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Structural and Functional Characterization of Newcastle Disease Virus Polycistronic RNA Species

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Upon infection, the Newcastle disease virus (NDV) genome is transcribed to produce 18S, 22S, and 35S RNAs (M. Bratt, and W. Robinson, J. Mol. Biol. 23:1–21, 1967). The 22S RNA has been shown to contain 18S sequences and is thought to represent polycistronic transcripts generated by transcriptional readthrough of adjacent genes (Varich et al., Acta Virol. 23:341–343, 1979). With improved extraction procedures, the 22S RNA was found to represent up to 25% of the total transcription in NDV-infected cells. This RNA was resolved into at least five discrete species on formaldehyde-agarose gels. All but one of these molecules contain 3′ polyadenylate sequences but not internal polyadenylate sequences. These transcripts are found on polyribosomes of infected cells, suggesting that they are functional mRNAs.

Newcastle disease virus (NDV), a paramyxovirus, is a negative-stranded RNA virus. The genome of 5.1×10^6 to 5.7×10^6 daltons (7) encodes six known viral proteins (3, 7, 16). Upon infection, the genome is transcribed by a virion-associated polymerase to produce viral mRNA. These transcripts have been separated on sucrose gradients into three size classes with sedimentation coefficients of 18S, 22S, and 35S (4, 27, 32). The 18S RNA has been shown to contain five electrophoretically distinct monocistronic polyadenylate [poly(A)]-containing mRNAs (10, 13, 17, 22, 33) that encode the viral nucleocapsid protein (NP), a nucleocapsid-associated phosphoprotein (P), a nonglycosylated membrane protein (M), the hemagglutinin-neuraminidase glycoprotein (HN), and the fusion glycoprotein (Fo) (9, 12, 13, 26). The 35S RNA contains a single RNA species which encodes the viral transcriptase (L) (12, 24). Thus the 18S RNA and the 35S RNA encode all six known viral proteins.

RNA-RNA hybridization experiments have also shown that the 18S and 35S RNA species account for the coding capacity of the genome (4, 22, 29): the 35S RNA anneals to 34 to 40% of the genome, whereas the 18S species anneal to the remainder of the genome (4, 18, 22, 27, 29). On the basis of hybridization competition experiments, the 22S-size class contains sequences present in the 18S RNA species (28). Two to four 22S species have been resolved electrophoretically (13, 28, 33). It has been suggested (13, 28) that these species may be covalently linked 18S transcripts, perhaps generated by transcriptional readthrough of adjacent genes. Sendai virus- and measles virus-infected cells also contain RNA species which appear analogous to the NDV 22S RNA (6, 18).

Vesicular stomatitis virus (VSV) in vitro transcription reactions, as well as infected cells, also contain RNA molecules which are the result of transcription across adjacent genes (14, 15). For the most part, these transcripts contain long stretches of poly(A) at the intercistronic regions (14, 15). These species account for ca. 10% of the in vitro transcription products but are found as less than 1% of the in vivo transcripts (14, 15).

In contrast to VSV-infected cells, we have found that the NDV 22S RNA may account for up to 25% of NDV transcription in infected cells. Since this RNA represents a significant amount of NDV transcription, we were interested in characterizing the structural and functional properties of these RNA molecules. We resolved these RNAs into at least five species. All but one of these molecules contain 3′ poly(A) but not internal poly(A) sequences. These RNA molecules are found on polyribosomes in infected cells.

MATERIALS AND METHODS

Cells and virus. Chinese hamster ovary cells were used. The Australia-Victoria strain of Newcastle disease virus was grown and purified as previously described (8, 16, 31). VSV (obtained from D. Summers) was grown and purified as described previously (30).

Isolation, labeling, and fractionation of viral RNAs. Chinese hamster ovary cells were grown in suspension in minimal essential medium, supplemented with nonessential amino acids and 7.5% fetal calf serum. Cells were infected with virus (either NDV or VSV) at a multiplicity of 10 PFU per cell. At 60 min postinfection, cells were washed and resuspended in phosphate-free minimal essential medium, supplemented with nonessential amino acids, 7.5% dialyzed fetal calf serum, and actinomycin D (2 μg/ml). After a 15-min incubation in the presence of actinomycin D, 10 μCi of ^{32}P (New England Nuclear Corp.) per ml was added. At 4 h postinfection, cells were washed in phosphate-buffered saline (0.15 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4) containing 0.01 M adenosine vanadyl complex (2) and then were suspended in RSE (0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, 0.02 M EDTA) containing 1% Triton X-100 and 0.5% deoxycholate. Cell lysates were extracted with phenol-isoamyl alcohol-chloroform (5). RNA was ethanol precipitated.

Gel electrophoresis. Formaldehyde agarose (1%) gels were prepared as described previously (20). RNA was denatured for 10 min at 60°C in 50% formamide and 6% formaldehyde before loading onto a slab gel (10 by 18 by 0.5 cm) and then was electrophoresed for 18 h at 100 V (25 mA) at 4°C.

Oligo (dT) cellulose chromatography. Total ^{32}P-labeled NDV RNA in a 100-μl volume was denatured in 50% formamide at 60°C for 15 min, diluted to 10 ml in binding

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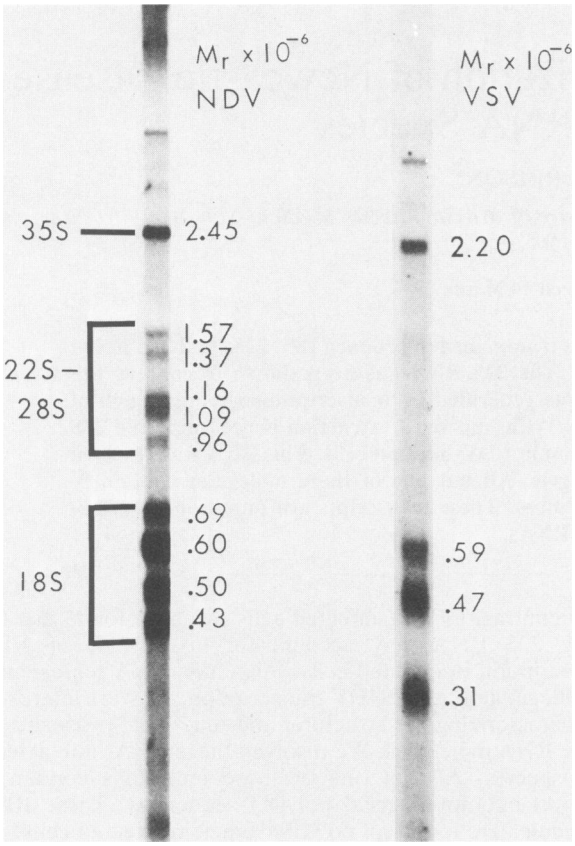

FIG. 1. Determination of molecular weights of intracellular NDV RNA species by comparative electrophoresis with VSV RNAs. Labeling and isolation of NDV RNAs was done as described in the text. Labeled VSV RNA was prepared by an identical protocol. With VSV RNAs as standards, molecular weights for each band were calculated according to the method of Lehrach (19).

buffer (0.4 M NaCl, 20 mM Tris-hydrochloride, pH 7.6, 1 mM EDTA, 0.1% sodium dodecyl sulfate), and loaded onto an oligodeoxythymidylate [oligo(dT)] cellulose column (Collaborative Research). Poly(A)-containing RNA was eluted in elution buffer (10 mM Tris-hydrochloride, pH 7.5, 1 mM EDTA, 0.05% sodium dodecyl sulfate). Unbound and bound RNA was ethanol precipitated.

RNase H digestion. ^{32}P-labeled total RNA extracted from NDV-infected cells (5 μg) was incubated with 5 μg of oligo(dT) (12–18 nucleotides) for 3 min at 50°C and then for 30 min at 32°C in 0.02 M Tris (pH 7.5)–0.1 M KCl–0.01 M $MgCl_2$–0.1 mM dithiothreitol–5% sucrose. RNase H (6.8 U, *Escherichia coli*; Bethesda Research Laboratories) was added, and incubation was continued for 20 min at 37°C. RNA was phenol extracted and ethanol precipitated. The RNA pellet was resuspended in sample buffer (50% formamide, 6% formaldehyde, 0.02 M MOPS [morpholinepropanesulfonic acid] buffer, pH 7.0, 0.005 M sodium acetate, 0.001 M EDTA, 0.1% bromphenol blue) and loaded onto a 1% formaldehyde-agarose gel.

Polyribosomes. Infected cells radioactively labeled as described above were washed in phosphate-buffered saline, resuspended in either RSB (0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$) or RSE containing 1% Triton X-100, 0.5% deoxycholate, and 50 μg of cycloheximide per ml. Nuclei were removed by centrifugation, and the resulting cell extracts were layered on top of an 8 to 52% (wt/wt) sucrose gradient buffered by either RSB or RSE. Gradients were centrifuged for 16 h at 16,000 rpm in a Beckman SW27 rotor.

To isolate RNA from the gradient factions, the appropriate regions of the gradients were pooled, made 20 mM with respect to EDTA, and phenol extracted, and the RNA was ethanol precipitated.

RESULTS

Gel analysis of intracellular NDV RNA species. To characterize the NDV 22S RNA, total cellular RNA was resolved on formaldehyde-agarose gels (Fig. 1). This RNA was prepared from NDV-infected Chinese hamster ovary cells in the presence of actinomycin D. Viral-specific RNA was radioactively labeled with ^{32}P from 2 to 4 h postinfection, and total cytoplasmic RNA was extracted for electrophoresis. Near the bottom of the gel, four RNA species were resolved. These species sedimented at 18S in sucrose gradients (not shown). Six larger species were also resolved. Five of them had sedimentation values of 22S-28S, whereas the sixth sedimented at 35S (not shown).

The molecular weights of these NDV RNAs were estimated by electrophoresis of [^{32}P]RNA isolated from NDV-

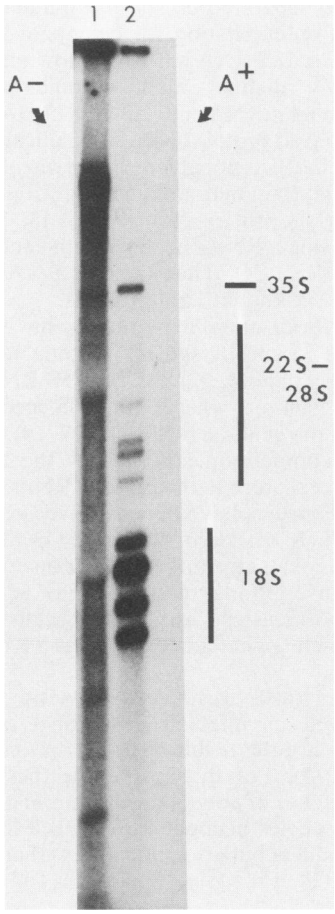

FIG. 2. Poly(A) content of NDV RNA. Labeled total RNA from NDV-infected cells was applied to oligo(dT) columns as described in the text. Lane 1, poly(A)$^-$ RNA; lane 2, bound or poly(A)$^+$ RNA.

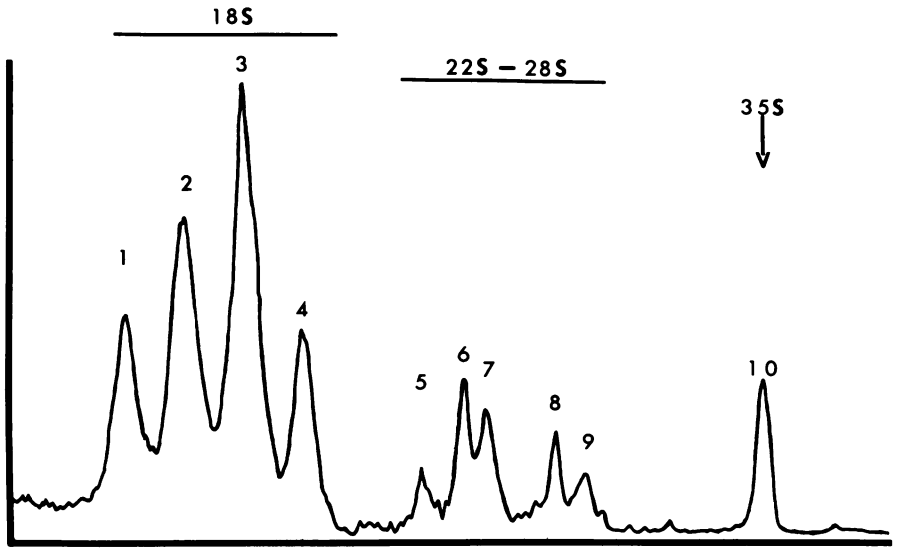

FIG. 3. Densitometric analysis of intracellular NDV RNA. Autoradiograms similar to that shown in Fig. 1 were scanned with an Ortec Microdensitometer. Shown is a representative tracing. Multiple exposures of the gel to X-ray film (with no intensifying screen) determined the exposure to be within the linear range of the film response.

infected cells in parallel with the well-characterized VSV mRNA species (34). By using VSV mRNA as a marker, the molecular weights ($\times 10^6$) of the NDV 18S RNA species were estimated to be 0.43. 0.50, 0.60, and 0.69 (Fig. 1). These values are similar to the molecular weight estimates made from polyacrylamide-formamide gels (33). Whereas the polyacrylamide-formamide gel system, as well as acid urea-agarose gels (13), reveals five 18S-sized RNA species, formaldehyde gels clearly resolved only four species in this region (Fig. 1). The 35S RNA was estimated to have a molecular weight of 2.45×10^6, also similar to that found in the other gel systems (10, 12, 13). The five RNA species which sediment at 22S-28S have estimated molecular weights ($\times 10^6$) of 0.96, 1.09, 1.16, 1.37, and 1.57. Also seen as minor amounts are species with molecular weights of 1.86×10^6 and 3.02×10^6 (Fig. 1 and 2).

Densitometric analysis of autoradiographs similar to that shown in Fig. 1 revealed that the 22S-28S RNA species account for 15 to 25% of the total [^{32}P]RNA on a weight basis. Different preparations of RNA yielded values from 15 to 25% of the total. Fig. 3 shows a densitometer tracing from an RNA preparation which yielded 24% of the total in the form of 22S-28S RNA. A quantitation of these results is shown in Table 1. The molar ratios of the intracellular RNA species are also shown in Table 1. On a molar basis, these species account for ca. 13% of the total intracellular RNA.

To eliminate the possibility that these species result from aggregation of 18S-sized molecules or incomplete denaturation of RNAs, the electrophoretic pattern of total NDV RNA was compared after treatment with formamide-formaldehyde or dimethyl sulfoxide-glyoxal (21). In both cases, five species in the 22S-28S size range were evident. Moreover, the single-stranded nature of RNAs observed under these conditions is supported by their disappearance after treatment with RNase A (data not shown). Since the resolution of the 22S-28S species obtained after treatment with formaldehyde-formamide was superior to that obtained after treatment with glyoxal, subsequent experiments used formaldehyde-formamide-treated RNA resolved on formaldehyde agarose gels.

Poly(A) content of 22S-28S RNA. As a means of determining the poly(A) content of the 22S-28S RNA, their retention by an oligo(dT) cellulose column was determined. To eliminate the nonspecific binding of RNAs by intermolecular hybridization, RNA was denatured in formamide (33) before loading the column. As expected, the 18S and 35S species were retained by the column (Fig. 2). In addition, four of the five 22S-28S species (molecular weights [$\times 10^6$] of 0.96, 1.09, 1.16, and 1.37) as well as the species with molecular weights of 1.82×10^6 and 3.02×10^6 were also retained by the

TABLE 1. Quantitation of NDV RNA species

RNA species[a]	Mol wt ($\times 10^6$)	Amt[b]	% of total wt	Molar ratio[c]	% of total molar amt
18S					
1	0.43	162	10.9%	1.00	17.2%
2	0.50	235	15.8%	1.25	21.6%
3	0.60	435	29.3%	1.90	32.8%
4	0.69	170	11.5%	0.65	11.2%
22-28S					
5	0.96	54	3.6%	0.14	2.4%
6	1.09	105	7.1%	0.25	4.3%
7	1.16	97	6.5%	0.21	3.6%
8	1.37	53	3.6%	0.10	1.7%
9	1.57	52	3.5%	0.09	1.6%
35S 10	2.45	120	8.1%	0.21	3.6%

[a] Numbers refer to RNA species shown in Fig. 3.

[b] The amount of RNA present in each RNA species was quantitated from the scan shown in Fig. 3 in densitometer units.

[c] Molar ratios were calculated by dividing the amount of each species by its molecular weight and setting the value obtained for species 1 as 1.

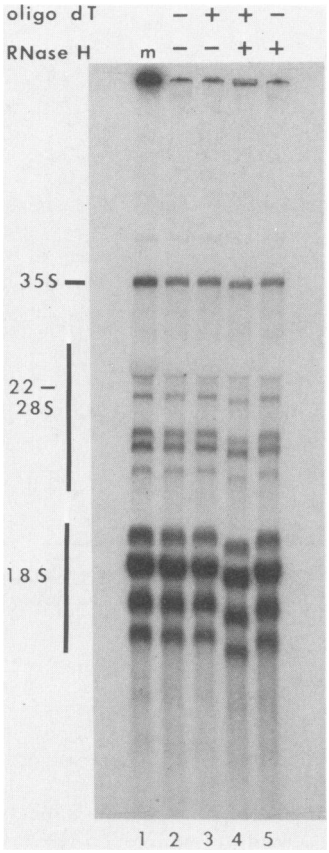

FIG. 4. NDV RNAs do not contain internal poly(A). Labeled total RNA isolated from NDV-infected cells was incubated with oligo(dT) and then digested with RNase H as described in the text. Lane 1, marker RNA (M); lane 2, RNA subjected to reaction conditions in the absence of oligo(dT) and RNase H; lane 3, RNA subjected to reaction conditions in the presence of oligo(dT) only; lane 4, RNA subjected to reaction conditions in the presence of oligo(dT) and RNase H; lane 5, RNA subjected to reaction conditions in the presence of RNase H only.

column. Interestingly, the 1.57×10^6-dalton species was present only in the unbound fraction.

Internal polyadenylation. To determine whether these 22S-28S RNA species contain internal poly(A), as do VSV polycistronic transcripts, NDV RNA was hybridized to oligo(dT), and then the hybrid structures were digested with RNase H. Any double-stranded structure should be susceptible to digestion (25). If there is internal poly(A), then the 22S-28S RNA should disappear after digestion. Fig. 4 shows the result of such an experiment. When both RNase H and oligo(dT) are present, the sizes of the 18S RNAs are reduced slightly, consistent with the removal of 3′ poly(A). In addition, the sizes of the 22S-28S RNA species, with the exception of the 1.57×10^6-dalton species, are also slightly reduced, consistent with the removal of 3′ poly(A). Size analysis of the undigested and digested 18S RNAs is consistent with the removal of ca. 80 bases of poly(A). Similarly, the 22S-28S RNA is reduced in size by ca. 100 bases upon RNase H digestion.

However, the 22S-28S RNA species remain intact. Thus, by this criterion, these RNA species do not contain internal poly(A). The 1.57×10^6-dalton species apparently contains no poly(A), as suggested by its inability to bind to oligo(dT) cellulose column.

Association with polyribosomes. To determine whether these polyadenylated 22S-28S RNA transcripts can function as mRNAs in infected cells, we asked whether these RNAs can be found on polyribosomes. Infected cells were radioactively labeled with ^{32}P from 1.5 to 4 h postinfection, cells were lysed with detergent, and cytoplasmic extracts were centrifuged through sucrose gradients to resolve polyribosomes. Fig. 5 shows the result of such an experiment. The solid line represents the optical density across the gradients. The polyribosome region can be resolved, as well as the 80S monoribosomes (Fig. 5, bottom panel). The broken line shows the distribution of [^{32}P]RNA in the gradient. After disruption of polyribosomes with EDTA (top panel), both the optical density and the ^{32}P label no longer sediment in the polyribosome region of the gradient.

To identify the RNA species present in polyribosomes, as well as the RNA that is not polyribosome associated,

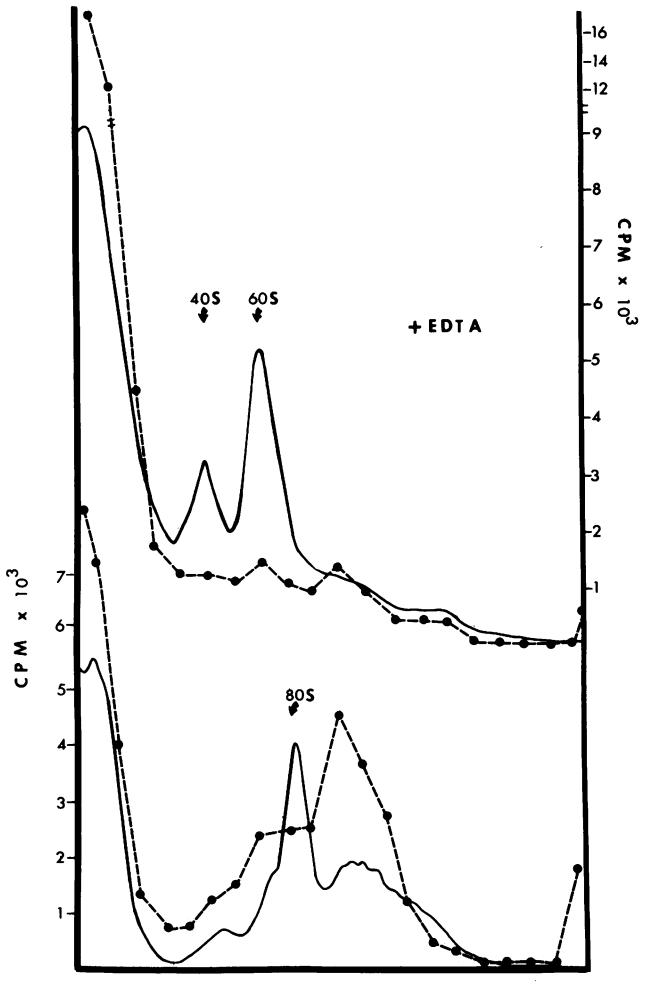

FIG. 5. Polyribosomes isolated from NDV-infected cells. Top panel, A cell extract from NDV-infected cells was treated with 20 mM EDTA and then centrifuged through a sucrose gradient containing 20 mM EDTA. Bottom panel, A cell extract from NDV-infected cells was centrifuged through a sucrose gradient to resolve polyribosomes as described in the text. Symbols: ——, optical density at 260 nm; - - - - -, trichloroacetic acid-precipitable [^{32}P]RNA.

fractions from the polyribosome region of the gradient, the subunit region, and the top of the gradient were pooled. The RNA present in each fraction is shown in Fig. 6. The polyribosome region of the gradient (left panel, lane P) contains the expected 18S and 35S RNA species. In addition, all the 22S-28S RNA species are also found in the polyribosome fraction. That the 22S-28S RNA species are associated with polyribosomes and are not in cosedimenting ribonucleoprotein structures is shown by the fact that upon EDTA disruption of the polyribosomes, the 22S-28S RNA species no longer sediment in the polyribosome region of the gradient, but rather they are found in the top of the gradient with the 18S and 35S RNA species (Fig. 6, right panel, lane T).

DISCUSSION

The genomes of negative-stranded, nonsegmented viruses are transcribed to produce monocistronic mRNAs (3, 7, 30). However, Herman et al. (14, 15) have reported the existence of minor amounts of polycistronic transcripts in transcription reactions of VSV in vivo in vitro. These transcripts, which represent covalently linked sequences from adjacent genes with an intervening stretch of poly(A) at the intercistronic boundary (14, 15), are thought to represent an infrequent failure to terminate synthesis after formation of the poly(A) sequences at the 3′ end of the monocistronic transcript (14, 15).

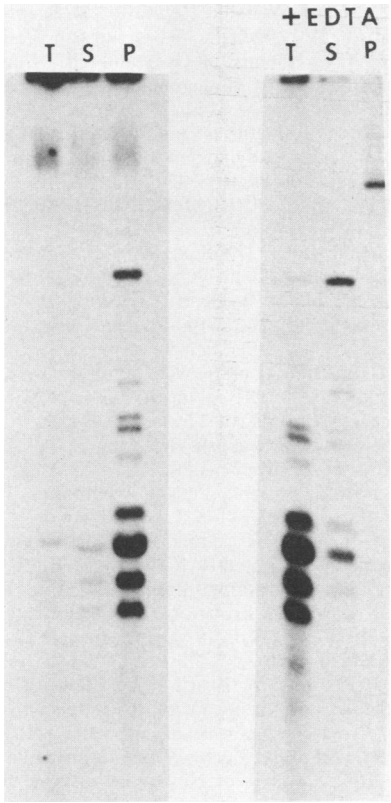

FIG. 6. RNA species present in the polyribosome fraction of NDV-infected cells. Fractions of the gradients shown in Fig. 5 were pooled and phenol extracted, and the RNA present was ethanol precipitated. Fractions 1 through 13 (Fig. 5) were pooled and are labeled P (polyribosomes). Fractions 14 through 18 were pooled and are labeled S (subunits), whereas fractions 19 through 25 were pooled and are labeled T (top). Fractions from EDTA-treated cell extracts are labeled +EDTA.

Other investigators have reported the existence of RNA species in paramyxovirus-infected cells which may represent analogous RNA transcripts (6, 17, 18, 28). The coding capacity of the paramyxovirus genome is accounted for by the 18S and 35S monocistronic RNAs (3, 10, 18, 28, 29); thus, the 22S RNAs cannot represent unique sequences. Furthermore, RNA hybridization experiments have shown that the 22S RNAs contain sequences present in the monocistronic RNA species (28, 29). In addition, using cDNA clones derived from the individual respiratory syncytial virus genes, Collins and Wertz (11) have shown the existence of di- and tricistronic RNAs which represent transcription across adjacent genes. Using cDNA clones derived from the individual NDV genes, we also found that the 22S-28S RNA species represent di- and tricistronic RNA molecules (manuscript in preparation).

However, the paramyxovirus polycistronic transcripts appear to occur at a higher frequency than the VSV polycistronic transcripts. In contrast to the VSV polycistronic transcripts, the paramyxovirus transcripts have readily been detected in sucrose gradients and in various gel systems (12, 13, 18, 33). Using improved extraction conditions, we were able to detect up to 25% by weight of the total NDV transcription in a polycistronic form. It was of immediate interest, therefore, to determine whether these transcripts were, in fact, analogous to the VSV polycistronic transcripts; that is, do these transcripts contain intervening poly(A) sequences? All but one of these transcripts (1.57×10^6-dalton species) clearly contain poly(A) sequences, since they are retained by an oligo(dT)-cellulose column. To ask whether these sequences were internal, we made use of a protocol reported previously to detect internal polycytidylate sequences in the foot and mouth virus genome (25). This protocol takes advantage of the susceptibility of RNA to RNase H in RNA-DNA hybrids. We asked whether polycistronic RNAs could be reduced to 18S-sized molecules after hybridization to oligo(dT) and digestion with RNase H. With the exception of the 1.57×10^6-dalton species, all the 22S-28S RNAs, as well as the 3.02×10^6-dalton species, were reduced slightly in size after this protocol, consistent with the presence of 3′ poly(A) sequences. However, RNase H digestion did not reduce the size of these species to 18S-sized molecules. Thus, the generation of polycistronic transcripts in NDV-infected cells occurs by a mechanism somewhat different than that responsible for the VSV polycistronic transcripts.

By analogy with VSV, paramyxovirus transcription may occur by sequential start-stop action of the transcriptase (1). The existence of polycistronic transcripts is consistent with this model. The transcriptase may fail, at a certain frequency, to recognize a polyadenylation signal or termination signal, or both. However, the existence of these transcripts containing no intervening poly(A) is also consistent with a transcription mechanism involving synthesis of a precursor, followed by nucleolytic cleavage and subsequent polyadenylation.

Thus, the origin of these transcripts is not clear. However, since significant amounts of the NDV transcripts occur in the form of polycistronic transcripts, it was of interest to determine whether these molecules have any functional activity. In fact, they appear to be functional mRNAs. At 4 h postinfection, all detectable polycistronic RNAs were found on polyribosomes, including the RNA which contains no

detectable poly(A). Thus, these forms of viral transcripts contribute to the expression of viral genetic information.

In summary, NDV-infected cells synthesize up to 25% by weight of their RNA in at least five different polycistronic RNA species. Poly(A) sequences are present in all but one of these species; however, in none of these species are the poly(A) sequences contained at the intercistronic boundaries. Since these polycistronic transcripts are associated with polyribosomes, they likely function as mRNAs in infected cells.

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