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Multiple Roles for ATP in the Synthesis and Processing of mRNA by Vaccinia Virus: Specific Inhibitory Effects of Adenosine (β,γ-Imido)Triphosphate

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Adenosine (β,γ-imido)triphosphate (AMP-PNP) and guanosine (β,γ-imido)-triphosphate (GMP-PNP) are analogs of ATP and GTP with non-hydrolyzable γ-phosphates. Although both AMP-PNP and GMP-PNP were used in place of ATP and GTP by *Escherichia coli* RNA polymerase to transcribe vaccinia virus DNA, only GMP-PNP was used by the transcriptase present within vaccinia virus cores. AMP-PNP specifically prevented initiation of transcription, since RNA initiated in the presence of ATP, GTP, and CTP was subsequently elongated by incubating the washed cores in the presence of AMP-PNP, GTP, CTP, and UTP. The RNA formed in this manner, however, was (i) several times longer than normal transcripts, indicating a defect in chain termination and/or cleavage of nascent RNA, (ii) was not polyadenylylated (although free polyadenylic acid formed), and (iii) was not extruded from the virus cores. Nearest neighbor analysis demonstrated that AMP-PNP was incorporated adjacent to all four nucleotides, and hybridization to restriction endonuclease fragments of vaccinia virus DNA indicated that the high-molecular-weight RNA was transcribed from representative fractions of the entire genome. The possibility of a block in processing rather than or in addition to a block in chain termination was suggested by the cleavage of the high-molecular-weight RNA within the core after replacement of AMP-PNP with ATP. Cleavage of purified high-molecular-weight RNA by a soluble endoribonuclease extracted from vaccinia virus cores, however, was not dependent upon ATP, nor was it inhibited by AMP-PNP. The latter results suggest that AMP-PNP blocks a step preceding cleavage.

In vitro synthesis of RNA by vaccinia virus cores depends on relatively high ATP concentrations (8, 16). That this requirement might not simply reflect a high K_m of the DNA-dependent RNA polymerase was suggested by the detection of core-associated adenosine triphosphatase activities (3, 17). Further studies led to the characterization of several enzymes that use ATP, including two immunologically distinct nucleic acid-dependent ribonucleoside triphosphatases (21, 23, 24), a protein kinase (9), and a polyadenylic acid [poly(A)] polymerase (15). The findings that RNA synthesis did not occur when adenosine (β,γ-methylene)triphosphate was substituted for ATP (29) and that the same analog prevented extrusion of RNA from vaccinia virus cores (28) pointed to the importance of a hydrolyzable β,γ-phosphodiester linkage. Using another ATP derivative, adenosine (β,γ-imido)triphosphate (AMP-PNP), we now report that a hydrolyzable γ-phosphate is required for initiation of RNA synthesis by vaccinia virus cores but not for chain elongation. However, the RNA elongated in the presence of AMP-PNP remains core associated, is not polyadenylylated, and is several times longer than vaccinia virus mRNA, indicating interference with chain termination and/or processing. Although the latter possibility is suggested by subsequent cleavage of the high-molecular-weight RNA after replacement of the analog with ATP, AMP-PNP does not inhibit the activity of a soluble endoribonuclease extracted from vaccinia virus cores.

MATERIALS AND METHODS

Virus. Vaccinia virus (strain WR) was purified from the cytoplasm of infected HeLa cells by sedimentation through a cushion of sucrose followed by two cycles of sucrose gradient centrifugation essentially as described by Joklik (6).

RNA synthesis. Under standard conditions for RNA synthesis, purified virus (1.3 to 2 units of absorbance [at 260 nm] per ml) was incubated in 50 mM Tris-hydrochloride (pH 8.5)-10 mM dithiothreitol-0.05% Nonidet P-40 detergent-10 mM $MgCl_2$-2 to 5

mM ATP-0.2 to 2 mM GTP-0.2 mM CTP-0.05 mM [^{3}H]UTP (0.8 μCi/mmol)-10 μM *S*-adenosylmethionine at 37°C.

To activate virus particles and initiate RNA chains, purified virus (4 units of absorbance [at 260 nm] per ml) was incubated in 50 mM Tris-hydrochloride (pH 8.5)-10 mM dithiothreitol-0.5% Nonidet P-40 detergent for 10 min at 37°C. After this, ATP (2 to 5 mM), GTP (1 mM), and CTP (0.2 mM) were added, and the incubation was continued for 5 min at 37°C. In later experiments, the activation step was simplified by adding the three ribonucleoside triphosphates at the same time as the detergent and dithiothreitol and incubating the mixture for 15 min. The activated cores were then diluted with cold buffer A (50 mM Tris-hydrochloride [pH 8.5], 10 mM dithiothreitol, and 0.05% Nonidet P-40 detergent) and sedimented at 8,000 or 12,000 × *g* for 1 min in an Eppendorf microcentrifuge. This is a critical step; if centrifugation is prolonged, the particles are difficult to disperse and have low transcriptase activity. The virus cores were gently resuspended in buffer A and washed once again by centrifugation to remove remaining ATP. In a control experiment with [^{3}H]ATP, we determined that 99.96% of the ATP was removed by the second wash. Chain elongation was then measured in reaction mixtures similar to the standard one used for RNA synthesis except that ATP was replaced by AMP-PNP. No contaminating ATP was detected in the AMP-PNP by polyethyleneimine-cellulose thin-layer chromatography.

Sucrose gradient sedimentation. RNA synthesis was terminated by additions of sodium dodecyl sulfate and EDTA to final concentrations of 0.5 to 1% and 20 mM, respectively. In some experiments, core-associated RNA was separated from released RNA by centrifugation at 8,000 or 12,000 × *g* for 2 min before sodium dodecyl sulfate-EDTA treatment. RNA was analyzed directly by sucrose gradient sedimentation or after phenol-chloroform extraction and ethanol precipitation. In either case, before sedimentation, RNA was always heated at 100°C for 2 min to dissociate any aggregates. Sucrose gradients, 5 to 20% in 10 mM sodium acetate (pH 6.0)-0.1 M NaCl-0.1% sodium dodecyl sulfate, were centrifuged at 18°C in an SW41 rotor at 40,000 rpm for 270 min or in an SW60 rotor at 50,000 rpm for 110 min. Fractions were collected from the bottom of the tube, and portions were precipitated with trichloroacetic acid and collected on Whatman GF/C glass fiber filters for scintillation counting.

RNA sedimenting between 18 and 28S was precipitated with ethanol and dissolved in water, and the solution was adjusted to final concentrations of 90% dimethyl sulfoxide, 10 mM LiCl, and 0.5 mM EDTA (pH 7.5). After heating at 60°C for 10 min, the RNA was loaded onto a 5 to 20% sucrose gradient in 99% dimethyl sulfoxide-0.5 mM EDTA (pH 7.5)-10 mM LiCl and centrifuged in an SW50.1 rotor at 189,000 × *g* for 20 h at 23°C.

Nearest neighbor analysis. RNA labeled with [α-^{32}P]AMP-PNP was phenol-chloroform extracted, ethanol precipitated, fractionated by sucrose gradient centrifugation, ethanol precipitated, and passed through a Sephadex G-50 column equilibrated with 0.05 M ammonium acetate. The RNA was lyophilized and then digested with 2.5 U of RNase T_2 in 50 μl of 50 mM ammonium acetate (pH 4.5) for 16 h at 37°C. After addition of carrier mononucleotides, the products were separated by high-voltage paper electrophoresis at pH 3.5. Labeled mononucleotides were located by UV absorption of internal markers and by fluorography with DuPont Cronex intensifier screens. The spots were cut out, and the amounts of radioactivity were determined by scintillation counting.

Preparation of soluble core extracts. Purified vaccinia virus particles were treated with Nonidet P-40 detergent and dithiothreitol, and the resulting cores were sedimented through a cushion of sucrose. The cores were disrupted by deoxycholate treatment, after which the insoluble structural proteins were removed by centrifugation and the DNA was removed by passage through a DEAE-cellulose column (24). Endoribonuclease activity was measured in a 0.1-ml reaction containing 50 mM Tris-hydrochloride (pH 8.5) and 1 mM dithiothreitol.

Sources of materials. Ribonucleoside triphosphates were from P-L Biochemicals except for AMP-PNP and GMP-PNP, which were obtained from Boehringer Mannheim Biochemicals. [^{3}H]UTP (55 Ci/mmol), [α-^{32}P]UTP (341 Ci/mmol), and [α-^{32}P]AMP-PNP were from Amersham/Searle, New England Nuclear, and ICN Pharmaceuticals, respectively.

RESULTS

Inhibition of RNA synthesis by AMP-PNP. The analogs AMP-PNP and GMP-PNP differ from ATP and GTP in the substitution of an NH group for the terminal bridge oxygen. Since the α-β-phosphodiester linkage is unaltered, such analogs are generally suitable for RNA synthesis (4). Indeed, when vaccinia virus was incubated in a standard reaction mixture containing GMP-PNP in place of GTP, transcription occurred at only a modestly reduced rate (Fig. 1A). In contrast, substitution of AMP-PNP for ATP prevented any detectable RNA synthesis by vaccinia virus cores (Fig. 1A). Nevertheless, both AMP-PNP and GMP-PNP were utilized by *Escherichia coli* RNA polymerase for transcription of vaccinia virus DNA, although at reduced rates compared with the natural ribonucleoside triphosphates (Fig. 1B). Thus, the inability of AMP-PNP to substitute for ATP appears to be specific for the vaccinia virus transcriptional system.

Elongation of RNA chains with AMP-PNP. We considered that ATP with a hydrolyzable γ-phosphate might be required for initiation of RNA synthesis but not for chain elongation. Accordingly, virus particles were preincubated for varying times with all four ribonucleoside triphosphates, washed by repeated sedimentation, which removed 99.96% of the ATP, and suspended in a reaction mixture containing AMP-PNP in place of ATP. Such experiments indicated that chain elongation occurred after a

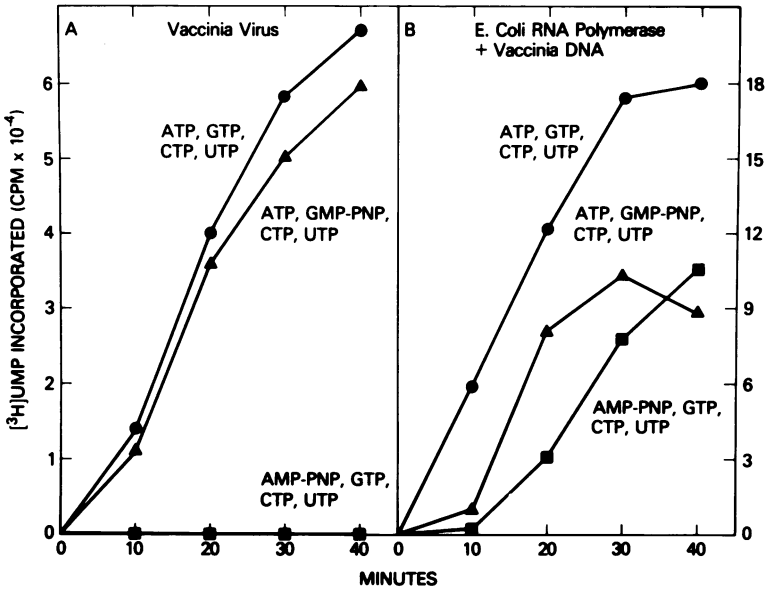

FIG. 1. *Effects of AMP-PNP and GMP-PNP on transcription. (A) Vaccinia virus transcription was measured in a standard reaction mixture, substituting 2 mM AMP-PNP for 2 mM ATP or 2 mM GMP-PNP for 2 mM GTP where indicated. (B) Transcription of vaccinia virus DNA by E. coli RNA polymerase was in a 0.25-ml reaction mixture containing 20 mM Tris-hydrochloride (pH 7.5), 0.15 M NaCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM ATP or AMP-PNP, 2 mM GTP or GMP-PNP, 0.2 mM CTP, 0.05 mM [^{3}H]UTP (0.4 μCi/mmol), 10 μM S-adenosylmethionine, 5 μg of vaccinia virus DNA, 14 μg of E. coli RNA polymerase, and 10 μg of bovine serum albumin.*

2- to 10-min preincubation at 37°C (Fig. 2). Additional studies (not shown) indicated that preincubation of vaccinia virus with ATP, GTP, and either CTP or UTP was as good as or better than preincubation with all four ribonucleoside triphosphates. An ATP concentration of 5 mM provided the maximal effect. Neither dATP nor adenosine (α,β-imido)triphosphate at 5 mM could substitute effectively for ATP in the preincubation step. That AMP-PNP was required for chain elongation and that RNA synthesis did not result simply from trace amounts of ATP trapped in the virus core is shown in Fig. 3. There was no significant incorporation when AMP-PNP was omitted, and the rate of RNA synthesis was proportional to AMP-PNP concentration. Incorporation of AMP-PNP into RNA will be shown below.

When the ATP concentration during preincubation and the AMP-PNP concentration during chain elongation were both 5 mM, the total amount of RNA synthesized by 30 min was about 34% of that made in the presence of the same concentration of ATP (Fig. 4). Most of the RNA made in the presence of AMP-PNP, however, was retained by the virus core (Fig. 4), whereas 68% of the RNA made in the presence of ATP was released. We concluded from these experiments that AMP-PNP could support

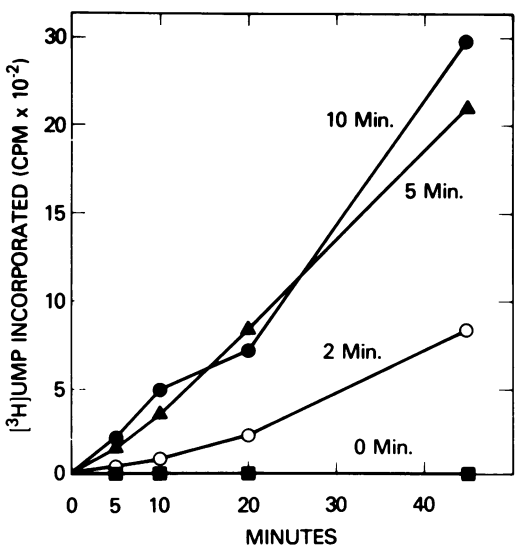

FIG. 2. *Effect of preincubation with all four ribonucleoside triphosphates on subsequent RNA synthesis with AMP-PNP. Virus particles were preincubated in a mixture containing ATP, GTP, CTP, and UTP as described in the text. After the indicated intervals, cores were sedimented and washed, and [^{3}H]UTP incorporation was measured in a standard transcription mixture containing AMP-PNP in place of ATP.*

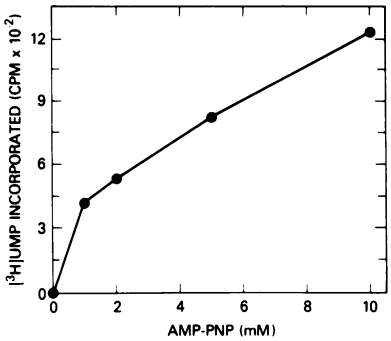

FIG. 3. *Effect of AMP-PNP concentration on the rate of RNA synthesis. After a preincubation step with ATP, GTP, and CTP, cores were washed and incorporation of [^{3}H]UTP was measured in standard transcription mixtures containing no ATP and 0 to 10 mM AMP-PNP.*

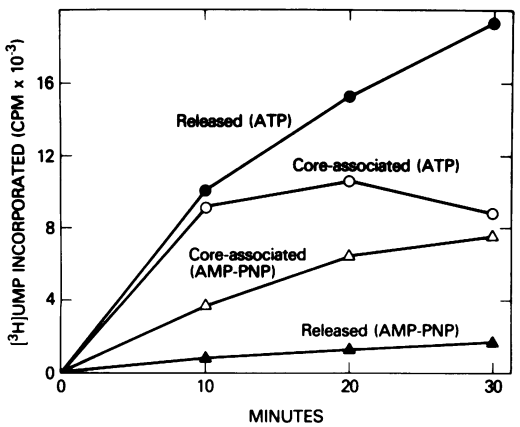

FIG. 4. *Comparison of rates of RNA synthesis in presence of ATP or AMP-PNP. Vaccinia virus cores were preincubated in the presence of 5 mM ATP, 1 mM GTP, and 0.2 mM CTP for 5 min, sedimented, washed, resuspended, and divided into two portions. One portion received 5 mM ATP and the other received 5 mM AMP-PNP in addition to the other components required for transcription. At intervals, portions were sedimented, and the amounts of core-associated and released RNA were determined by trichloroacetic acid precipitation.*

RNA chain elongation but that ATP was required for initiation of RNA synthesis and for extrusion of RNA.

RNA sedimentation properties. After a preincubation period to initiate RNA synthesis, the total RNA made during 30 min in the presence of AMP-PNP was analyzed by sucrose gradient sedimentation. In contrast to the RNA made in the presence of ATP, which sedimented at 8 to 12S, the RNA made in the presence of the analog sedimented mainly between 18 and 28S (Fig. 5). RNA labeled from 20 to 30 min (not shown) was similar in size to that labeled from 0 to 30 min, suggesting that new chains were not being initiated at the later times.

To confirm that the rapid sedimentation of RNA in sucrose gradients was a consequence of size, RNA in fractions between 18 and 28S was heated at 60°C in 90% dimethyl sulfoxide and then sedimented in a 99% dimethyl sulfoxide-sucrose gradient. Under denaturing conditions, the RNA still sedimented between 18 and 28S rRNA markers (Fig. 6).

The high-molecular-weight RNA could also be labeled with *S*-adenosyl[*methyl*-^{3}H]methionine. Analysis of the RNA after enzyme digestion and paper chromatography of the products as previously described (14) indicated the presence of the 5′ ends m^{7}G(5′)pppA^m and m^{7}G(5′)pppG^m in a ratio of 0.9. Corresponding ratios for core-associated and released RNA made under the same conditions, except for the use of ATP instead of AMP-PNP, were 1.0 and 0.9, respectively.

Incorporation of [α-^{32}P]AMP-PNP and nearest neighbor analysis. The incorporation of AMP-PNP into RNA was demonstrated by using the α-^{32}P-labeled compound. Virus particles were preincubated with ATP, GTP, and CTP to initiate RNA chains and then washed and suspended in a reaction mixture containing [α-^{32}P]AMP-PNP, [^{3}H]UTP, and unlabeled GTP and CTP. The core-associated RNA was then purified, denatured, and analyzed by su-

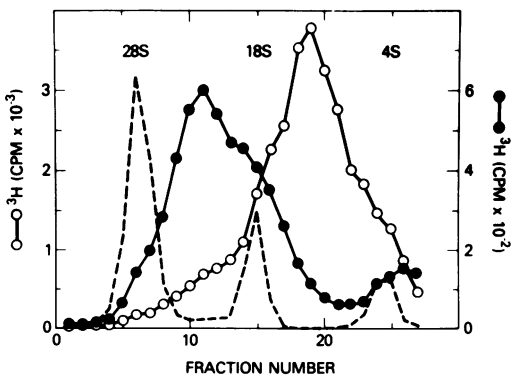

FIG. 5. *Sucrose gradient sedimentation of RNA made in the presence of ATP or AMP-PNP. After preincubation with all four ribonucleoside triphosphates, virus cores were incubated in reaction mixtures containing ATP (○) or AMP-PNP (●). Incorporation of [^{3}H]UTP was measured for 30 min, and the total RNA was analyzed by sucrose gradient sedimentation after extraction with sodium dodecyl sulfate and heating at 100°C for 2 min. [^{14}C]rRNA and [^{14}C]tRNA markers (- - -) were included in each gradient.*

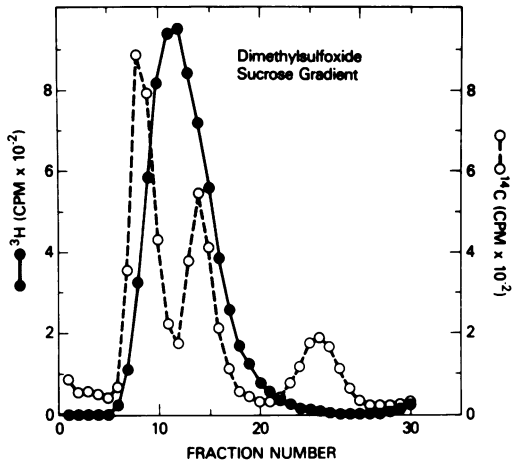

FIG. 6. *Sedimentation of high-molecular-weight RNA in dimethyl sulfoxide-sucrose gradients. RNA elongated in a transcription mixture containing AMP-PNP and [^{3}H]UTP was isolated from vaccinia virus cores by sodium dodecyl sulfate-phenol-chloroform extraction and ethanol precipitation. After heating at 100°C for 2 min, the RNA was sedimented as in Fig. 5. RNA sedimenting between 18 and 28S rRNA markers was ethanol precipitated, dissolved in water, heated at 60°C in 90% dimethyl sulfoxide, and then analyzed on a 99% dimethyl sulfoxide-sucrose gradient. [^{14}C]rRNA and [^{14}C]tRNA markers were included.*

crose gradient sedimentation (Fig. 7A). As expected, the [^{3}H]RNA sedimented as a broad peak primarily between 18 and 28S. In contrast, part of the ^{32}P-labeled material sedimented slightly faster than 4S, and the remainder sedimented as a broad peak from 18 to 28S. We suspected that the ^{32}P-labeled material sedimenting at 4S was poly(A), since [^{3}H]uridine was not incorporated. In agreement with this, 85% of the 4S ^{32}P-labeled material bound to a polyuridylic acid-Sepharose column, whereas less than 15% of the 18 to 28S RNA bound. When the virus cores were incubated directly in a mixture containing [^{3}H]UTP, [α-^{32}P]AMP-PNP, and unlabeled GTP and CTP without a prior incubation with ATP, there was no incorporation of ^{3}H, and ^{32}P was incorporated exclusively into 4S material (Fig. 7B).

The products isolated by sucrose gradient sedimentation in Fig. 7 were further characterized by nearest neighbor analysis. Upon RNase T_2 digestion of RNA, 3'-mononucleotides were released. Since the RNA was synthesized with [α-^{32}P]AMP-PNP, only those 3'-mononucleotides adjacent to incorporated AMP were labeled. In the case of the 4S material, significant amounts of radioactivity were associated only with AMP, providing proof that this was indeed poly(A) (Table 1). In contrast, all four mononucleotides were labeled upon digestion of the 18 to 28S RNA (Table 1). From these experiments we concluded that AMP derived from AMP-PNP is incorporated next to each of the four mononucleotides in high-molecular-weight RNA and that the majority of the high-molecular-weight RNA is not polyadenylated, even though AMP-PNP does not prevent initiation of free poly(A) synthesis.

Hybridization of core-associated RNA with DNA restriction endonuclease fragments. To rule out the possibility that only a very limited segment of the vaccinia genome is transcribed in the presence of AMP-PNP, hybridization to restriction endonuclease fragments was performed. After a standard preincubation to initiate RNA chains, RNA labeled with [α-^{32}P]UTP was synthesized in the presence of AMP-PNP or ATP. The core-associated RNA was isolated, denatured, and sedimented on sucrose gradients, where the difference in size

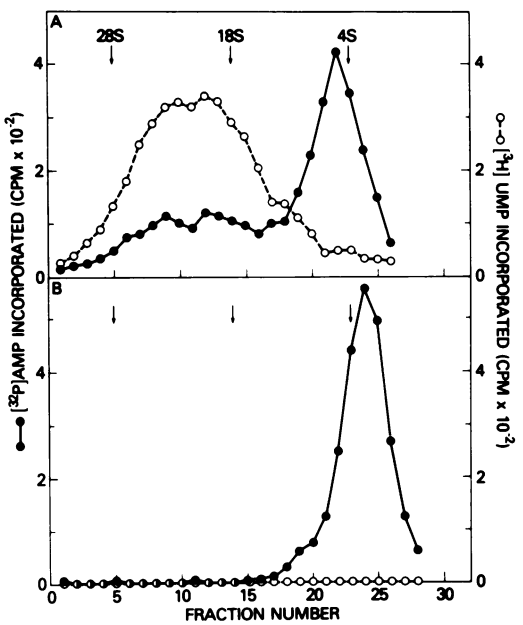

FIG. 7. *Sucrose gradient sedimentation of RNA labeled with [α-^{32}P]AMP-PNP. (A) After a standard preincubation, RNA was formed in the presence of [α-^{32}P]AMP-PNP and [^{3}H]UTP. After sodium dodecyl sulfate-phenol-chloroform extraction and ethanol precipitation, the RNA was heated at 100°C for 2 min and sedimented on a sucrose gradient. Portions of each fraction were precipitated with trichloroacetic acid and counted in a scintillation spectrophotometer. (B) Incorporation of [α-^{32}P]AMP-PNP and [^{3}H]UTP was performed as above except that the preincubation step with ATP, GTP, and CTP was omitted.*

TABLE 1. *Nearest neighbor analysis*[a]

Expt	RNA fraction	^{32}P activity associated with:							
		CMP		AMP		GMP		UMP	
		cpm	%	cpm	%	cpm	%	cpm	%
1	>18S	121.9	15.4	282.6	35.6	143.9	18.1	245.6	30.9
	4S	4.3	3.4	109.6	86.9	6.9	5.5	5.3	4.2
2	4S	18.1	0.3	6,280	99.6	1.8	0.0	5.4	0.1

[a] RNA contained in pooled fractions 4 to 13 (4S) and 20 to 24 (>18S) from Fig. 7A were used in experiment 1; RNA in fractions 22 to 26 from Fig. 7B were used in experiment 2. After digestion with RNase T_2, mononucleotides were separated by paper electrophoresis at pH 3.5.

between core-associated RNAs made in the presence and absence of AMP-PNP was evident (Fig. 8). The RNA made in the presence of AMP-PNP that sedimented at greater than 18S and the RNA made in the presence of ATP that sedimented at less than 18S were pooled separately. A complete *Hin*dIII restriction endonuclease digest of vaccinia virus DNA was prepared, and the fragments were separated by electrophoresis on a composite 0.6%–1.5% agarose slab gel. The pattern obtained was similar to that previously described for rabbitpox (31). The DNA fragments were then transferred to a nitrocellulose sheet by the procedure of Southern (27). Although all fragments were transferred, the procedure was not quantitative for the three largest segments of DNA. Significantly, the high-molecular-weight RNA formed in the presence of AMP-PNP hybridized to all of the DNA fragments (Fig. 9). Although there appear to be some differences in the relative amounts of high- and low-molecular-weight RNA hybridizing to individual restriction fragments, a more detailed analysis would be required to substantiate this.

Cleavage of high-molecular-weight RNA. The large size of the RNA made in the presence of AMP-PNP suggested that the ATP analog interfered with either chain termination or processing. To determine whether the high-molecular-weight [^{3}H]RNA formed during a 45-min period in the presence of AMP-PNP could be cleaved, the virus cores were sedimented and then incubated for 30 min in a standard reaction mixture containing ATP, GTP, CTP, and UTP. At the end of this time, both core-associated and released RNAs were analyzed by sucrose gradient sedimentation, which indicated that most of the RNA was reduced in size (Fig. 10). The major peak of core-associated RNA sedimented at about 12S with a smaller peak at about 18S, whereas the released RNA was mostly smaller than 12S. The finding that a major reduction in size occurred while the RNA was still core associated suggests that the endonuclease is within the core. Previously, Paoletti detected high-molecular-weight RNA synthesized in vitro under suboptimal conditions and reported that this RNA was reduced in size after a chase (19, 20) or after incubation with soluble core extracts (21a).

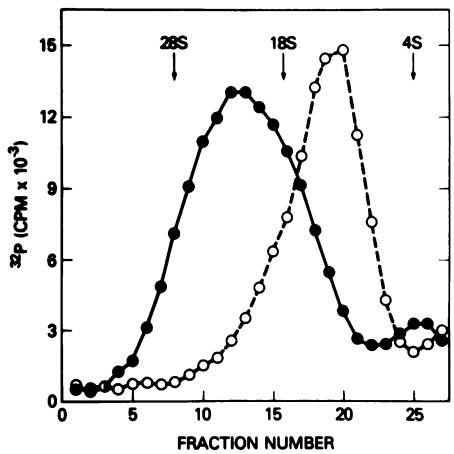

FIG. 8. *Sucrose gradient sedimentation of [α-^{32}P]UTP-labeled core-associated RNA. After a standard preincubation with ATP, GTP, and CTP, virus cores were washed and suspended in transcription mixtures containing [α-^{32}P]UTP (0.4 μCi/mmol) and either AMP-PNP (●) or ATP (○) for 45 min at 37°C. RNA was extracted from the virus cores with sodium dodecyl sulfate-phenol-chloroform and then ethanol precipitated, heat denatured, and sedimented on a 5 to 20% sucrose gradient. RNA was detected by Cerenkov radiation.*

Cleavage of high-molecular-weight RNA by a soluble endoribonuclease. Core-associated RNA synthesized in the presence of AMP-PNP and labeled with [α-^{32}P]UTP was isolated by sucrose gradient sedimentation and then incubated with soluble core extract for 30 min at 37°C. Cleavage of RNA, apparently to a limit size with increasing extract, occurred (Fig. 11). However, ATP was not required, and AMP-

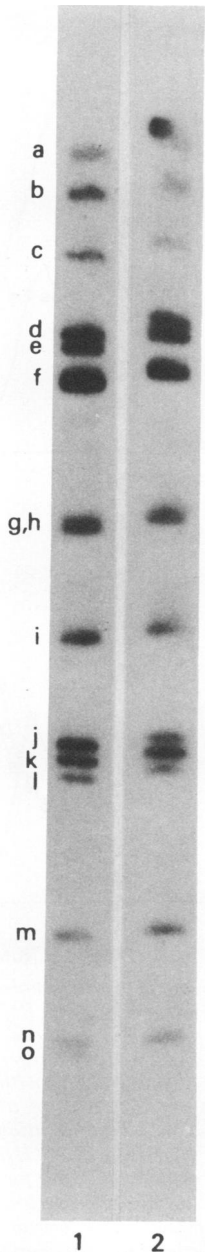

FIG. 9. *Hybridization of core-associated RNA with restriction endonuclease fragments of vaccinia virus DNA. The RNA made in the presence of AMP-PNP and sedimenting faster than 18S (lane 1) and RNA made in the presence of ATP and sedimenting slower than 18S (lane 2), as indicated in Fig. 8, were hybridized to HindIII restriction fragments of vaccinia virus DNA that were separated by agarose gel electrophoresis and transferred to a nitrocellulose sheet. The hybridization conditions were 0.9 M NaCl-0.09 M sodium citrate-0.1% sodium dodecyl sulfate-0.3 mg of salmon sperm DNA in a volume of 3 ml at 60°C for 48 h. The nitrocellulose sheets were treated with RNase T_1 (2.5 μg/ml) and RNase A (50 μg/ml) in 0.3 M NaCl-0.03 M sodium citrate for 1 h at 37°C and washed. Fluorography was performed with DuPont Cronex intensifier screens.*

PNP at 5 mM (plus 5 mM $MgCl_2$) did not prevent cleavage.

DISCUSSION

Although considerable information is available regarding the steps involved in mRNA biosynthesis in bacteria (1), relatively little is known for eucaryotic systems. Indeed, no in vitro system that can accurately initiate and synthesize defined eucaryotic mRNA from a DNA template is currently available. For this reason, vaccinia virus provides a unique model system. The purified DNA virus particle contains all of the enzyme and factors needed to synthesize capped, methylated, and polyadenylylated mRNA in vitro. Furthermore, the mRNA can be translated into authentic early proteins (2, 5, 18, 26; J. A. Cooper and B. Moss, Virology, in press).

As one approach to defining some of the steps involved in the biosynthesis of vaccinia virus mRNA, we have used AMP-PNP, a compound that closely resembles ATP stereochemically (32) but which has a non-hydrolyzable γ-phosphate. These studies indicate that AMP-PNP can be used for RNA chain elongation but that it blocks normal vaccinia virus transcription at four points: (i) initiation of RNA synthesis by virus cores, (ii) chain termination and/or cleavage of nascent RNA, (iii) polyadenylylation of the 3′ end of RNA [but not the synthesis of free poly(A)], and (iv) extrusion of RNA from the core. Significantly, transcription is not arrested at any of these steps when GMP-PNP is substituted for GTP.

The inability of the vaccinia virus DNA-dependent RNA polymerase to use AMP-PNP to form the first phosphodiester bond could readily explain the block in initiation of RNA chains beginning with adenosine. However, vaccinia virus RNA begins with $m^7G(5')pppG^m$ as well as $m^7G(5')pppA^m$ (30). If, as seems likely, RNA chains are initiated with GTP as well as ATP, it is difficult to understand why AMP-PNP prevents initiation of both types of RNA chains and why GMP-PNP has a rather small effect on transcription. A more attractive hypothesis is that ATP is required for some activation step that precedes initiation of all RNA chains. The protein kinase (9) and DNA-dependent adenosine triphosphatase (22) are examples of enzymes isolated from vaccinia virus cores that use

ATP, specifically require a hydrolyzable γ-phosphate, and have unknown biological functions. We noted previously (22) that phosphorylation of proteins in vaccinia virus cores precedes the linear synthesis of RNA and suggested a possible causal relationship between the two events. Watanabi et al. (29) have also proposed that protein phosphorylation might be linked to transcription.

After the initiation of RNA synthesis with ATP, GTP, and CTP, AMP-PNP can substitute for ATP in chain elongation. However, the RNA formed in the presence of this analog sediments from 18 to 28S, as compared with 8 to 12S for RNA made in the presence of ATP. That RNA made in the presence of AMP-PNP is several times longer than normal mRNA was confirmed by sedimentation in dimethyl sulfoxide-sucrose gradients. Of the three possibilities that we considered to explain the large size of the RNA, (i) failure of proper chain termination, (ii) inhibition of endonucleolytic cleavage, and (iii) addition of a very long poly(A) segment, only the last could be ruled out. Less than 15% of the high-molecular-weight RNA bound to polyuridylic acid-Sepharose, and nearest neighbor analysis, after [α-^{32}P]AMP-PNP labeling, did not reveal an unusual proportion of adenylate

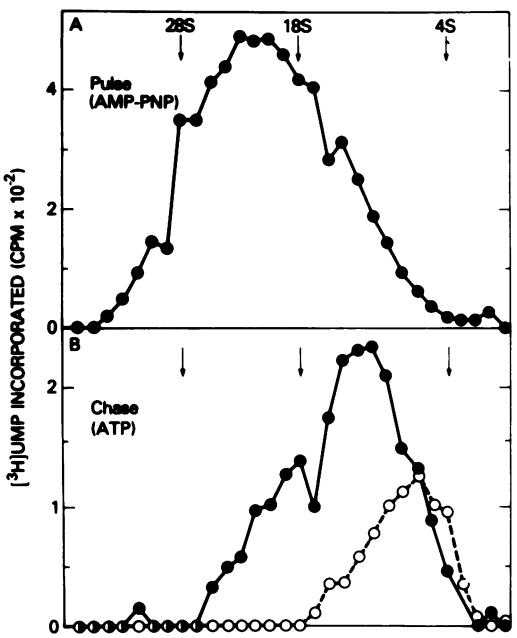

FIG. 10. *Cleavage of core-associated high-molecular-weight RNA. After chain initiation, virus cores were incubated in a complete transcription mixture containing AMP-PNP and [^{3}H]UTP for 45 min. At the end of this time, the cores were sedimented and washed, and half of them were incubated further at 37°C for 30 min with unlabeled ATP, GTP, CTP, and UTP under standard conditions. Core-associated and released RNAs were extracted with sodium dodecyl sulfate, heat denatured, and analyzed by sucrose gradient centrifugation. (A) Pulse-labeled core-associated RNA made in the presence of AMP-PNP. (B) Core-associated (●) and released (○) RNAs after chase with ATP, GTP, CTP, and UTP.*

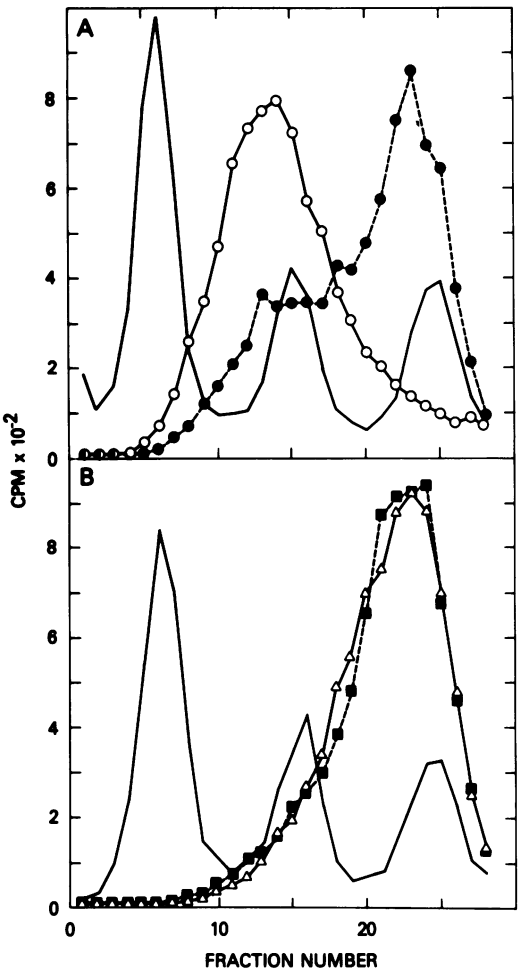

FIG. 11. *Cleavage of high-molecular-weight RNA by a soluble endoribonuclease. RNA was labeled with [α-^{32}P]UTP, purified by sucrose gradient sedimentation as described in the legend to Fig. 8, and incubated for 30 min at 37°C with buffer (○) or with 5 (●), 10 (■), or 20 (△) μl of a soluble extract of vaccinia virus cores. After addition of sodium dodecyl sulfate and [^{3}H]-rRNA and [^{3}H]tRNA markers, the mixture was heated at 100°C for 2 min and centrifuged on a 5 to 20% sucrose gradient. Each entire fraction was counted in a Triton X-100 and toluene-based scintillation fluid. Marker 28, 18, and 4S RNAs are indicated by the solid line.*

sidered to explain the large size of the RNA, (i) failure of proper chain termination, (ii) inhibition of endonucleolytic cleavage, and (iii) addition of a very long poly(A) segment, only the last could be ruled out. Less than 15% of the high-molecular-weight RNA bound to polyuridylic acid-Sepharose, and nearest neighbor analysis, after [α-^{32}P]AMP-PNP labeling, did not reveal an unusual proportion of adenylate

residues. Failure of chain termination is an attractive hypothesis, since *E. coli* ρ factor has an associated RNA-dependent ATPase activity (11) and transcription with *E. coli* RNA polymerase cannot be terminated by ρ factor in the presence of the four ribonucleoside [β,γ-imido]triphosphates unless ATP is added (4). Unlike the apparent situation with vaccinia virus, however, RNA is terminated by ρ factor with high concentrations of GTP, CTP, and UTP in the presence of AMP-PNP. Thus, we would have to postulate that the putative vaccinia virus ρ factor has a stringent requirement for ATP. Conceivably, the vaccinia virus core-associated DNA-dependent ATPase activity (23) could have such a role in RNA chain termination.

The reduction in size of the high-molecular-weight RNA after the substitution of ATP for AMP-PNP indicates the presence of a core-associated endoribonuclease and is consistent with an inhibitory effect of AMP-PNP on processing. Paoletti previously described the labeling of 18 to 28S, core-associated, non-polyadenylylated RNA at high virus concentration and suboptimal conditions of RNA synthesis and reported that cleavage of this RNA occurred after addition of ribonucleoside triphosphates (19, 20). More recent experiments (21a) indicate a relationship between decreased ATP concentration and formation of high-molecular-weight RNA. Thus, it seems likely that similar underlying mechanisms are responsible for formation of the high-molecular-weight RNA under the conditions used by Paoletti (depleted ATP) and those described here (substitution of AMP-PNP for ATP). The formation of high-molecular-weight RNA has also been detected after heat inactivation of virus particles (G. Darby, personal communication).

A soluble endoribonuclease activity extracted from vaccinia virus cores can cleave high-molecular-weight RNA made at reduced ATP concentrations (21a), as well as high-molecular-weight RNA made in the presence of AMP-PNP, to an apparent limit product. However, this endoribonuclease, which has not yet been purified or extensively characterized, is not dependent on ATP, nor is it inhibited by AMP-PNP. Possibly, AMP-PNP blocks a step preceding endonuclease cleavage.

To what extent cleavage of RNA is involved in processing under normal conditions, i.e., high ATP and no AMP-PNP, is not known. If cleavage of RNA leads to the formation of new 5′ ends, then either additional phosphates must be added or a second mechanism of formation of the $m^7G(5')pppN^m$ cap unlike that already described for vaccinia virus (12–14) must exist. Alternatively, if RNA processing involves excision of internal RNA segments followed by splicing, the initial 5′-terminal sequence would be preserved and there would be no need to propose a new capping mechanism. Still another possibility is that the endoribonuclease is normally involved in excising a small segment near the 3′ end of vaccinia virus mRNA before polyadenylation. According to this model, if chain termination is prevented, e.g., with AMP-PNP, subsequent cleavage at the usual site would result in a major size reduction. Although entirely speculative, the latter mechanism fits in well with established information regarding capping as well as our inability to detect significant amounts of high-molecular-weight RNA under optimal conditions of transcription and with UV data indicating that primary transcripts are translated in a coupled cell-free system (25).

The lack of a poly(A) tail on the 3′ end of most of the high-molecular-weight RNA may be secondary to the failure of proper termination or cleavage. In fact, even after a 45-min incubation, the RNA still appeared to be slowly increasing in size (data not shown). AMP-PNP did not prevent the synthesis by vaccinia virus cores of 4 to 5S poly(A) unattached to mRNA, and prior initiation with ATP was not required.

The failure of the RNA formed in the presence of AMP-PNP to be extruded from the virus core was consistent with earlier experiments of Kates and co-workers (7, 28). They noted that extrusion of nascent RNA from virus cores is stimulated by ATP but not by adenosine (β,γ-methylene)triphosphate or certain other analogs of ATP.

The present studies indicate that the transcription of DNA by vaccinia virus cores is a complex process that can be inhibited at specific steps by AMP-PNP. Analogs of this type also inhibit transcription by vesicular stomatitis virus (29) and by cytoplasmic polyhedrosis virus (Y. Furuichi, personal communication). Further work is needed to determine how the protein kinase (9, 10), nucleic acid-dependent ribonucleoside triphosphatases (22, 23), or still-unknown factors that hydrolyze the γ-phosphate of ATP might be involved in transcription by vaccinia virus particles.

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