble N protein accumulates to initiate encapsidation of the nascent RNA product, which somehow switches the polymerase into a read-through replicative mode. This model was examined for respiratory syncytial virus (RSV) using a reconstituted transcription and RNA replication system that involves a minireplicon and viral proteins that are expressed intracellularly from transfected plasmids. Preliminary experiments showed that reconstituted RNA replication was highly productive, such that on average each molecule of plasmid-supplied minigenome that became encapsidated was amplified 10- to 50-fold. N protein was increased on its own or in concert with the phosphoprotein P and in the presence or absence of the M2 ORF1 transcription elongation factor. The maximum level of N and P protein expression achieved from plasmids equalled or exceeded that obtained in RSV-infected cells. Increased levels of N protein stimulated RNA replication. This is consistent with the idea that RNA replication is dependent on the availability of N protein for encapsidation, which is one postulate of the model. The M2 ORF1 protein had no detectable effect on RNA replication under the various conditions of expression of N and P, which confirmed and extended previous results. However, there was no evidence of a significant switch in positive-sense RNA synthesis from transcription (synthesis of mRNAs) to RNA replication (synthesis of antigenome). The synthesis of positive-sense antigenome and mRNA appeared to occur at a fixed ratio, with mRNA being by far the more abundant product.

INTRODUCTION

Human respiratory syncytial virus (RSV), a paramyxovirus, is the leading viral agent of severe pediatric respiratory tract disease worldwide. Its genome is a single negative-sense strand of RNA of 15,222 nucleotides that encodes 10 major mRNAs. Characterization of RSV molecular biology has been impeded by the lability of the virus and its poor growth *in vitro.*

Most of our knowledge of transcription and RNA replication of nonsegmented negative-strand RNA viruses is derived from studies with the prototype viruses such as the rhabdovirus vesicular stomatitis virus (VSV) and the paramyxovirus Sendai virus (SeV) (Colonno and Banerjee, 1976; Iverson and Rose, 1981; Emerson, 1982; for reviews see Wagner and Rose, 1996; Lamb and Kolakofsky, 1996). The genomes of these prototype viruses are tightly encapsidated by the major nucleocapsid N (or NP) protein and are associated with the phosphoprotein P and the polymerase subunit L to form the functional nucleocapsid, which contains the RNA polymerase. The polymerase enters the genome at the 3′ end and copies the extragenic leader region to produce a leader RNA. The polymerase can then execute transcription, whereby the leader RNA is terminated and the linear array of nonoverlapping genes is copied by a sequential, stop–start mechanism during which the polymerase remains template-bound and is guided by short consensus gene-start (GS) and gene-end (GE) signals to produce a series of subgenomic mRNAs (Collins *et al.,* 1996b; Kuo *et al.,* 1996). Alternatively, the polymerase can execute the first step in RNA replication, whereby it continues uninterrupted elongation beyond the leader region and across the genome to produce a complete encapsidated positive-sense replicative intermediate, the antigenome. This serves as the template for the second step in RNA replication, namely the production of progeny genomes.

The regulation of transcription and RNA replication by nonsegmented negative-strand RNA viruses is incom-

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0042-6822/97 $25.00

pletely understood. A widely accepted model proposes that both processes are executed by the same polymerase complex and that the two processes are balanced by the availability of N protein (Kingsbury, 1974; Lamb and Kolakofsky, 1996). This model has two postulates: (i) that RNA replication requires soluble N protein, and (ii) that the availability of soluble N protein for encapsidation acts as a switch to shift the polymerase from transcription to RNA replication.

Several lines of evidence strongly support the first postulate, namely that soluble N protein is required for RNA replication. For example, the products of RNA replication are found only in encapsidated form, and RNA replication is dependent on ongoing protein synthesis (Robinson, 1971; Wertz and Levine, 1973; Perlman and Huang, 1973; Blumberg *et al.,* 1981; Wertz, 1983; Patton *et al.,* 1984; Vidal and Kolakofsky, 1989). The introduction of N protein to an *in vitro* RNA synthesis system programmed with defective interfering (DI) nucleocapsids resulted in a shift from the synthesis of leader RNA to that of full-length antigenome (Wertz, 1983; Patton *et al.,* 1984). Conversely, depletion of soluble N protein *in vitro* or in infected cells by the introduction of antibody-blocked RNA replication but not transcription (Wertz and Levine, 1973; Hill and Summers, 1982; Arnheiter *et al.,* 1985). The site of initiation of VSV nucleocapsid assembly was mapped to a short sequence within the leader RNA (Blumberg *et al.,* 1983). In these experiments, the maintenance of N protein monomers in a soluble form competent for nucleocapsid assembly was found to depend on association with the P protein, which acts as a chaperone (Masters and Banerjee, 1988; Horikami *et al.,* 1992, Curran *et al.,* 1995).

It should be emphasized that the requirement for soluble N protein for RNA replication does not, on its own, constitute evidence that this involves a switch between transcription and replication. For example, soluble N protein might be required for replicase processivity as suggested by Vidal and Kolakofsky (1989) but have no influence on transcription. The evidence for a switch is based mainly on experiments in which treatment of VSV- or SeV-infected cells with cycloheximide resulted in a cessation of RNA replication with a concomitant increase in transcription (Portner and Kingsbury, 1972; Wertz and Levine, 1973). However, it must be recognized that an increase in transcription associated with cessation of protein synthesis might have causes other than polymerase redeployment, such as reduction in the levels of a negative regulatory protein such as the C protein (Curran *et al.,* 1992). In other studies a shift was inconsistent or was not observed (Robinson, 1971; Perlman and Huang, 1973). RNA replication directed by standard VSV nucleocapsids *in vitro* was too inefficient to document a switch (Hill and Summers, 1982; Davis and Wertz, 1982), and a comparable system employing a DI particle nucleocapsid was not informative on this point because the DI RNA lacked the standard 3′ genome end (Wertz, 1983). Conversely, an *in vitro* system for SeV directed abundant RNA replication, but inhibition of replication with cycloheximide did not result in an increase in transcription (Carlsen *et al.,* 1985). Other lines of evidence suggest that transcription and replication might be distinct rather than interconvertible processes (Discussion).

In this study, we sought to demonstrate a switch between transcription and RNA replication for RSV using a previously described plasmid-encoded minireplicon system (Grosfeld *et al.,* 1995; Collins *et al.,* 1996a), which, as described herein, directs highly productive transcription and RNA replication. We anticipated that this regulatory switch would be readily reconstituted and could then be analyzed with regard to *cis*-acting RNA signals and *trans*-acting proteins. Increased expression of the N protein did enhance minireplicon RNA replication. Surprisingly, however, the ratio between transcription (mRNA synthesis) and RNA replication (antigenome synthesis) remained essentially constant. These results are fully consistent with the idea that RNA replication depends on the availability of N protein, but provide no evidence of a switch between transcription and replication mediated by the N, P, or M2 ORF1 proteins.

MATERIALS AND METHODS

cDNAs

Two minigenomes (i.e., negative-sense genomic analogs), C2 and C2/t5A, and one miniantigenome (i.e., a positive-sense antigenome analog), C4, were used in this study (Fig. 1). cDNA encoding the negative-sense C2 minigenome was constructed in previous work (Grosfeld *et al.,* 1995). This minigenome contains, in 3′ to 5′ order, the 44-nucleotide RSV leader region, the 10-nucleotide NS1 gene-start (GS) signal, the upstream 29 nucleotides of the nontranslated region of the NS1 gene, a 669-nucleotide negative-sense copy of the CAT translational open reading frame (ORF), the last 12 nucleotides of the nontranslated region of the L gene and 12-nucleotide L gene-end (GE) signal, and the 155-nucleotide trailer region. The cDNA is bordered at the 5′ end relative to the encoded minigenome by three G residues and the T7 RNA polymerase promoter (the G residues improve efficiency of initiation by the T7 RNA polymerase) and at the 3′ end with a self-cleaving hammerhead ribozyme. The C2 minigenome has a C rather than a G at position 4 of the leader which appears to provide a several-fold enhancement of antigenome synthesis (M.E.P. and P.L.C., unpublished data). Both position 4 assignments have been described in biologically derived virus: the G assignment is that of the sequenced A2 strain wild-type virus (Mink *et al.,* 1991; Connors *et al.,* 1995), whereas the C assignment has been detected in some attenuated derivatives

of strain A2 of subgroup A and strain B1 of subgroup B (Crowley *et al.,* 1994; Firestone *et al.,* 1996), although it is not thought to be involved in the attenuation phenotype. A second minigenome, C2/t5A, was constructed from C2 by changing the fifth nucleotide from the 5′ (trailer) end from G (in negative-sense) to A, which was done for reasons described later. The previously described C4 miniantigenome RNA is synthesized from a cDNA in which the positions of the T7 promoter and the ribozyme were interchanged to yield the positive-sense complement of the C2 minigenome (Grosfeld *et al.,* 1995).

pTM1 plasmids containing the ORFs of the N, P, M2 ORF1, or L protein under the transcriptional control of the promoter for T7 RNA polymerase and the translational control of the internal ribosome entry site of encephalomyocarditis virus were constructed in previous work (Grosfeld *et al.,* 1995).

Transfections

Monolayers of HEp-2 cells in 6-well dishes were simultaneously transfected with plasmids and infected with 10 PFU per cell of vaccinia virus vTF7-3 (provided by Drs. Thomas Fuerst and Bernard Moss), which expresses the T7 RNA polymerase (Fuerst *et al.,* 1986), by the addition of 1 ml per well of a mixture prepared as follows: 0.1 ml of OptiMem containing the plasmids was mixed with 0.1 ml of OptiMem containing 12 μl of Lipofectase (Life Technologies), incubated at room temperature for 15 min, and mixed with 0.8 ml of OptiMem containing 2% fetal bovine serum and the vaccinia virus inoculum. Eighteen hours later, the transfection–infection mixture was replaced with MEM containing 2% fetal bovine serum, and cells were harvested at 42 to 48 hr postinfection as indicated in the figure legends. The total amount of transfected plasmid per well was kept at 1.2 μg or below, conditions under which the response of expressed protein to transfected plasmid was approximately linear.

Western blot analysis

Cells were lysed in 2% SDS, 50 m*M* Tris, pH 7.0, 0.63 *M* β-mercaptoethanol, and clarified by passage through a QIAshredder column (Qiagen). Lysate representing 1/20th of one well of cells was subjected to electrophoresis through a 4–20% gradient gel and the separated polypeptides were transferred to nitrocellulose using conventional techniques. RSV-specific proteins were detected by incubation with rabbit antiserum raised against gradient-purified RSV virions, followed by incubation with anti-rabbit IgG conjugated with alkaline phosphatase (Vector Laboratories) followed by an alkaline phosphatase reaction using a BCIP/NBT color development system (Promega). Densitometry was carried out using a Personal Densitometer SI (Molecular Dynamics).

RNA isolation, Oligo(dT) chromatography, and Northern blot hybridization

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 with phenol-chloroform following the isopropanol precipitation. Oligo(dT) chromatography was performed using a minibatch method (Grosfeld *et al.,* 1995). RNA representing one-fifth of one well of cells, approximately 5–10 μg in the case of unfractionated RNA, was analyzed by electrophoresis in 1.5% agarose gel containing 0.44 *M* formaldehyde, transferred to nitrocellulose, and fixed by UV crosslinking (Stratagene). Negative-sense or positive-sense RSV-CAT riboprobe was synthesized *in vitro* from *Xba*I–digested C2 cDNA or *Nco*I-digested C4 cDNA, respectively. Phosphorimage analysis was carried out using a Phosphorimager 445 SI (Molecular Dynamics).

Micrococcal nuclease (MCN) treatment of RNA

For MCN analysis of RNA, each transfection reaction was set up in duplicate wells. Following harvest, one cell pellet from each duplicate well was treated directly with Trizol reagent (Life Technologies) and RNA was extracted as described above (untreated RNA). The other cell pellet was treated with MCN using a protocol adapted from Baker and Moyer (1988). Briefly, the cell pellet was resuspended in 100 μl of lysis buffer: 10 m*M* NaCl, 10 m*M* Tris, pH 7.5, 1.5 m*M* $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 10 m*M* $CaCl_2$ containing 0.9 K.I.U. of aprotinin (Calbiochem) and 30 U of MCN S7 (Boehringer Mannheim). The lysate was incubated at 30° for 1 hr, following which 1 ml of Trizol was added and RNA was isolated as described above.

RESULTS

Expression of RSV proteins from transfected plasmids

The effect of changes in the level of intracellular synthesis of the N protein, or of the N and P proteins together, on RSV transcription and RNA replication was investigated using the previously described RSV-CAT minireplicon system (Grosfeld *et al.,* 1995). In this system, a plasmid-encoded analog of negative-sense genomic RNA, or one of positive-sense antigenomic RNA (Fig. 1), is synthesized intracellularly in the presence of viral proteins encoded by cotransfected support plasmids. Plasmid expression is mediated by T7 RNA polymerase supplied by the vaccinia virus recombinant vTF7-3.

It has been shown that conditions can be established in which the amount of each expressed protein is proportional to the amount of its input plasmid (Pattnaik and Wertz, 1991). Figure 2 shows Western blot analysis of RSV proteins synthesized in cells transfected with sup-

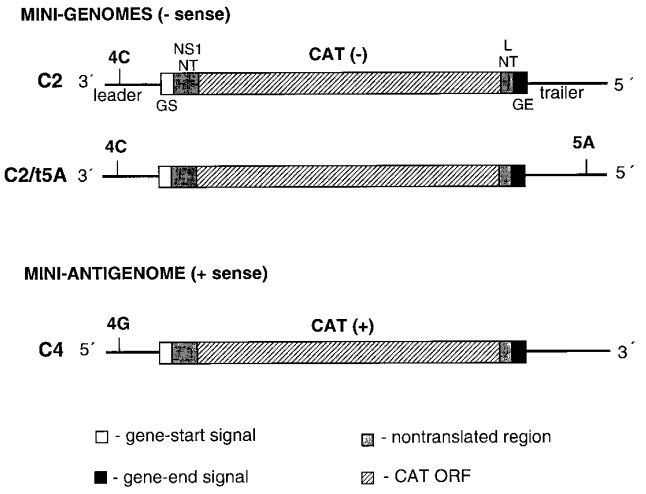


FIG. 1. A diagram of the different minireplicons used in this study. The negative-sense minigenomes, C2 and C2/t5A, contain in 3′ to 5′ order; the RSV leader, the NS1 gene-start signal, the nontranslated region of NS1, the complement of the translational open reading frame (ORF) for CAT, the nontranslated region of L, the L gene-end signal and the RSV trailer. The RSV-specific sequence of the C2 minigenome is identical to the published prototype sequence for strain A2 except that it has a nucleotide substitution at position 4 of the leader (G to C in negative-sense), which has been identified in certain RSV isolates. C2/t5A is similar to C2 but contains in addition a nucleotide substitution (G to A in negative-sense) at position 5 from the 5′ end of the trailer, which greatly reduces the synthesis of progeny minigenome (see text). C4 is a positive-sense miniantigenome, which is the complement of C2 and so has a G at position 4 relative to its 5′ terminus. The figure is not drawn to scale.

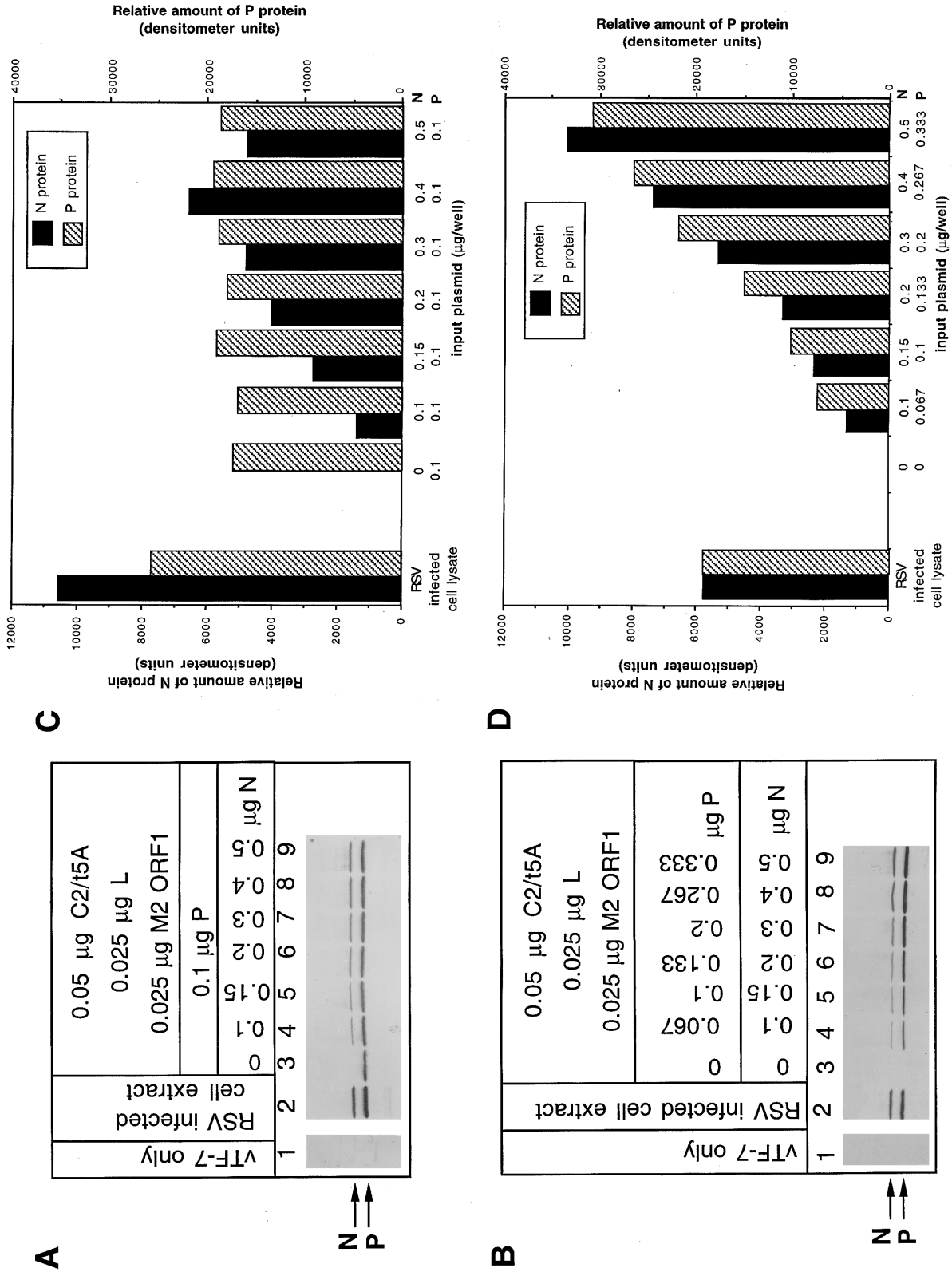
port plasmids under conditions where the N plasmid alone was increased (Fig. 2A) or where the N and P plasmids were increased coordinately at a fixed ratio of 1.5:1 (Fig. 2B). Cell lysates were analyzed by Western blotting with anti-virion antibodies in parallel with proteins harvested from RSV-infected cells late in infection when the bulk of viral protein synthesis is complete, thus representing the maximum level of viral proteins achieved during RSV infection. Densitometry analysis was carried out on the Western blots and is depicted graphically in Figs. 2C and 2D.

This analysis showed that, in general, the levels of expression of the N and P proteins increased in response to an increase in the amount of transfected plasmid. Importantly, the amounts of the N and P proteins expressed at the relatively higher levels of input plasmids equalled or exceeded the maximum levels obtained in RSV-infected cells. When N plasmid was increased alone with the others held fixed, the increase in expression of N protein reached a plateau at the higher levels of input plasmid (Figs. 2A and 2C). This might be due to instability or altered antigenicity of N protein when expressed in unilateral excess. When N was increased in tandem with P, reflecting more closely the situation in RSV-infected cells, protein expression increased with each increment in input plasmid (Figs. 2B and 2D). Some experimental variability in levels of expressed proteins was observed. In some cases (e.g., Figs. 2B and 2D), N increased to a slightly greater extent than P when both plasmids were increased in tandem, whereas in other experiments N and P increased in parallel, or P increased to a slightly greater extent than N. In each case, the variation in the N:P ratio was minor (<twofold). We accommodated the variability by repeating each experiment several times and by performing Western blot analysis as part of each experiment. We found that these minor differences did not significantly influence RNA synthesis.

RNA encapsidation and replication

Because minireplicon RNA is synthesized intracellularly from plasmid by T7 polymerase for the duration of each experiment, the possibility existed that excessive quantities of this RNA might accumulate and perhaps act as a sink for soluble N protein or otherwise perturb RNA replication. Therefore, the intracellular accumulation and encapsidation of negative-sense C2 minigenome (Fig. 1) were examined under typical experimental conditions where it was supplied from transfected plasmid and complemented by support proteins lacking or including the polymerase L protein. RNA was purified directly (Fig. 3A) or, alternatively, cells were lysed and treated with micrococcal nuclease (MCN) followed by RNA purification (Figs. 3B and 3C), a standard procedure which destroys unencapsidated RNA. RNA was analyzed by Northern blot hybridization with a positive-sense riboprobe. When L protein was omitted, the intracellular accumulation of minigenome would be solely that encoded by transfected plasmid, whereas with the inclusion of L, this would be augmented by RSV-mediated RNA replication. Replication of progeny minigenome from plasmid-supplied template by the RSV polymerase would require the complete replication cycle, namely synthesis of miniantigenome followed by synthesis of progeny minigenome. Plasmid encoding the M2 ORF1 protein happened to be included in this experiment, but its presence was incidental because it has no detectable effect on encapsidation or RNA replication (unpublished data).

Analysis of RNAs prepared without MCN treatment (Fig. 3A) showed that cells transfected with the C2 plasmid accumulated an abundant RNA species (designated "uncleaved T7 transcript"), which was of slightly lower electrophoretic mobility than minigenome. As described previously (Grosfeld *et al.,* 1995), this appears to be the minigenome which contains uncleaved hammerhead ribozyme and T7 terminator and is stabilized by these folded structures at its 3′ end. Apparently, most of the minigenome which was cleaved correctly (which makes up the vast majority of the product when the C2 cDNA is transcribed *in vitro*) was quickly degraded (see below).


A
vTF-7 only
RSV infected cell extract
1 2 3 4 5 6 7 8 9
0.05 µg C2/t5A
0.025 µg L
0.025 µg M2 ORF1
0.1 µg P
µg N
0 0.1 0.15 0.2 0.3 0.4 0.5
N
P
B
vTF-7 only
RSV infected cell extract
1 2 3 4 5 6 7 8 9
0.05 µg C2/t5A
0.025 µg L
0.025 µg M2 ORF1
µg N
µg P
0 0.1 0.15 0.2 0.3 0.4 0.5
0 0.067 0.1 0.133 0.2 0.267 0.333
N
P
C
Relative amount of P protein
(densitometer units)
N protein
P protein
RSV infected cell lysate
input plasmid (µg/well)
Relative amount of N protein
(densitometer units)
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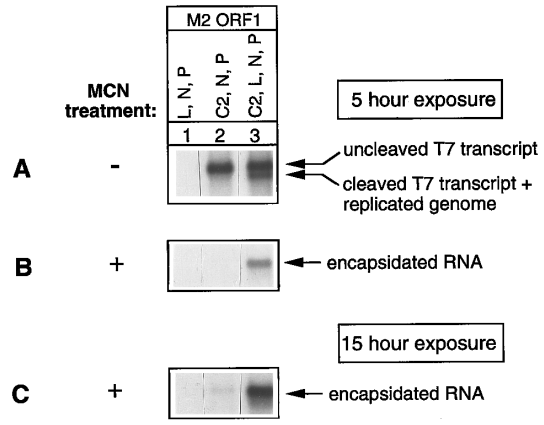

FIG. 3. Accumulation and encapsidation of intracellular negative-sense minigenome expressed from transfected plasmid in the absence or presence of the complete reconstituted RSV polymerase. Cells were infected with vaccinia virus vTF7 and duplicate wells were transfected with M2 ORF1 and various combinations of C2, L, N, and P plasmids, as indicated in the figure. Cells were harvested at 48 hr posttransfection. The RNA from the cells of 1 well of each duplicate was isolated directly (A), the cell pellet from the other well was treated with MCN prior to RNA extraction (B and C). RNA was analyzed by Northern blot hybridization using positive-sense (C4) riboprobe. C shows a longer exposure of the Northern blot shown in B.

When plasmids encoding N, P, and L proteins (i.e., the complete RSV replicase) were included in addition to C2, substantial amounts of correctly sized minigenome also accumulated, designated "cleaved T7 transcript + replicated genome" in Fig. 3A. When cell lysates were treated with MCN (Fig. 3B), the uncleaved T7 transcript was destroyed (i.e., lanes 2 and 3), whereas the minigenome-sized material made in the presence of the complete RSV replicase was protected (lane 3). Longer film exposures showed that a small amount of protected RNA was present in cells expressing N and P without L (Fig. 3C, lane 2), evidence of a low level of encapsidation of plasmid-derived minigenome.

These experiments showed that plasmid-derived minigenome RNA indeed did accumulate intracellularly, mostly in the larger uncleaved form, but that almost all of this RNA was unencapsidated and thus did not serve as a sink for nucleocapsid-associated proteins. The fraction of plasmid-derived minigenome which was cleaved and encapsidated was amplified 10- to 50-fold by the RSV polymerase (compare lanes 2 and 3 in Fig. 3C), depending on the experiment, indicating that many rounds of full-cycle replication had occurred. This experiment demonstrates that reconstituted RSV replication is highly productive.

Effect of increased N protein expression on minireplicon transcription and RNA replication

cDNA encoding the C2 minigenome was transfected with plasmids expressing the P, L, and M2 ORF1 proteins held at a constant level and N plasmid added at increasing levels. Cells were harvested at 42 hr posttransfection, a small aliquot of cells was processed for analysis by Western blotting (not shown), as was done in every experiment to confirm that the level of protein expression was appropriate, and the remainder was processed for RNA purification. Previous studies showed that the major positive-sense RNAs synthesized from the C2 minigenome template are the miniantigenome and subgenomic CAT mRNA (Grosfeld *et al.,* 1995). To distinguish between these two species, RNA was subjected to oligo(dT) chromatography. The resulting fractions were analyzed by Northern blot hybridization using a negative-sense riboprobe (Figs. 4A and 4B). The unbound fraction (Fig. 4A) contains the miniantigenome and a second RNA species of slightly greater electrophoretic mobility which was identified previously as nonpolyadenylated subgenomic mRNA (Collins *et al.,* 1996, and unpublished data). The oligo(dT)-bound fraction contains the polyadenylated subgenomic mRNA (Fig. 4B).

When the N plasmid was increased over a fivefold range (0.1 to 0.5 μg relative to P at a constant 0.1 μg), the synthesis of mRNA and miniantigenome was enhanced slightly at intermediate levels and was inhibited slightly (relative to the maximum levels) at higher levels. Surprisingly, the higher levels of N expression did not result in a shift from the synthesis of mRNA to the synthesis of mini-antigenome, as confirmed by phosphorimager analysis (Fig. 4D). At each of the tested levels of N protein,

FIG. 2. Western blots showing the intracellular RSV proteins from cells transfected with minigenome plasmid and support plasmids where the amount of the N plasmid (A and C) or the N and P plasmids together (B and D) was increased incrementally. Cells were infected with vaccinia virus vTF7 and transfected with, per single well of a six-well dish, 0.05 μg of RSV-CAT C2/t5A minigenome plasmid (note that the nature of the minigenome is not relevant to this experiment, which measures levels of support proteins), 0.025 μg L plasmid, 0.025 μg M2 ORF1 plasmid, and N and P plasmids as follows: in A, cells received 0.1 μg P plasmid, the indicated amount of N plasmid, and the appropriate amount of empty pTM1 plasmid to bring the total amount of input plasmid to 0.7 μg per well. Alternatively, in B, cells received in addition the indicated amount of N and P plasmids (in a ratio of 1.5:1) and the appropriate amount of empty pTM1 plasmid to bring the total amount of plasmid input to 1.03 μg. Cell lysates were prepared 42 hr posttransfection and proteins were separated by SDS–PAGE on a 4–20% gel and transferred to nitrocellulose. As marker, cells were infected with RSV at an input m.o.i. of 10 PFU/cell and were harvested at 26 hr postinfection and analyzed in parallel (lane 2). As a negative control, cells received the Lipofectase and vTF7–3 mixture lacking any plasmids (lane 1). RSV specific proteins were detected as described under Materials and Methods using antiserum raised against gradient-purified virions. The blots were analyzed by densitometry, the results are shown graphically for increasing N (C) and increasing N and P (D). It should be noted that the N and P values are plotted against different y-axes.

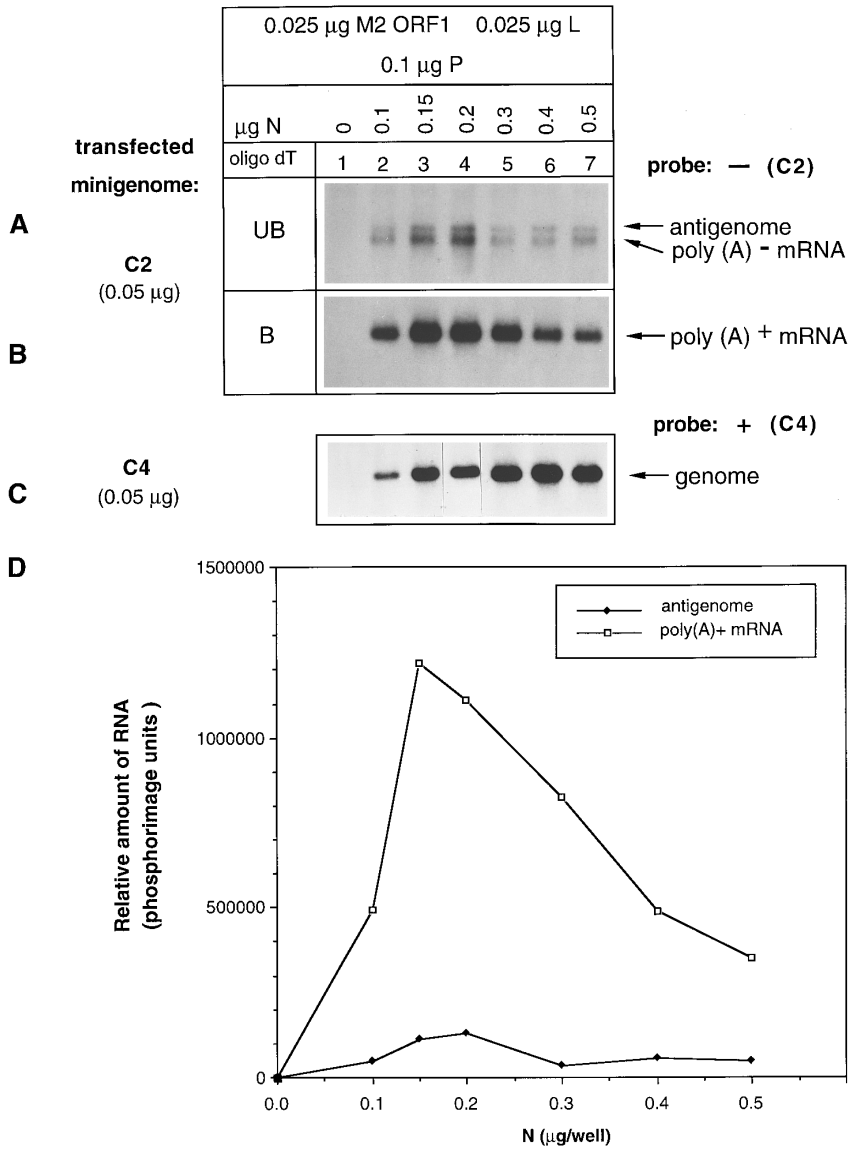

FIG. 4. Northern blot analysis of intracellular minireplicon RNA synthesis under conditions where the level of N plasmid was increased incrementally. Following the conditions of the experiment described in the legend to Fig. 2A, cells were infected with vaccinia virus vTF7 and transfected with 0.05 μg of minigenome C2 plasmid (A and B) or miniantigenome C4 plasmid (C) together with 0.1 μg P plasmid, 0.025 μg L plasmid, 0.025 μg of M2 ORF1 plasmid, the indicated amount of N plasmid, and the appropriate amount of empty pTM1 to bring the total plasmid input to 0.7 μg per well. Total intracellular RNA was extracted at 42 hr posttransfection, and the RNA from cells transfected with the C2 minigenome template was subjected to oligo(dT) chromatography. RNAs were separated by electrophoresis on formaldehyde agarose gels and transferred to nitrocellulose. A and B show positive-sense RNAs. A shows RNA which did not bind to oligo(dT) cellulose (unbound, UB), B shows RNA which did bind (bound, B). C shows negative-sense RNAs. The blots were hybridized with negative-sense C2 (A and B) or positive-sense C4 (C) riboprobe. Phosphorimager analysis of the blots in A and B is shown in D.

the subgenomic mRNA was the predominant positive-sense RNA product.

The effect of increased expression of the N protein on minigenome synthesis was examined in parallel using a plasmid-supplied C4 miniantigenome template (Materials and Methods, Fig. 1). Total intracellular RNA was isolated at 42 hr posttransfection and analyzed by Northern blot hybridization using a positive-sense riboprobe (Fig. 4C). This analysis showed that each increase in the level of N protein resulted in an increase in the synthesis of minigenome.

Effect of increasing the N and P proteins together

A number of studies have shown that the N proteins of rhabdoviruses and paramyxoviruses have a propensity for self-aggregation and that association with the P protein is important for N to be maintained in a soluble state,

capable of participating in RNA encapsidation (Masters and Banerjee, 1988; Horikami *et al.,* 1992; Buchholz *et al.,* 1993; Curran *et al.,* 1995). Therefore, the experiment described above was repeated under conditions where synthesis of the N and P proteins was increased coordinately. The ratio of N and P plasmids was maintained at 1.5:1, which was determined in preliminary experiments to be optimal for RNA synthesis (e.g., Fig. 4).

Northern blot analysis (Figs. 5A to 5C) and phosphorimager analysis (D) showed that synthesis of each of the minireplicon-encoded RNAs, namely the subgenomic CAT mRNA (Fig. 5B), the miniantigenome (Fig. 5A), and the minigenome (Fig. 5C), increased in response to increasing amounts of the N and P proteins. Whereas increasing the expression of N alone resulted in a partial inhibition of miniantigenome RNA at the higher plasmid levels (Fig. 4A), increased expression of N and P together was not inhibitory to miniantigenome synthesis at any of the tested levels, consistent with the idea that N and P function cooperatively in replication. The highest level of expressed N and P, which was in excess of the maximum level observed in RSV-infected cells, resulted in a modest reduction in mRNA synthesis, but there was no evidence of a shift by the RSV polymerase from the synthesis of mRNA to that of miniantigenome.

Effect of increasing the N protein, or the N and P proteins together, in the absence of the M2 ORF1 protein

The M2 ORF1 protein was included in the experiments described above because it has been shown to be a transcription elongation factor that is necessary for the efficient synthesis of full-length mRNA. In previous work it had no discernable effect on the synthesis of minigenome or miniantigenome and also did not appear to affect the frequency of transcription initiation. However, we could not exclude the possibility that the M2 ORF1 protein might somehow be responsible for the failure of increasing levels of N, or of N and P together, to shift synthesis from mRNA to antigenome. Therefore, we examined RNA synthesis directed by plasmid-supplied C2 minigenome or C4 miniantigenome under conditions of increasing N alone, or N and P together, in the absence of M2 ORF1.

Consistent with previous studies, the positive-sense RNAs synthesized from C2 minigenome template in the absence of the M2 ORF1 protein include the miniantigenome, a small amount of complete CAT mRNA, and a smear of prematurely terminated CAT mRNA (Figs. 6A and 6C). Under these conditions, the miniantigenome could be distinguished from the diminished band of mRNA without oligo(dT) chromatography. Increased expression of N protein alone (Fig. 6A), or of N and P proteins together (Fig. 6C), resulted in slightly increased synthesis of both miniantigenome and mRNA but did not shift the balance in favor of miniantigenome. Thus, omission of M2 ORF1 did not facilitate a shift from transcription to replication.

The synthesis of minigenome in cells programmed with the C4 miniantigenome template was enhanced by increased expression of N alone (Fig. 6B) or of N and P together (Fig. 6D). These results were similar to those observed in the presence of M2 ORF1 (Figs. 4 and 5).

Studies with a minigenome that is limited to the first step in RNA replication

In the experiments described above, increased expression of N protein alone, or of N and P proteins together, did not shift the polymerase from the synthesis of mRNA to that of miniantigenome. However, in most cases it did increase the synthesis of all RNAs. As was shown in Fig. 3, the complete cycle of RNA replication (i.e., minigenome to miniantigenome to minigenome) is very efficient in this reconstituted system, and typically 90 to 98% of the minigenome that has accumulated at 48 hr posttransfection is the product of replication by the RSV polymerase rather than synthesis from plasmid. One possibility was that increased availability of N for encapsidation increased the rate of RNA replication, which also would have the effect of increasing mRNA synthesis since more templates would be available. Increased RNA replication could reduce the pool of N and P protein and nullify the effect of increased protein expression. Therefore it was important to analyze synthesis of mRNA versus miniantigenome under conditions where the complete cycle of replication did not occur. In work to be described elsewhere (M.E.P., J. Cristina, and P.L.C., unpublished data), a series of point mutations in the trailer region was examined. One mutant minigenome (C2/t5A), which contains the substitution of G to A (negative-sense) at trailer position 5 relative to the 5′ end (Fig. 1), was competent for encapsidation and synthesis of positive-sense RNAs but produced only very small amounts of progeny minigenome (data not shown). Since this mutation would result in a substitution in the encoded miniantigenome at position 5 relative to its 3′ end, a likely explanation is that this reduces the activity of the antigenome promoter. Thus, the C2/t5A mutant provides the basis for analysis of the synthesis of mRNA and miniantigenome under conditions where the amount of minigenome template is not augmented efficiently by full-cycle RNA replication.

Transfections involving the C2/t5A mutant were carried out similarly to those described above for C2. As shown in Fig. 7, N plasmid was increased alone while the others were held fixed (Figs. 7A and 7B), or was increased in tandem with P (Figs. 7C and 7D), and each condition was performed in the presence (Figs. 7A and 7C) or

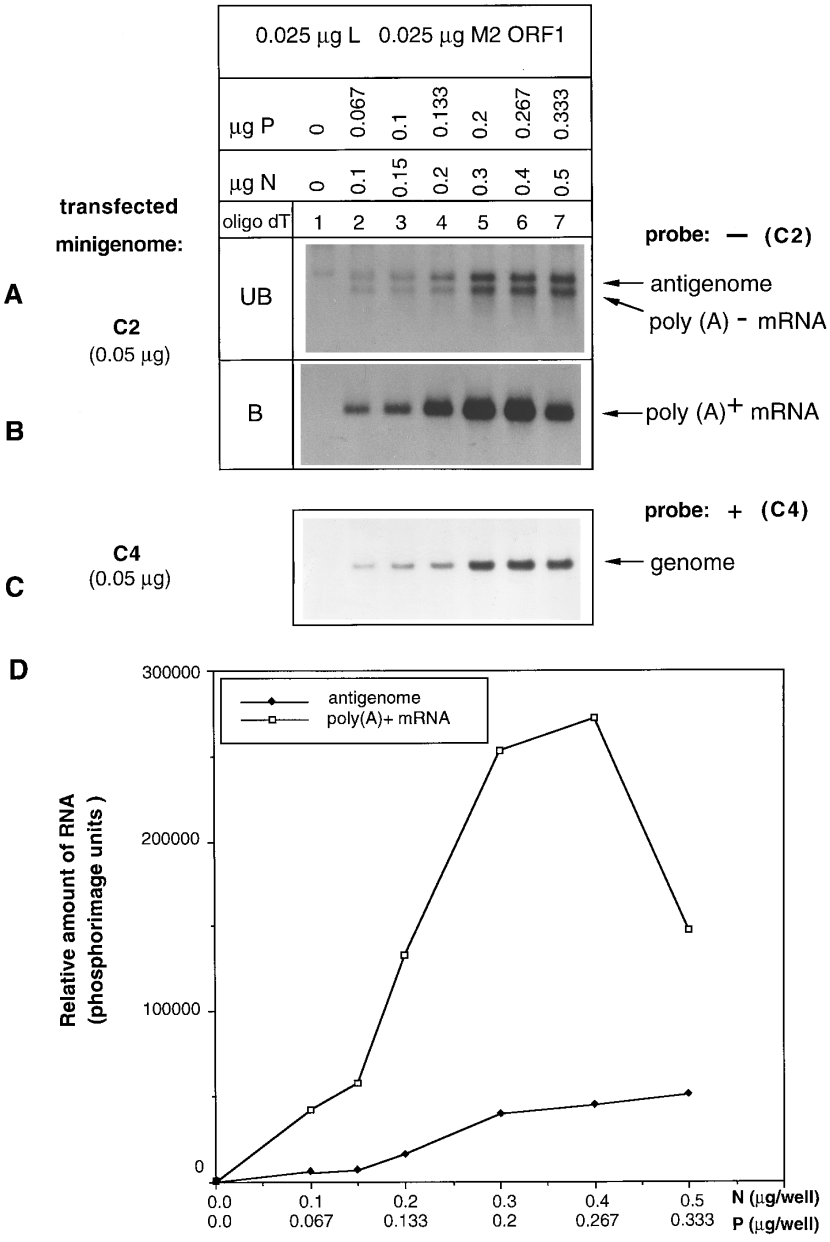

FIG. 5. Northern blot analysis of intracellular minireplicon RNA synthesis under conditions where the levels of N and P plasmids were increased coordinately. Following the conditions of the experiment described in the legend to Fig. 2B, cells were infected with vaccinia virus vTF7 and transfected with 0.05 μg of minigenome C2 cDNA (A and B) or miniantigenome C4 cDNA (C) together with 0.025 μg L, 0.025 μg of M2 ORF1, the indicated amounts of N and P plasmids (in a ratio of 1.5:1), and sufficient empty pTM1 to bring the total plasmid input to 1.03 μg per well. A and B show positive-sense RNAs from the unbound (UB) and bound (B) RNA fractions following oligo(dT) chromatography; C shows negative-sense RNA. The blots were hybridized with negative-sense C2 (A and B) or positive-sense C4 (C) riboprobe. Phosphorimager analysis of the blots shown in A and B is shown in D.

absence (Figs. 7B and 7D) of M2 ORF1. Because the mutation in the C2/t5A minigenome greatly reduced full-cycle replication, the overall accumulation of positive-sense RNA was approximately 5% that of the C2 minigenome. Thus, the background was higher, including a band which migrated slightly more slowly than the miniantigenome. The reduced accumulation of positive-sense RNA made it possible to distinguish between miniantigenome and mRNA without oligo(dT) chromatography. The results showed that increased expression of N alone or of N and P together had little effect on the total amount of positive-sense RNA synthesis by the C2/t5A mutant. This indicated that the increase in RNA synthesis observed under comparable conditions by the C2 minigenome is due primarily to an increase in full-cycle RNA replication. Increases in N alone resulted in modest de-

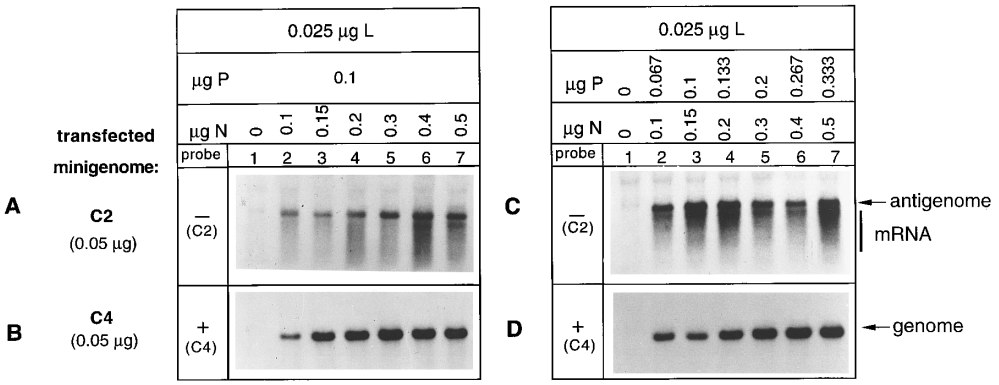

FIG. 6. Northern blot analysis of intracellular minireplicon RNA synthesis in response to increasing levels of N (A and B) or N and P together (C and D) in the absence of M2 ORF 1. Cells were infected with vaccinia virus vTF7 and transfected with 0.05 μg minigenome C2 plasmid (A and C) or miniantigenome C4 plasmid (B and D) together with 0.025 μg L plasmid. In addition, A and B received the indicated amount of N plasmid and sufficient empty pTM1 to bring the total input plasmid to 0.7 μg per well. Alternatively, C and D received the indicated amounts of N and P plasmids (in a ratio of 1.5:1) and sufficient empty pTM1 to bring the total plasmid input to 1.03 μg per well. The blots were hybridized with negative-sense RSV-CAT C2 riboprobe (A and C) or positive-sense C4 riboprobe (B and D).

creases in mRNA synthesis in the presence of M2 ORF1 (Fig. 7A) but not in its absence (B), which had been seen previously with the C2 minigenome. Overall, increased expression of N or N and P did not shift synthesis from mRNA to antigenome, even under these conditions where the overall amount of RNA replication had been reduced 20-fold and thus the relative molar amount of soluble N protein was greatly increased.

Actinomycin-D treatment of transfected cells

We were concerned that the unregulated accumulation of T7 transcripts might somehow interfere with the regulation of RNA replication by N protein. Although this concern was mitigated by the results shown in Fig. 3, we used another measure to preclude possible artifacts due to overexpression of T7 transcripts. Specifically, actinomycin D was added to terminate DNA-dependent transcription midway through the experiment. In this experiment, a series of transfection reactions was carried out similarly to those described above, using C2 as a template with N and P increased in tandem in the presence of M2 ORF1. Twenty-four hours following transfection, the cells were incubated for 2 hr with medium containing actinomycin D at 2 μg/ml, after which fresh medium not containing actinomycin D was added and the cells were incubated for an additional 20 hr. Under these conditions DNA-templated RNA synthesis is strongly inhibited (data

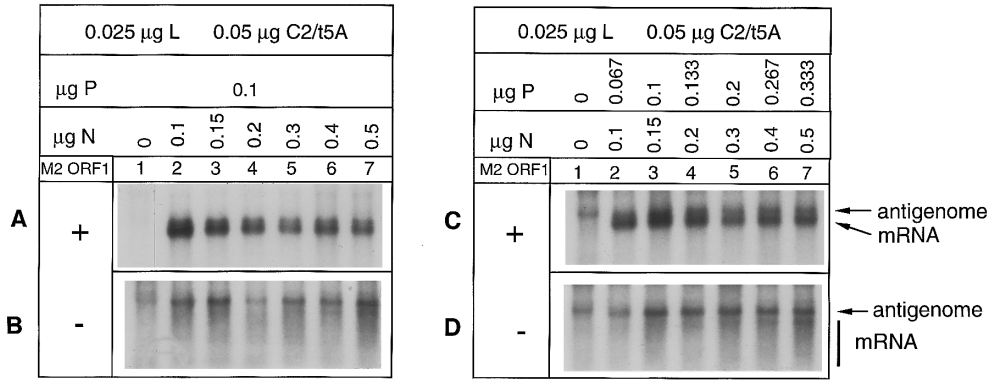

FIG. 7. Northern blot analysis of intracellular RNAs produced by the C5/t5A minigenome, which contains a mutation at trailer position 5 that inhibits the synthesis of negative-sense RNA by the RSV polymerase. RNA synthesis was examined under conditions of increasing N protein in the presence (A) or absence (B) of M2 ORF1 or under conditions of increasing N and P at a ratio of 1:1.5 in the presence (C) or absence (D) of M2 ORF1. Cells were infected with vaccinia virus vTF7-3 and transfected with 0.05 μg minigenome C2/t5A cDNA together with 0.025 μg L plasmid and the following additions: (A) 0.1 μg of P plasmid, 0.025 μg of M2 ORF 1 plasmid, and sufficient empty pTM1 to bring the total amount of input plasmid to 0.7 μg; (B) 0.1 μg of P plasmid, the indicated amounts of N plasmid and sufficient empty pTM1 to bring the amount of input plasmid to 0.7 μg; (C) the indicated amounts of N and P, 0.025 μg of M2 ORF1, and sufficient empty pTM1 to bring the total amount of input plasmid to 1.03 μg; and (D) the indicated amounts of N and P plasmid and sufficient empty pTM1 to bring the total amount of input plasmid to 1.03 μg. Total intracellular RNA was analyzed by Northern blot hybridization using negative-sense C2 riboprobe.

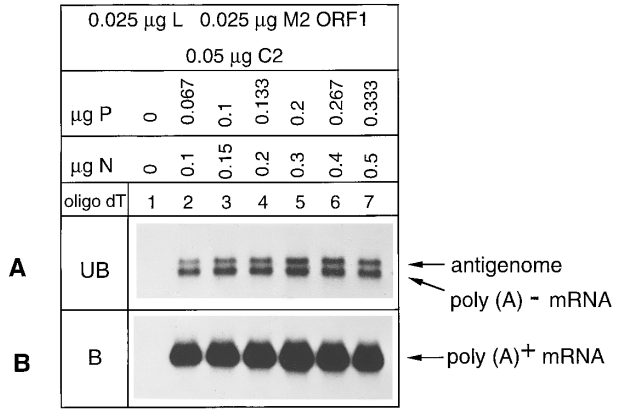

FIG. 8. Northern blot analysis of intracellular minireplicon RNAs produced in cells treated with actinomycin D. Following the conditions of the experiment described in the legend to Fig. 5, cells were infected with vaccinia virus vTF7 and transfected with 0.05 μg of minigenome C2 cDNA together with 0.025 μg L, 0.025 μg M2 ORF1, and the indicated amounts of N and P plasmids and sufficient pTM1 to bring the total amount of input plasmid to 1.03 μg. At 24 hr posttransfection the cells were treated with actinomycin D at 2 μg/ml for 2 hr, and then fresh medium was added and the cells were incubated for a further 20 hr. A shows the C2-encoded RNA fractions which did not bind to oligo(dT) cellulose and B shows the fractions which did bind. The blots were hybridized with a negative-sense C2 riboprobe.

not shown). Thus RNA synthesis from transfected plasmids would be strongly inhibited but the reconstituted RSV polymerase would remain active and RSV protein synthesis would continue from preexisting mRNAs. As was observed in the previous experiments, miniantigenome and mRNA levels paralleled each other at the various N and P concentrations and mRNA levels were consistently greater than miniantigenome (Fig. 8).

DISCUSSION

RNA synthesis by nonsegmented negative strand RNA viruses involves two distinct processes: transcription, during which cis-acting transcription signals are utilized to direct stop–start synthesis of subgenomic mRNAs, and RNA replication, during which the signals are ignored and genome-length encapsidated RNAs are produced. The relationship between these processes is not well understood. A widely accepted model is that there is a balance between the two processes which is mediated by encapsidation of the nascent RNA product by soluble N protein (Introduction). According to this model, each polymerase molecule which enters the genomic nucleocapsid has the potential to commit to either process, with the decision being made by the N-mediated "switch." This proposed switch might be envisioned as a conformational change induced in the polymerase. There was ample evidence that RNA replication indeed is dependent on the availability of soluble N protein. We argue that the idea that N protein mediates a switch between transcription and RNA replication is a separate issue which has little direct experimental support (Introduction).

This model was examined with a reconstituted transcription and replication system based on minireplicons and proteins supplied from transfected plasmids. Initial experiments were performed to show that this was a suitable approach. For example, we examined whether sufficient viral protein was made to support efficient RNA replication and whether the synthesis of minireplicon RNA from plasmid by the T7 polymerase was inhibitory to RSV-mediated RNA replication. When minigenome was synthesized from plasmid in the presence of N and P alone, only a very small amount became encapsidated and the remainder evidently was rapidly degraded. In the case of the C2 construct, which contains the hammerhead ribozyme, there was an accumulation of minigenome containing uncleaved ribozyme, which apparently rendered it more stable. However, this material was not encapsidated and thus would not affect the availability of soluble viral protein. We also performed experiments with another construct called C41 which is identical to C2 except that the hammerhead ribozyme was replaced with that of hepatitis delta virus (Perrotta and Been, 1991) which cleaves with slightly greater efficiency *in vitro* (not shown). With C41, the larger band was not present and thus very little minigenome accumulated in the presence of N and P alone (data not shown). With either the C41 or C2 template, the fraction of plasmid-supplied minigenome, which did become encapsidated, was subsequently amplified 10- to 50-fold by the reconstituted RSV polymerase. This very high level of reconstituted RNA replication implies that the supply of soluble viral protein was abundant and that other characteristics of the system, such as the continued synthesis of minireplicon from plasmid and the vaccinia virus infection did not significantly perturb RNA replication.

N protein expression was varied alone or in concert with the P protein and in the presence and absence of the M2 ORF1 transcription elongation factor. Our findings show that increased synthesis of N protein (alone or in concert with P) resulted in most cases in an increase in the synthesis of all RNAs, namely minigenome, miniantigenome, and mRNA. However, there was no evidence of a switch from transcription (synthesis of mRNA) to RNA replication (synthesis of antigenome). In agreement with previous studies, the presence or absence of the M2 ORF1 protein appeared to have little or no influence on RNA replication.

The overall increase in RNA synthesis in response to increased N protein might have been due to two factors which are not mutually exclusive: (i) an increase in the efficiency of encapsidation of plasmid-derived minireplicon, and (ii) an increase in the rate of RNA replication by the reconstituted RSV polymerase. In either case, the

resultant increase in minigenome template would account for the concordant increase in transcription. These two possibilities could be distinguished by comparing the effect of N protein on RNA synthesis by the parental C2 minigenome, which is competent for multiple cycles of RNA replication, versus the C2/t5A minigenome, which is restricted mostly to the synthesis of positive-sense RNAs and produces very little progeny minigenome. RNA synthesis by the mutant minigenome was not enhanced by increased expression of N protein, implying that the enhancement observed with the parental C2 minigenome is due primarily to increased RSV-specific replication rather than increased encapsidation of the plasmid-supplied minigenome.

The finding that increasing levels of N protein augment RNA replication is not surprising since there is considerable evidence for an intimate link between encapsidation and the synthesis of genome and antigenome (Introduction). For example, soluble N protein might enhance replicase processivity or increase the stability of the product, or both. However, the requirement for N protein in RNA replication does not address the issue of a switch between transcription and RNA replication. Here, the critical observation is that none of the tested conditions induced a substantial increase in RNA replication (synthesis of antigenome) at the expense of transcription (synthesis of mRNA). Instead, transcription usually increased coordinately with RNA replication, as if the proportion of the two synthetic processes was fixed rather than interchangeable. Or, when full-cycle RNA replication was greatly reduced with the use of the above-mentioned C2/t5A mutant minigenome, the amounts of miniantigenome and mRNA remained essentially unchanged by increases in N, with or without P and M2 ORF1.

We note that there were circumstances where the increased expression of N protein sometimes resulted in a decrease in the accumulation of mRNA. However, this was not associated with a switch to replication, as there was not a corresponding increase in the level of miniantigenome and indeed in some cases miniantigenome also decreased. This effect was the most pronounced when N alone was increased (Figs. 4A and 4B). Since N protein expression is not thought to increase unilaterally during RSV infection, these effects probably are artifactual. When N and P were increased together, inhibition occurred only at the highest level of expression (Fig. 5B), which exceeded the maximum level of expression in RSV-infected cells and thus might also be artifactual. Also, high levels of expression of N alone or of N and P together did not always cause this inhibition (Figs. 7A, 7C, and 8). Moreover, this inhibition usually occurred only when M2 ORF1 protein was present; in its absence, the nonprocessive-type of mRNA synthesis that occurs under these conditions was reduced little or not at all by high levels of N protein (Figs. 6A, 6C, 7B, and 7D). Since the N protein has been shown to bind the M2 ORF1 protein in RSV-infected cells (Garcia *et al.,* 1993; Samal *et al.,* 1993), the apparent reduction in transcription might be an indirect effect of sequestration of M2 ORF1. Taken together, the inhibition of mRNA synthesis by N was never associated with a shift to the synthesis of miniantigenome and instead appeared to occur when the conditions of the support proteins were inconsistent with conditions of RSV-infected cells.

Efforts were made to exclude the possibility that the inability to reconstitute a switch between transcription and RNA replication reflects some deficiency in the minireplicon system. We note that the highest levels of plasmid-encoded N and P protein equalled or exceeded the maximum levels observed in RSV-infected cells (Fig. 2). Thus, insufficient expression of the RSV proteins probably was not a problem, which also is suggested by the high level of amplification of encapsidated RNA achieved by the reconstituted polymerase. Nor is the minireplicon system insensitive to changes in protein concentration. For example, increase of P protein relative to other components has a profound inhibitory effect on minigenome RNA synthesis (Grosfeld *et al.,* 1995, and data not shown) and RNA synthesis is also sensitive to levels of M2 ORF1 or ORF2 proteins or L protein (Grosfeld *et al.,* 1995). Furthermore, the overall accumulation of minireplicon RNA replication products responded positively and sensitively to changes in the level of protein synthesis.

Specific aspects of the minireplicon system which might have been imagined to compromise reconstitution of a switch between transcription and replication were addressed and modified. For example, the unregulated nature of the transient expression system was addressed by adding actinomycin D partway through the experiment to block DNA-encoded synthesis (Fig. 8). This would terminate the input of cDNA-encoded minireplicon while protein synthesis would continue. We also utilized the mutant (C2/t5A) which is deficient in full-cycle RNA replication and so would not deplete the cell of soluble N protein (Fig. 7). Additionally we examined RNA synthesis in transfections when RNA was isolated at an earlier time point, 24 hr, and we also tested a minigenome containing a G residue at position 4 in the leader region rather than C, which represents the other naturally occurring assignment (data not shown). However, the results were unchanged: miniantigenome and mRNA levels paralleled each other at the various N and P concentrations and mRNA levels were consistently greater than miniantigenome.

One interpretation of our findings is that the model of a switch between transcription and RNA replication has some validity but needs to be restated. The revised model would continue to postulate that, in the absence of soluble N protein, the polymerase engages only in transcription. Encapsidation of the nascent leader RNA

by soluble N protein redirects the polymerase into RNA replication. However, even at the highest levels of N protein, RNA replication involves only a small fraction of the viral polymerase, and thus the "switch" is only partial. A complete switch to replication never occurs and transcription is always the major synthetic event, at least for the genomic nucleocapsid.

An alternative view is that transcription and RNA replication are separate processes, probably employing functionally distinct polymerases. Leader RNA could be involved in both processes. The binding of N protein to nascent leader might mediate a shift from the synthesis of leader RNA to that of antigenome, but would affect only that fraction of leader RNA that is associated with the replicase and would not have an effect on transcription. The transcriptase and replicase complexes could be functionally distinct due to, for example, a posttranslational covalent modification. For example, phosphorylation of the VSV and RSV P proteins has been shown to be important for transcription (Kingsford and Emerson, 1980; Barik and Banerjee, 1992; Takacs *et al.,* 1992; Mazumder and Barik, 1994), and there is experimental support for a model in which phosphorylation of P or of one of the other nucleocapsid proteins switches the polymerase from a replicase to a transcriptase (Chanda *et al.,* 1980; Testa *et al.,* 1980). Another model, founded on studies utilizing mutants of VSV is that the control of transcription and replication is mediated by a regulatory modification of template-associated N protein (Perrault *et al.,* 1983). The idea that transcription and replication are separate processes recently received additional support from the report that the VSV transcriptase can enter the genome internally at the beginning of the N gene (Chuang and Perrault, 1997).

In summary our findings support the idea that soluble N protein is required for RNA replication and confirm the idea that the M2 ORF1 protein is not involved. But there was no evidence for a dynamic, quantitative switch between transcription and replication. This suggests that a dynamic balance between these two processes is not a feature of regulation of RSV RNA synthesis. Our findings indicate that the fraction of polymerase committed to RNA replication is small, at least for synthesis of the miniantigenome nucleocapsid. Further evaluation of the regulation of RNA synthesis of nonsegmented negative-strand RNA viruses will be facilitated by the ability to manipulate the template and *trans*-acting proteins in reconstituted minireplicon systems such as described here.

ACKNOWLEDGMENTS

The authors thank Ena Camargo for technical assistance and Drs. Robert Chanock, Brian Murphy, Sue Emerson, John Patton, and Michael Teng for critical comments on the manuscript.

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