cDNA cloning and transcriptional mapping of nine polyadenylylated RNAs encoded by the genome of human respiratory syncytial virus

(recombinant DNA/RNA transfer blots/polycistronic RNAs/genome organization/paramyxoviruses)

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ABSTRACT   We have isolated cDNA clones representing nine unique poly(A)$^+$ RNAs transcribed from the genome of human respiratory syncytial virus, a paramyxovirus. A cDNA library was constructed by using poly(A)$^+$ RNA from virus-infected cells as template and the *Escherichia coli* plasmid pBR322 as vector. Viral cDNA clones were identified by hybridization with cDNA probes prepared from viral genomic RNA. The viral clones were grouped into nine different families by hybridization with individual size-selected reverse transcripts representing the major classes of poly(A)$^+$ RNA from virus-infected cells. The largest clone from each family was selected for analysis. These nine clones, molecular sizes ranging from 520 to 2,600 base pairs, were shown to be unrelated on the basis of reciprocal hybridization using dot-blots. These cDNA clones were then used as hybridization probes to analyze intracellular viral RNAs that had been separated by gel electrophoresis and transferred to diazobenzyloxymethyl-paper. All nine clones hybridized with intracellular viral genomic RNA, confirmation of virus specificity. Nine unique intracellular viral poly(A)$^+$ RNAs were identified [molecular sizes ranging from 720 to 7,500 nucleotides, including poly(A)]. Comparison of the sizes of these major RNAs and the cDNA clones indicated that a number of the clones represented nearly complete copies of the corresponding RNAs. Several other intracellular viral poly(A)$^+$ RNAs appeared to be polycistronic by the criteria of molecular weights and homologies to various combinations of cDNA clones. The sizes and sequence contents of these polycistronic RNAs were used to prepare a transcriptional map whose significance is discussed.

Human respiratory syncytial (RS) virus is an enveloped, RNA-containing cytoplasmic virus that is a leading cause of respiratory tract infection in young children (1). RS virus has been classified as a member of the paramyxovirus family (2, 3). However, a detailed characterization of its molecular biology has been hindered by low yields of virus in cell culture and virion instability. As described in this paper, our approach to the analysis of the RS virus genome and gene products has been to use molecular cloning techniques.

The RS virus genome is a single negative strand of RNA, molecular mass at least 5×10^6 daltons (4), which is transcribed approximately in its entirety *in vivo* to generate transcripts that consist predominantly of monocistronic messengers (4–6). Genome replication, but not transcription, is dependent upon protein synthesis (4). Preliminary UV mapping studies suggested that transcription of most or all of the RS virus genes is dependent upon sequential read-through from a single promoter (ref. 7; unpublished data). These observations suggest that the molecular biology of RS virus shares several general features

with other paramyxoviruses and rhabdoviruses.

RNA from RS virus-infected cells, labeled in the presence of actinomycin D and analyzed by gel electrophoresis, includes genome-length RNAs (RNA 8) of positive and negative polarities and at least seven major species of poly(A)$^+$ RNA (RNAs 1–7), ranging from 0.24 to 2.50×10^6 daltons (4–6). The viral specificity of these RNAs has been confirmed by blot hybridization with ^{32}P-labeled genomic RNA (4) and the coding assignments of several RNAs have been determined (5). The smallest RNA band, RNA 1, appeared to contain at least two components (4, 5). In addition to the major poly(A)$^+$ species, other distinct but less abundant RNAs were detected (4, 5); these species are described in greater detail in this paper.

There is general agreement on the existence of six RS virus proteins, which are structural components of the virion and appear to be analagous to the six structural proteins, designated L, NP, P, M, F, and HN or H, of other paramyxoviruses (refs. 2, 3, 5, 8–13; unpublished data). Several additional, small (9,000–25,000 daltons) proteins have been detected in extracts of RS virus-infected cells. Seven or eight complementation groups of temperature-sensitive mutants have been reported for RS virus (9). Therefore, it seemed likely that RS virus encodes more unique gene products than the six known structural proteins.

Here we report the preparation and cloning of DNA copies of RS virus poly(A)$^+$ RNAs. These cDNA clones were used as hybridization probes to analyze viral RNAs that had been separated by gel electrophoresis and transferred to diazobenzyl-oxymethyl-paper (RNA transfer blotting). The results demonstrated that the RS virus genome is transcribed into at least nine unique poly(A)$^+$ RNA species. Additionally, these results demonstrated the existence of several polycistronic poly(A)$^+$ RNAs. Analysis of the sequence homologies of the polycistronic RNAs provided a method for identifying neighboring genes, and in this way a transcriptional map was prepared.

MATERIALS AND METHODS

Virus and Cells. The A$_2$ strain of RS virus was propagated in HEp-2 cells as described (4).

mRNA Purification. Viral [^{3}H]mRNAs were purified from cytoplasmic extracts of actinomycin D-treated, virus-infected cells (4, 5). For RNA transfer blotting, RNA purification was by phenol/chloroform extraction (14). For reverse transcription, RNA was purified by centrifugation through CsCl (15), resulting in a preparation free of detectable genomic RNA. In both cases, poly(A)$^+$ RNA was selected by chromatography on

Abbreviations: RS, respiratory syncytial; bp, base pair(s); VSV, vesicular stomatitis virus.

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oligo(dT)-cellulose (15). mRNA from vesicular stomatitis virus (VSV)-infected baby hamster kidney cells was prepared as described (15).

cDNA Synthesis. Twenty-five micrograms of poly(A)$^+$ RNA from RS virus-infected cells was transcribed into cDNA by using 40 μg of oligo(dT) as primer and 140 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) (16, 17). The RNA templates were dissociated by boiling (18) and the cDNAs were made double-stranded by using 55 units of *Escherichia coli* DNA polymerase I, Klenow fragment (P-L Biochemicals). The cDNAs were purified with phenol and passed through a column of Sepharose 6B. Following published procedures (16, 17, 19), the cDNAs were treated with nuclease S1 (P-L Biochemicals, 45 units/μg of cDNA), and calf thymus DNA terminal transferase (P-L Biochemicals, 40 units/μg of cDNA) was used to add 10–15 dCMP residues per 3' end. Fifty nanograms of this preparation was used for molecular cloning.

Molecular Cloning. The conditions for digestion of pBR322 with restriction endonuclease *Pst* I (Boehringer Mannheim), addition of 3' oligo(dGMP) tails, hybridization of vector and insert, and transformation of competent (20) *E. coli* strain HB101 cells followed published procedures (21).

Detection of Transformants Containing Viral Sequences. Transformants were analyzed by colony hybridization (22) with two hybridization probes. (*i*) ^{32}P-Labeled cDNAs were prepared by reverse transcription of poly(A)$^+$ RNAs from actinomycin D-treated infected cells, the RNA templates were hydrolyzed with alkali (17), and the cDNAs were purified by extraction with phenol and filtration through Sephadex G-75. Prior to use the cDNAs were prehybridized (62°C for 4 hr) with a 100-fold excess of poly(A)$^+$ RNA from uninfected cells (17) in 0.45 M NaCl/0.045 M Na citrate, pH 7.0. (*ii*) ^{32}P-Labeled cDNAs were synthesized by reverse transcription, primed with calf thymus DNA fragments (23), of RNA purified from viral nucleocapsids. The viral nucleocapsids were purified from cytoplasmic extracts (4) of infected cells by two rounds of banding in linear gradients of 20–40% (wt/wt) CsCl in TNE buffer (25 mM Tris·HCl/50 mM NaCl/2 mM EDTA, pH 7.5), in a Beckman SW 40 rotor (35,000 rpm for 16 hr at 4°C). Nucleocapsids banded as described above were sedimented through 35% (wt/vol) sucrose in TNE buffer into a cushion containing 60% (wt/vol) sucrose (Beckman SW 50.1 rotor, 40,000 rpm for 4 hr at 4°C), diluted, and pelleted. The RNA was recovered by phenol extraction and ethanol precipitation. The transcription reaction contained 50 μg of actinomycin D per ml. The products were treated with alkali, passed through Sephadex G-75, and boiled before colony hybridization.

Preparation of ^{32}P-Labeled cDNA Clones. Plasmids were purified by using a Triton X-100/lysozyme-cleared lysate procedure, followed by two rounds of equilibrium centrifugation in CsCl containing ethidium bromide (24). Insert cDNAs were excised from cloned recombinant plasmids by digestion with *Pst* I, separated from the vector by gel electrophoresis, recovered by electroelution, purified with phenol and chloroform, and labeled by nick-translation (25).

RNA Transfer Blotting. [^{3}H]RNAs were separated by electrophoresis on 1.5% agarose gels containing 6 M urea, transferred to diazobenzyloxymethyl-paper (Schleicher & Schuell), and analyzed by hybridization with ^{32}P-labeled cDNAs and cDNA clones (26).

Molecular Mass Estimates. Estimates were made relative to marker DNAs (prepared by digestion of pBR322 with nuclease *Pst* I, *Hinf*I, *Taq* I, or *Alu* I) by using a linear relationship between the log of the distance migrated during gel electrophoresis and the square root of the molecular mass (27).

RESULTS

Preparation of cDNAs. The source of RS virus poly(A)$^+$ RNAs for cDNA synthesis and molecular cloning was virus-infected HEp-2 cells. The relative proportions of viral and cellular cDNAs obtained with such RNA preparations were assessed by comparison, with gel electrophoresis, of [^{3}H]cDNAs synthesized by reverse transcription of mRNAs extracted from uninfected (Fig. 1, lane a) and RS virus-infected (Fig. 1, lane b) HEp-2 cells. Because both gel patterns contained a disperse, heterogeneous continuum of cDNAs, this background appeared to be cell-specific. The pattern of DNAs copied from infected-cell mRNAs also contained a number of discrete, more intense bands (marked with arrows in Fig. 1, lane b). Therefore, these abundant cDNAs were candidates to be virus-specific, although it was possible that some might be copies of virus-stimulated cellular mRNAs (28, 29).

Treatment of the RS virus-infected cells with actinomycin D for 6 hr prior to mRNA purification and reverse transcription resulted in a substantial reduction in the background of presumptive cell-specific cDNAs (Fig. 1, lane d). The same experiment was performed with mRNA from VSV-infected, actinomycin D-treated baby hamster kidney cells. Because the pattern of VSV-specific reverse transcripts has been published (16), this served as a positive control. As is shown in Fig. 1, lane c, the abundant bands contained in the gel pattern were of the appropriate sizes, complexity, and abundances to be complete copies of the VSV mRNAs, whereas the background of reverse transcripts presumed to be cellular appeared minimal. Thus, actinomycin D treatment prior to RNA purification appeared to result in a subsequent enrichment for these viral cDNAs.

cDNA Cloning. Reverse transcripts of poly(A)$^+$ RNAs from RS virus-infected cells, such as shown in Fig. 1, lane d, were made double-stranded, inserted at the *Pst* I site in the β-lactamase gene of pBR322 by homopolymer tailing, and cloned by transformation of *E. coli* (see *Materials and Methods*). This generated a cDNA library consisting of $\approx$2,500 ampicillin-sensitive transformants. Approximately 1,000 members were analyzed for the presence of viral sequences by colony hybridization with ^{32}P-labeled cDNAs made by reverse transcription of (*i*) mRNA extracted from RS virus-infected cells and (*ii*) RNA extracted from RS virus nucleocapsids (see *Materials and Methods*). About 35% hybridized strongly with both probes, presumptive evidence for virus-specific cDNA clones.

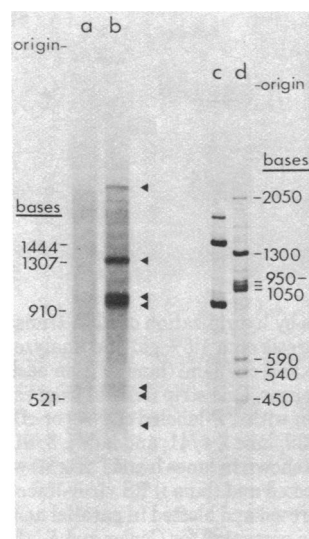Cloned recombinant plasmids from 43 transformants that were

FIG. 1. Comparison, by gel electrophoresis, of cDNAs synthesized by reverse transcription of poly(A)$^+$ RNAs extracted from (lanes a, b, and d) HEp-2 cells that were (lane a) uninfected, (lane b) RS virus-infected, and (lane d) RS virus-infected and actinomycin D-treated and from (lane c) virus-infected, actinomycin D-treated baby hamster kidney cells. The ^{3}H-labeled reverse transcripts were separated by electrophoresis on (lanes a and b) an alkaline 1.5% agarose gel and (lanes c and d) a 1.75% agarose gel containing 6 M urea and were visualized by fluorography (15). The positions and sizes of molecular mass markers are shown next to lane a. The arrows adjacent to lane b indicate cDNA bands that lack counterparts in lane a. The estimated sizes of the principal bands in lane d are also shown.

positive for colony hybridization were partially purified by a small-scale rapid method (30), sized by gel electrophoresis, and characterized by replicate dot-blot hybridization (31). The dot-blot hybridizations were performed with individual ^{32}P-labeled reverse transcripts, copied from infected cell poly(A)$^+$ RNAs, which had been separated and eluted from gels such as shown in Fig. 1, lane d. In this way, the clones were organized into groups related to the six cDNA hybridization probes (refer to Fig. 1, lane d): (probe i) the 450-base cDNA; mixtures of (probe ii) the 540- and 590-base and (probe iii) the 950- to 1,050-base cDNAs; (probe iv) the 1,300-base cDNA; (probe v) 2,050-base cDNA; and (probe vi) a mixture containing a series of nonabundant cDNAs of >2,500 bases. This last probe hybridized to only a single clone; the other groups each contained 4–17 clones. Interestingly, one clone hybridized with both probes iii and iv. That clone (clone 6/55) was subsequently found to be a cDNA of a polycistronic RNA (see *Discussion*).

The largest recombinant plasmid from each of groups i–vi was purified preparatively for further analysis. Because the cDNA hybridization probes used to define groups ii and iii were complex, the cDNA clone selected from each of these two groups was cut from the plasmid, labeled by nick-translation, and compared to the other members of the same group by dot-blot hybridization. In each of the two groups several clones lacked homology and were presumed to contain different cDNA inserts. The largest nonhomologous cDNA clone from each of the two groups was purified preparatively, labeled, and compared to the rest of the same group by a second round of dot-blot hybridization. In group iii, a single remaining clone lacked homology and therefore was also purified preparatively. Thus, nine different representative cDNA clones were obtained, one each from groups i, iv, v, and vi (clones 3/39, 6/63, 4/41, and 3/5, respectively), two from group ii (clones 3/55 and 6/9), and three from group iii (clones 6/6, 3/26, and 3/51). The lack of detectable sequence homology among all nine representative cDNA clones was confirmed by reciprocal hybridization of dot-blotted plasmids and nick-translated insert cDNAs. The results for the
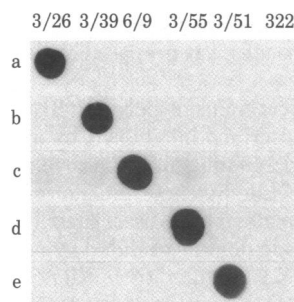

FIG. 2. Hybridization of replicate dot-blots of plasmid pBR322 and recombinant plasmids containing cDNA clones 3/26, 3/39, 6/9, 3/55, and 3/51 with the following ^{32}P-labeled purified insert cDNAs: (a) 3/26, (b) 3/39, (c) 6/9, (d) 3/55, and (e) 3/51. Autoradiograms of the hybridized filters are shown.

five clones from groups ii and iii are shown in Fig. 2. Preliminary restriction site mapping for clones 4/41, 3/55, 6/9, and 3/39 also showed that these clones are unrelated (unpublished data).

RNA Transfer Blot Hybridization. The nine representative cloned cDNAs, labeled by nick-translation, were identified by hybridization to RNAs that had been extracted from RS virus-infected cells, separated by gel electrophoresis, and transferred to diazobenzyloxymethyl-paper.

The pattern of poly(A)-selected RS virus [^{3}H]RNAs separated by gel electrophoresis (Fig. 3 *Left*) consisted of the previously reported (4) RNAs 1–7 and several additional, relatively less abundant RNAs, designated A–H. In these experiments it was desirable to have RNA 8, which we have previously identified as intracellular nonpolyadenylylated RS virus genomic RNA (4), present in the gel pattern for blot transfer as an internal control for viral-specificity of the cDNA clones. When the RNA was resuspended in a small volume and held on ice prior to passage through oligo(dT)-cellulose, a substantial amount of RNA 8 remained in the poly(A)-selected fraction (Fig. 3 *Left*). This appeared to be due to hybridization of genomic RNA to mRNAs, which, in turn, bound to the column: after denaturation with heat, RNA 8 was found exclusively in the poly(A)$^-$ fraction (not shown). In contrast, with or without heat denaturation, RNAs 1–7 and A–H bound to oligo(dT)-cellulose, with
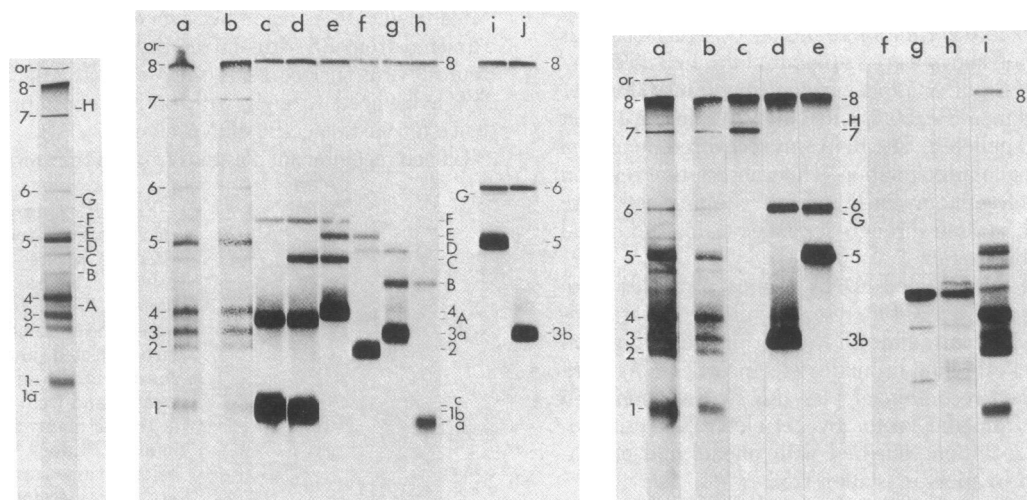

FIG. 3. Analysis of RS virus RNAs by hybridization of RNA transfer blots with ^{32}P-labeled cDNA clones. The pattern of viral [^{3}H]RNAs from infected cells, separated by electrophoresis on a 1.5% gel and analyzed by fluorography, is shown in *Left*. For *Center* and *Right*, viral [^{3}H]RNAs were electrophoresed on separate gels and one lane (lane a) from each gel was analyzed by fluorography. The remaining lanes of each gel were transferred to diazobenzyloxymethyl-paper. One strip (lane b) from each blot was analyzed by fluorography. The remaining strips were analyzed by autoradiography after hybridization with ^{32}P-labeled cDNAs or cDNA clones. For *Center*, the clones were: lane c, 3/55; lane d, 6/9; lane e, 6/63; lane f, 6/6; lane g, 3/26; lane h, 3/39; lane i, 4/41; and lane j, 3/51. For *Right*, the clones were: lane c, 3/5; lane d, 3/51; lane e, 4/41; and lane g, a 940-bp cellular cDNA clone. Strips shown in lanes h and i (*Right*) were hybridized with ^{32}P-labeled cDNAs synthesized by reverse transcription of poly(A)$^+$ RNAs from (lane h) uninfected and (lane i) RS virus-infected cells. Finally, the strip shown in lane f (*Right*) contained RNA from uninfected cells that had been electrophoresed and blotted in parallel and analyzed by hybridization with clone 3/51 and autoradiography in parallel with lane d (*Right*). Overexposures are presented for *Center* and *Right* to show RNAs B–H. or, Origin.

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Table 1. cDNA clone sizes and relationships with viral RNAs

cDNA clones		Hybridized with	
		Major polyadenylylated RNAs[†]	Presumptive polycistronic RNAs[‡]
Clone	Size, bp*		
3/39	620	1a	B
6/9	520	1b	A, C, F
3/55	620	1c	A, F
6/6	770	2	D, E
3/26	520	3a	B, D
3/51	990	3b	6, H
6/63	1,330	4	C, E, F
4/41	1,950	5	G, 6
3/5	2,600	7	H

* Estimated by gel electrophoresis in 1.5% agarose or 6% polyacryl-amide gels.

[†] RNA molecular sizes (in nucleotides), calculated from molecular mass estimates (refs. 4 and 5; unpublished data) and including poly(A) are: RNA 1a, 720; RNAs 1b and 1c, 770; RNA 2, 1,170; RNA 3, 1,240; RNA 4, 1,420; RNA 5, 2,240; and RNA 7, 7,500.

[‡] RNAs A–H and 6 appeared to be polycistronic by the criteria of se-quence homologies and molecular masses (Fig. 4).

only trace amounts eluting as unbound material (refs. 4 and 5; unpublished data).

The results of hybridization of individual ^{32}P-labeled cDNA clones with RNA transfer blots of viral RNAs are shown in Fig. 3 *Center* and *Right*. The relationships between the cDNA clones and viral RNAs, based on the data in Fig. 3, are outlined in Table 1. cDNA clone sizes are also shown in Table 1. In summary, each of the nine cDNA clones hybridized at the positions of one of the major viral RNAs 1–5 and 7 and to one or more of RNAs A–H and 6. Three cDNA clones hybridized to RNA 1, and each appeared to be distinct because of differing patterns of hybridization to RNAs A–C and F (Fig. 3 *Center*, lanes c, d, and h, and Table 1). On this basis, RNA 1 appeared to contain three unique transcripts (see *Discussion*). The RNA 1 components, designated 1a–c, appeared to differ slightly in molecular mass, with the increasing order being 1a, 1b, and 1c. Similarly, the two representative clones that hybridized to RNA 3 differed in the patterns of hybridization to RNAs B, D, 6, and H (Fig. 3 *Center*, lanes g and j, Fig. 3 *Right*, lane d, and Table 1). As will be discussed below, RNA 3 appeared to contain two components, designated 3a and 3b, that could not be distinguished by electrophoretic mobility.

Finally, all nine clones hybridized strongly at the position of intracellular genomic RNA, RNA 8 (Fig. 3 *Center*, lanes c–j, and Fig. 3 *Right*, lanes c–e). In contrast, RNA 8 failed to hybridize with cellular cDNA clones (representative results are shown in Fig. 3 *Right*, lane g) and hybridized with cDNAs made by reverse transcription of mRNAs extracted from infected (Fig.

3 *Right*, lane i) but not uninfected (Fig. 3 *Right*, lane h) cells. Furthermore, viral cDNA clones failed to hybridize with RNA transfer blots prepared with mRNA from uninfected cells (representative results are shown in Fig. 3 *Right*, lane f). These results firmly established that the nine representative cDNA clones were specific to the genome of RS virus.

DISCUSSION

Nine representative cDNA clones were identified, which each hybridized both to RS virus genomic RNA and to one of the major RS virus RNAs 1–5 and 7. None of the clones shared detectable sequence homology with each other. Size comparisons of the cDNA clones and their respective RNAs (Table 1) seemed to exclude the trivial possibility that any of the nine clones, such as the three RNA 1 clones, might be derived from nonoverlapping segments of a single transcript. Furthermore, all of the nine cDNA clones except the RNA 7 clone could be shown to select by hybridization mRNAs encoding different, unique polypeptides (unpublished data). Therefore, the nine unique cDNA clones were evidence that the RS virus genome encodes at least nine unique polyadenylylated transcripts—RNAs 1a, 1b, 1c, 2, 3a, 3b, 4, 5, and 7.

The nine unique cDNA clones also hybridized in various combinations with RNAs A–H and 6. These RNAs appeared to be polycistronic on the basis of their sequence homologies and molecular masses (Fig. 4). Polycistronic transcripts have been demonstrated for the rhabdovirus VSV (33, 34) and appear to exist for the paramyxovirus Newcastle disease virus (13, 35). The VSV polycistronic transcripts have been shown to be the products of transcriptional read-through of adjacent genes (33, 34). The existence of RS virus polycistronic transcripts would be entirely consistent with these observations because RS virus, like VSV and Newcastle disease virus, appears to have a sequential mode of transcription (ref. 7; unpublished data).

As shown in Fig. 4, the sequence contents and molecular masses of RNAs A–H and 6 were interpreted to prepare a transcriptional map. For example, RNA C hybridized exclusively with cDNA clones of RNAs 1b (clone 6/9, Fig. 3 *Center*, lane d) and 4 (clone 6/63, Fig. 3 *Center*, lane e). The estimated molecular mass of RNA C (0.62×10^6 daltons, Fig. 4) was approximately equal to the combined molecular masses of RNAs 1b and 4 (0.64×10^6 daltons). Our interpretation was that the genes encoding RNAs 1b and 4 are adjacent in the transcriptional map and that RNA C is a dicistronic transcript of the two genes (Fig. 4). By extending this line of reasoning to the other polycistronic RNAs, the nine RS viral genes were arranged into a map containing two clusters of genes: the six genes encoding RNAs 1a–c, 2, 3a, and 4 and the three genes encoding RNAs 3b, 5, and 7 (Fig. 4). This analysis did not provide information on the 3′ to 5′ orientation of either group of genes or their relative 3′ to 5′ placements. Nor did these data show whether the
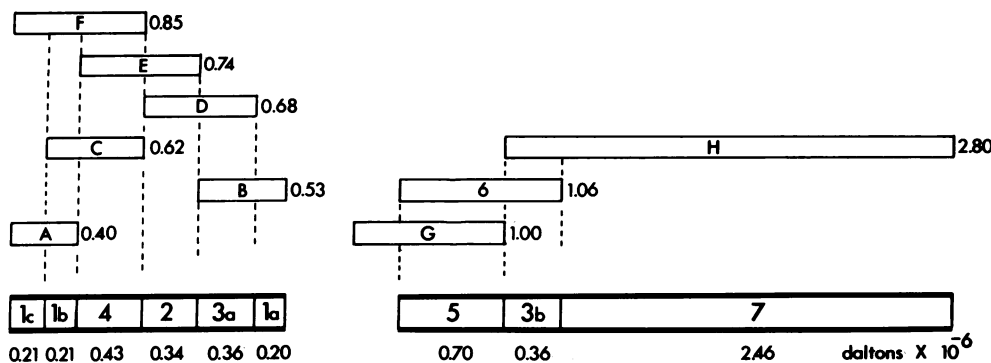

FIG. 4. Transcriptional map for RS virus. The horizontal placements reflect sequence homologies among RS virus RNAs 1–7 and A–H, based on the RNA transfer blot analyses shown in Fig. 3. The length of each box is proportional to the RNA molecular mass after subtraction of a contribution of 125 adenylate residues [the average size of poly(A) estimated for the paramyxovirus Newcastle disease virus (32)]. These adjusted molecular masses (daltons × 10⁻⁶) are indicated.

RS virus genome consists of two transcriptional units or a single unit containing an apparent gap. These questions could be resolved by transcriptional mapping based on UV inactivation studies. An advantage of transcriptional mapping by the analysis shown in Fig. 4 was that map positions were obtained for the multiple components of RNAs 1 and 3, which were not separated by gel electrophoresis. Furthermore, the pattern of overlapping polycistronic RNAs, together with the molecular mass estimations, provided evidence that within each block of genes there were no additional, intervening genes.

The apparent gap in the transcriptional map might be due to the presence of a second promoter. However, this is inconsistent with preliminary UV mapping data (ref. 7; unpublished data). Instead, the apparent gap might simply reflect the failure to detect one or more polycistronic RNAs, such as a hypothetical RNA containing the sequences of RNAs 1a and 5. Alternatively, the gap might indicate the presence of an additional, unidentified gene(s). This latter interpretation is consistent with the detection of RNA G, which contained sequences in common with RNA 5 and was larger by about 0.30×10^6 daltons but failed to hybridize with any of the other eight representative cDNA clones.

Hybridization of two or more unique cDNA clones with each of RNAs A–F, H, and 6, together with the molecular mass estimates, was presumptive evidence that these RNAs were polycistronic. But for this interpretation to be convincing, it will be necessary to prove that the two or three cDNA clones that hybridized to each RNA band hybridized with a single polycistronic RNA species. In preliminary work to that end, we have identified a cDNA clone, clone 6/55 (650 bp), which hybridized to both RNAs 2 and 4 (not shown). Reciprocal hybridization with the other cDNA clones confirmed that clone 6/55 was homologous to clones 6/6 and 6/63, the cDNA clones of RNAs 2 and 4 (not shown). This indicated that clone 6/55 was a partial copy of a polycistronic RNA, presumably RNA E. This provided independent evidence that (*i*) a polycistronic RS virus RNA exists and (*ii*) the genes encoding RNAs 2 and 4 are adjacent in the RS virus transcriptional map.

Evidence for nine poly(A)$^+$ RNAs for RS virus was unexpected because for other paramyxoviruses there is evidence for six or seven unique viral transcripts (12, 13), excluding the leader RNA (36). Use of the cDNA clones to select mRNAs by hybridization for translation *in vitro* showed that RNA 4 encoded the major nucleocapsid protein (unpublished data). This confirmed results obtained by translation of RNA 4 separated by gel electrophoresis (5). Therefore, the major nucleocapsid protein gene, which is first in the transcriptional order of other paramyxoviruses (37, 38), is internal in the RS virus transcriptional map (Fig. 4). The differences in gene number and transcriptional map may be indicative of important differences between RS virus and other paramyxoviruses.

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