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Construction and characterization of cDNA clones for four respiratory syncytial viral genes

(cDNA library/hybrid selection and cell-free translation/respiratory syncytial virus proteins)

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ABSTRACT **Cytoplasmic poly(A)-containing RNA from respiratory syncytial virus-infected cells was used as a template to synthesize oligo(dT)-primed cDNAs. Discrete size classes of single-stranded cDNAs, resolved by alkali agarose gel electrophoresis, were used separately to construct double-stranded cDNAs that were subsequently inserted into the plasmid vector pBR322 at the *Pst* I site by means of oligo(dG)-oligo(dC) tailing. After transfection of *Escherichia coli*, recombinant plasmids were screened mostly by serial rounds of hybrid selection of mRNAs from virus-infected cells and subsequent *in vitro* translation of the selected mRNAs. Comparative peptide mapping of the translation products with those of authentic virion proteins served to establish the viral origin of the cDNA recombinants. In this manner, four distinct classes of recombinant plasmids were identified. These encode sequences corresponding to those of respiratory syncytial virus nucleocapsid protein, matrix protein, phosphoprotein, and a nonstructural protein.**

Respiratory syncytial virus (RS virus) is a pleomorphic enveloped virus with properties similar to the paramyxoviruses but has been placed in a separate genus, *Pneumovirus*, on the basis of its morphology and lack of hemagglutinin and neuraminidase activities. It is a major etiologic agent of severe respiratory tract infection, especially in the pediatric age group (1). Despite its clinical importance, biologic and molecular studies of this virus have been hindered, partly as a result of its poor growth in tissue culture systems, and the polypeptide composition and mechanism of gene expression have been described only recently (2, 3). RS virus has been reported to contain a negative-stranded genomic RNA of 5,000 kilodaltons (kDal) encoding seven or eight polyadenylylated mRNAs (3). Earlier genetic analysis with *ts* mutants identified seven or eight complementation groups (4, 5), consistent with the presence of seven or eight virus-encoded polypeptides. These include a 160-kDal protein (probably the virion polymerase by analogy with the paramyxoviruses), an 84-kDal envelope glycoprotein, a 68-kDal envelope glycoprotein that may represent the fusion factor responsible for entry and cell-to-cell spread, a 46-kDal nucleocapsid protein (NC protein), a 36-kDal phosphoprotein (P protein), a 28-kDal matrix protein (M protein), and two additional proteins of 18 and 16 kDal (refs. 2, 6–9 and this work). The functional roles of the surface glycoproteins in cell penetration and spread are poorly understood as are the functions of internal viral proteins.

Our interest in RS virus genetics stems from its public health importance as a human pathogen. At present there is no satisfactory vaccine for prevention of serious RS virus disease. Candidate live vaccine virus strains have proved to be unsatisfactory because of overattenuation or genetic instability (1). Recent attempts to protect against RS virus disease by parenteral administration of wild-type virus also have failed because of inadequate antigenicity (10). Therefore, it was decided to redirect our efforts toward gaining a better understanding of RS virus and then to use this information to develop a more effective strategy for immunoprophylaxis. Recombinant DNA technology was employed to analyze the different transcriptional units of this virus and the mechanism of their regulation. In this communication, we describe the construction and characterization of RS viral recombinant cDNA clones for four viral genes.

MATERIALS AND METHODS

Virus and Cells. RS virus, strain A_2 (11), was propagated in Hep-2 cell monolayers in Eagle's minimal essential medium with 2% fetal calf serum. Extracellular virus was purified from clarified culture fluid as described (3). The visible virus band at the 60% sucrose interphase was suspended in 5 vol of TE buffer (10 mM Tris·HCl, pH 7.2/1.0 mM EDTA) and was concentrated by centrifugation in an SW 41 rotor at 35,000 rpm for 60 min. The pelleted virus was suspended in TE buffer.

Cells were infected at a multiplicity of 5 plaque-forming units/cell and actinomycin D was added at 12 hr after infection (2 μg/ml), followed by addition of [5,6-^{3}H]uridine (10 μCi/ml; 1 Ci = 3.7×10^{10} Bq) 1 hr later. The cells were harvested and cytoplasmic RNA was isolated 12 hr later (12). Alternatively, at a time when cytopathic effect was first evident RNA was isolated from infected cells not treated with actinomycin D. mRNAs were recovered by two cycles of oligo(dT)-cellulose chromatography (13).

Synthesis and Cloning of cDNAs in *Escherichia coli*. Single-stranded cDNAs were synthesized by reverse transcription of oligo(dT)-primed cytoplasmic mRNAs [20 μg of oligo(dT); 100 μg of mRNAs] from infected cells as a template (14). cDNAs were rendered free of mRNAs by treatment with 0.1 M NaOH at 68°C for 30 min and were resolved by electrophoresis through alkali agarose gels (15). Five discrete bands of cDNAs with approximate lengths of 3,500, 2,100, 1,375, 640, and 550 bases produced when mRNAs from infected cells were used as template were extracted from gels by electroelution (16). Approximately 20 deoxycytidylate residues were added at the 3′ ends of gel-fractionated single-stranded cDNAs by using terminal transferase (17). Oligo(dG)-primed second strand synthesis, oligo(dC) addition to double-stranded cDNA, and subsequent insertion into oligo(dG)-tailed plasmid pBR322 at the *Pst* I site were as described (14). Recombinant cDNA plasmids were used to transform *E. coli* K-12 strain HB101 (18). A total of 2,900 tetracycline-resistant and ampicillin-sensitive colonies was screened by colony hybridization for the presence of RS viral sequences by using ^{32}P-labeled infected cellular mRNAs synthesized in the

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Abbreviations: RS virus, respiratory syncytial virus; kDal, kilodalton(s); NC, P, M, and NS proteins, nucleocapsid, phospho-, matrix, and nonstructural proteins, respectively; bp, base pairs.

presence of actinomycin D (5 μg/ml) (19). Two-thirds of the colonies reacted positively with the probe. Seventy-five individual colonies exhibiting the strongest reaction were picked. They were screened in duplicate by colony hybridization (20) by using as a probe ^{32}P-labeled single-stranded cDNAs reverse-transcribed from mRNAs of infected or mock-infected cells. All of the 75 colonies reacted with cDNAs made from infected cell mRNA, whereas none did so with an equivalent amount of cDNA made from uninfected cell mRNA. Recombinant plasmids were isolated by the mini-isolation procedure (21) and were analyzed for the size of the cDNA inserts. Many cDNA plasmids lacked one or both *Pst* I sites. However, most released inserts ranging from 580 to 2,200 base pairs (bp) upon digestion with *Hpa* II, which cleaves the vector DNA 50 bp on either side of the *Pst* I site. All of the cloning experiments were conducted in compliance with the current prescribed National Institutes of Health guidelines.

Screening of Recombinant cDNA Clones. Recombinant plasmids (80 μg) in groups of 10 were immobilized on nitrocellulose filters, and the DNA filters were used to hybrid-select mRNAs from infected cells (22). Specific mRNAs eluted from the DNA filters were translated in a messenger-dependent rabbit reticulocyte lysate system and the polypeptides were resolved by electrophoresis through 15% polyacrylamide gel (23). Subsequent screening by this procedure was performed by immobilizing single cDNA clones on filters.

Peptide Mapping. Two-dimensional tryptic peptide mapping and one-dimensional gel electrophoretic analysis of *Staphylococcus aureus* V8 protease products of the viral proteins were carried out as described (24, 25).

Reagents. Restriction enzymes were purchased either from Bethesda Research Laboratories or from New England BioLabs. Avian myeloblastosis virus reverse transcriptase was a generous gift from J. W. Beard (Life Sciences, St. Petersburg, FL). Terminal transferase and *E. coli* DNA polymerase I and its large subunit were purchased from Boehringer Mannheim. Nucleoside triphosphates and T4 polynucleotide kinase were purchased from P-L Biochemicals. Radioactive materials were purchased from Amersham.

RESULTS

Identification of RS Virus Proteins. Hep-2 monolayer cells were infected at a ratio of 5 plaque-forming units/cell and the proteins were labeled at 12 hr after infection with [^{3}H]leucine, [^{35}S]methionine, or [^{3}H]glucosamine for 18 hr. Cytoplasmic extracts and extracellular virus were analyzed by electrophoresis on NaDodSO$_4$/polyacrylamide gels. For extracellular virus, five major and two minor (arrows) bands labeled with [^{3}H]leucine (Fig. 1, lane 4) had apparent molecular masses of 84, 49, 46, 36, 28, 22, and 16 kDal. In several other experiments the 22-kDal protein was found to have a mobility corresponding to ≈18 kDal. [^{3}H]Glucosamine was incorporated into 84-, 49-, and 15-kDal proteins (Fig. 1, lane 5) and, in accordance with other reports (2, 6, 8), probably represents the major envelope glycoprotein (84 kDal) and the two subunits (49 and 15 kDal) of the putative fusion factor (9). The smaller subunit was not labeled with leucine or methionine and hence was not seen in virions (Fig. 1, lanes 4 and 6). Fusion factor of paramyxoviruses has been shown to be synthesized as a precursor that is cleaved proteolytically into two subunits that are linked by disulfide bonds (26). The other viral polypeptides represent the major NC protein (46 kDal), P protein (36 kDal), M protein (28 kDal), and the two additional proteins of 22 or 18 and 16 kDal seen in Fig. 1, lane 4 (2, 8). In addition, intracellular nucleocapsids purified by two cycles of cesium chloride centrifugation (27) were found to contain predominantly NC protein (46 kDal) and minor quantities of 160-kDal protein, presumably the virion polymerase (L protein; Fig. 1, lane 7).

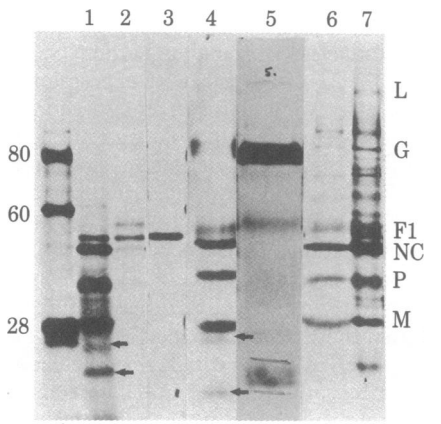

FIG. 1. Analysis of RS virus proteins by electrophoresis on 15% polyacrylamide gels in NaDodSO$_4$ under reducing conditions. Lanes 4–6 illustrate extracellular virus-associated proteins labeled *in vivo* with [^{3}H]leucine, [^{3}H]glucosamine, or [^{35}S]methionine. Lane 7 is the profile of intracellular proteins labeled *in vivo* in infected cells with [^{35}S]methionine. [^{35}S]Methionine-labeled cell-free translation products of poly(A)$^+$ RNA from actinomycin D-treated (5 μg/ml) infected cells (lane 1) or mock-infected actinomycin D-treated cells (lane 2) are shown. As a control exogenous RNA was not added to the cell-free translation system (lane 3). Molecular mass markers (far left lane; shown in kDal) are ^{35}S-labeled influenza viral proteins.

The viral origin of the proteins shown in lane 4 of Fig. 1 was established further by specific immunoprecipitation with rabbit hyperimmune RS virus antiserum (data not shown). Cell-free

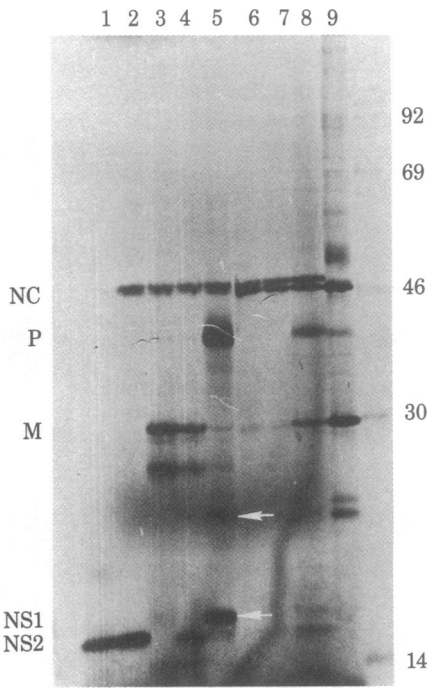

FIG. 2. Cell-free translation products of mRNAs from RS virus-infected cells selected by hybridization to putative recombinant RS virus cDNA plasmids. The different recombinant plasmids used were pRSB$_8$ (lane 1), pRSC$_6$ (lane 2), pRSB$_7$ (lane 3), pRSD$_3$ (lane 4), pRSC$_1$ (lane 5), pRSB$_{10}$ (lane 6), and pRSB$_{11}$ (lane 7). Translation products of polyadenylylated RNA from infected cells are displayed in lane 8. RS virion polypeptides labeled *in vivo* with [^{35}S]methionine are shown in lane 9. Molecular mass markers (far right lane; shown in kDal) were obtained commercially from Amersham.

translation of polyadenylylated cytoplasmic RNA from infected cells yielded products corresponding to the NC, P, and M proteins as well as the 22- or 18- and 16-kDal proteins (arrows, Fig. 1, lane 1). The latter two proteins were observed variably in purified virions and are referred to as nonstructural proteins (NS1 and NS2) for lack of a better definition. Several paramyxoviruses are known to encode two nonstructural proteins designated as C and S proteins (28–33). In one case, it was shown by tryptic mapping that the 22-kDal C protein of Sendai virus was unrelated to other virion proteins (29, 31). In contrast, two minor proteins encoded by Newcastle disease virus were shown to be derived from the P protein of the virus (34).

Screening of cDNA Library by Hybrid Selection and *in Vitro* Translation of mRNAs. The initial screening procedure yielded too many clones to analyze simultaneously. Fortunately, the translational profile of mRNAs from infected cells was reasonably unambiguous and this allowed us to hybrid-select mRNAs for translation by using groups of putative RS virus cDNA clones bound to nitrocellulose filters. By serial hybrid selection of mRNAs and subsequent cell-free translation of the selected mRNAs, plasmids encoding distinct viral mRNA sequences were recovered (Fig. 2). Two cDNA recombinants hybrid-selected mRNA encoding NS2 protein (Fig. 2, lanes 1 and 2). Other recombinants encoded M protein (Fig. 2, lanes 3 and 4), P protein (Fig. 2, lane 5), or NC protein (Fig. 2, lanes 6 and 7). An endogenous band migrating slower than 46 kDal (NC protein) was variably present (Fig. 2, lanes 2–8). Additional translational products seen with mRNAs hybrid-selected by putative M ($pRSD_3$; Fig. 2, lane 4) and P ($pRSC_1$; Fig. 2, lane 5) gene recombinants did not disappear with increasing stringency of hybridization. It is possible that the larger of the two additional bands selected by the M gene recombinants is a premature termination product of M protein because it shares tryptic peptide spots with the M protein (data not shown). It might reflect certain inherent artifacts of cell-free translation with large inputs of specific mRNAs. The smaller of the two bands comigrates with a nonstructural protein (NS2). For technical reasons, we were unable to show by peptide mapping that it was indeed identical to the NS2 protein. However, in several experiments, M gene mRNA was not selected by hybridization with the putative NS2 recombinant DNA. Conversely, another putative M gene recombinant ($pRSB_7$) did not hybrid-select NS2 mRNA (Fig. 2, lane 3). It should be noted that two additional minor bands, 21.5 and 18 kDal, were visualized among translation products of P gene recombinant DNA hybrid-selected mRNA (Fig. 2, lane 5, arrows). Both of these bands also were observed among translational products of total viral mRNA.

Peptide Mapping. To establish that the major translation products of hybrid-selected mRNAs were of viral origin, the P and M proteins were purified after $NaDodSO_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled virions. Two-dimensional tryptic mapping patterns of the virion proteins were compared with those of translation products of hybrid-selected mRNAs (Fig. 3). The tryptic peptide map of the P protein of viral origin was indistinguishable from that of a 36-kDal protein translated from the mRNA selected by the putative P gene recombinant DNA (Fig. 3 *Upper*). Similar analysis of the putative M gene recombinant DNA revealed that the coding sequences for this gene were contained within this plasmid (Fig. 3 *Lower*).

We also compared the staphylococcal V8 protease cleavage products of NC and M proteins from the virions with those of the 46- and 28-kDal polypeptides translated from the mRNAs

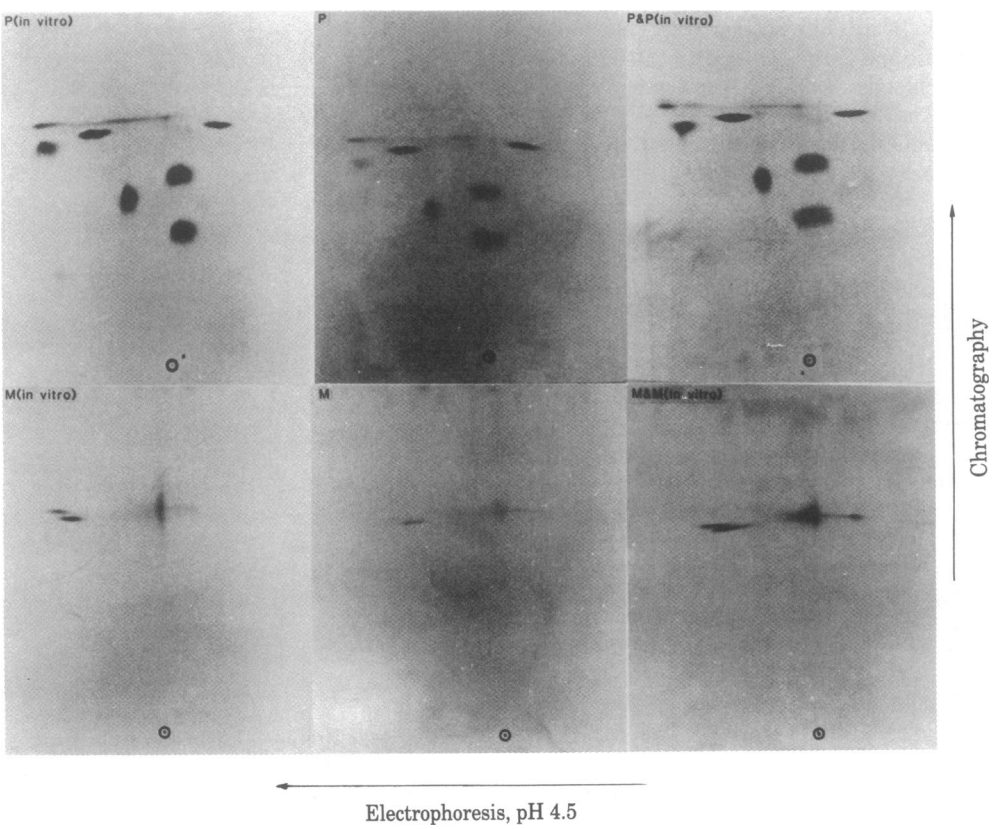

FIG. 3. (*Upper*) Two-dimensional tryptic peptide mapping of [^{35}S]methionine-labeled RS virus P protein (P), a corresponding translation product of mRNA hybrid-selected by a RS cDNA plasmid (P *in vitro*), and a mixture of both (P and P *in vitro*). (*Lower*) Similar analysis was done with virus M protein, translation product of hybrid-selected mRNA, and a mixture of both. The digests were spotted on cellulose thin layers at sites marked by ⊙ and were electrophoresed in the horizontal dimension (anode to left), followed by ascending chromatography.

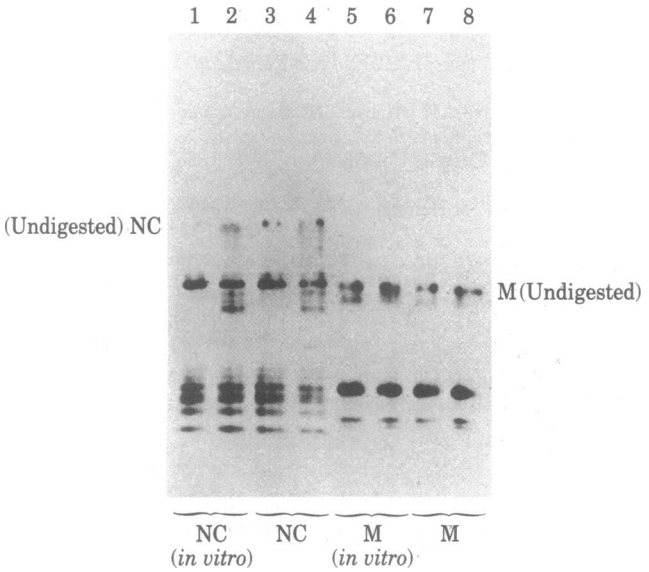

FIG. 4. *Staphylococcus aureus* V8 protease digestion patterns of viral NC protein (NC) and a corresponding polypeptide translated *in vitro* from mRNA hybrid-selected by a RS cDNA plasmid (NC *in vitro*) and viral M protein (M) and a corresponding *in vitro* translation product of mRNA hybrid-selected by a M gene recombinant DNA (M *in vitro*). Digestions were done with 25 ng of enzyme (lanes 1, 3, 5, and 7) or 50 ng of enzyme (lanes 2, 4, 6, and 8).

hybrid-selected by the NC and M gene plasmids (Fig. 4). The profiles of the virus-associated proteins were identical to those of the cell-free translation products.

Analyses of Recombinant cDNA Plasmids. Many of the clones lacked the *Pst* I site. However, on cleavage with *Hpa* II, most of them released inserts ranging from 580 to 2,200 bp in size (Fig. 5). Two recombinant DNAs of each gene were analyzed,

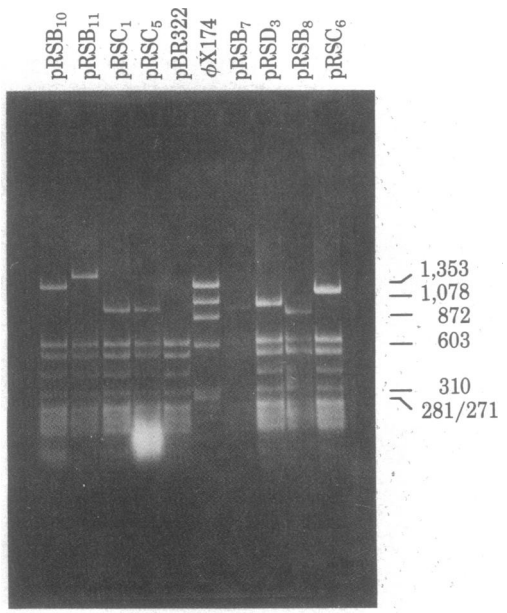

FIG. 5. Analysis by neutral agarose (1.5%) gel electrophoresis of *Hpa* II restriction fragments of representative recombinant plasmids. Presented are two plasmids ($pRSB_{10}$ and $pRSB_{11}$) encoding NC gene, two ($pRSC_1$ and $pRSC_5$) encoding P gene, two for the M gene ($pRSD_7$ and $pRSB_3$), and two representing the NS2 protein gene ($pRSB_8$ and $pRSC_6$). *Hpa* II fragments of vector plasmid DNA are illustrated by the lane labeled pBR322. *Hae* III restriction fragments of form I φX174 DNA serve as markers with the sizes of the relevant fragments in bp shown on the right.

the smaller one probably representing the result of abortive first- or second-strand synthesis during construction of cDNA. Thus, $pRSB_{10}$ and $pRSB_{11}$ contain RS viral sequences of 1,250 and 1,550 bp, respectively, and represent cDNA clones of the NC gene. Preliminary DNA sequence analysis (35) established that $pRSB_{11}$ has a poly(A) tail and has sequence homology with $pRSB_{10}$ at the 5′ end. $pRSB_{10}$ lacks a poly(A) tail, implying that $pRSB_{10}$ was defective in second-strand cDNA synthesis. Two P gene recombinant plasmids ($pRSC_1$ and $pRSC_5$) with *Hpa* II inserts of ≈950 bp might be longer because they have *Pst* I and *Hpa* II sites within the insert. Recombinant plasmids $pRSB_8$ and $pRSC_6$ have 870 and 1,100 bp of cDNA, respectively. The latter has been subjected to sequence analysis to a limited extent and was found to contain a poly(A) tail. The sizes of the larger cDNA inserts are compatible with full-length copies of their respective mRNAs.

DISCUSSION

We report the construction of cDNA clones containing RS virus NC, P, M, and NS2 gene sequences. The cloning strategy avoided the self-priming reaction for the second-strand synthesis that requires the use of nuclease S1 to remove the resulting hairpin. This method has been shown previously to yield nearly full-length copies of the template with retention of the 5′ sequences of mRNAs (14). Because discretely sized single-stranded cDNAs were used separately for construction of the recombinant plasmids, the cDNA library so prepared is representative of RS virus sequences, as was indeed shown by initial screening.

The recombinant plasmids were screened by successive cycles of hybrid selection of mRNA from infected cells followed by cell-free translation of selected mRNAs. By this approach and the use of isomorphous restriction enzyme cleavage patterns, gene sequences for NC protein were identified in independent plasmids. Similarly, plasmids bearing sequences for P, M, or NS2 protein also were identified.

Some viral-specific clones retained the 3′ end of mRNA, whereas others did not. Also, the lengths of the inserts of the larger recombinants are adequate to accommodate the coding sequences of the relevant gene products. However, proof that the recombinants represent full-length copies of the different transcripts must await further DNA sequence analysis to establish the proper reading frames and mapping of the 5′ ends of the individual mRNAs by cDNA extension on viral mRNAs (36).

Under stringent conditions RS virus M-gene recombinant plasmids ($pRSC_{12}$ and $pRSD_3$) hybrid-selected mRNA from infected cells that yielded upon translation both M protein and a protein that comigrated with viral NS2. There also were other recombinant plasmids that hybrid-selected mRNA for M protein only ($pRSB_7$). Conversely, two cDNA clones ($pRSB_8$ and $pRSC_6$) selected mRNA under nonstringent hybridization conditions that yielded only NS2 protein as a translation product.

RS virus has been demonstrated to be a negative-stranded RNA virus encoding seven, or probably eight, transcriptional units, consistent with the presence of seven or eight virion proteins (3). Although the precise gene order is not available, it is reasonable to assume that it is similar to that of the paramyxoviruses—i.e., 3′ NC F_0 M P HN L 5′ or 3′ NC P (F_0M) HN L 5′ (37, 38). If this pattern holds true for this virus, we have obtained clones containing sequences adjacent and 3′ to F_0 and the HN equivalent. Hence, use of our recombinant DNAs should permit us to "walk" across genomic RNA and obtain cDNAs encoding the F_0 and the HN equivalent. It is not clear whether the transcriptional and replicative mechanisms of RS virus involve synthesis of untranslatable leader RNAs, as occurs in the case of the best-studied negative-stranded RNA virus, vesicular stomatitis virus (39, 40). Availability of cDNA clones that represent

probably the first gene—namely, NC—should enable us to determine the 5′-end sequence of the antigenomic RNA and reveal the presence of untranslated leader RNA if it exists. Construction of cDNA clones containing the 3′ end of the genomic RNA should allow us to study the mechanism of RS virus replication as well as analyze the structures of DI particles generated during virus replication (41).

Seven or eight different complementation groups have been identified among the various *ts* mutants of RS virus (4, 5). The cDNA clones presently described could be transferred to a eukaryotic expression vector to facilitate identification of the lesion in each of these groups by complementation. Attenuated strains that belong to a specific complementation group and that are capable of eliciting active immunity thus can be analyzed at a molecular level with respect to their specific gene defect(s). Presently we are unable to obtain by cell-free translation putative precursors to the two viral glycoproteins. Shotgun cloning in yeast or eukaryotic expression vectors of cDNA products synthesized from the genomic RNA by using P or NC gene 3′-end fragments as primers might yield RS viral glycoproteins and thus allow us to identify the desired clones.

This report provides preliminary characterization of the gene organization of RS virus by use of recombinant DNA technology. The cloned genes will serve as a starting point for biochemical analysis of the structure and nature of expression of RS virus genome.

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