Polyadenylate Sequences on Newcastle Disease Virus mRNA Synthesized In Vivo and In Vitro

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Polyadenylate [poly(A)] sequences are associated with the 35 and 50S Newcastle disease virus (NDV)-specific RNAs as well as all six to seven of the 18-22S NDV-specific messenger RNAs extracted from infected chicken embryo cells. The poly(A) associated with the 18-22S RNA has an average size of 120 to 130 nucleotides. The 18-22S RNA synthesized in vitro by NDV's virion-bound polymerase contains six to seven species of the same size and relative proportions as its intracellular counterpart. This in vitro synthesized 18-22S RNA also contains covalently linked poly(A) sequences which, although variable in size, are usually larger and more heterogeneous than those from the infected cell. In vitro RNA synthesis is supported not only by magnesium (at an optimal concentration of 4 mM) but by manganese (at an optimal concentration of 0.5 to 1.0 mM) as well. However, the major product made in the presence of manganese, although sedimenting at 18 to 22S, differs somewhat from the product made in the presence of magnesium.

Polyadenylate [poly(A)] sequences have been found on the 3' end (28, 30) of most eukaryotic mRNA and heterogeneous nuclear RNA (1, 4, 16, 26). Synthesis of this poly(A) occurs in the nucleus, probably by a post-transcriptional process (13, 23, 29).

Poly(A) has also been found on the messenger RNAs of many animal viruses, including several RNA viruses. Among these are representatives of the "negative-stranded viruses," Sendai virus (30), Newcastle disease virus (NDV) (S. R. Weiss and M. A. Bratt, *Abstr. Annu. Meet. Amer. Soc. Microbiol.*, p. 202, 1973), and vesicular stomatitis virus (VSV) (2, 17, 18, 33, 34). The messenger RNA of these viruses is complementary to RNA contained in the virion (4, 8, 12, 22, 25). Since these viruses replicate completely in the cytoplasm of cells, it was expected that their poly(A) is synthesized by a process distinct from cellular poly(A) synthesis in the nucleus.

We report here that messenger RNA synthesized in vitro by the virion transcriptase of NDV (21) also contains poly(A) sequences. Further characterization of the NDV-specific RNAs synthesized in the infected cell and in vitro and of the poly(A) associated with them is presented. The poly(A) synthesized in vitro appeared to be larger and more heterogeneous than that from

infected cells. These findings are discussed in terms of the mechanism of poly(A) synthesis.

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MATERIALS AND METHODS

Virus purification and infectivity assays. NDV-HP (Israel HP, 1935) and NDV-AV (Australia-Victoria, 1932) (7) were grown in embryonated hen eggs and then concentrated from the allantoic fluid by centrifugation as previously described (10). Virus was then further purified by centrifugation in a Spinco SW25.1 rotor for 1 h at 4°C and 24,000 rpm (59,000 × g) in standard buffer through 20% sucrose onto a 65% sucrose-D₂O pad. Virus at the interface of the two sucrose layers was then diluted in Tris saline containing 2% calf serum and stored at -70°C. Infectivity titrations by plaque formation (PFU per milliliter) were carried out as described previously (10) but with standard medium (see below).

Virus to be used in studies on in vitro RNA synthesis were further purified by equilibrium centrifugation in linear 20 to 65% sucrose-D₂O gradients containing low-salt buffer for 16 h at 4°C and 24,000 rpm (74,800 × g) in a Spinco SW27 rotor. The virus band was diluted in low salt buffer and stored at -70°C.

Cell culture procedures. Secondary cultures of chicken embryo cells were prepared and grown in a 5% CO₂ atmosphere as previously described (7) but in

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standard medium. Cultures were plated at 5×10^6 cells per 100-mm tissue culture plate and used after 48 h of incubation at 40 C.

Intracellular RNA. Virus-specific RNA was labeled in the following manner. Cultures were infected at an input multiplicity of 5 PFU/cell with NDV-HP or NDV-AV (as designated for each experiment) and incubated at 40 C. (NDV-AV is usually used in studies on 18-22S RNA because in NDV-AV-infected cells considerably more of these species is made than in NDV-HP-infected cells (6). Although the electrophoretic mobilities of the six to seven species of 18-22S RNA are slightly different for the two strains (12), the 18-22S RNA of both strains contains the same percentage of RNase resistance and the same size poly(A). Significant differences in RNase resistance of the intracellular 50S RNAs of these strains are currently under investigation. NDV-HP is preferred for *in vitro* RNA synthesis because of its growth to higher titers in eggs and its higher polymerase activity (21).) Actinomycin D (10 μ g/ml) was added 3.25 h postinfection and was followed 45 min later by the addition of 50 to 100 μ Ci of either [^{3} H]uridine or [^{3} H]adenosine per ml. At 9 h postinfection the cells were solubilized in solubilizing buffer, phenol extracted (with buffer-saturated phenol), and precipitated twice in ethanol as previously described (8).

Host cell RNA was labeled during a 30-h incubation with 2 μ Ci of [^{14} C]uridine per ml and then purified as described for virus-specific RNA.

In vitro RNA. Reaction mixtures, modified from previously described procedures (11, 21), contained 50 mM Tris (pH 7.3), 3.3 mM dithiothreitol, 0.1 M NaCl, 0.8 mg of Triton N-101 per ml, 0.7 mM UTP, 0.7 mM CTP, 0.7 mM GTP (reduced to 0.02 mM when [^{3} H]GTP was used as label), 1.4 mM ATP (reduced to 0.17 mM when [^{3} H]ATP was used as label), 33 to 67 μ Ci of [^{3} H]GTP per ml or 100 to 200 μ Ci of [^{3} H]ATP per ml, and 4 mM $MgCl_2$ (except when noted). Virus was used at 125 to 375 mg/ml of protein (26). The specific activity in the standard reaction mixtures was 400 to 800 counts per min per pmol for GTP or 190 to 380 counts per min per pmol for ATP. Reactions were carried out for 2 to 3 h at 31 C and then terminated as previously described (11). RNA was phenol extracted in the presence of uninfected and unlabeled chicken embryo cell RNA as described above for intracellular RNA.

Fractionation and analysis of RNA. Velocity sedimentation analyses were performed as follows. RNA dissolved in SDS (sodium dodecyl sulfate)-standard buffer was layered on 12-ml linear 15 to 30% sucrose gradients containing the same buffer. Centrifugation was carried out at 22 C and 39,000 rpm ($180,000 \times g$) in a Spinco SW41 rotor for various lengths of time as designated in the figure legends. Fractions (0.4 ml) were collected from the top by using an ISCO (Instrumentation Specialties Co.) fractionator, which measured adsorbancy at 260 nm, coupled to an ISCO fraction collector. Trichloroacetic acid-precipitable counts were determined by previously described procedures (11).

RNase digestion was done by incubating RNA at 37 C for 1 h in $2 \times$ SSC, 50 μ g of pancreatic RNase per

ml, and 25 U of T1 RNase per ml. T2 RNase digestion was done by incubating samples of RNA with 0.5 U of T2 RNase per ml in acetate buffer. Alkaline digestion was done by incubating RNA at 37 C in 0.5 ml of 0.3 N KOH for 18 h. The solution was then neutralized with an equivalent amount of HCl before trichloroacetic acid precipitation. Denaturation of RNA was accomplished by boiling for 5 to 7 min in 0.0025 M Tris, pH 7.4, and 0.001 M EDTA, followed immediately by quenching in ice water.

Acrylamide gel electrophoresis was carried out by using 10-cm ethylene diacrylate cross-linked 4 or 8% polyacrylamide gels according to the procedures, including gel slicing and counting, of Collins and Bratt (12). Determination of RNase resistance of RNA fractionated by gel electrophoresis was made by boiling 1-mm gel slices for 1 h in 0.8 ml of SDS-standard buffer and then incubating equal samples of each fraction in the presence or absence of T1 and pancreatic RNases, followed by trichloroacetic acid precipitating each sample.

Solutions, chemicals, and abbreviations. Standard buffer was 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 0.002 M EDTA. SDS-standard buffer was standard buffer with 0.5% SDS. Solubilizing buffer was 0.1 M NaCl, 0.01 M Tris (pH 8.5), 0.002 M EDTA, 1% SDS, and 1% mercaptoethanol. Phenolbuffer was solubilizing buffer without SDS. Tris saline was 0.15 M NaCl, 0.005 M KCl, 0.025 M Tris (pH 7.3), and 1 g of glucose per liter. Low-salt buffer was 0.03 M NaCl and 0.01 M Tris (pH 7.4). SSC was 0.15 M NaCl and 0.015 M sodium citrate. Acetate buffer (pH 4.6) was 0.1 M NaCl, 0.05 M acetic acid, and 0.49 M sodium acetate. Standard medium was Eagle minimal essential medium supplemented with 2.5% calf serum, 2.5% tryptose phosphate broth, and 0.07% $NaHCO_3$. Actinomycin D was generously provided by Merck, Sharp & Dohme, Rahway, N.J. [^{3} H]uridine (28 Ci/mmol), [^{3} H]adenosine (15.5 to 27.1 Ci/mmol), [^{3} H]GTP (5.66 Ci/mmol), [^{3} H]ATP (34.2 Ci/mmol), [^{14} C]uridine (52.7 Ci/mol) and Omnifluor were obtained from New England Nuclear Corp. Bovine pancreatic RNase was obtained from Schwarz BioResearch, and T1 and T2 RNases and dithiothreitol (Cleland reagent) were from Calbiochem.

RESULTS

Intracellular virus-specific poly(A). Figure 1 compares velocity sedimentation patterns of NDV-specific RNA labeled with [^{3} H]uridine (panel A) and [^{3} H]adenosine (panel B). In each case, the 50, 35, and 18-22S viral RNA species are seen (8). A fourth major peak found at 4S only when RNA was labeled with [^{3} H]adenosine (panel B) is probably not virus specific since it is also found in uninfected cells labeled in the presence of actinomycin D (S. R. Weiss, unpublished data).

Treatment of equal samples of each fraction with a combination of T1 and pancreatic RNases revealed significant resistance of the 18-22S RNA only when it was labeled with

FIG. 1. Comparison of [^3H]uridine- and [^3H]adenosine-labeled NDV-specific RNA. Intracellular NDV-HP-specific RNA was labeled with [^3H]uridine (A) or [^3H]adenosine (B) and fractionated by velocity sedimentation for 4 h in sucrose gradients as described in Materials and Methods. Equal samples (0.025 ml) were trichloroacetic acid-precipitated before ($\bullet$) and after ($\blacktriangledown$) treatment with T1 and pancreatic RNases. Arrows designate the position of the A_{260} peaks of cellular RNA.

[^3H]adenosine. In contrast, RNA in the 35 and 50S regions showed some RNase resistance regardless of label. Relative to the 35 and 50S RNAs, the 18–22S RNA appeared to be enriched for adenosine. These results and the fact that the 18–22S RNA binds to polyuridylic acid fiber-glass filters (S. R. Weiss, unpublished data) provide preliminary evidence for the presence of poly(A) sequences in the 18–22S RNA.

To determine what the T1 and pancreatic RNase resistance in all of the species represents, [^3H]uridine- and [^3H]adenosine-labeled NDV-specific RNAs were pooled from gradients similar to those in Fig. 1 and subjected to various treatments as described in Table 1. Complete alkali sensitivity and DNase resistance (S. R. Weiss, unpublished data) of all of the RNAs showed that there was no labeled DNA contaminating them. Therefore, all of the observed T1 and pancreatic RNase resistance must represent base-paired RNA or poly(A).

The 18–22S RNA was almost completely single stranded, although it contained a small amount of RNase resistance (2%) which was lost upon denaturation. When labeled with [^3H]adenosine, the 18–22S RNA was 17% resistant to T1 and pancreatic RNases (which is only slightly reduced by denaturation) but almost completely sensitive to T2 RNase, providing further evidence for the presence of poly(A).

RNA in the 35 and 50S regions contained both base-paired RNA and poly(A) sequences.

About 6 to 9% of the 35S RNA and 15 to 20% of the 50S RNA was base paired (Table 1). The greater T1 and pancreatic RNase resistance of the [^3H]adenosine-labeled RNA, compared with [^3H]uridine-labeled RNA from both regions, suggests the presence of poly(A) sequences in these RNAs as well. This is confirmed by the partial loss of resistance to T1 and pancreatic RNases and nearly complete loss of resistance to T2 RNase after denaturation of [^3H]adenosine-labeled RNA. These results suggest that approximately 12% of the [^3H]adenosine label in the 35S RNA and 10 to 11% in the 50S RNA was contained in poly(A) sequences.

We wanted to further characterize the poly(A) associated with 18–22S RNA because, as will be discussed below, these RNAs probably serve as the virus-specific messenger RNAs and these species are better defined than the heterogeneous RNAs of the 35 and 50S regions.

To estimate the size of poly(A) sequences of the 18–22S RNA, the products of T1 and pancreatic RNase digestion of this RNA were analyzed by velocity sedimentation (Fig. 2A). The product of digestion of the [^3H]adenosine-labeled RNA sedimented slightly slower than, and appears to be as homogeneous as, yeast tRNA run in a parallel gradient. As expected, all of this [^3H]adenosine label was resistant to T1 and pancreatic RNases (not shown). The digest of [^3H]uridine-labeled RNA run on another parallel gradient (not shown) contained

TABLE 1. Nuclease and alkali sensitivities of NDV-specific RNAs

Treatment	Resistance (%) of					
	$[^3\text{H}]$ uridine-labeled RNA ^a			$[^3\text{H}]$ adenosine-labeled RNA ^a		
	50S	35S	18-22S	50S	35S	18-22S
Alkali	0.0	0.0	0.0	0.0	0.0	0.1
T1 and pancreatic RNases before denaturation	18.8	9.5	1.9	30.2	15.8	17.2
T1 and pancreatic RNases after denaturation	0.0	0.2	0.2	10.6	11.8	16.0
T2 RNase before denaturation	15.4	8.0	1.4	20.3	5.9	2.0
T2 RNase after denaturation	0.3	0.0	0.2	2.0	1.2	0.4

^a Fractions were pooled from a gradient similar to that in Fig. 1 (but containing NDV-AV RNA), and duplicate samples were treated as described in Materials and Methods. Total counts for each sample ranged from 2,500 to 5,300 counts/min except where the samples were denatured prior to treatment. In the latter case, three to five times as much radioactivity was used to increase accuracy. Percentage of resistance is defined as the ratio of trichloroacetic acid-precipitable counts before and after treatment, times 100.

FIG. 2. Analysis of poly(A) by velocity sedimentation. Products of T1 and pancreatic RNase digestion of 18-22S RNA (●) were sedimented for 20 h and fractions were analyzed for trichloroacetic acid-precipitable counts. A marker of yeast tRNA (Δ) was run in a parallel gradient. (A) Intracellular NDV-AV 18-22S RNA labeled with $[^3\text{H}]$ adenosine; (B) NDV-HP 18-22S RNA labeled in vitro by using $[^3\text{H}]$ ATP in the presence of 4 mM MgCl_2 ; (C) NDV-HP 18-22S RNA labeled in vitro by using $[^3\text{H}]$ ATP in the presence of 0.5 mM MnCl_2 .

almost no trichloroacetic acid-precipitable radioactivity. The $[^3\text{H}]$ adenosine-labeled digest was further purified by phenol extraction and co-electrophoresed in an 8% gel with chicken embryo cell RNA from the 4S region of a sucrose gradient (containing 4, 5, and 7S RNA) labeled with $[^{14}\text{C}]$ uridine. This procedure revealed considerable heterogeneity of the poly(A) with a size range of 4 to 7S (Fig. 3A).

Collins and Bratt (12) described a procedure which separates 18-22S NDV RNA into six to seven species. This makes it possible to examine all species of the 18-22S RNA for the presence of poly(A). Figure 4 shows the gel patterns of $[^3\text{H}]$ uridine- and $[^3\text{H}]$ adenosine-labeled intracellular NDV RNA. The patterns are similar to those of Collins and Bratt (12). The 35 and 50S RNAs did not move into the gels beyond the first seven slices; only the 18-22S RNA was

resolved on these gels (12). Treatment of each fraction with T1 and pancreatic RNases revealed that, although $[^3\text{H}]$ uridine-labeled RNA was almost completely RNase sensitive, when labeled with $[^3\text{H}]$ adenosine, all of the species contained from 13 to 18% RNase resistance. Thus, all of the species contained poly(A). [The RNase-resistant counts have been plotted on a sixfold-expanded scale to show the relative amounts of poly(A) among the species.]

Poly(A) synthesized in vitro. An excellent system for studying poly(A) synthesis would be available if poly(A) were synthesized during in vitro RNA synthesis employing detergent-activated virions (11, 21). The usefulness of this procedure would depend upon the ability of this system to transcribe with a high degree of fidelity. It has previously been shown that the in vitro transcription product is complementary to

Fig. 3. Electrophoretic analysis of poly(A). The T1 and pancreatic RNase-resistant fragments of 18–22S RNA (●) were extracted with phenol, precipitated with alcohol, and then resolubilized and co-electrophoresed with [^{14} C]uridine-labeled 4, 5, and 7S chicken embryo cell RNA (□) on 8% gels for 5 h. (A) Intracellular NDV-AV poly(A) labeled with [^{3} H]adenosine; (B) NDV-HP poly(A) labeled in vitro by using [^{3} H]ATP and 0.5 mM $MnCl_2$.

virion RNA (21) and sediments at approximately 18–22S (11, 21) like its intracellular counterpart (8). By using SDS-acrylamide gel electrophoresis, it is now possible to more carefully examine the product of in vitro transcription.

Figure 5 compares [^{3} H]uridine-labeled 18–22S RNA from infected cells (A) with 18–22S [^{3} H]GMP-labeled RNA made in vitro (B). The pattern of the in vitro RNA appears to contain all of the intracellular species in approximately the same proportions although the peaks are not as distinct, particularly in the low-molecular-weight region of the gel.

Having established the similarity of the intracellular and in vitro 18–22S RNAs, we com-

pared the in vitro RNA labeled with [^{3} H]GMP (Fig. 6A) and [^{3} H]AMP (Fig. 6B) by velocity sedimentation. In each case there was a major peak in the 18–22S region. A peak at 4S was also observed. The latter peak varied in amount from one experiment to the next and was not further characterized. In addition, there was a small amount of RNA which sedimented between 40 and 50S (and was pelleted in these gradients), contained some base-paired RNA, and presumably was a transcriptive intermediate (11). We did not analyze the poly(A) content of the 4 and 40–50S RNAs because our main interest was in the 18–22S in vitro product that corresponds to intracellular messenger RNA.

As shown for the intracellular 18–22S RNA (Fig. 1, Table 1), the in vitro 18–22S RNA appeared to be almost completely sensitive to T1 and pancreatic RNase digestion when labeled with [^{3} H]GMP (Fig. 6A) but about 20% resistant when labeled with [^{3} H]AMP (Fig. 6B). This suggests that poly(A) sequences are associated with RNA synthesized in vitro. Supporting evidence is provided by the data in Table 2. Denaturation had only a very small effect on the T1 and pancreatic RNase resistance of the [^{3} H]AMP-labeled RNA. This finding suggests that the vast majority of this RNA is single stranded. Also, [^{3} H]GMP- and [^{3} H]AMP-labeled RNAs were completely sensitive to T2 RNase, proving that all of the label is in RNA. (As with 18–22S RNA synthesized in vivo, there may have been a small amount of base-paired RNA present, since the 2% resistance of [^{3} H]AMP- and [^{3} H]GMP-labeled RNAs to T2 RNase was reduced by denaturation.)

To obtain an estimate of the size of the in vitro poly(A), the T1 and pancreatic RNase digest of 18–22S in vitro RNA was analyzed by velocity sedimentation as shown in Fig. 2B and 2C. The digest of [^{3} H]AMP-labeled RNA sedimented as large as or larger than 4S and looked more heterogeneous than marked RNA run on a parallel gradient. It also looked larger and more heterogeneous than intracellular NDV-specific poly(A) (Fig. 2A). The digest of [^{3} H]GMP-labeled RNA run in a parallel gradient (not shown) contained no trichloroacetic acid-precipitable radioactivity.

We also wanted to determine whether the poly(A) associated with the in vitro synthesized 18–22S RNA was covalently linked to it. This was done by pooling [^{3} H]AMP-labeled 18–22S RNA from a sucrose gradient, boiling for 2 min, and then resedimenting the RNA. After this treatment, T1 and pancreatic RNase resistance was still sedimented in association with the 18–22S RNA (not shown, but similar to the

FIG. 4. Electrophoretic analysis of [^3H]uridine- and [^3H]adenosine-labeled NDV-specific 18-22S RNA. NDV-AV intracellular RNA labeled with [^3H]uridine (A) or [^3H]adenosine (B) was electrophoresed for 11 h in 4% gels. Each gel fraction was boiled as described in Materials and Methods. Equal samples of each fraction were trichloroacetic acid-precipitated before ($\bullet$) and after ($\blacktriangledown$) treatment with T1 and pancreatic RNases. Arrows designate the peaks of ^{14}C -labeled chicken embryo cell RNA electrophoresed in a parallel gel. The numbering system for these 18-22S RNA species is taken from Collins and Bratt (12).

FIG. 5. Electrophoretic analysis of 18-22S intracellular and in vitro RNA. Virus-specific 18-22S ($\bullet$) [^3H]uridine-labeled intracellular RNA (A) and [^3H]GMP-labeled RNA synthesized in vitro in the presence of MgCl_2 (B) were co-electrophoresed with ^{14}C -labeled chicken embryo cell RNA ($\square$) for 11 h in 4% gels.

pattern in Fig. 6B) and was not released as free poly(A). Therefore, we conclude that the poly(A) is covalently linked to the 18-22S RNA.

Finally, we showed that the poly(A) synthesizing activity was associated with viral cores. This was done by treating virus with 2% triton N-101 for 10 min at room temperature and then sedimenting the cores through 20% sucrose onto a 65% sucrose- D_2O pad as described in Materials and Methods for the final purification of complete virions. The products of standard reactions using these cores showed patterns similar to those in Fig. 6.

Cation and nucleotide triphosphate dependence of in vitro RNA synthesis. Maximal [^3H]GMP incorporation in NDV's in vitro transcriptase reaction has previously been reported to occur at magnesium ion concentrations of 4 to 6 mM (21). Figure 7 shows that for maximal velocity in the presence of 4mM magnesium when the other nucleotides are excess, GTP [as well as UTP and CTP (not shown)] is required at a concentration of 0.1 to 0.2 mM, whereas ATP is required at a concentration of 1.0 to 1.2 mM. This finding is similar to those obtained for both influenza virus and VSV (3). As a consequence,

FIG. 6. Comparison of [^3H]GMP- and [^3H]AMP-labeled products of *in vitro* synthesis. RNA was synthesized in the *in vitro* reaction in the presence of magnesium and labeled with [^3H]GMP (A) or [^3H]AMP (B) and fractionated by velocity sedimentation for 9 h as described in Materials and Methods. Equal aliquots of each fraction were TCA precipitated before (●) or after (▼) treatment with T1 and pancreatic RNases. Arrows designate the positions of the A_{260} peaks of cellular RNA.

TABLE 2. Nuclease sensitivities of 18–22S RNAs synthesized *in vitro*

Treatment	Resistance (%) of RNA ^a			
	Magnesium		Manganese	
	[^3H]GMP	[^3H]AMP	[^3H]GMP	[^3H]AMP
T1 and pancreatic RNases before denaturation	4.6	22.7	1.7	57.9
T1 and pancreatic RNases after denaturation	0.2	20.1	0.2	56.4
T2 RNase before denaturation	1.7	2.1	1.8	1.7
T2 RNase after denaturation	0.2	0.6	0.2	0.2

^a 18–22S RNAs labeled *in vitro* in the presence of 4 mM MgCl_2 or 1 mM MnCl_2 ([^3H]GMP-labeled RNA) or 0.5 mM MnCl_2 ([^3H]AMP-labeled RNA) were treated as in the experiment described in Table 1. Total counts were 2,500 and 1,200, respectively, for [^3H]GMP and [^3H]AMP RNAs labeled in the presence of MgCl_2 and 3,000 counts for both [^3H]GMP and [^3H]AMP RNAs labeled in the presence of MnCl_2 .

maximal incorporation of a small but fixed amount of radioactivity is achieved at a concentration of 0.02 mM GTP (when [^3H]GMP incorporation is measured) or 0.1 to 0.2 mM ATP (when [^3H]AMP incorporation is measured). Thus, high specific activity [^3H]AMP incorporation is difficult to achieve because of the slow reaction velocity at low ATP concentrations.

As originally described, manganese ion at concentrations of 2 to 26 mM will not substitute for magnesium in this *in vitro* reaction (21). However, Henry O. Stone (personal communication) found that manganese will support the reaction at concentrations of less than 1 mM. Taking advantage of this finding, we measured the velocity of [^3H]AMP incorporation (at a con-

centration of 0.17 mM ATP) and [^3H]GMP (at a concentration of 0.02 mM GTP) as a function of magnesium or manganese concentration (Fig. 8). Optimal [^3H]GMP incorporation (of the same magnitude) occurred in 4 mM magnesium or 1.0 mM manganese. In contrast, for [^3H]AMP optimal incorporation occurred at a magnesium concentration of about 4 mM but at a level which was four to six times less than for the optimum, which occurred at 0.5 mM manganese. Thus, the presence of manganese instead of magnesium had no effect on gross incorporation of [^3H]GMP but provided a more efficient means of measuring [^3H]AMP incorporation.

When reaction products labeled with

FIG. 7. Dependence of *in vitro* RNA synthesis on nucleotide triphosphate concentration. Reaction mixes were incubated in the presence of 4 mM magnesium incorporating [^3H]GMP (A) or [^3H]AMP (B) in the presence of varying amounts of GTP (A) or ATP (B). Closed and open circles represent two different experiments.

[^3H]GMP in the presence of 1.0 mM manganese or [^3H]AMP in the presence of 0.5 mM manganese were analyzed by velocity sedimentation, the overall patterns were similar to those obtained with magnesium (Fig. 5). However, when the 18-22S RNA made in the presence of manganese was pooled from a gradient and analyzed by SDS-gel electrophoresis (not shown), the distinct peaks seen for RNA made in the presence of magnesium as in Fig. 5B were not found (S. R. Weiss, unpublished data). Furthermore, the 18-22S RNA labeled with [^3H]AMP in the presence of manganese showed resistance to T1 and pancreatic RNases which varied from 20 to 60% in different experiments (whereas [^3H]GMP-labeled RNA was consist-

ently about 2% resistant). The high RNase resistance often encountered during [^3H]AMP labeling in the presence of manganese was probably poly(A) since it was not lost during denaturation and was completely sensitive to digestion by T2 RNase (Table 2). Finally, poly(A) made in the presence of manganese (Fig. 2C) appeared larger and more heterogeneous than that made intracellularly (Fig. 2A) or *in vitro* in the presence of magnesium (Fig. 2B). This greater size and heterogeneity was seen more clearly when the RNase-resistant fraction was phenol extracted and co-electrophoresed with ^{14}C -labeled 4, 5, and 7S (Fig. 3B) chicken embryo cell RNA.

DISCUSSION

There are now several criteria by which the 18-22S RNA synthesized *in vitro* by NDV's virion-bound polymerase has been shown to resemble its intracellular counterpart. It was previously shown that both intracellular (8) and *in vitro* (21) 18-22S NDV-specific RNAs are complementary to virion RNA. Herein, we have demonstrated that poly(A) sequences are associated with the 18-22S RNA synthesized *in vitro* as well as with all six to seven species of the 18-22S NDV-specific RNA from infected cells. We have also shown that the 18-22S *in vitro* RNA contains six to seven species of approximately the same sizes and proportions as those from the infected cell. We did not,

FIG. 8. Dependence of *in vitro* RNA synthesis on divalent cations. *In vitro* reactions were done incorporating [^3H]GMP (●) or [^3H]AMP (■). Magnesium (—) or manganese (---) concentrations were varied as indicated on the abscissa.

however, demonstrate that each of these *in vitro* RNA species corresponds in base sequence to the intracellular species of approximately the same size.

There is also considerable evidence suggesting that these 18-22S RNAs and comparable species in cells infected by Sendai virus, another paramyxovirus, serve as viral messenger RNA. This evidence includes: the presence of 18-22S RNA in polyribosomes and its release from them by EDTA (5, 8); the presence of poly(A) sequences on the 18-22S species (this study; ref. 30; S. R. Weiss and M. A. Bratt, *Abstr. Annu. Meet. Amer. Soc. Microbiol.*, p. 202, 1973; and Bratt et al., *in Negative strand viruses*, in press); the correlation of size and number of species contained in the 18-22S RNA (12; Bratt et al., *in Negative strand viruses*, in press) with those of virus-specific polypeptides from infected cultures (20; Bratt et al., *in Negative strand viruses*, in press); and Kingsbury's more direct demonstration that 18S RNA from Sendai virus-infected cells can direct the synthesis of virus-specific polypeptides in an *in vitro* translation system (25). Definitive proof that the 18-22S NDV-specific RNAs are messengers comes from the demonstration that 18-22S RNA synthesized *in vitro* can direct the synthesis of NDV-specific polypeptides in an *in vitro* translation system (T. Morrison, B. S. Collins, L. E. Hightower, S. R. Weiss, and M. A. Bratt, unpublished data).

Poly(A) is also associated with the 35 and 50S NDV-specific RNAs from infected cells. However, because of the heterogeneity of these RNAs, they have not been considered in depth here. They contain both single-stranded species (including 50S virion RNA) and partially base-paired species which may serve as intermediates for the synthesis of single-stranded species (8, 9; B. S. Collins and M. A. Bratt, unpublished data). RNA from NDV virions does not contain poly(A) sequences (19). At least part of the poly(A) in the 35 and 50S regions may be associated with 18S RNA (perhaps as a part of the presumptive intermediates), because after denaturation a large portion of the 35 and 50S RNA sediments at 18S (B. S. Collins and M. A. Bratt, unpublished data).

The size of the poly(A) associated with intracellular 18-22S NDV-specific RNA can be estimated in several ways. This poly(A) sediments somewhat slower than 4S transfer RNA, suggesting a size of less than 80 nucleotides. However, on 8% polyacrylamide gels, the poly(A) migrates heterogeneously from about 4 to 7S, indicating a size range of 25,000 to 55,000 daltons, which corresponds to 70 to 170 nucleotides.

These sizes must only be considered approximate since conformational differences between poly(A), transfer RNA, and chicken embryo cell RNA may affect their relative rates of migration or sedimentation. A similar size estimate is obtained by using the value for RNase resistance. If it is assumed that only one poly(A) segment is associated with each RNA molecule (an assumption which is currently under investigation), the facts that an average of 16% of the 18-22S RNA (after denaturation) is T1 and pancreatic RNase resistant, that this RNA contains 32 to 33% AMP (8), and that the average size of these species is about 800,000 daltons (12) suggest a size of 120 to 130 nucleotides for the average poly(A) sequence.

Size estimates using the same criteria for the poly(A) on the *in vitro* synthesized RNA suggest that this poly(A) is longer and more heterogeneous than its intracellular counterpart. Even the *in vitro* poly(A) made in the presence of magnesium (Fig. 2B) (which tends to be smaller than that made in the presence of manganese; Fig. 2C) often sediments more rapidly than the intracellular poly(A) (Fig. 2A). It sediments more like HeLa cell poly(A) (14, 26), which has a size of 150 to 200 nucleotides (14, 16, 29). By using the value of about 20% RNase resistance (after denaturation) of the [³H]AMP-labeled 18-22S *in vitro* RNA (made in the presence of magnesium), the assumptions used above, and the further assumption that the non-poly(A) portion of the *in vitro* RNA has the same base composition as the intracellular RNA, a size estimate of 160 to 170 nucleotides is obtained for the poly(A).

Under our conditions of labeling, *in vitro* synthesized poly(A) appears to be larger than intracellular poly(A). This could be due to an imprecision of the *in vitro* system in poly(A) synthesis. Another explanation is suggested by the evidence that poly(A) isolated from long-labeled cellular mRNA is shorter than that from pulse-labeled RNA (29, 31). The NDV-specific intracellular poly(A) that we have isolated was labeled for 5 h (a relatively long time) and, like cellular poly(A), might have been processed or degraded and therefore appear shorter than newly synthesized poly(A). Experiments measuring intracellular poly(A) size as a function of labeling time are in progress.

We do not understand the great variation in RNase resistance of the 18-22S *in vitro* RNA made in the presence of manganese and the size of the RNase resistant fragment derived from it. Nor do we understand the four- to sixfold difference in maximal AMP incorporation in the presence of magnesium and manganese that

does not exist for GMP incorporation.

NDV-specific RNA synthesis occurs exclusively in the cytoplasm (8), and the synthesis of the 18-22S RNA and its associated poly(A) is insensitive to the adenosine analogue cordecypin (S. R. Weiss, unpublished data). These two facts strongly suggest that poly(A) is not synthesized by the nuclear cordecypin-sensitive process by which cellular poly(A) is synthesized (13, 22, 29). This is confirmed by the finding that RNA synthesized *in vitro* by Triton-treated virus and by cores isolated from the virus contains poly(A) sequences. H. O. Stone has also found poly(A) sequences associated with *in vitro* synthesized NDV and Sendai virus messenger RNA (personal communication). Similar results have recently been reported for vesicular stomatitis virus (2, 34).

Several possibilities for the mechanism of NDV-specific poly(A) synthesis can be considered. The poly(A) could be transcribed from polyuridylic acid sequences as suggested by the high (29%) UMP content of virion RNA (15, 24). Mediating against this possibility is Marshall and Gillespie's finding that [³H]poly(A) does not hybridize with NDV virion RNA under conditions where it can hybridize with polyuridylic acid (28). Furthermore, since about 16% of the 32 to 33% AMP, or 5% of the total nucleotides, is in poly(A) sequences, then 28 to 29% of the non-poly(A) portion of the 18-22S RNA is AMP. This number is close to the 29% UMP content of the virion RNA. It is probable that all of the UMP of the virion RNA can be accounted for by AMP not contained in poly(A). Our finding that the size of the poly(A) synthesized *in vitro* can vary according to the reaction conditions also suggests that its synthesis is not completely transcriptive. Another possibility is that poly(A) is synthesized and then added to the RNA. This is unlikely since no [³H]AMP incorporation into trichloroacetic acid-precipitable material occurs in the *in vitro* reaction in the absence of the other nucleotide triphosphates (S. R. Weiss, unpublished data). It therefore seems likely that poly(A) is added without transcription by the transcriptase itself or another virion-bound enzyme.

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LITERATURE CITED

- Adesnik, M., M. Salditt, W. Thomas, and J. E. Darnell. 1972. Evidence that all messenger RNA molecules (except histone messenger RNA) contain poly(A) sequences and that poly(A) has a nuclear function. *J. Mol. Biol.* 71:21-30.
- Banerjee, A. K., and D. P. Rhodes. 1973. *In vitro* synthesis of RNA that contains polyadenylate by virion-associated RNA polymerase of vesicular stomatitis virus. *Proc. Nat. Acad. Sci. U.S.A.* 12:3566-3570.
- Bishop, D. H. L., J. Objeski, and R. W. Simpson. 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for *in vitro* activity of the ribonucleic acid-dependent ribonucleic acid polymerase. *J. Virol.* 8:66-73.
- Blair, C. D., and W. S. Robinson. 1968. Replication of Sendai virus. I. Comparison of viral RNA and virus specific RNA synthesis to Newcastle disease virus. *Virology* 35:537-549.
- Blair, C. D., and W. S. Robinson. 1970. Replication of Sendai virus. II. Steps in virus assembly. *J. Virol.* 5:639-650.
- Bratt, M. A. 1969. RNA synthesis in chick embryo cells infected with different strains of NDV. *Virology* 38:485-488.
- Bratt, M. A., and W. R. Gallaher. 1969. Preliminary analysis of the requirements for fusion from within and fusion from without by Newcastle disease virus. *Proc. Nat. Acad. Sci. U.S.A.* 64:536-543.
- Bratt, M. A., and W. S. Robinson. 1967. Ribonucleic acid synthesis in cells infected with Newcastle disease virus. *J. Mol. Biol.* 23:1-23.
- Bratt, M. A., and W. S. Robinson. 1971. Evidence for an RNA replicative intermediate in cells infected with Newcastle disease virus. *J. Gen. Virol.* 10:139-145.
- Bratt, M. A., and H. Rubin. 1967. Specific interference among strains of Newcastle disease virus. I. Demonstration and measurement of the interference. *Virology* 33:598-608.
- Clavell, L. A., and M. A. Bratt. 1971. Relationship between the ribonucleic acid-synthesizing capacity of ultraviolet-irradiated Newcastle disease virus and its ability to induce interferon. *J. Virol.* 8:500-508.
- Collins, B. S., and M. A. Bratt. 1973. Separation of the messenger RNAs of Newcastle disease virus by gel electrophoresis. *Proc. Nat. Acad. Sci. U.S.A.* 70:2544-2548.
- Darnell, J., L. Philipson, R. Wall, and M. Adesnik. 1971. Polyadenylic acid sequences: role in conversion of nuclear RNA into messenger RNA. *Science* 174:507-510.
- Darnell, J. E., R. Wall, and R. J. Tushinski. 1971. An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA. *Proc. Nat. Acad. Sci. U.S.A.* 68:1321-1325.
- Duesberg, P. H., and W. S. Robinson. 1965. Isolation of the nucleic acid of Newcastle disease virus (NDV). *Proc. Nat. Acad. Sci. U.S.A.* 54:794-800.
- Edmonds, M., M. H. Vaughan, and H. Nakazato. 1971. Polyadenylic acid sequence in the heterogeneous nuclear RNA and rapidly-labelled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship. *Proc. Nat. Acad. Sci. U.S.A.* 68:1336-1340.
- Ehrenfeld, E., and D. F. Summers. 1972. Adenylate-rich sequences in vesicular stomatitis virus messenger ribonucleic acid. *J. Virol.* 10:683-688.
- Galet, H., and L. Prevec. 1973. Polyadenylate synthesis by extracts of L cells infected with vesicular stomatitis virus. *Nature N. Biol.* 243:200-203.
- Gillespie, D., S. Marshall, and R. C. Gallo. 1972. RNA of RNA tumor viruses contains poly(A). *Nature N. Biol.* 236:227-231.
- Hightower, L. E., and M. A. Bratt. 1974. Protein synthesis in Newcastle disease virus-infected chick embryo cells. *J. Virol.* 13:788-800.
- Huang, A. S., D. Baltimore, and M. A. Bratt. 1971.

- Ribonucleic acid polymerase in virions of Newcastle disease virus: comparison with the vesicular stomatitis virus polymerase. *J. Virol.* **7**:389-394.
22. Huang, A. S., D. Baltimore, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. *Virology* **42**:947-957.
23. Jelinek, W., M. Adesnik, M. Salditt, D. Sheiness, R. Wall, G. Molloy, L. Philipson, and J. E. Darnell. 1973. Further evidence on the nuclear origin and transfer to the cytoplasm of polyadenylic acid sequences in mammalian cell RNA. *J. Mol. Biol.* **75**:515-532.
24. Kingsbury, D. W. 1968. Newcastle disease virus. I. Isolation and preliminary characterization of RNA from virus particles. *J. Mol. Biol.* **18**:195-203.
25. Kingsbury, D. W. 1973. Cell-free translation of paramyxovirus messenger RNA. *J. Virol.* **12**:1020-1027.
26. Lee, S. Y., J. Mendecki, and G. Brawerman. 1971. A polynucleotide segment rich in adenylic acid in the rapidly-labelled polyribosomal RNA component of mouse sarcoma 180 ascites cells. *Proc. Nat. Acad. Sci. U.S.A.* **68**:1331-1335.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
28. Marshall, S., and D. Gillespie. 1972. Poly U tracts absent from viral RNA. *Nature N. Biol.* **240**:43-45.
29. Mendecki, J., S. Y. Lee, and G. Brawerman. 1972. Characteristics of the polyadenylic acid segment associated with messenger ribonucleic acid in mouse sarcoma 180 ascites cells. *Biochemistry* **11**:792-798.
30. Pridgen, C., and D. W. Kingsbury. 1972. Adenylate-rich sequences in Sendai virus transcripts from infected cells. *J. Virol.* **10**:314-317.
31. Sheiness, D., and J. E. Darnell. 1973. Polyadenylic acid segment in mRNA becomes shorter with age. *Nature N. Biol.* **241**:265-268.
32. Sheldon, R., J. Kates, D. E. Kelley, and R. P. Perry. 1972. Poly(A) sequences on 3' termini of vaccinia messenger ribonucleic acid and mammalian nuclear and messenger ribonucleic acid. *Biochemistry* **11**:3829-3834.
33. Soria, M., and A. S. Huang. 1973. Association of poly(A) with messenger RNA of vesicular stomatitis virus. *J. Mol. Biol.* **77**:449-455.
34. Villarreal, L. P., and J. J. Holland. 1973. Synthesis of poly(A) *in vitro* by purified virions of vesicular stomatitis virus. *Nature N. Biol.* **246**:17-19.