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Transcription of Human Respiratory Syncytial Virus Genome RNA In Vitro: Requirement of Cellular Factor(s)

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Extracts made from human respiratory syncytial virus (RSV)-infected Hep-2 cells synthesized mRNAs encoded by all known viral genes. In contrast, RSV ribonucleoproteins purified from infected cells failed to transcribe in vitro; transcription was restored by addition of a cytoplasmic extract of uninfected Hep-2 cells, demonstrating that a cellular factor(s) has a role in RSV gene expression. Quantitation of the individual gene mRNAs transcribed in vitro revealed polarity of transcription of the genome.

Human respiratory syncytial virus (RSV) is the single most important viral agent responsible for respiratory tract diseases in young children, often resulting in serious complications, such as bronchiolitis and pneumonia, and even death. The virus is classified in the genus *Pneumovirus* (which also includes bovine RSV and mouse pneumonia virus) within the *Paramyxoviridae* family. Sequencing and cloning studies have resulted in the identification of conserved mRNA termini, intergenic sequences, and coding regions for each of the 10 genes of the virus (6, 7, 9, 10–13, 15, 16, 22–25, 27, 34). Like other members of the *Paramyxoviridae* family (2), RSV is a nonsegmented negative-strand RNA virus containing an RNA genome about 15,221 nucleotides (nt) long. The gene order is 3′(leader)-1C-1B-N-P-M-SH-G-F-22K-L-(trailer)5′; alternative names of some genes are NS1 (1C), NS2 (1B), 1A (SH), and M2 (22K). All 10 RSV proteins have been identified, and most of them are structural components of the virion; interestingly, the two most abundant ones (1C and 1B) appear to be nonstructural (17, 22, 31, 32, 36). The nucleocapsid core of the virion consists of the viral genomic RNA and the N, P, and L proteins (22, 31, 36). By analogy to other paramyxoviruses (2), as well as the well-studied prototype rhabdovirus vesicular stomatitis virus (VSV; reviewed in references 1 and 2), it is generally believed that the RSV nucleocapsid is the transcription complex of the virus and that it encodes an RNA-dependent RNA polymerase activity; however, this has yet to be demonstrated. In addition, the various activities of the proteins that constitute the functional ribonucleoprotein (RNP) complex remain uncharacterized.

Thus, it became increasingly obvious that elucidation of the molecular mechanisms of RSV gene expression would require an in vitro transcription reaction reconstituted from purified macromolecules. In this communication, I report a cell-free transcription system for RSV derived from partially purified viral nucleocapsids. Synthesis of viral mRNAs in this system was found to be completely dependent on addition of cell extracts, suggesting an essential role of a host factor(s) in RSV transcription. The relative molar yields of the different transcripts showed a gradation in accordance with the order of the genes in the genome, 1C mRNA being the most and L mRNA the least abundant.

Synthesis of RSV RNA by infected-cell extract in vitro. To establish an in vitro transcription system for RSV, extracts were prepared from RSV-infected cells as follows. The long strain of RSV was grown in Hep-2 cells in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Monolayers of Hep-2 cells, grown in T flasks to about 80% confluency, were infected with RSV at a multiplicity of infection of about 1.0. At 5 h postinfection (p.i.), media in the flasks were replaced with fresh prewarmed media. The infected cells were exposed to actinomycin D (2 μg/ml) between 14 and 16 h p.i. At 16 h p.i., the cells were washed with phosphate-buffered saline and then treated with lysolecithin (250 μg/ml) at 4°C (5). The permeabilized cells were scraped into ice-cold buffer A (50 mM Tris-acetate [pH 8.0], 100 mM K-acetate, 1 mM dithiothreitol [DTT], 2 μg of actinomycin D per ml; 0.5 ml of buffer was used per 150-cm^2 flask) and then disrupted by a Dounce homogenizer (in ice). The cell lysate was centrifuged at 2,000 × *g* for 10 min to pellet nuclei, cell debris, and unlysed cells. The supernatant, referred as cell extract (3 mg of protein per ml), was collected and portions of it were used directly in transcription reactions. Extracts of mock-infected cells were prepared in an identical manner.

Standard viral transcription reactions (20 μl) contained 50 mM Tris-acetate (pH 8.0), 120 mM K-acetate, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, actinomycin D (2 μg/ml), 20 μM [α-^{32}P]UTP (20 μCi), CTP and GTP each at 400 μM, 1 mM ATP, and 12 μg of infected-cell extract. Reaction mixtures were incubated at 30°C for 2 h. Synthesis of labeled RNA was quantitated by subjecting 10 μl of the reaction mixture to a DE-81 paper-binding assay as previously described (4). Initially, the reaction conditions were standardized by varying the temperature, pH, and monovalent (K^+) and divalent (Mg^{2+}) cation concentrations. Results presented in Fig. 1 show the following optimum values for these parameters, respectively: 30 to 32°C, pH 7.5 to 8.5, 100 to 140 mM K-acetate, and 5 mM $MgCl_2$. Standardization of any given parameter was performed by maintaining the others at or near the optimum value. All subsequent reactions were carried out under this set of conditions.

To determine the nature of the RNA species synthesized in vitro, labeled transcripts were affinity chromatographed in oligo(dT)-cellulose (28). For comparison, [^{3}H]uridine-labeled RSV mRNAs were prepared from actinomycin D-treated RSV-infected cells essentially as described before, except that cycloheximide was omitted (23); poly(A)$^+$ mRNAs were then selected by affinity chromatography through oligo(dT)-cellulose. Results presented in Fig. 2 demonstrate the close similarity of the in vitro RNA profile

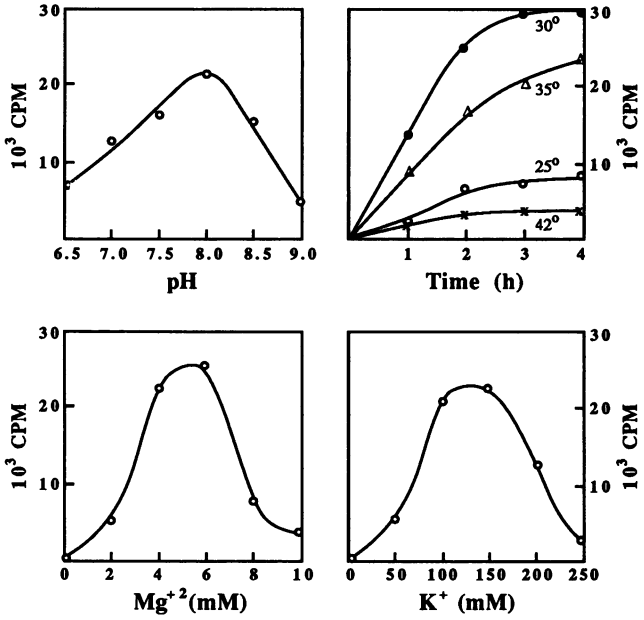


FIG. 1. Optimization of RSV transcription in vitro. In vitro transcription reactions with extracts of RSV-infected cells were carried out as described in the text; 10-μl reaction mixture was spotted on DE81 paper and processed for scintillation counting (4).

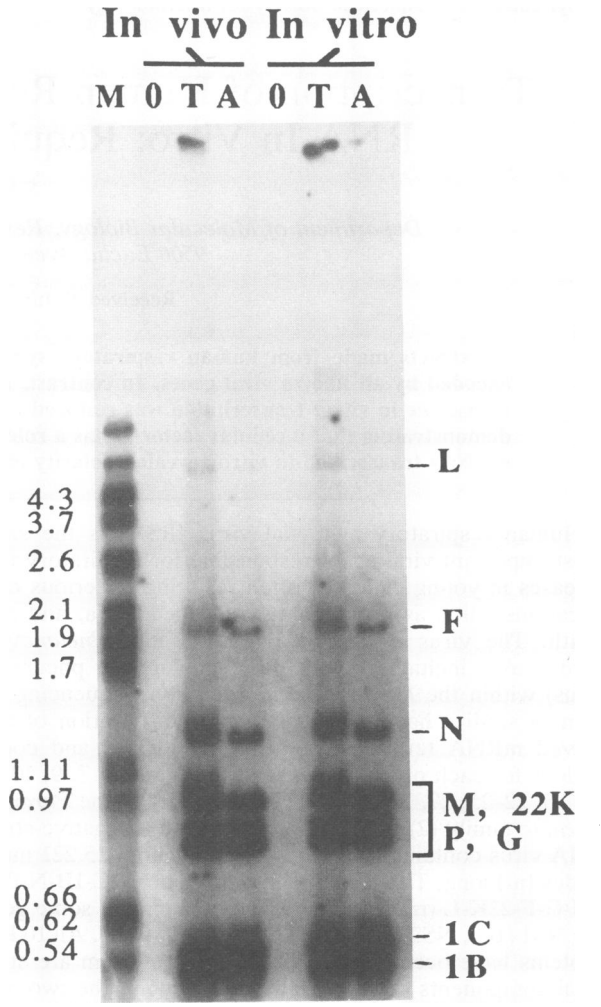


FIG. 2. Electrophoretic analysis of RSV transcripts. The in vivo side shows RSV transcripts purified from virus-infected cells labeled with [^{3}H]uridine in the presence of actinomycin D between 11 and 14 h p.i. The in vitro side shows labeled transcripts synthesized by an extract of RSV-infected cells in vitro. Both kinds of RNAs were deproteinized by phenol-chloroform and fractionated through oligo(dT)-cellulose column chromatography (28). RNAs bound to oligo(dT)-cellulose are shown in lanes A; an approximately equivalent amount of unfractionated RNA was analyzed in lanes T; lanes zero contained RNA from mock-infected cells. All RNA samples were finally treated with oligo(dT) and RNase H (3), denatured in glyoxal, and analyzed by electrophoresis in 1.5% agarose–6 M urea gels in 25 mM Na-citrate, pH 3.0 (26, 35). An autoradiograph of the dried gel is shown. The in vivo side was exposed to the film for longer periods to make the band intensities comparable to those of the in vitro side. M, ^{32}P-labeled λ-*Cla*I DNA fragments of the indicated lengths (kilobases) used as size markers. The various RSV mRNAs are shown on the right (10, 23).

to that made in vivo. As mentioned before, RSV is known to code for at least 10 species of mRNA in vivo; in order of decreasing size, the calculated lengths of the mRNAs, excluding the nontemplated poly(A) tail, are as follows: L, 6,578 nt; F, 1,903 nt; N, 1,203 nt; 22K, 961 nt; M, 958 nt; G, 923 nt; P, 914 nt; 1C, 532 nt; 1B, 503 nt; and SH, 410 nt (6). While the 410-nt-long SH mRNA ran off the gel, mRNAs of other size classes were readily observed (23). The L mRNA was produced in very small amounts and could be seen only upon overexposure of the autoradiogram. Since most of the labeled RNA bound to oligo(dT), it can be concluded that the transcripts are polyadenylated in vivo as well as in vitro. The single major RNA species that failed to bind to oligo(dT) and barely entered the gel was not characterized but appears to be a genome size replication product (23).

To optimize the in vitro transcription system further, the cell harvesting time was standardized. Poly(A)$^+$ viral RNAs were isolated from infected cells labeled with [^{3}H]uridine at various times (8, 14, and 20 h) p.i. Parallel infected, unlabeled cell cultures were lysed at the same time points, and the extracts of these cells were tested for RNA-synthetic activity in vitro. Results of this comparison (Fig. 3) show that intracellular viral transcription increased up to about 14 h p.i. and then decreased; similarly, the ability of the cell extract to synthesize viral RNA in vitro leveled off at about 14 h p.i. and also decreased afterwards. This time point was therefore chosen for preparation of infected-cell extracts or viral RNP for use in all subsequent in vitro experiments.

Transcription by viral RNP in vitro: requirement of a cellular factor(s). It was obvious that one of the first steps toward biochemical dissection of the in vitro system would involve establishment of a transcription reaction based on a purified viral RNP complex. Studies of a number of other negative-strand RNA viruses have identified the RNP as the minimal viral transcription complex (2). RSV RNP was isolated by centrifugation of the infected-cell extract at 150,000 × *g* for 4 h through a layer of 40% glycerol in 50 mM Tris-acetate (pH 8.0) and 1 mM DTT, with a 100% glycerol cushion at the bottom of the tube. The RNP complexes, pelleted on the cushion, were collected by dissolution in small volumes of TE (20 mM Tris-acetate [pH 8.0], 1 mM EDTA) and used immediately for transcription. The final protein concentration of the RNP suspension was adjusted to 4 mg/ml. Uninfected-cell extract was processed similarly,

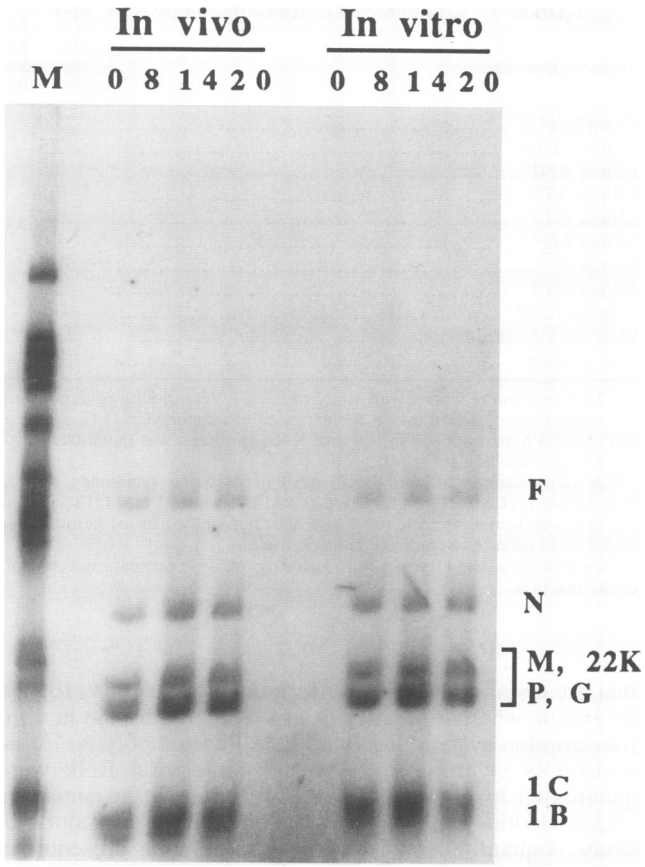


FIG. 3. RSV RNA synthesis in vivo and in vitro. Autoradiograph showing labeled poly(A)$^+$ RSV RNA isolated from Hep-2 cells at the indicated times (in hours) p.i. (in vivo) and labeled RNA synthesized in extracts of RSV-infected cells at the same times (in vitro). The RNAs were processed and analyzed in 1.5% agarose gels containing urea as described for Fig. 2. The labeled standard markers (M) and the various mRNA species are as described in the legend to Fig. 2.

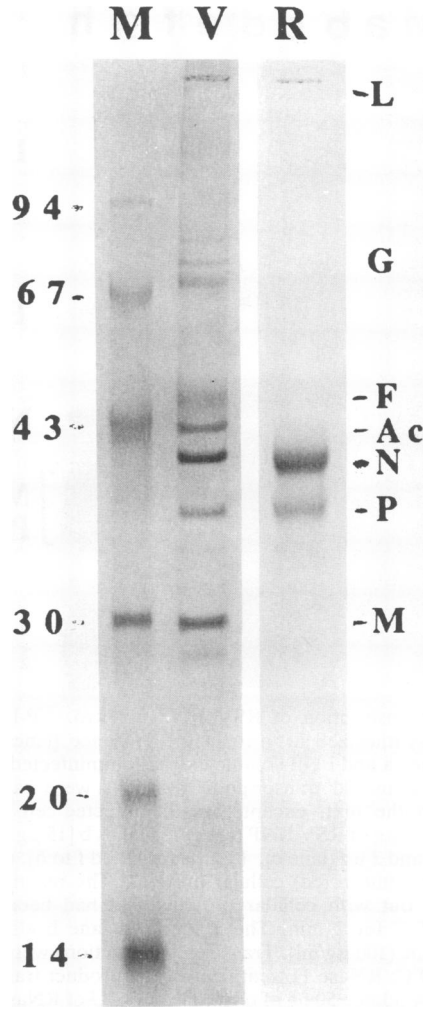


FIG. 4. Polypeptide profile of RSV RNP. Viral RNP (lane R) isolated from RSV-infected Hep-2 cells labeled with [^{35}S]methionine and [^{35}S]cysteine was analyzed by electrophoresis in SDS–12% polyacrylamide gels followed by autoradiography. Purified virions (lane V) and standard protein markers (lane M) were electrophoresed in parallel lanes for comparison and stained with Coomassie blue. The various polypeptides of the RSV virion have already been described (17, 22, 31, 32, 36). The numbers on the left are molecular sizes in kilodaltons.

and the top layer of the glycerol gradient, containing soluble cytoplasmic proteins (2 mg/ml), was collected and saved for use in transcription reactions. To examine the viral polypeptides in the RNP, radiolabeled RNP was prepared in a similar manner, except that following actinomycin treatment at 14 to 16 h p.i., complete medium was replaced by prewarmed minimal medium lacking methionine and cysteine and containing 50 μCi of L-[^{35}S]methionine per ml and 25 μCi of L-[^{35}S]cysteine per ml. After another 6 h of growth, the cells were lysed and RNP was purified as described above. The labeled RNP was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and compared with the polypeptide profile of purified RSV virions. Results presented in Fig. 4 showed the presence of N and P polypeptides as the major components of the RNP, while L was present only in trace amounts. As mentioned earlier, RNP complexes purified from RSV virions have been shown to consist primarily of the viral genome RNA, nucleocapsid protein N, phosphoprotein P, and large protein L (22, 31, 36). A polypeptide with an M_r of about 44,000, believed to be cellular actin (19), was found to be tightly associated with the virion but was barely detectable in the labeled RNP (Fig. 4).

When employed in transcription reactions (Fig. 5), the viral RNP was found to be completely inactive, suggesting loss of its transcription function during purification (lanes a and b). To study the mechanism of this defect, portions of the soluble cytoplasmic extract of uninfected cells were added to the transcription reaction. This resulted in restoration of transcription by the RNP (lanes c to e), suggesting that the cell extract supplied a factor(s) essential for RSV transcription. Control reactions containing the uninfected-cell extract but lacking viral RNP did not produce any transcript (lane f). Heating of the soluble extract at 65°C for 2 min totally destroyed its stimulatory activity (lane g), suggesting that the active component(s) is probably a protein. Cycloheximide, a protein synthesis inhibitor, had no effect on transcription (lane h), indicating that the stimulation was not due to de novo synthesis of cellular or viral proteins. As with the infected-cell extract, a transcription reaction based on purified viral RNP produced polyadeny-

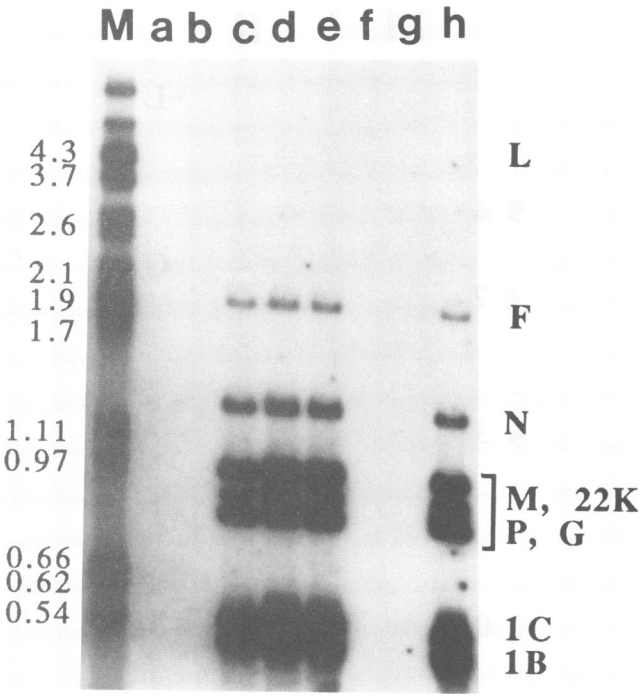


FIG. 5. Transcription of RSV RNP in vitro. ^{32}P-labeled RSV transcripts synthesized in vitro in the presence (lanes c to h) or absence (lanes a and b) of soluble extract of uninfected Hep-2 cells are shown. Standard transcription reactions were carried out as described in the text, except that the infected-cell extract was replaced by 10 μg of RSV RNP (except in lanes b [15 μg of RNP] and f [no RNP]) and 2 μg (lane c), 4 μg (lanes d and f to h), or 8 μg (lane e) of soluble (uninfected) cellular proteins. The reaction in lane g was carried out with cellular proteins that had been previously heated at 65°C for 2 min. The reaction in lane h also contained cycloheximide (100 μg/ml). Transcription reactions were carried out for 2 h at 30°C; RNase H treatment of the product transcripts was performed by adding 50 ng of oligo(dT) and 2 U of RNase H directly to the transcription mixture 10 min before the end of transcription (3). An autoradiograph of the gel is presented. The labeled standard markers (lane M) and the various mRNA species are as described in the legend to Fig. 2.

lated mRNAs as judged by their affinity to oligo(dT)-cellulose (data not shown).

A more detailed study of the requirements of RNP-mediated transcription is presented in Table 1. Transcription is clearly dependent on the various small molecules, such as ribonucleotides, monovalent and divalent cations, and DTT. Also, as shown in Fig. 5 (lane h) and Table 1, cycloheximide had little effect on viral transcription. However, omission of cellular proteins, heat treatment of viral RNP, or the presence of trypsin completely abolished transcription, confirming the involvement of viral and cellular proteins in the transcription process. Taken together, the results presented in Fig. 5 and Table 1 demonstrate the essential role of a cellular component(s) in the transcription of RSV RNP in vitro.

Polarity of RSV transcription in vitro. Although the relative amounts of RSV mRNAs have never been quantitated directly, blot hybridization studies and the general pattern of intracellular mRNAs have suggested a gradation in their amounts, i.e., mRNAs of genes proximal to the 3′ end of the genome are produced in greater molar amounts than those

TABLE 1. Requirements of transcription by RSV RNP

Transcription reaction	pmol of UMP incorporated/ mg of RNP/h
Complete[a]	25.0
Minus extract	2.5
Minus RNP	0.5
Minus ATP	0.6
Minus GTP	0.7
Minus Mg^{2+}	1.0
Minus K^+	1.2
Minus DTT	2.2
Heated RNP[b]	0.6
Plus cycloheximide[c]	23.0
Plus trypsin[c]	2.4

[a] Transcription reactions with 10 μg of RSV RNP and 5 μg of uninfected-cell extract were carried out in the presence of actinomycin D (2 μg/ml) at 30°C for 1.5 h as described in the text. RNA synthesis was quantitated by a DE 81 paper-binding assay (4).

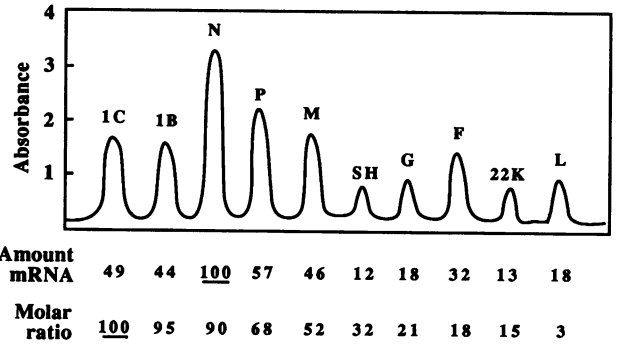
[b] A 10-μg sample of viral RNP in 10 μl of buffer containing 50 mM Tris-acetate (pH 8.0), 5 mM $MgCl_2$, 120 mM K-acetate, 1 mM DTT, and 5% glycerol was heated at 65°C for 2 min and then chilled in ice before being added to an otherwise complete reaction mixture.

[c] Cycloheximide and trypsin, both at 100 μg/ml, were present from the start of the reaction.

that are distal, indicating transcriptional polarity (9, 10). To determine whether polarity is also exhibited by the in vitro transcription system described here, labeled poly(A)$^+$ viral transcripts synthesized in vitro by the viral RNP were quantitated by hybridization to an excess of an antisense oligonucleotide specific for each gene mRNA in a slot blot assay. A quantitative analysis of the slot blot is presented in Fig. 6, in which the relative molar amounts of the various gene mRNAs clearly show polarity of RSV transcription, 1C mRNA being the most and L mRNA the least abundant (~3% of 1C mRNA). A control oligonucleotide probe complementary to the first 45 nt of the VSV P gene mRNA did not hybridize to RSV transcripts (data not shown).

In conclusion, I have described a detailed characterization of an in vitro transcription system for RSV that uses either infected-cell extract or viral RNP purified from infected cells, the latter requiring a heat-labile cellular cytosolic factor(s) for transcriptional activity. The in vitro system appears to be a faithful representation of viral transcription as judged by the following criteria. (i) All 10 viral mRNAs were produced (Fig. 6). (ii) The mRNAs were polyadenylated (Fig. 2), as well as capped and methylated at their 5′ ends (unpublished data), although capping of RSV mRNAs in vivo has yet to be shown. (iii) The molar gradation of various gene mRNAs indicates polarity of transcription (Fig. 6) and also suggests (but does not prove) the existence of a single transcriptional promoter at the 3′ end of the genome and polymerase fall-off at intergenic junctions. Essentially similar conclusions were reached through measurement of the kinetics of RSV gene inactivation by UV light (15). (iv) Synthesis of viral mRNAs in vitro, like that in vivo, was resistant to actinomycin D. The characteristics of in vitro RSV transcription reported here are indeed regarded as the hallmarks of transcription of all nonsegmented negative-strand RNA viruses (2).

Earlier (1989), Herman reported a cell-free transcription system for RSV that employed a total extract of virus-infected cells for transcription in vitro (21). The biochemical parameters of his transcription system were very similar to those of the infected-cell extract described here (Fig. 1). On



Amount of mRNA	49	44	100	57	46	12	18	32	13	18
Molar ratio	100	95	90	68	52	32	21	18	15	3

FIG. 6. Quantitation of RSV gene transcripts by slot blot hybridization. Oligonucleotide probes (45 nt long) complementary (antisense) to each gene mRNA from codons 2 through 16 (both inclusive) were synthesized in an Applied Biosystems 380B DNA synthesizer by using standard phosphoramidite chemistry. The available gene sequences of the Long strain of RSV (used in this study) was used to design the oligonucleotides; these genes are N, P, G, and F (24, 25, 27). However, for all other genes, for which the sequence of the Long strain is not known, the corresponding sequences of the A2 strain (18) were used since the two strains are nearly identical in sequence. Approximately 4 μg of each oligonucleotide was applied to a GeneScreen membrane (Bio-Rad) in a Bio-Rad slot blot apparatus and cross-linked with shortwave UV light. ^{32}P-labeled poly(A)$^+$ RNA purified from 100-μl in vitro transcription reactions using RSV RNP and uninfected-cell extract was applied to each slot. Prehybridization and hybridization were carried out in accordance with the manufacturer's protocol, and the membrane was washed sequentially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 2% SDS at room temperature, 2× SSC plus 2% SDS at 60°C, and 0.1× SSC at room temperature. The membrane was air dried and exposed to X-ray film for autoradiography without using an intensifying screen. A densitometric scan of the autoradiograph is shown; the units of absorbance are arbitrary. Hybridization showed a linear response up to at least five times as much viral RNA as was used here (data not shown). The various RSV gene probes are indicated at the tops of the peaks. The relative amount of each class of mRNA was estimated by integrating the area under each peak and is shown as a percentage of the largest peak area (that of N mRNA). This value was then divided by the calculated length of the corresponding mRNA in nucleotides (6). The resultant value, representing the relative molar amount of each mRNA, is shown as a percentage of 1C mRNA, which was the most abundant.

the basis of the relative mobilities of the transcripts, it appeared that all of the viral mRNAs were synthesized (21), although the identity of the transcripts or their relative abundance was not analyzed with specific probes. Attempts to transcribe purified RSV nucleocapsids in his study failed; it was not determined whether cellular proteins could restore transcription. Furthermore, mRNAs (especially the smaller ones, i.e., SH, 1B, and 1C) synthesized in vitro exhibited slightly slower electrophoretic mobilities than their intracellular counterparts; this was postulated to be due to a longer poly(A) tail of in vitro mRNAs. Although the exact reason for the mobility difference was not determined, I was not able to detect such differences to an appreciable extent (Fig. 2), even before removal of the poly(A) tail of the mRNAs (data not shown). It is possible that some of the minor variations between the two studies resulted from the different cell lines (HeLa versus Hep-2) used, reflecting the complexity of RSV transcription at the same time.

In my studies of RSV transcription in vitro, the exact time of harvest of the viral RNP appeared to be a critical determinant of the efficiency of transcription. Results presented in Fig. 3 demonstrate that the ability of the infected-cell extract to produce viral transcripts starts decreasing at about 14 h p.i.; extracts made at 30 h p.i. or later transcribed extremely poorly or not at all (data not shown). Viral RNP lost its transcription property even earlier; while RNP made at 14 to 16 h p.i. transcribed well (in the presence of cell extract), that made at 24 h p.i. was highly defective (data not shown). The mechanism of this loss of transcriptional competence remains unknown. However, one possibility is that the RNP at later periods tightly associates with nucleases or with a specific inhibitor of transcription.

The restoration of transcription of viral RNP by uninfected-cell extract as shown here (Fig. 5) strongly suggests involvement of a cellular factor in RSV transcription. However, the alternative possibility that the cell extract merely compensates for the activity of a viral protein lost during purification of the RNP cannot be ruled out. It is also not known whether more factors than one are responsible for the observed stimulation. Recently, in vitro transcription systems of various degrees of purity have been reported for a number of paramyxoviruses. In all cases in which it has been studied, highly purified virus or viral RNP has been found to be transcriptionally defective; transcription in these systems could be restored by addition of various cellular components (8, 14, 20, 29, 30, 33). The cell extract-dependent transcription system described here should be helpful in identifying the exact nature of the cellular factor(s) required for RSV transcription.

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