Poly(A) Polymerase from Vaccinia Virus-Infected Cells

I. Partial Purification and Characterization

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Poly(A) polymerase activity is induced during vaccinia virus infection of HeLa cells. The enzyme is maximally induced at 3.5 h postinfection. Partial purification frees the preparation of RNase activity and RNA polymerase activity. ATP is the substrate for poly(A) synthesis. A small amount of poly(A) is produced from added adenosine diphosphate due to the production of ATP by an adenylate kinase present in the preparation. The incorporation of ATP into poly(A) is dependent on divalent cations (Mg^{2+} or Mn^{2+}) and is not inhibited by UTP, CTP, or GTP. Poly(U) stimulates ATP incorporation; poly(A) and poly(C) have little effect on ATP incorporation, and poly(dT) is extremely inhibitory. RNA prepared from HeLa cells and from the partially purified poly(A) polymerase (the enzyme preparation contains endogenous RNA [Brakel and Kates]) stimulates ATP incorporation by poly(A) polymerase which was subjected to DEAE-cellulose chromatography. RNase's, pancreatic and T_1 , inhibit the production of poly(A). DNase has little effect. Poly(U) is able to stimulate poly(A) production in the presence of T_1 RNase.

Polyadenylic acid is present on the 3' end of eukaryotic messenger and heterogeneous nuclear RNA (4, 7, 20, 24). Virus-specific RNAs also contain poly(A) (1, 8, 10, 13, 17, 18, 23, 27, 28). Poly(A) may be added post-transcriptionally to cellular and viral RNAs (3, 23, 27).

Cellular enzymes capable of extending poly(A) chains have been partially characterized (5, 6, 14, 15, 25). An Ehrlich ascites cell enzyme is stimulated by added RNA (11, 12) as is an enzyme isolated from yeast (26). Galet and Prevec (9) have recently demonstrated a poly(A) polymerase activity associated with the RNA-dependent RNA polymerase complex from vesicular stomatitis virus-infected cells. This enzyme produced poly(A) which was associated with larger RNA.

The early results of Kates and Beeson (18) and Kates (17) suggested that vaccinia virus cores formed poly(A) in a DNA-dependent reaction. Ethidium bromide and proflavin sulfate were capable of inhibiting poly(A) synthesis by vaccinia cores. Recent experiments of Sheldon and Kates (24a) indicate that there are no extensive regions of dT in vaccinia virus DNA. The data presented in this and the following

paper suggest that poly(A) synthesis by a vaccinia virus-induced enzyme is dependent on RNA primers and not dependent on a DNA template.

This report describes the induction of poly(A) polymerase activity during vaccinia virus infection of HeLa cells. The partial purification of the enzyme is described. The properties of the enzymatic reaction and its RNA dependence are demonstrated. In the following paper (1a) evidence is presented that poly(A) is synthesized on endogenous RNA and on added poly(U).

While this work was in progress Moss and co-workers (22) described the purification and properties of a poly(A) polymerase isolated from purified vaccinia virions. The production of poly(A) was stimulated either by poly(dA)-poly(dT) or by poly(rC). The virion enzyme (22) appears to be distinct from the enzyme described here on the basis of in vitro response to added polynucleotides. More conclusive evidence concerning their relationship awaits further purification of the in vivo enzyme and further in vitro studies with both enzymes.

MATERIALS AND METHODS

Virus and infection procedures. The WR strain of vaccinia virus was grown in HeLa S₃ cells in spinner cultures. The virus was purified by the method of Joklik (16).

For most experiments cells were infected with 200

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to 300 particles per cell (4 to 6 PFU per cell). Cultures were incubated with virus for 30 min at 5×10^6 cells per ml in F-14 (Gibco). After the adsorption period cultures were diluted to 10^6 cells per ml in F-14 plus 5% calf serum (Gibco) and the infection was allowed to proceed at 37 C for the time indicated for the individual experiment.

Preparation of cytoplasmic extracts. Infected or mock-infected cells were harvested by centrifugation and washed in 0.05 M Tris-hydrochloride, pH 8.5, 0.15 M NaCl, 0.005 M EDTA. Cells were broken by Dounce homogenization after allowing them to stand for 5 min at 2.5×10^7 cells per ml in 0.01 M Tris-hydrochloride, pH 8.5, 0.01 M KCl, 0.005 M EDTA at 4 to 5 C. Nuclei were removed by centrifugation. Further treatment of the cytoplasmic extracts is described in the text (see Table 1).

Poly(A) polymerase reaction conditions. To 0.15 ml of substrates, 0.05 ml of enzyme preparation was added; the final concentration of substrates were 0.125 mM ATP, 2.5 μ Ci of [3 H]ATP per ml (New England Nuclear Corp.) in 0.05 M Tris-hydrochloride, pH 8.5, 5 mM MgCl₂, and 10 mM 2-mercaptoethanol. When [14 C]ATP was substituted for [3 H]ATP, 0.4 μ Ci was used per 0.2-ml reaction mixture. Reactions were incubated at 37 C for the indicated time and incorporation was stopped by addition of cold 5% trichloroacetic acid. Acid-insoluble material was collected on Whatman GF/C filter disks and incorporation was determined by liquid scintillation spectrometry in PPO:toluene (5 g/liter).

RESULTS

Enzyme induction. To determine the time of induction of poly(A) polymerase activity HeLa cells were infected as described in Materials and Methods and cytoplasmic extracts were prepared at various times after virus adsorption. Cytoplasmic extracts were assayed for RNA polymerase and poly(A) polymerase activities in

the presence of 2-mercaptoethanol and Nonidet P-40. The results (Fig. 1) show that the time of induction is different for the two activities.

Poly(A) polymerase activity increased within an hour after infection and decreased after 4 h. This decrease was not pronounced if an ATP regenerating system was included in the reaction mixtures (data not shown). RNA polymerase activity induction is similar to that found by Kates et al. (19). These results suggested that the mechanism of induction of poly(A) polymerase may differ from that of RNA polymerase. Cytosine arabinoside, a drug which inhibits the induction of RNA polymerase in vaccinia virus-infected cells (19), had no effect on poly(A) polymerase induction (Fig. 1). These results suggest but do not prove that the poly(A) polymerase and RNA polymerase are not the same enzymes. Preliminary data suggest that poly(A) polymerase is not induced when protein synthesis is inhibited after virus adsorption.

Enzyme induction was multiplicity dependent. Enzyme induction was most rapid in cells infected at very high multiplicities (500 particles per cell), although the amount of activity in cytoplasmic preparations reached similar maximum levels. All of the following experiments were performed at a multiplicity of 200 to 300 particles (4 to 6 PFU per cell) per cell. Enzyme preparations were made at 3.5 h postinfection at which time the activity in the cytoplasmic extract was highest.

Enzyme purification. A summary and the details of the steps used to partially purify the enzyme are presented in Table 1. The active fraction was excluded from the agarose bed and eluted in the void volume. The specific activity

TABLE 1. *Partial purification of poly(A) polymerase*

Enzyme fraction ^a	Protein (mg)	Enzyme units (counts per min per h)	Sp act (nmol per mg per h)
I. Cytoplasmic extract	640 [100]	2.5×10^7 [100]	3.6
II. Supernatant fraction	360 [56]	2.5×10^7 [100]	6.4
III. (NH ₄) ₂ SO ₄ fraction	152 [24]	2.6×10^7 [100]	15.1
IV. Agarose chromatographed	70 [11]	2.4×10^7 [94]	31.1

^a The cytoplasmic extract was prepared as described in Materials and Methods from 24×10^6 cells infected for 3.5 h with 200 particles per cell. The cytoplasm was centrifuged for 1 h at $100,000 \times g$ at 5 C and the supernatant fraction (II) was taken. The material precipitating between 35 and 60% saturation with (NH₄)₂SO₄ was dialyzed versus TKEE (0.01 M Tris-hydrochloride, pH 8.5, 0.01 M KCl, 0.01 M 2-mercaptoethanol, 0.005 M EDTA) and assayed (fraction III). One-half of fraction III was applied to a 50×1.2 cm agarose column (agarose A-5m, 6%, 100 to 200 mesh, 5×10^6 molecular weight exclusion limit) in TKEE. Fractions of 2.5 ml were collected and enzyme activity was located in fractions 12, 13, and 14 of the eluate. These fractions were combined and assayed as fraction IV. Numbers in brackets give the recovery as percentage of the cytoplasmic extract. Recovery data are given for the entire preparation. Enzyme assays were performed under conditions of limiting enzyme to ensure linearity. One enzyme unit is defined as one count per minute incorporated per hour in the standard 0.2-ml reaction mixture.

FIG. 1. Induction of RNA and poly(A) polymerase activities in vaccinia virus-infected HeLa cells. HeLa cells were infected with vaccinia virus at 500 particles per cell. Samples were taken at the end of the adsorption period (0 h) and at the indicated times thereafter. Cytoplasmic fractions were prepared in breakage buffer. Samples (0.1 ml each) were assayed with 0.3 ml of poly(A) polymerase substrates or with RNA polymerase substrates (see Table 2) in the presence of 0.5% Nonidet P-40 (Shell) and 0.01 M 2-mercaptoethanol. Symbols: ●, [³H]UMP incorporation from RNA polymerase substrates; ■, [³H]AMP from poly(A) polymerase substrates; ○, [³H]AMP from poly(A) polymerase substrates from cells infected in the presence of cytosine arabinoside (10^{-5} M).

increased approximately 8 to 10-fold after agarose chromatography. Recovery of enzyme through this step was usually 90 to 100%. Further attempts to purify the enzyme by size (glycerol gradient centrifugation) or by ion exchange chromatography [DEAE-cellulose, phosphocellulose, carboxymethylcellulose, hydroxylapatite, or oligo(dT)-cellulose] resulted in large losses of activity and very little further purification.

Contaminating enzymic activities. Little, if any, exonucleolytic activity was present in fraction IV since incubation of fraction IV with [³H]uridine-labeled HeLa cell RNA caused no reduction in the acid-insoluble tritium label. Tests for endonucleolytic activity were performed using poly(U)GF/C filters (24) and [³H]uridine-labeled vaccinia virus in vivo (isolated 3.5 h after infection) RNA. Since RNA

interacts with poly(U)GF/C filters by virtue of covalently linked poly(A) it is possible to detect low levels of endonuclease by observing the interaction of uridine- (or guanosine- or cytosine-) labeled RNA with the poly(U)GF/C filters. Endonucleolytic cleavage would result in a rapid reduction in the amount of uridine-labeled material binding to the poly(U)GF/C filter. Incubation of [³H]uridine vaccinia virus RNA with fraction IV under conditions used to produce poly(A) in vitro resulted in no reduction of the amount of label annealing to poly(U)GF/C filters, thus excluding the possibility that fraction IV contained active endonuclease.

Tests for RNA polymerase were negative (Table 2). Ribonucleoside triphosphates other than ATP did not inhibit the incorporation of [³H]ATP into acid-insoluble material (see Table 4 below). Within the limits of the assay (10 to 30 pmol), uridine triphosphate was not incorporated into acid-insoluble material when the enzyme was incubated with labeled UTP and the other three ribonucleoside triphosphates (Table 2). This excluded the presence of an active RNA polymerase-template complex. This was expected as most of the RNA polymerase activity is pelleted by centrifugation of cytoplasmic extracts of infected cells at 100,000 × g for an hour (19).

To test for the presence of template-dependent RNA polymerase activity, the enzyme was incubated with ATP and [³H]UTP and increasing concentrations of poly(dAT). Although the incorporation of 30 to 40 pmol of UMP from these reaction mixtures would have been detected, no incorporation above background levels was observed.

Substitution of [³H]ADP and ADP for [³H]ATP and ATP resulted in lowered, but considerable, incorporation of label into acid-insoluble material. The level of incorporation was about one-fourth that with ATP substrates (Table 2). These results suggest either that ADP was the immediate substrate for poly(A) synthesis in a polynucleotide phosphorylase-like reaction or that ADP was converted to ATP, which acts as the immediate substrate. If ATP were the true substrate, the incorporation from ADP substrates would be expected to be very low while ATP accumulated after the initiation of the incubation.

The rates of incorporation of label from ATP and ADP were compared. Figure 2 shows that the ATP reaction was linear from time zero but the incorporation of label from ADP was initially low and then increased. This suggested that there was a step in the ADP reaction that is not necessary in the ATP reaction.

TABLE 2. Tests for RNA polymerase and polynucleotide phosphorylase

Substrate mixture	Counts/min incorporated per 30 min	pMol per 30 min
Expt 1		
A. [^3H]ATP, ATP	5,686; 5,272	450
B. [^3H]UTP, UTP, CTP, GTP and ATP	130; 92	10
Expt 2		
A. [^3H]ATP, ATP	3,753; 2,984	280
B. [^3H]UTP, UTP and ATP		
1. No Template	127; 107	30
2. Poly(dAT) (0.04 A_{260})	72; 91	30
3. Poly(dAT) (0.08 A_{260})	103; 74	30
4. Poly(dAT) (0.10 A_{260})	64; 83	30
Expt 3		
A. [^3H]ATP, ATP	5,686; 5,272	450
B. [^3H]ADP, ADP	1,557; 1,105	110

^a Poly(A) polymerase substrates are described in Materials and Methods. RNA polymerase mixtures contained 2 μmol of ATP, 1 μmol each of CTP and GTP, 0.2 μmol of UTP and 4 μCi of [^3H]UTP, 4 μmol of MgCl_2 , and 25 μmol of Tris-hydrochloride, pH 8.0, per 0.4-ml reaction mixture (0.3-ml substrates plus 0.1 ml of enzyme). Poly(dAT) was added at the concentration indicated to mixtures which contained 1 μmol of ATP, 0.1 μmol of UTP, 1 μCi of [^3H]UTP, 0.01 M MgCl_2 , 0.05 M Tris-hydrochloride, pH 8.5, in a final volume of 0.2 ml (0.15-ml substrates plus 0.05 ml of enzyme). In section 3, ADP and [^3H]ADP were substituted for ATP and [^3H]ATP at equivalent specific activities. Experiments 1 and 3 were performed with 0.1 ml of enzyme plus 0.3-ml substrates. Experiment 2 was performed with 0.2-ml reaction mixtures.

FIG. 2. Kinetics of incorporation of [^3H]AMP from [^3H]ATP and [^3H]ADP. Reaction mixtures were prepared with either normal ATP substrates (■) or with ADP replacing ATP (see Table 2) (●) and portions of a fraction IV preparation. Samples (0.1 ml each) were withdrawn and acid-precipitable radioactivity was determined at the indicated times. Ordinate values have been multiplied by 10^{-3} .

The possibility that an adenylate kinase was involved in the ADP reaction was tested in several ways. If an adenylate kinase were responsible for the production of ATP from ADP, the side product, 5'-AMP would be expected to act as an inhibitor of the incorporation of label into acid-insoluble material from ADP substrates but not from ATP substrates. To test this, increasing amounts of 5'-AMP were added to either ATP or ADP reaction mixtures (Table 3). The ATP reaction is insensitive to 5'-AMP but the ADP reaction is 50% inhibited at an AMP:ADP ratio of 0.2:1.0. These results suggest that an adenylate kinase produces ATP and AMP from added ADP and the poly(A) polymerase then makes poly(A) from the resulting ATP.

In another experiment, ADP reaction mixtures were tested for the presence of AMP and ATP at various times after the initiation of the reaction. The AMP level rose throughout the 100 min of incubation and the ATP level initially rose and then fell. The amount of poly(A) increased. These results were consistent with the hypothesis that ATP was the immediate substrate for poly(A) synthesis and suggested that the reaction was not a polynucleotide phosphorylase-like reaction.

Properties of the poly(A) polymerase: magnesium optimum. Incorporation of ATP into acid-insoluble material was absolutely dependent on divalent metal ions. Both Mg^{2+} and

TABLE 3. Effect of 5'-AMP on poly(A) synthesis from [^3H]ADP and [^3H]ATP

[AMP]/[ADP] or [AMP]/[ATP] ^a	Percent incorporation with	
	ATP substrates	ADP substrates
0	100	100
0.02	101	88
0.04	114	84
0.10	104	78
0.20	95	51
1.00	99	28
2.00	109	18

^a Increasing concentrations of 5'-AMP were added to normal poly(A) polymerase reactions which contained either ATP or ADP as the substrate. The incorporation of label into acid-insoluble material was determined after incubation at 37 C for 30 min.

Mn^{2+} were capable of supporting comparable levels of poly(A) synthesis. The optimum concentration of Mg^{2+} was very sharp and occurred at 0.005 M MgCl_2 . The dependence on Mn^{2+} was bimodal with maximum incorporation occurring at 0.002 M MnCl_2 . Magnesium chloride was routinely used.

Effect of inhibitors, other substrates, and oligonucleotides. DNA-dependent RNA polymerase inhibitors have no effect on the enzyme activity (Table 4). Neither actinomycin D nor α -amanitin inhibit ATP incorporation.

Other ribonucleotides do not inhibit the incorporation of ATP into acid-insoluble material. Addition of the three other ribonucleoside triphosphates, each at four times the concentration of ATP, caused only partial inhibition (21%).

Adenosine triphosphate analogues have various effects. The analogues AMPPcP and AMPcPP are not inhibitory to ATP incorporation. At a concentration equal to that of ATP, both analogues are stimulatory and at a 10-fold excess no effect is evident for either compound. If unlabeled ATP is replaced in the reaction mixture by 2'-dATP, incorporation is inhibited by 85%.

Oligoriboadenylic acid, (rAp) $_4\text{A}_{\text{OH}}$, was added to a reaction mixture and caused a slight inhibition of incorporation of ATP (Table 4). Oligomers of deoxyribonucleotides were also tested for their effect on poly(A) synthesis. Oligo(dT), (dT) $_{14}$, was without effect and (dC) $_{14}$ was slightly stimulatory. The inclusion of KCl at 0.1 M was inhibitory (37%). The inclusion of (dT) $_{14}$ and oligo(A) in the 0.1 M KCl reaction mixture had an even greater inhibitory effect.

Effect of polynucleotides on poly(A) synthesis. Increasing concentrations of polynucleo-

TABLE 4. Effect of inhibitors, nucleotides, and oligonucleotides on poly(A) synthesis

Additions ^a	Counts/min incorporated	Un-treated (%)
Expt 1		
Control—no additions	11,560	100
A. Inhibitors		
Actinomycin D (25 $\mu\text{g}/\text{ml}$)	11,110	96
α -Amanitin (5 $\mu\text{g}/\text{ml}$)	10,708; 12,660	100
B. Other substrates (0.2 $\mu\text{mol}/\text{mix}$)		
UTP	10,960	95
CTP	10,420	91
CTP	11,300	98
UTP, CTP and GTP	9,140	79
C. ATP analogues		
AMPPcP		
0.05 $\mu\text{mol}/\text{mix}$	20,855	180
0.10 $\mu\text{mol}/\text{mix}$	13,206	114
0.50 $\mu\text{mol}/\text{mix}$	11,425	99
AMPcPP		
0.05 $\mu\text{mol}/\text{mix}$	20,920	180
0.10 $\mu\text{mol}/\text{mix}$	20,050	180
0.50 $\mu\text{mol}/\text{mix}$	11,363	99
Expt 2		
Control—no additions	5,470	100
A. Substitute dATP for ATP	357	6
B. Oligonucleotides		
10 μM oligo (rA)	4,626	85
dT $_{14}$ (100 $\mu\text{g}/\text{ml}$)	5,197	95
dC $_{14}$ (100 $\mu\text{g}/\text{ml}$)	6,832	125
0.1 M KCl	3,430	63
dT $_{14}$, oligo(rA) in 0.1 M KCl	2,473	45

^a Additions to usual 0.4-ml (section 1) or 0.2-ml (section 2) reaction mixtures.

tides were added to reaction mixtures and the effect on ATP incorporation was determined (Fig. 3). Poly(A) and poly(C) are slightly stimulatory at low concentrations. Poly(C) appears to be without effect at high concentrations and poly(A) is slightly inhibitory at 100 $\mu\text{g}/\text{ml}$. Poly(dT) inhibits the incorporation of [^3H]ATP into acid-insoluble material at all concentrations tested.

Poly(U) stimulates incorporation up to three- to fourfold at concentrations from 20 to 50 $\mu\text{g}/\text{ml}$ but is inhibitory at 250 $\mu\text{g}/\text{ml}$. Poly(A) inhibits the stimulation by poly(U) presumably by interacting with poly(U). This is suggested since the activity at a given excess of poly(U) over poly(A) is similar to the activity in the presence of that concentration of poly(U) alone. Poly(C) has no effect on the poly(U) stimulation of poly(A) synthesis (data not shown). It is shown in the following paper (1a) that poly(U) is able to act as a "primer" for ATP incorporation into poly(A).

Effect of nuclease on incorporation of ATP into poly(A). If the enzyme activity depended

FIG. 3. Effect of polynucleotides on poly(A) synthesis. Increasing concentrations of the indicated polynucleotide were added to standard 0.2-ml reaction mixtures. The acid-precipitable radioactivity was determined after 60 min at 37 C. In the inset in the left-hand frame, is shown the effect of adding increasing amounts of poly(A) to reaction mixtures that contained 25 µg of poly(U) per ml. Symbols: ●, poly(U); □, poly(A) at 25 µg of poly(U) per ml; ■, poly(A) alone; ○, poly(C); ▲, poly(dT).

only on added substrate and metal ions, pancreatic RNase treatment at 0.2 M KCl should have no effect on the incorporation of label into acid-insoluble material. Likewise, T_1 RNase should not inhibit if there is no requirement other than that for ATP and Mg^{2+} . Pretreatment of the enzyme with either RNase was inhibitory, suggesting that endogenous RNA of usual base composition was involved in the *in vitro* synthesis of poly(A) by fraction IV (Table 5).

DNase pretreatment had little effect on the incorporation of ATP into acid-insoluble material. These results suggested that RNA, but not DNA, was involved in the synthesis of poly(A) by the enzyme preparation.

RNA dependence of enzyme activity. To examine the RNA dependence of the poly(A) polymerase reaction, an attempt was made to stimulate poly(A) synthesis by adding RNA to a DEAE-cellulose chromatographed portion of fraction IV. A portion of fraction IV was adsorbed to DEAE-cellulose in TKEE and stepwise elution was performed with increasing concentrations of KCl. Enzyme activity was located in the 0.2 M KCl wash. Two RNA preparations were tested for their ability to stimulate poly(A) synthesis by this preparation (Fig. 4). HeLa cell cytoplasmic RNA and RNA isolated by phenol:CHCl₃ extraction of a fraction IV preparation were both able to stimulate poly(A) synthesis. There was, however, a 10- to 20-fold difference in the stimulatory efficiency of the two RNA preparations. Although RNA isolated from fraction IV was able to stimulate

TABLE 5. Effect of RNase and DNase on *in vitro* poly(A) synthesis

Pretreatment	Counts/min incorporated	Untreated (%)
None	14,115; 11,981	100
Pancreatic RNase ^a	100; 100	0.76
0.2 M KCl	2,478; 3,364	22
Pancreatic RNase in 0.2 M KCl	152; 110	0.90
T_1 RNase	207; 194	1.50
MgCl ₂ (0.01 M)	8,636; 8,724	67
DNase in 0.01 M MgCl ₂	8,437; 6,675	59

^a Pancreatic and T_1 RNase were added to 5 µg/ml. DNase was added to 20 µg/ml. Preincubation was performed on 50-µl portions of fraction IV enzyme at 37 C for 30 min prior to the addition of substrates. Substrates were added and acid-insoluble material was determined after 60 min at 37 C.

poly(A) synthesis at very low concentrations, it also appeared to inhibit poly(A) synthesis when added at higher concentrations. The RNA present in fraction IV is described in greater detail in the following paper.

Effects of T_1 RNase on poly(A) synthesis. Since T_1 RNase is incapable of degrading poly(A), it was reasonable to predict that, after starting *in vitro* synthesis of poly(A), T_1 RNase would have no effect on continued poly(A) synthesis. To test this possibility poly(A) polymerase reactions were allowed to incubate at 37 C and T_1 RNase was added at various times after the initiation of the incubation. Acid-insoluble label was determined at 60 min total incubation

FIG. 4. RNA dependence of eluates from DEAE-cellulose. Fraction IV was applied to DEAE-cellulose (0.5 by 20 cm) in TKEE and washed with TKEE and then step-wise eluted with 0.1, 0.2, 0.5, and 0.8 M KCl in 0.01 M Tris-hydrochloride, pH 8.5, 0.0005 M EDTA, 0.01 M 2-mercaptoethanol. The protein-containing fractions of each wash were dialyzed overnight versus 0.01 M Tris-hydrochloride, pH 8.5, 0.0005 M EDTA, 0.01 M KCl. Enzyme activity of each wash was determined by adding 250 μ g of HeLa cytoplasmic RNA per ml; activity was located in the dialyzed 0.2 M KCl wash. Increasing amounts of either HeLa cytoplasmic RNA or RNA isolated by CHCl_3 /phenol extraction and ethanol precipitation of a fraction IV preparation were added to the dialyzed 0.2 M KCl wash. Incorporation of [^3H]AMP into acid-insoluble material was determined after 60 min at 37 C and is expressed as the stimulation above activity in the absence of added RNA. Symbols: $\bullet$, RNA isolated from a fraction IV preparation; $\circ$, HeLa cytoplasmic RNA.

time. Figure 5 shows the results of this experiment. The amount of label incorporated increased as the time of incubation prior to RNase addition was increased. T_1 RNase was, therefore, able to inhibit the incorporation of ATP into acid-insoluble material at any time during the reaction.

This effect was not due to the production of 3' phosphates as the inclusion of bacterial alkaline phosphatase did not reduce the effect of T_1 RNase. The rate at which poly(A) synthesis was inhibited was dependent on the concentration of T_1 RNase; 1 $\mu\text{g}/\text{ml}$ inhibited incorporation after 2 to 3 min but 0.1 $\mu\text{g}/\text{ml}$ inhibited incorporation only after 10 to 15 min. Complete inactivation of the poly(A) polymerase preparation with T_1 RNase required a preincubation period, the length of which varied with the concentration of T_1 RNase used. These experiments suggested that both the initiation and continued *in vitro* synthesis of poly(A) is dependent on some intact endogenous RNA.

FIG. 5. Effect of T_1 RNase on continued incorporation of [^3H]AMP by fraction IV poly(A) polymerase. Eight 0.2-ml reaction mixtures were made as described in Materials and Methods. At various times after the initiation of incubation at 37 C, T_1 RNase was added to 1 $\mu\text{g}/\text{ml}$. The incorporation of [^3H]AMP into acid-insoluble material in each mixture was determined after 60 min total incubation time.

The inclusion of poly(U) in the reaction mixture allows incorporation of ATP in the presence of T_1 RNase (Fig. 6). The amount of incorporation in the presence of T_1 RNase is dependent on the concentration of poly(U). Neither the addition of poly(A) nor the addition of poly(C) stimulated incorporation in the presence of T_1 RNase (Fig. 6). Preincubation of the enzyme preparation with T_1 RNase (4.0 $\mu\text{g}/\text{ml}$) for up to 4 h does not result in complete inhibition of ATP incorporation provided poly(U) is added with the substrate mixture (see following paper). Under these conditions poly(U) acts as a primer for poly(A) synthesis.

DISCUSSION

Although poly(A) has been found on most cellular and viral mRNAs and on heterogeneous nuclear RNA, little is known about the mechanism of poly(A) polymerization on RNA. Numerous poly(A) polymerase activities have been described (5, 6, 14, 15, 25) but no clear demonstration that any of these enzymes are capable of polymerizing poly(A) on RNA has been made. The poly(A) polymerases previously described are responsive to poly(A) (6, 11, 12) or to oligomers of AMP (25). Although some poly(A) polymerase preparations are responsive to added RNA, the ability of various RNAs to serve as primers and preference for messenger-like RNAs have not been unequivocally established.

FIG. 6. Protection of poly(A) polymerase activity from T_1 RNase by poly(U). Portions of 0.15-ml substrates were combined with T_1 RNase (0.2 μ g) and varying amounts of polynucleotide. Enzyme (50 μ liters of fraction IV) was added and incubation at 37°C was started immediately and the incorporation of label was determined at 60 min.

Since vaccinia virus-specific RNAs synthesized in the cytoplasm of infected cells contain poly(A) (17, 18) we expected that an enzyme capable of polymerizing poly(A) from ATP would be found in the infected cell cytoplasmic fraction. Indeed, a poly(A) polymerase activity is induced in the cytoplasm. This enzyme is probably distinct from RNA polymerase since it is maximally induced when RNA polymerase activity is low and partial purification of the poly(A) polymerase frees it of RNA polymerase activity.

In some respects this enzyme differs markedly from the cellular poly(A) polymerases, from poly(A) polymerase isolated from vaccinia virions (22) and from the terminal transferase of calf thymus (25) and of vaccinia virions (2). The three other ribonucleoside triphosphates have little effect on poly(A) synthesis by fraction IV (Table 4) but significantly inhibit terminal transferases. Short polymers of AMP [e.g., $r(Ap)_4A_{OH}$] are necessary for terminal transferase activity. This primer has little effect on

poly(A) synthesis by the vaccinia virus induced enzyme (Table 4). Terminal transferase is more active with Mn^{2+} ion, while the enzyme described in this report is equally active with either Mn^{2+} or Mg^{2+} ion.

The poly(A) polymerase from calf thymus nuclei (6) is stimulated by poly(A) as is the enzyme isolated from ascites (11, 12). Moss et al. (22), found that poly(rC) and poly(dA)-poly(dT) stimulated poly(A) synthesis by the poly(A) polymerase isolated from vaccinia virions. In contrast to these results, the enzyme preparation induced in vaccinia virus infected HeLa cells was minimally affected by poly(A) or poly(C), strongly inhibited by poly(dT) and maximally stimulated by poly(U). Poly(A) was an efficient inhibitor of the stimulation afforded by poly(U). In contrast to this, Giron and Huppert (12) observed that the ascites cell enzyme was stimulated by added poly(A) and this stimulation was inhibited by poly(U).

Giron and Huppert (12) showed that RNA or poly(A) had a stimulatory effect on poly(A) synthesis after DEAE-Sephadex chromatography of the enzyme preparation from ascites cells. Results of DEAE-cellulose chromatography of fraction IV suggested that this preparation contained bound nucleic acid which was required for poly(A) synthesis. Indeed, RNA obtained by $CHCl_3$:phenol extraction of a fraction IV preparation was able to stimulate poly(A) synthesis by a DEAE-cellulose eluate (Fig. 4). HeLa cell cytoplasmic RNA was also stimulatory although maximum stimulation was obtained at higher RNA concentrations than with RNA from fraction IV.

The RNA dependence of poly(A) synthesis was further demonstrated by nuclease treatment (Table 5). Pretreatment with pancreatic or T_1 RNase completely inhibited poly(A) synthesis, whereas DNase pretreatment was without effect. Continued synthesis of poly(A) was dependent on intact RNA since T_1 RNase was able to inhibit on-going poly(A) synthesis. These results strongly suggested that poly(A) synthesis by this preparation was dependent on RNA which closely resemble in vivo RNA. The following paper (1a) demonstrates that this enzyme adds poly(A) to endogenous vaccinia virus mRNA as well as to added poly(U).

LITERATURE CITED

1. Armstrong, J. A., M. Edmonds, H. Nakazato, B. A. Phillips, and M. H. Vaughn. 1972. Polyadenylic acid sequences in the virion RNA of poliovirus and eastern encephalitis virus. *Science* 176:526-528.
- 1a. Brakel, C., and J. R. Kates. 1974. Poly(A) polymerase from vaccinia virus-infected cells. II. Product and primer characterization. *J. Virol.* 14:724-732.
2. Brown, McK., J. W. Dorson, and F. J. Bollum. 1973. Terminal riboadenylate transferase: a poly(A) polymer-

- ase in purified vaccinia virus. *J. Virol.* **12**:203-208.
3. Darnell, J. E., Jr., L. Philipson, R. Wall, and M. Adesnik. 1971. Polyadenylic acid sequence: role in conversion of nuclear RNA into messenger RNA. *Science* **174**:507-510.
 4. Darnell, J. E., Jr., R. Wall, and R. J. Tushinski. 1971. An adenylate acid-rich sequence in mRNA of HeLa cells and its possible relationship to reiterated sites in DNA. *Proc. Nat. Acad. Sci. U.S.A.* **68**:1321-1325.
 5. Edmonds, M., and R. Abrams. 1960. Polynucleotide biosynthesis: formation of a sequence of adenylate units from adenosine triphosphate by an enzyme from thymus nuclei. *J. Biol. Chem.* **235**:1142-1149.
 6. Edmonds, M., and R. Abrams. 1962. Nature of a polynucleotide required for polyribonucleotide formation from adenosine triphosphate with an enzyme from thymus nuclei. *J. Biol. Chem.* **237**:2636-2642.
 7. Edmonds, M., M. H. Vaughn, and H. Nakazato. 1971. Polyadenylic acid sequences 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.
 8. Ehrenfeld, E., and D. F. Summers. 1972. Adenylate-rich sequences in vesicular stomatitis virus messenger ribonucleic acid. *J. Virol.* **10**:683-688.
 9. Galet, H., and L. Prevec. 1973. Polyadenylate synthesis by extracts from L cells infected with vesicular stomatitis virus. *Nature N. Biol.* **243**:200-203.
 10. Gillespie, D., S. Marshall, and R. C. Gallo. 1972. RNA of RNA tumour viruses contains poly(A). *Nature N. Biol.* **236**:227-231.
 11. Giron, M. L., and J. Huppert. 1972. Polyadenylate synthetase des cellules D' Ascites de Souris I. Purification et caracterisation de L' enzyme. *Biochim. Biophys. Acta* **287**:438-447.
 12. Giron, M. L., and J. Huppert. 1972. Polyadenylate synthetase des cellules D' Ascites de Souris II. Etude de la reaction enzymatique. *Biochim. Biophys. Acta* **287**:448-455.
 13. Green, M., and M. Cartas. 1972. The genome of RNA tumor viruses contain polyadenylic acid sequences. *Proc. Nat. Acad. Sci. U.S.A.* **69**:791-794.
 14. Hyatt, E. A. 1967. Polyriboadenylate synthesis by nuclei from developing sea urchin embryos I. Characterization of the ATP polymerase reaction. *Biochim. Biophys. Acta* **142**:246-253.
 15. Hyatt, E. A. 1967. Polyriboadenylate synthesis by nuclei from developing sea urchin embryos. II. Polyriboadenylic acid priming of ATP polymerase. *Biochim. Biophys. Acta* **142**:254-262.
 16. Joklik, W. K. 1964. The intracellular uncoating of pox virus DNA. *J. Mol. Biol.* **8**:263-288.
 17. Kates, J. 1970. Transcription of the vaccinia virus genome and the occurrence of polyriboadenylic acid sequences in messenger RNA. *Cold Spring Harbor Symp. Quant. Biol.* **35**:743-752.
 18. Kates, J., and J. Beeson. 1970. Ribonucleic acid synthesis in vaccinia virus. II. Synthesis of polyriboadenylic acid. *J. Mol. Biol.* **50**:19-33.
 19. Kates, J., R. Dahl, and M. Mielke. 1968. Synthesis and intracellular localization of vaccinia virus deoxyribonucleic acid-dependent ribonucleic acid polymerase. *J. Virol.* **2**:894-900.
 20. 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.
 21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
 22. Moss, B., E. N. Rosenblum, and E. Paoletti. 1973. Polyadenylate polymerase from vaccinia virions. *Nature N. Biol.* **245**:59-63.
 23. Philipson, L., R. Wall, R. Glickman, and J. E. Darnell, Jr. 1971. Addition of polyadenylate sequences to virus specific RNA during adenovirus replication. *Proc. Nat. Acad. Sci. U.S.A.* **68**:2806-2809.
 24. Sheldon, R., C. Jurale, and J. Kates. 1972. Detection of polyadenylic acid sequences in viral and eukaryotic RNA. *Proc. Nat. Acad. Sci. U.S.A.* **69**:417-421.
 - 24a. Sheldon, R., and J. Kates. 1974. Mechanism of poly(A) synthesis by vaccinia virus. *J. Virol.* **14**:214-224.
 25. Tsiapalis, C. M., J. W. Dorson, D. M. De Sante, and F. J. Bollum. 1973. Terminal riboadenylate transferase: a polyadenylate polymerase from calf thymus gland. *Biochem. Biophys. Res. Commun.* **53**:737-743.
 26. Twu, J. S., and R. K. Bretthauer. 1971. Properties of a polyriboadenylate polymerase isolated from yeast ribosomes. *Biochemistry* **10**:1576-1582.
 27. Weinberg, R. A., Z. Ben-Ishai, and J. E. Newbold. Poly(A) associated with SV-40 messenger RNA. *Nature N. Biol.* **238**:111-113.
 28. Yogo, Y., and E. Wimmer. 1972. Polyadenylic acid at the 3'-terminus of poliovirus RNA. *Proc. Nat. Acad. Sci. U.S.A.* **69**:1877-1882.