Respiratory Syncytial Virus mRNA Coding Assignments

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The polypeptide coding assignments for six of the respiratory syncytial virus-specific mRNAs were determined by translation of the individual mRNAs *in vitro*. The coding assignments of the RNAs are as follows. RNA band 1 is complex and can be separated into at least two components on the basis of electrophoretic mobility (molecular weights [MWs] $\approx 0.21 \times 10^6$ and 0.31×10^6 , respectively) that code for three polypeptides of 9.5, 11, and 14 kilodaltons (K). RNA 2 (MW, 0.39×10^6) codes for a 34K polypeptide; RNA 3 (MW, 0.40×10^6) codes for a 26K polypeptide; RNA 4 (MW, 0.47×10^6) codes for a 42K polypeptide; and RNA 5 (MW, 0.74×10^6) codes for a 59K polypeptide. By limited-digest peptide mapping, the 34, 26, and 42K polypeptides synthesized *in vitro* appeared to be unique. Additionally, peptide mapping showed that the 34, 26, and 42K polypeptides synthesized *in vitro* were indistinguishable from their counterparts synthesized in infected cells. Thus, the 34, 26, and 42K polypeptides coded for by mRNAs 2, 3, and 4, respectively, were identified as the respiratory syncytial virus phosphoprotein (34K), matrix protein (26K), and nucleocapsid protein (42K), respectively. RNA 5 was shown to code for a 59K polypeptide. The 59K polypeptide synthesized *in vitro* did not comigrate with any polypeptide specific to infected cells, suggesting that it is a candidate for co- or post-translational modification.

Respiratory syncytial (RS) virus, the prototype virus of the genus *Pneumovirus* of the *Paramyxoviridae*, is the most important causative agent of serious lower respiratory tract illness in young children. Despite the significance of RS virus as a pathogenic agent, a clear enumeration of the viral-specific gene products is not currently available.

There has been lack of agreement concerning both the number and the size of RS virus-specific RNAs (9-11, 13, 14, 20) and proteins (1-3, 7, 8, 15-17, 21). To approach this problem, we isolated RNAs from RS virus-infected cells and assayed their viral specificity by hybridization with highly purified virion RNA. Recently, we identified eight major RNA species in RS virus-infected HEP-2 cells. The largest RNA (molecular weight; ca. 5×10^6) comigrated with RNA from purified virions and was not polyadenylated, and its synthesis was dependent upon concurrent protein synthesis (10, 11). The subgenomic-sized RNAs synthesized in actinomycin D-treated cells had approximate molecular weights ranging from 0.2×10^6 to 2.6×10^6 , and all (i) were polyadenylated, (ii) were synthesized in the presence of cycloheximide, and (iii) hybridized specifically to purified virion RNA, as determined by Northern blot hybridization analysis. (11). These results substantiated the classi-

fication of RS virus as negative stranded and indicated that the polyadenylated subgenomic-sized RNAs were candidates to be viral RNAs. In this study, we fractionated these RS virus-specific RNAs by preparative electrophoresis and identified the polypeptide coding assignments for the six smallest RNA bands by translation of the separated RNAs *in vitro*.

RNA was isolated from cytoplasmic extracts of RS virus-infected HEP-2 cells after metabolic labeling with [3 H]uridine in the presence of actinomycin D. Total polyadenylated RNA was selected by chromatography on oligodeoxythymidylic acid-cellulose. The RNAs were then separated by agarose-urea gel electrophoresis, using a preparative apparatus for simultaneous elution and recovery as described previously (19). Samples of the fractionated RNAs were analyzed in comparison with the unfractionated RNA by electrophoresis in an analytical scale agarose-urea gel. The data presented in Fig. 1 show, as reported previously (10, 11), that seven major polyadenylated RNA species can be separated by electrophoresis in this gel system. Fractions substantially enriched for RNA bands 1 through 5 were recovered by preparative electrophoresis. Band 1 RNA was separated into faster (1a) and slower (1b) migrating components. This is consistent with our previous find-

FIG. 1. Analytical gel electrophoresis of RS virus mRNAs fractionated by preparative gel electrophoresis. Total [^3H]uridine-labeled polyadenylic acid containing RS virus mRNAs from HEp-2 cells infected with the A₂ strain of RS virus (11) were isolated (6) and separated by preparative electrophoresis (19) as described previously. The fractionated RNAs were subsequently analyzed by analytical agarose-urea gel electrophoresis (18). The ^3H -labeled RNAs were visualized by fluorography of the dried gel. Lanes A and H, Unfractionated RNAs; lanes B through G, sequential fractions from preparative gel.

ing which showed that band 1 can be separated into at least two electrophoretic components by denaturation with glyoxal before electrophoresis (11). Because RNA bands 2, 3, and 4 have very similar electrophoretic mobilities (molecular weights, 0.39×10^6 , 0.40×10^6 , and 0.47×10^6 , respectively), it was difficult to obtain clean separation of these species by this method of preparative electrophoresis. Therefore, a second method was also used to obtain individual RNAs. RNA bands were cut directly from analytical gels, and the RNA was recovered from the gel slices by electroelution. Separation by this method resulted in minimal contamination of closely migrating RNA species; however, recovery was poor.

The RNA fractions separated by preparative electrophoresis were analyzed in comparison

with unfractionated polyadenylated RNA isolated from infected or uninfected cells for their ability to direct synthesis in an mRNA-dependent rabbit reticulocyte cell-free protein synthesis system. Samples of each RNA fraction as shown in Fig. 1 were added to a reticulocyte lysate in the presence of [^{35}S]methionine or a ^3H -amino acid mixture. The products of the *in vitro* translation of each of the separated RNAs and of the total RNA were analyzed by electrophoresis in 15% polyacrylamide gels. Figure 2A shows the results for ^{35}S -labeled products; similar results were obtained by labeling with a ^3H -amino acid mixture (data not shown).

Total polyadenylated RNA (Fig. 2A, lane c) isolated from infected cells directed the synthesis of seven polypeptides of estimated molecular weights of 9.5, 11, 14, 26, 34, 42, and 59 kilodaltons (K), respectively, which were specific to RNA from infected cells. Polyadenylated RNA from mock-infected cells showed synthesis of only faint amounts of two polypeptides which did not comigrate with the major polypeptides synthesized in infected cells (Fig. 2A, lane b). These data show that under the conditions of infection used here (i.e., in the presence of actinomycin D from 14 h postinfection), host mRNA synthesis was inhibited significantly, thereby allowing visualization of viral-specific products with essentially no background of host products.

Fractions containing both RNA 1a and 1b directed the synthesis of 9.5, 11, and 14K polypeptides (Fig. 2a, lanes d and e). It appeared that RNA 1a directed the synthesis of proportionally more 9.5K polypeptide than did RNA 1b, whereas RNA 1b directed the synthesis of more 11 and 14K polypeptide. This observation was confirmed by densitometric scanning of fluorograms of the dried gels (data not shown). The broadness of the band at approximately 11K may be due to the presence of a high concentration of globin in the reticulocyte lysate. Translation of RNA 1 in a wheat germ lysate, in which this problem was avoided, resulted in the synthesis of three polypeptides which migrated as sharp bands at 9.5, 11, and 14K (data not shown).

RNA 2 directed the synthesis of a 34K polypeptide, with small amounts of a 26K polypeptide (Fig. 2A, lane f). Fractions enriched for RNA 3 directed the synthesis of a large amount of a 26K polypeptide as well as a 34K polypeptide (Fig. 2A, lane g). As mentioned above, RNAs 2 and 3 are very similar in size and therefore were difficult to separate. A cleaner separation of these RNAs was achieved by excision of bands (see below). Fractions enriched for RNA 4 predominantly directed the synthesis of a 42K polypeptide, although small amounts of 34 and 26K polypeptides were also

FIG. 2. Analysis by gel electrophoresis of polypeptides synthesized in vitro in response to RS virus mRNAs fractionated by preparative electrophoresis. In vitro protein synthesis reactions programmed with individual mRNA fractions as listed below were carried out in a micrococcal nuclease-treated rabbit reticulocyte lysate at 30°C for 90 min in the presence of [35 S]methionine as described previously (6). Samples of each reaction were analyzed by electrophoresis in a 15% polyacrylamide gel, using the buffer system of Laemmli (12). (A) Translation products from reactions programmed with mRNAs separated by preparative electrophoresis (19). Reactions contained the following additions: lane a, no added mRNA; lane b, polyadenylated mRNA from mock-infected, actinomycin D-treated HEP-2 cell cytoplasmic extracts; lane c, polyadenylated mRNA from RS virus-infected, actinomycin D-treated HEP-2 cell cytoplasmic extracts; lanes d, e, f, g, h, and i, the RNA fractions 1a, 1b, 2, 3, 4, and 5, respectively, as shown in Fig. 1; lanes j and l, proteins from RS virus-infected, actinomycin D-treated HEP-2 cell extracts labeled with [35 S]methionine; lane k, proteins from mock-infected, actinomycin D-treated HEP-2 cell extracts labeled with [35 S]methionine. The fluorogram of the products in (A) was exposed for 5 days (lanes a through h and j and k) or 10

days (Fig. 2A, lane h). Fractions enriched for RNA band 5 directed the synthesis of a 59K polypeptide, in addition to 42, 34, and 26K polypeptides (Fig. 2A, lane i). The finding that RNA from band 5 directed the synthesis of three of the smaller polypeptides as well as the 59K polypeptide was determined to be the result of contamination of the larger RNA with smaller RNAs during the preparative electrophoresis recovery procedure (19).

Because of problems with cross-contamination of RNAs, these results allowed only a tentative correlation of polypeptides with mRNAs as described above. To confirm the coding assignments, we used the RS virus RNAs 1 through 5 that had been isolated by electroelution from bands cut directly from analytical gels to program protein synthesis in vitro. The results (Fig. 2B) show the synthesis of proteins directed by individual RNA bands in the reticulocyte lysate in the presence of [35 S]methionine. RNA 1a directed the synthesis of 9.5, 11, and 14K polypeptides. Band 1b directed the synthesis of 11 and 14K polypeptides (Fig. 2B, lanes c and d). The individual RNA bands 2, 3, 4, and 5 directed the synthesis, respectively, of a 34K protein, a 26K protein, a 42K protein, and a 59K protein. As can be seen from the data in Fig. 2B, use of RNAs isolated by electroelution resulted in separation of RNAs with only a very slight level of cross-contamination from either higher or lower molecular weight RNA species, as evidenced by the discriminating test of in vitro translation. Thus, the data presented in Fig. 2 allow the following messenger RNA and protein coding assignments: RNA 1 (a and b), 9.5, 11, and 14K polypeptides; RNA 2, 34K polypeptide; RNA 3, 26K polypeptide; RNA 4, 42K polypeptide; RNA 5, 59K polypeptide.

The proteins synthesized in vitro were compared with proteins synthesized in RS virus-infected cells. The data (Fig. 2A and B) show

days (lanes i and l). (B) Polypeptides synthesized in vitro in response to individual mRNA bands which were excised from analytical gels and recovered by electroelution. Samples a through d were electrophoresed in one gel and samples e through j in a separate gel. Lanes a and j, Proteins from mock-infected, actinomycin D-treated HEP-2 cell extracts labeled with [35 S]methionine; lanes b and i, proteins from RS virus-infected, actinomycin D-treated HEP-2 cell extracts labeled with [35 S]methionine; lane c, translation products of RNA band 1a (faster migrating portion of band 1); lane d, translation products of RNA band 1b (slower migrating portion of band 1); lanes e, f, g, and h, translation products of RNA from bands 2, 3, 4, and 5, respectively. Molecular weights were estimated in a parallel lane of each gel by electrophoresis of protein standards obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

that six of the products synthesized in vitro by the separated mRNAs comigrated with six of the proteins specific to RS virus-infected cells. Of the infected cell proteins, the 42K polypeptide is the most abundant viral-specific protein and has been identified as the major nucleocapsid protein, N, by Peeples and Levine (16), Cash et al. (2), and ourselves (unpublished data). Similarly, proteins with approximate molecular weights of 34 and 26K have been identified as the RS virus phosphoprotein and matrix protein, respectively (2, 16). The 9.5, 11, and 14K polypeptides all comigrated with proteins specific to RS virus-infected cells. The 59K polypeptide, however, did not comigrate with a counterpart specific for infected cells.

Limited digest peptide mapping with *Staphylococcus aureus* V8 protease was used to determine the relationships of the 42, 34, and 26K polypeptides synthesized in vitro to their counterparts synthesized in RS virus-infected cells. The data (Fig. 3) show that the pattern of peptides generated by digestion of the proteins synthesized in vitro is indistinguishable from that of the proteins synthesized in infected cells in the case of these three proteins. Additionally, the peptide pattern of each of the three individual proteins was unique. Therefore, this data established the identity of (i) the 34K polypeptide synthesized in vitro as the phosphoprotein (P), (ii) the 26K polypeptide as the matrix protein (M), and (iii) the 42K polypeptide as the nucleocapsid protein (N).

Attempts to carry out limited-digest peptide mapping on the 9.5, 11, and 14K polypeptides and their counterparts from infected cells were not successful because the digestion products generated by V8 protease were not sufficient to establish identity.

Previously, we identified at least seven major polyadenylated RNA species from RS virus-infected cells which could be separated on the basis of electrophoretic mobility (11). These RNAs were shown to be RS virus specific by their ability to anneal to RS virion RNA (11). In this study, we separated these RNA species by electrophoresis and analyzed the protein products encoded by translation of each in vitro.

The data presented here show that the five smallest RNA species separated by electrophoresis in agarose-urea gels code for at least seven polypeptides. RNA band 1 appeared to be complex. It could be separated into slower and faster migrating components by electrophoresis. Three polypeptides, having molecular weights of 9.5, 11, and 14K, were produced by in vitro translation of RNA band 1. These three polypeptides comigrated with three polypeptides that are present in RS virus-infected cells but not in mock-infected cells. The finding that these three poly-

FIG. 3. Comparison by peptide mapping of proteins synthesized in vitro and in infected cells. The three RS virus polypeptides M (26K), P (34K), and N (42K), identified by autoradiography, were cut from a dried gel, rehydrated, and digested with 5 μ g of *S. aureus* V8 protease per ml by the method of Cleveland et al. (4). Peptides were analyzed by electrophoresis in a 15% polyacrylamide gel as described in the legend to Fig. 2. Radioactivity present in [35 S]methionine-labeled peptides was visualized by fluorography. Lanes 1, 2, and 3 show digestion of the polypeptides M, P, and N, respectively. Lane A shows the peptides generated by digestion of the proteins synthesized in RS virus-infected cells. Lane B shows the peptides generated by digestion of the proteins synthesized in vitro.

peptides were coded for specifically by RNA band 1 and were not present in preparations of the larger mRNAs indicates that these polypeptides are unique and are not degradation products of larger viral proteins and suggests that RNA band 1 contains at least three closely migrating RNA species. Additional evidence that RNA band 1 contains three unique viral specific components comes from the identification of three unique, nonhomologous, cloned RS virus-specific cDNAs, all of which hybridize to RNA band 1 (P. L. Collins and G. W. Wertz,

unpublished data). Additionally, data presented previously (11) and results from the cloning of the cDNAs of RNA band 1 show that the three band 1 RNAs are virus specific and therefore indicate that the protein products of these mRNAs are virus specific.

At present, however, because of the inability to separate band 1 RNA into its individual components, we cannot assign the small polypeptides to a specific RNA species within RNA 1. Previous reports concerning small RS virus-specific polypeptides have not been in agreement. There have been reports of a 10K polypeptide (2, 3, 17), a 12K polypeptide (8), and 15 and 18K polypeptides (7) specific to RS virus-infected cells. These small polypeptides have not been reported to be precipitated reproducibly with anti-RS virion antisera. Since these polypeptides have been observed only in infected cells and not in virions, this would be expected. The role of the 9.5, 11, and 14K polypeptides as RS virus nonstructural or structural proteins remains to be established. We postulate that one reason for the many discrepancies in reports of small RS virus proteins may be the fact that proteins of this size are frequently lost from lower percentage acrylamide gels and that molecular weight estimates in this lower range are frequently only approximate.

RNA bands 2, 3, 4, and 5, when added individually to the mRNA-dependent reticulocyte lysates, directed the synthesis of 34, 26, 42, and 59K polypeptides, respectively. The 26, 34, and 42K polypeptides all comigrated with proteins which were specific to RS virus-infected cells. These three proteins were shown to be unique and indistinguishable from their counterparts synthesized in infected cells on the basis of limited-digest peptide mapping. Additionally, since RNAs 2, 3, and 4 all hybridize specifically to RS virion RNA (11), these data indicate that these three proteins are all virus specified.

Previous reports have identified at least eight polypeptides associated with RS virions that have been shown to be virus specific (8, 17). Up to nine polypeptides specific to RS virus-infected cells have been identified (1, 2, 7), seven of which can be precipitated with anti-RS virus antiserum (1, 7). There has been some disagreement concerning the number and the exact sizes of several of the RS virus proteins. However, a recent paper by Pringle et al. (17) has correlated data from several laboratories and provided a unified nomenclature for eight of the RS virus polypeptides that incorporates much of the current knowledge concerning the function of the RS virus proteins. The identifications of three of the polypeptides that we have assigned have been firmly established. The polypeptide designated VP 41 has been identified as the nucleo-

capsid protein (N), VP 32 has been characterized as the viral phosphoprotein (P), and VP 27 as the RS virus matrix protein (M). Therefore, based on the observation that the *in vitro* translation products of RNAs 2, 3, and 4 comigrated with the 34, 26, and 42K polypeptides from RS virus-infected cells and had identical V8 protease peptide maps (Fig. 3), we make the following coding assignments: RNA 2, phosphoprotein (P); RNA 3, matrix protein (M); and RNA 4, nucleocapsid protein (N).

The 59K polypeptide synthesized in response to RNA 5 did not comigrate with any polypeptide specific for RS virus-infected cells (Fig. 2A, lanes i and j; Fig. 2B, lanes h and i). We have demonstrated previously that RNA 5 is virus specific (11). Therefore, we conclude that the 59K polypeptide synthesized *in vitro* in response to RNA 5 represents an RS virus-specific protein which normally undergoes co- or post-translational modification which is not carried out in this *in vitro* system. An example of one such modification would be glycosylation. Experiments to test this hypothesis are under way.

The results presented here on *in vitro* translation of mRNAs from RS virus-infected cells agree (within the limits of molecular weight estimates) with the previous report by Cash et al. (2), who identified 61, 41, 33, 32, 27, and 17K polypeptides by translation *in vitro* of unfractionated mRNA from RS virus-infected cells. The 33 and 32K polypeptides reported by these workers may represent two forms of the RS virus phosphoprotein (P) such as are observed with the rhabdovirus vesicular stomatitis virus (5) under certain gel conditions (e.g., in the presence of 7 M urea). We have observed that the 34K polypeptide can also migrate as a broad doublet band (data not shown). The work presented here, however, extends the observation of Cash et al. (2) by identifying two small RS virus-specific polypeptides and by assigning the synthesis of the individual polypeptides to individual mRNAs.

In summary, the coding assignments made here show that RNA 1 codes for three polypeptides of 9.5, 11, and 14K, RNA 2 codes for the 34K phosphoprotein, RNA 3 codes for the 26K matrix protein, RNA 4 codes for the 42K nucleocapsid protein, and RNA 5 codes for a 59K polypeptide. The work presented here, however, has not provided a coding assignment for the two largest RS virus-specific proteins, the 82K major surface glycoprotein and the large, ~200K polypeptide which is presumed to be the viral RNA polymerase. These studies are in progress.

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