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Effect of *S*-Adenosylmethionine on Human Rotavirus RNA Synthesis

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The characteristics of human rotavirus-associated RNA polymerase activity have been examined in relation to the effects of ribonucleoside triphosphate analogs and *S*-adenosylmethionine. These effects were analyzed by testing two forms of activated virus particles: EDTA- and heat-treated virions. The former lack outer shell proteins, and activation by means of heat treatment does not introduce any apparent modification in virion structure. Virus-associated RNA polymerase shows similar properties in both preparations, suggesting that outer proteins are not directly involved in RNA synthesis. Transcription in this virus is specifically dependent on a hydrolyzable form of ATP. Such a requirement is not overcome by preincubation or by the addition of *S*-adenosylmethionine, suggesting a hypothetical mechanism that couples transcription to ATP hydrolysis. The addition of *S*-adenosylmethionine stimulated transcription and diminished the K_m value not only for ATP but also for the other three ribonucleoside triphosphates. Analysis of methylated RNA products suggested that methyl groups were incorporated into all of the RNA species synthesized by virion-associated polymerase. Further analysis of those RNA molecules showed that they contained cap structures at the 5′ end. The results suggest that the cap structure at the end of RNA molecules may enable RNA polymerase to elongate transcripts more efficiently, in a reaction in which the hydrolysis of ATP is involved.

Rotavirus has been associated with acute diarrhea in a variety of mammals including humans (30). In infants it is the single most important cause of acute gastroenteritis (24, 25, 30). The viral particle contains as genome 11 double-stranded RNA (dsRNA) segment molecules with molecular weights running from 2.2×10^6 to 0.5×10^6, with slight variations among different virus strains (12, 24). The rotavirus particle contains six to seven structural polypeptides with definite locations in the virion, conforming to a characteristic double-shelled structure (6), in which one class of outer shell protein is glycosylated and defines some of the serological properties of the virus (14). The outer viral shell may be removed by chemical procedures, producing a single-shell virion which is noninfectious (3, 4). Both types of particles are normally shed by patients, and they may be separated by gradient centrifugation (2). Like other members of the *Reoviridae* family, rotaviruses catalyze RNA synthesis because of an associated RNA polymerase activity which transcribes the genome as RNA segments (23). This polymerase activity becomes active in vitro after a thermal shock or treatment with EDTA (3, 8, 23). Thermal shock does not seem to uncoat the virus; therefore it allows one to study the in vitro properties of viral genome transcription without major disruption of the particle (20). Human rotavirus-associated RNA polymerase synthesizes RNA in the presence of Mg^{2+} and all four ribonucleoside triphosphates (23). Previous results have suggested that transcription activity requires the presence of ATP in a reaction different from polymerization but closely related; it has been shown that the replacement of ATP by the analog β-γ-imido or methylene ATP inhibits transcription (23). This observation has previously been made in several systems including vesicular stomatitis virus and CPV (27, 28, 29). In those models, ATP exhibits a high K_m value which diminishes upon the addition of *S*-adenosylmethionine (9), a substrate required for methylation of the cap structure (15, 16). Furthermore, in those systems it has been shown that preincubation with ATP overcomes the inhibition by the analog, suggesting that ATP is involved in a reaction associated to transcription initiation in which *S*-adenosylmethionine may be involved (29). On the other hand, in vaccinia virus, preincubation with ATP does not overcome the requirement of this nucleotide for transcription (26). Furthermore, in vaccinia, the addition of *S*-adenosylmethionine is not essential for in vitro transcription, and the K_m for ATP is not altered by its addition. In this particular virus, ATP hydrolysis has been suggested to be associated with the elongation of preinitiated RNA chains by allowing RNA polymerase activity to proceed through the template DNA (22, 26). In other viruses, such as reovirus, *S*-adenosylmethionine is required neither for transcription nor to stimulate the process, but it seems to be involved in preventing abortive elongation of newly synthesized RNA chains (11).

In the present communication, we have examined the requirement of ATP hydrolysis as well as the effect of *S*-adenosylmethionine in human rotavirus transcription, in an attempt to understand the relationship between them.

MATERIALS AND METHODS

Virus. Human rotavirus particles were obtained from stool samples collected from a single patient with diagnosis of acute diarrhea during a period of 24 h. After testing for the presence of rotavirus by an enzyme-linked inmunosorbent assay, we purified virus as previously described (23). Purified virus was further characterized by analysis of viral genome RNA by polyacrylamide gel electrophoresis. The migration pattern for RNA segments corresponded to a "long" electropherotype (18). The virus was also analyzed by electron microscopy, showing only uniform particles with the characteristics of rotavirus (1, 30).

Purification of single-shelled rotavirus particles. Purified virus was treated with 10 mM EDTA for 45 min at 37°C. The viral suspension was then centrifuged in a 40 to 55% CsCl

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gradient as described by Flores et al. (8). The single-shelled particles were pelleted, suspended in 10 mM Tris-hydrochloride buffer (pH 8.4), and stored at −20°C. When RNA synthesis was studied by using these virus particles, they were added directly into the reaction mixtures (2 μg per reaction).

In vitro transcription of human rotavirus genome RNA. The RNA polymerizing activity was assayed by the incorporation of ^3H from [^3H]UTP into acid-insoluble material. The reaction mixtures (25 μl) contained 60 mM Tris-hydrochloride buffer (pH 8.4), 10 mM $MgCl_2$, 100 mM NaCl, 2 mM each ATP, CTP, and GTP, and 200 μM [^3H]UTP (specific activity [SA], 40 cpm/pmol). To this reaction mixture, 2 μg of purified virus was added after being heated for 1 min at 55°C in 10 mM Tris-hydrochloride buffer (pH 8.4). Some reaction mixtures included Adomet (*S*-adenosylmethionine), AdoHcy (*S*-adenosylhomocysteine), or ribonucleoside triphosphate analogs at concentrations noted in the table and figure legends. All reactions were performed by incubation at 45°C. Under these conditions, RNA synthesis was linear for at least 45 min. Incubation was terminated by the addition of 0.1 ml of denatured calf thymus DNA solution (1 mg/ml), 0.2 ml of 200 mM sodium pyrophosphate solution, and 5 ml of cold 5% trichloroacetic acid. The acid-insoluble fraction was collected in fiberglass filters which were washed with 15 ml of 5% trichloroacetic acid and 5 ml of cold ethanol, dried, and assayed for radioactivity in 5 ml of toluene-based scintillating liquid in a Nuclear-Chicago MK II scintillating counter (Nuclear-Chicago Corp.).

In vitro transcription in the presence of ^3H-labeled Adomet. For our study of Adomet incorporation into RNA, RNA polymerase reaction mixtures were made as described above and modified as follows. As labeled nucleotide, [^{32}P]GTP or [^{32}P]UTP was added at a concentration of 50 μM (SA, 4,000 cpm/pmol) and cold UTP or GTP at a concentration of 2 mM; ^3H-labeled Adomet was also added to a final concentration of 150 μM (SA, 1,875 cpm/pmol). The radioactivity associated with acid-insoluble material was measured as described above under conditions in which the counting of ^3H and ^{32}P had spillovers of less than 1%.

Identification of the modified 5′ terminus of RNA. For a determination of where the methyl groups from Adomet were introduced into the RNA chain, the following procedure was applied. Two reaction mixtures containing both labeled Adomet and GTP were scaled up 10 times and incubated as described above. After incubation for 30 min, the reaction was halted by centrifugation and the supernatant was deproteinized by the addition of a mixture of phenol-chloroform-isoamylalcohol (50:48:2). After centrifugation, the aqueous layer was precipitated with cold ethanol and the precipitate was collected by centrifugation and dried. The ethanol precipitation step was carried out three times to eliminate the unincorporated nucleotides. The pellet was then dissolved in 0.5 ml of 5 mM sodium acetate buffer (pH 6.0) and incubated for 17 h at 37°C with a mixture of RNase T2 (25 U) and penicillinum nuclease P1 (100 μg). The nuclease digests were deproteinized again as described above, washed with chloroform twice, desalted by passage through a column (1 by 24 cm) of Sephadex G-10 (previously equilibrated with a 0.05 M Tris-hydrochloride buffer [pH 8.0]), lyophilized, and dissolved in 0.1 ml of 0.05 M Tris-hydrochloride buffer (pH 8.0). Each sample was divided into two aliquots which were further digested, one with bacterial alkaline phosphatase (BAP) (60 U) and the other with BAP and nucleotide pyrophosphatase (80 μg) to release methylated nucleosides from possible cap structures. The digestion was carried out for 60 min, at 37°C in the presence of 2 mM $MgCl_2$. The digests were then subjected to phenol extraction, desalted, and lyophilized as described above. Each sample was dissolved in 20 μl of water, subjected to thin-layer chromatography in polyethyleneimine-cellulose plates, and developed in 1.2 M LiCl as previously described (21). After the solvent reached the top of the plate, it was washed in ethanol to desalt the chromatogram, and each sample was cut into 0.5-cm strips. Radioactivity on the strips was determined by counting in a toluene-based scintillation fluid. The selected channel setting allowed counting of ^3H and ^{32}P with spillovers of less than 1%, for which the samples were corrected. For identification of the spots, the following markers were run in the same plate: GTP, GDP, GMP, Adomet, P_i, PP_i, GpppA, 7-met-GpppA, GpppG, and 7-met-GpppG. Their positions are indicated with brackets or arrows in the figures.

Hybridization of RNA products to denatured viral dsRNA. To determine whether the RNAs synthesized in vitro were complete transcripts from all genome RNA segments, we hybridized the transcripts to viral denatured dsRNA obtained from phenol extraction of purified virions. Labeled transcripts with ^{32}P were obtained from rotavirus-associated RNA polymerase reactions in which [^{32}P]UTP was used at a concentration of 50 μM (SA, 4,000 cpm/pmol). These labeled transcripts were then hybridized to homologous denatured dsRNA. Similar experiments were carried out with in vitro transcripts obtained by labeling the RNA with ^3H-labeled Adomet. These transcripts were made by replacing the labeled nucleoside triphosphate with a cold one at a similar concentration to the others and by adding ^3H-labeled Adomet at a final concentration of 150 μM (SA, 1,875 cpm/pmol). The hybridization procedures were similar to the one previously described by Flores et al. (7), with the following modifications: (i) no tRNA carriers were added, (ii) the amount of radioactivity was 50,000 cpm of the in vitro [^{32}P]UMP-labeled transcript, and (iii) the amount of genome RNA was 0.3 μg. After hybridization, the mixture was subjected to electrophoresis in an 8% polyacrylamide discontinuous gel system by the method described by Laemmli (13). In this gel system, sodium dodecyl sulfate was omitted. After overnight electrophoresis at 20 mA, gels containing ^{32}P-labeled products were dried and autoradiographed on Fuji X-ray film. When ^3H-labeled hybrids were analyzed in gel electrophoresis, after the overnight electrophoresis they were cut into 1-mm slices, and radioactivity was determined by counting in a toluene-based scintillation fluid.

Materials. BAP and fiberglass filters were purchased from Enzo Biochemical Co. Nuclease S1, RNase T2, RNase P1, nucleotide pyrophosphatase, and cold ribonucleoside triphosphates were from Sigma Chemical Co. Cold Adomet and AdoHcy were also from Sigma. The ribonucleoside triphosphate analogs and the cap structure standards were from P-L Biochemicals, Inc. Polyethyleneimine-cellulose thin-layer plates were purchased form Merck & Co., Inc. [^{32}P]UTP, [^{32}P]GTP, [^3H]UTP, and ^3H-labeled Adomet were purchased from New England Nuclear Corp.

RESULTS

Human rotavirus in vitro transcription. Two methods were used for activation of the RNA polymerase: a heat shock, and incubation of the virus with EDTA. The latter seems to induce the loss of the outer shell proteins, leaving a structure called single-shelled virus (5, 8). The other procedure, heat shock, does not seem to alter the structure of the virus, leaving a double-shelled particle (23). In Fig. 1, the product

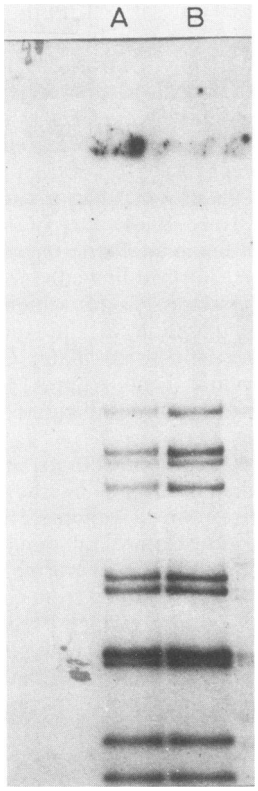

FIG. 1. Hybridization of in vitro-synthesized transcripts to denatured viral genome dsRNA. Reactions were carried out with EDTA-treated (A) and heat-treated (B) viruses. After RNA extraction, the samples were hybridized against denaturated viral genome RNA obtained from the same virus isolate and further digested with S1 nuclease. Each hybridization reaction contained 50,000 cpm of the in vitro transcripts and 0.3 μg of genome dsRNA. The samples were processed and described in the text. The direction of electrophoresis was from top to bottom.

of the associated RNA polymerase activity from heat- and EDTA-treated viruses is compared, using the same human rotavirus isolate. The product of both reactions hybridized with all RNA segments of the viral genome. These results suggest that both activation procedures allow the RNA polymerase activity to transcribe the 11 RNA segments of the viral genome. Furthermore, as the hybrid products were extensively treated with S1 nucleases, the electrophoretic mobility of the hybrids suggests that under the conditions of RNA synthesis, the products may correspond to full-length transcripts as compared with the mobility of the genome segments (data not shown).

The in vitro transcriptional characteristics of viral particles treated with both activation procedures were analyzed for optimal ribonucleoside triphosphates, Mg^{2+} and monovalent cation concentrations, and optimal pH. The results (Table 1) suggest that EDTA- and heat-treated viruses have similar optimal concentrations of ribonucleoside triphosphates and Mg^{2+} and are both stimulated by monovalent ions such as Na^+ and K^+. The optimal pHs are also equivalent. When K_m values for the ribonucleoside triphosphates were analyzed, they were also similar.

Effect of Adomet on human rotavirus RNA polymerase activity. The effect of Adomet in human rotavirus is shown in Fig. 2 and 3. Adomet stimulated the rate as well as the yield

TABLE 1. Comparison of requirements for human rotavirus-associated RNA polymerase activity of EDTA- and heat-treated virions

Parameter	Optimal value with the following activation procedure[a]:	
	EDTA	Heat shock
Ribonucleoside triphosphate (2 mM each)	8 mM	8 mM
$MgCl_2$	10 mM	10 mM
pH	8.5	8.5
NaCl or KCl	100 mM	100 mM
K_m ATP	133 μM	109 μM
K_m CTP	153 μM	158 μM
K_m UTP	76 μM	97 μM
K_m GTP	807 μM	794 μM
[^{3}H]UMP incorporation into acid-insoluble material	185 pmol/20 min	217 pmol/20 min

[a] Each optimal value was obtained after titration of the requirement under study, with the rest of the components of the reaction mixture in an "adequate" condition. The reaction mixture was as described in the text, and 2 μg of EDTA-purified or heat-treated virus was used.

of the reaction up to 2.5 times in both EDTA- and heat-treated viruses (Fig. 2). The addition of the product of the methylation reaction, AdoHcy, did not have any effect on the reaction in both virus preparations when Adomet was absent, but it seemed to overcome the Adomet stimulation,

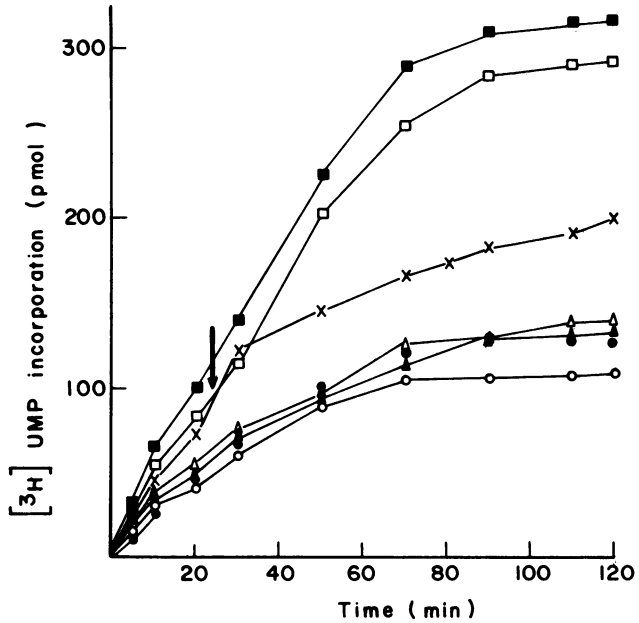

FIG. 2. Time course of the in vitro transcription in the presence of Adomet and AdoHcy catalyzed by heat- or EDTA-treated viruses. Reaction mixtures (25 μl) were incubated at 45°C for the indicated times, and the radioactivity was determined as acid-insoluble material. The reaction mixtures were as described in the text. In vitro transcription was determined by using heat-treated virus in the presence of 1 mM Adomet (■), 1 mM AdoHcy (▲), and with no addition (●). Similar experiments were done with EDTA-treated viruses in the presence of 1 mM Adomet (□), 1 mM AdoHcy (△), and in the absence of both (○). In an experiment in which 1 mM Adomet was added at the beginning of the reaction, AdoHcy was added to reach a final concentration of 1 mM after 25 min of incubation, as indicated by the arrow, and further incubated (×).

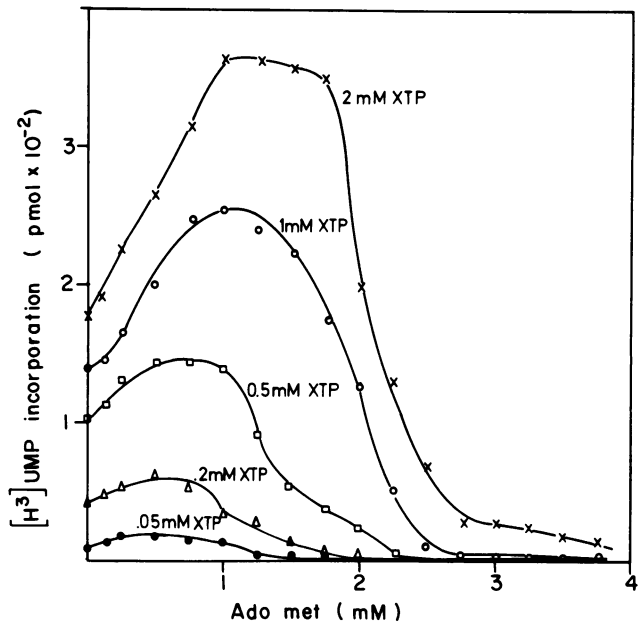

FIG. 3. Effect of increasing levels of Adomet on human rotavirus-associated RNA polymerase activity at different ribonucleoside triphosphate concentrations. RNA synthesis was determined in the presence of various Adomet concentrations. The enzymatic activity was measured at different concentrations of ribonucleoside triphosphate, and in each one of them the optimal $MgCl_2$ was taken. The reaction was carried out as described in the text, except that ATP, CTP, GTP, and $MgCl_2$ were added at the following concentrations: 2 mM each ATP, CTP, and 10 mM $MgCl_2$ (×), 1 mM XTP and 7 mM $MgCl_2$ (○), 0.5 mM XTP and 4 mM $MgCl_2$ (□), 0.2 mM XTP and 2.5 mM $MgCl_2$ (△), and 0.05 mM XTP and 1.5 mM $MgCl_2$ (●). The reactions were incubated for 30 min at 45°C in the presence of 7 μg of heat-treated human rotavirus, and radioactivity associated with acid-insoluble material was determined and expressed as picomoles of [^{3}H]UMP.

probably by competing with the methylating agent. A similar observation has been obtained with eucaryotic RNA polymerase II (10).

The effect of Adomet was also studied in relation to the ribonucleoside triphosphate requirement. In viruses in which transcription is partially dependent upon the addition of Adomet, the stimulatory effect is greater at a low concentration of ribonucleoside triphosphate (9). The results shown in Fig. 3 suggest that Adomet stimulates the reaction depending on the ribonucleoside triphosphate concentration used. At high concentrations of ribonucleoside triphosphates, Adomet stimulated at an optimal concentration of 1 mM; at lower ribonucleoside triphosphate concentrations, the optimal concentration of Adomet diminished to 0.5 mM; and at even lower amounts of ribonucleoside triphosphate, such an effect was not detected. The results suggest, therefore, that in human rotavirus, Adomet stimulates transcription; however, the reaction seems to be independent of the addition of the methylating agent.

Effect of ribonucleoside triphosphate analogs on transcription. The ability of rotavirus to perform transcription in the presence of β-γ-imido nucleotide analogs, and the effect of Adomet on it, were studied in the experiment shown in Fig. 4. Figure 4A shows the results for the case of β-γ-imido analogs, a molecule that may be incorporated into the RNA chain but cannot be hydrolyzed to ADP and P_i, and it shows that the replacement of ATP for the analog completely abolishes the reaction and that this effect is not reverted by the addition of Adomet.

The effects of analogs of the other three ribonucleoside triphosphates were also analyzed, and the results are shown in Fig. 4B, C, and D. In all of them, the corresponding β-γ-imido analog was able to support RNA synthesis, and the reaction was stimulated by Adomet. This was true with both EDTA- and heat-activated virus.

Effect of Adomet on the ribonucleoside triphosphate requirement for in vitro transcription. The effect of Adomet on the nucleotide requirement for RNA synthesis was studied in the experiments summarized in Fig. 5. Apparent K_m values for the ribonucleoside triphosphates were similar, except for GTP, which was higher, as we reported previously (23). The addition of Adomet decreased the apparent K_m value for all of the triphosphates, including GTP. Also, the apparent V_{max} of the reaction increased depending upon the concentration of Adomet tested; the effect did not seem to be associated exclusively with the requirement for ATP, as reported for other viruses (9).

For an investigation of whether Adomet and ATP hydrolysis are required only for initiation of RNA synthesis, the virus was preincubated under a variety of conditions. Virus particles were preincubated in the presence of all ribonucleoside triphosphates, with or without Adomet (Table 2). The virus from both preincubations was further reisolated, and the amount of [^{3}H]UMP incorporated into acid-insoluble material in the supernatant was measured. After viral reisolation, similar samples of the suspended virus were incubated for an additional 30 min with different combinations of ribonucleoside triphosphates in which [^{32}P]UTP and ^{3}H-labeled Adomet were included. The results showed that (i) the addition of Adomet during the preincubation did not overcome the requirement for a hydrolyzable form of ATP in a second incubation, (ii) lack of incorporation was not due to inactivation of the virus during reisolation, (iii) the radioactivity found was not carried over the preincubation, and (iv) specificity for ATP hydrolysis still remained after reisolation.

The effect of Adomet on the preincubated virus is shown in Table 2. Tritium-labeled Adomet was used to explore the possible methylation of RNA. The result suggested that Adomet stimulated transcription and was incorporated into acid-insoluble material only when RNA synthesis occurred. Under the assumption that each ribonucleoside is incorporated into RNA in a similar proportion, the ratio between incorporation of ^{3}H-methyl groups and ribonucleoside monophosphates is 1:494.

In vitro transcription in the presence of labeled Adomet. For a study of the nature of acid-insoluble Adomet incorporation, transcription was carried out with a standard mixture in the presence of ^{3}H-labeled Adomet.The RNA product was hybridized to total denatured genome viral RNA, and hybrids were subjected to polyacrylamide gel electrophoresis. The results (Fig. 6) suggest that the migration pattern corresponds to that obtained with [^{32}P]UMP-labeled transcripts hybridized to genome RNA (Fig. 1), implying that methyl groups from Adomet are incorporated into the same RNA species.

Analysis of methylated RNA. A 5′ cap structure has been shown to be present in eucaryotic mRNA as well as in several in vitro-synthesized viral mRNAs (16, 19), including reovirus. To study such a possibility, reaction mixtures were scaled up in the presence of [^{32}P]GTP and ^{3}H-labeled Adomet, using EDTA and heat or EDTA-activated viruses. After phenol extraction, RNA products were treated with

ribonucleases T2 and P1 and BAP and subjected to polyethyleneimine-cellulose chromatography. The radioactivity resolved in two peaks (Fig. 7A) that comigrated with two cap structure markers: unmethylated GpppG cap and 7-met-GpppG. ^{32}P-labeled digestion products comigrated with both cap structure markers, whereas ^{3}H label was associated only with the methylated cap marker position, suggesting that Adomet is required for methylation of a preformed cap. The product was further analyzed by assessing its sensitivity to nucleotidyl pyrophosphatase, an enzyme that cleaves cap

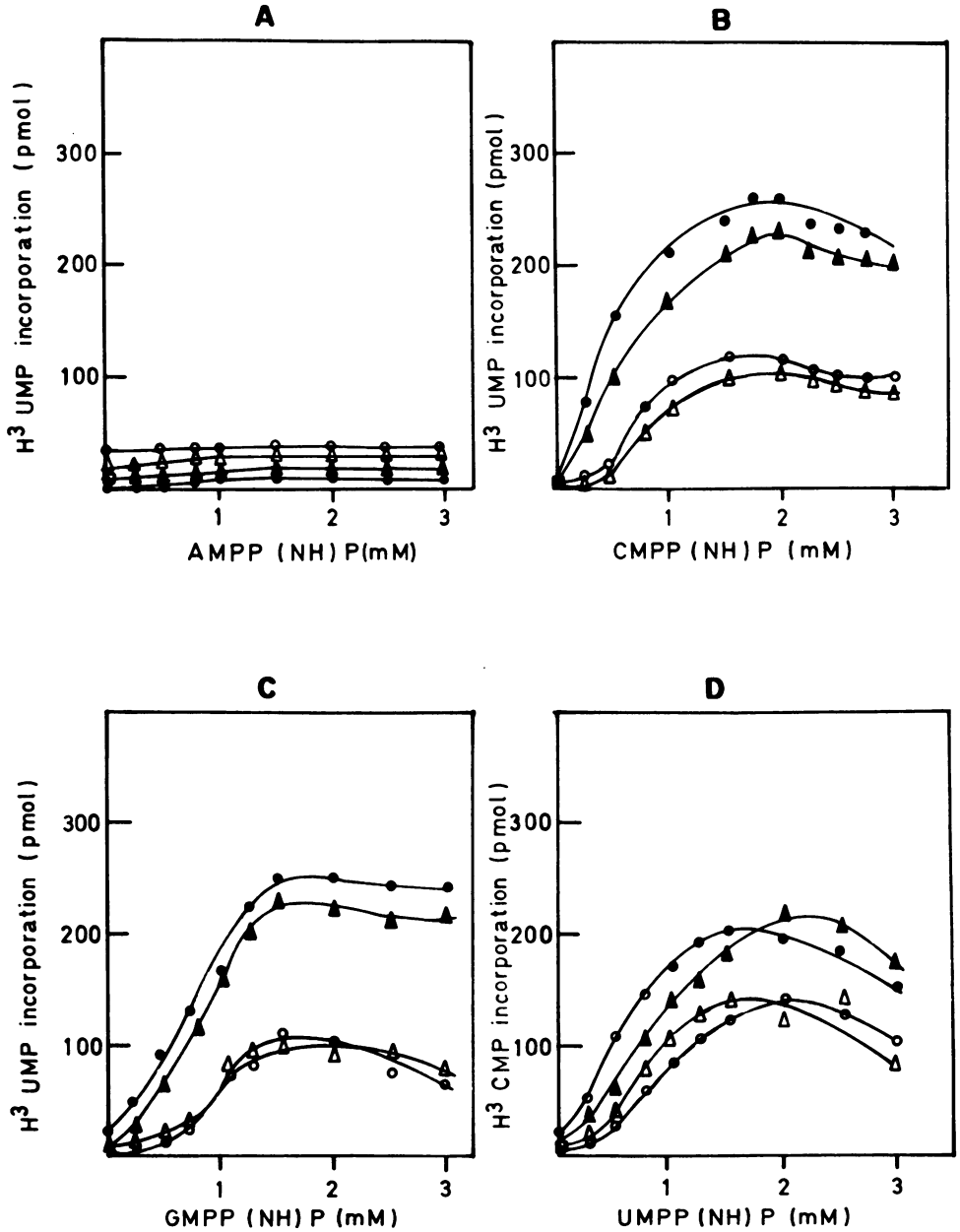

FIG. 4. Effect of ribonucleoside analogs on in vitro transcription. Standard reaction mixtures (25 μl) containing 60 mM Tris-hydrochloride buffer (pH 8.4), 10 mM $MgCl_2$, 100 mM NaCl, 2 μg of heat- or EDTA-treated virus, and the indicated ribonucleoside triphosphates or analogs were incubated at 45°C for 30 min, testing the combinations described below. In each case, closed symbols stand for the same reactions in the presence of 1 mM Adomet. (A) Mixtures contained 2 mM each CTP and GTP, 200 μM [^{3}H]UTP (SA, 40 cpm/pmol), and increasing concentrations of β-γ-imido ATP with heat-shocked (△) or EDTA-incubated (○) virus. (B) Mixtures contained 2 mM each ATP and GTP, 200 μM [^{3}H]UTP (SA, 40 cpm/pmol), and various concentrations of β-γ-imido CTP with heat- (△) or EDTA- (○) activated virus. (C) Mixtures contained 2 mM each ATP and CTP, 200 μM [^{3}H]UTP (SA, 40 cpm/pmol), and β-γ-imido GTP at the levels shown, with heated (△) or EDTA-subjected (○) virus. (D) Mixtures contained 2 mM each ATP and GTP, 200 μM [^{3}H]CTP (SA, 40 cpm/pmol), and various levels of β-γ-imido UTP with heat-shocked (△) or EDTA-incubated (○) virus.

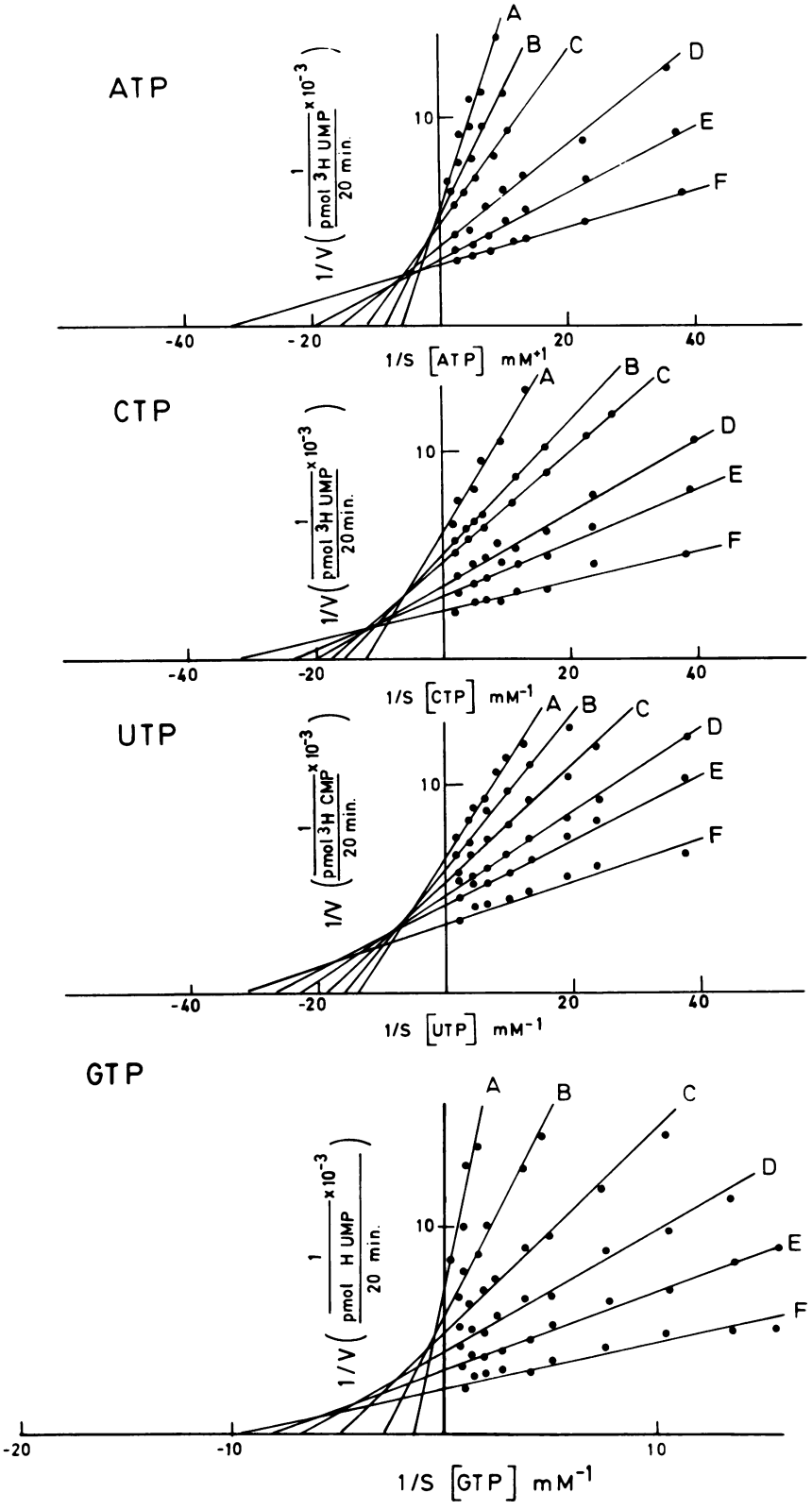

FIG. 5. Effect of Adomet on the ribonucleoside triphosphate requirement for in vitro transcription. The reaction mixture contained 60 mM Tris-hydrochloride buffer (pH 8.4), 10 mM $MgCl_2$, 100 mM $NaCl_2$, and 2 μg of heat-activated virus. [^{3}H]UTP was added at 200 μM, except where UTP dependence was measured. Other ribonucleoside triphosphates were included at 1.5 mM, except for the one being varied (XTP). Similar experiments were carried out with increasing concentrations of Adomet as follows: A, no addition; B, 0.05 mM; C, 0.1 mM; D, 0.4 mM; E, 0.7 mM; F, 1.0 mM. The rate of reaction (picomoles of [^{3}H]UMP incorporated per 15 min) was determined as described in the text and is expressed in the figure as a double reciprocal plot rate versus XTP concentration.

TABLE 2. In vitro RNA synthesis by preincubated virions[a]

Preincubation condition	Amt of [^{3}H]UMP incorporated into acid-insoluble material (pmol)	Additions after virus reisolation	Amt of [^{32}P]UMP incorporated into acid-insoluble material after an additional 30-min incubation (pmol)	Amt of ^{3}H-labeled Adomet incorporated into acid-insoluble material after an additional 30-min incubation (pmol)
Complete	284	None	2	
		ATP+CTP+GTP+[^{32}P]UTP	163	
		APP(NH)P+CTP+GTP+[^{32}P]UTP	2	
		ATP+GTP+CPP(NH)P+[^{32}P]UTP	128	
		ATP+CTP+GTP+[^{32}P]UTP+^{3}H-labeled Adomet	197	1.5
		APP(NH)P+CTP+GTP+[^{32}P]UTP+^{3}H-labeled Adomet	1	0.17
		ATP+GTP+CPP(NH)P+[^{32}P]UTP+^{3}H-labeled Adomet	157	1.32
Without Adomet	137	None	2	
		ATP+CTP+GTP+[^{32}P]UTP	137	
		APP(NH)P+CTP+GTP+[^{32}P]UTP	2	
		ATP+GTP+CPP(NH)P+[^{32}P]UTP	118	
		ATP+CTP+GTP+[^{32}P]UTP+^{3}H-labeled Adomet	159	5.2
		APP(NH)P+GTP+CTP+[^{32}P]UTP+^{3}H-labeled Adomet	2	0.19
		ATP+GTP+CPP(NH)P+[^{32}P]UTP+^{3}H-labeled Adomet	121	1.07

[a] Reaction mixtures (250 μl) containing 60 mM Tris-hydrochloride buffer (pH 8.4), 10 mM $MgCl_2$, 100 mM NaCl, 2 mM each ATP, GTP, and CTP, and 20 μg of heat-activated human rotavirus were incubated in the presence and absence of 1 mM Adomet for 30 min at 45°C. At the end of the incubation, 25-μl aliquots were taken and the acid-insoluble radioactivity was determined. The rest of the reaction mixture was centrifuged in an Eppendorf centrifuge, and the supernatant was discarded. The viral pellet was washed once with 100 mM Tris-hydrochloride buffer (pH 8.0) and suspended in 60 mM Tris-hydrochloride buffer (pH 8.4). Seven different aliquots, each equivalent to 25 μl of the original reaction mixture, were taken and further incubated with the following reagents: 10 mM $MgCl_2$, 100 mM NaCl, and 150 μM [^{32}P]UTP (SA, 400 cpm/pmol); the ribonucleoside triphosphate or the analog was added at a final concentration of 1.5 mM. In the experiment in which ^{3}H-labeled Adomet was added, it was present at 60 μM (SA, 1,900 cpm/pmol). These reaction mixtures were then incubated for an additional 30 min at 45°C. The reactions were halted, and the acid-insoluble material was collected and assayed for radioactivity as described for double label in the text. In control experiments in which ^{3}H-labeled Adomet was used in the presence or absence of the ribonucleotide triphosphate, no radioactivity remained associated with the virus particle after the preincubation.

structures at the pyrophosphate bridge. Digested products were treated with this enzyme and BAP to remove any phosphate associated with a nucleoside. The result (Fig. 7B) shows that both peaks are completely sensitive to the combination of enzymes; ^{32}P was rendered as inorganic phosphate and the ^{3}H migrated with the solvent front. This result is in agreement with the presence of a modified 5′ end structure in RNA molecules. The results also suggest the

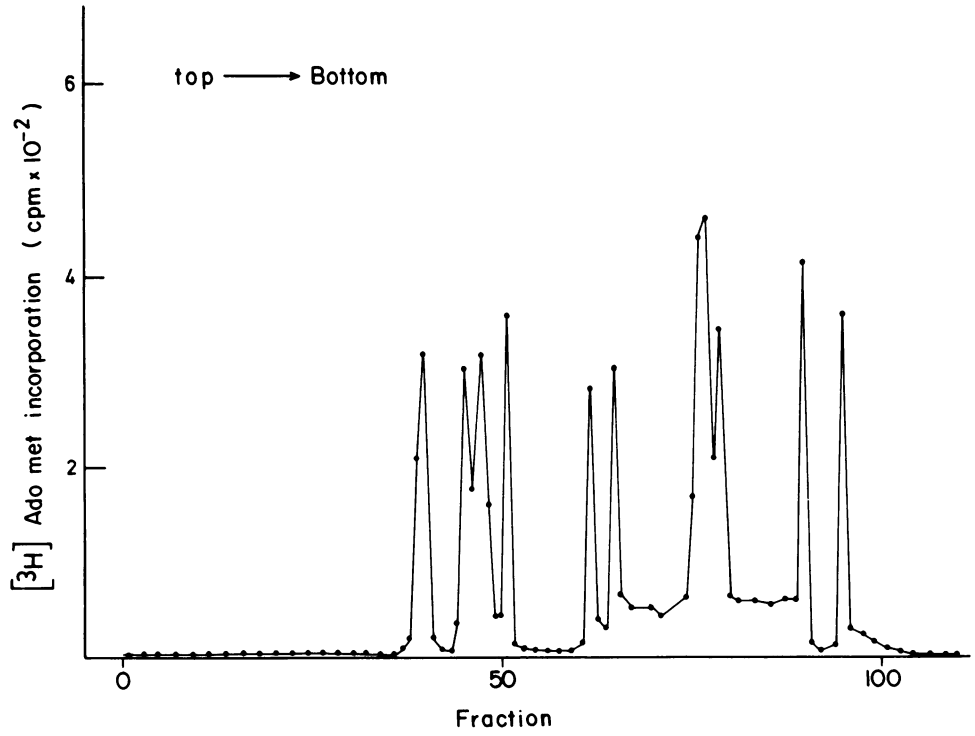

FIG. 6. Reaction mixtures (250 μl) contained 60 mM Tris-hydrochloride buffer (pH 8.4), 10 mM $MgCl_2$, 100 mM NaCl, 1.5 mM each ATP, CTP, GTP, and UTP, 150 μM ^{3}H-labeled Adomet (SA, 1,850 cpm/pmol), and 20 μg of heat-treated human rotavirus. The RNA was obtained by centrifugation at the end of 30 min of incubation at 45°C and processed as described in the text.

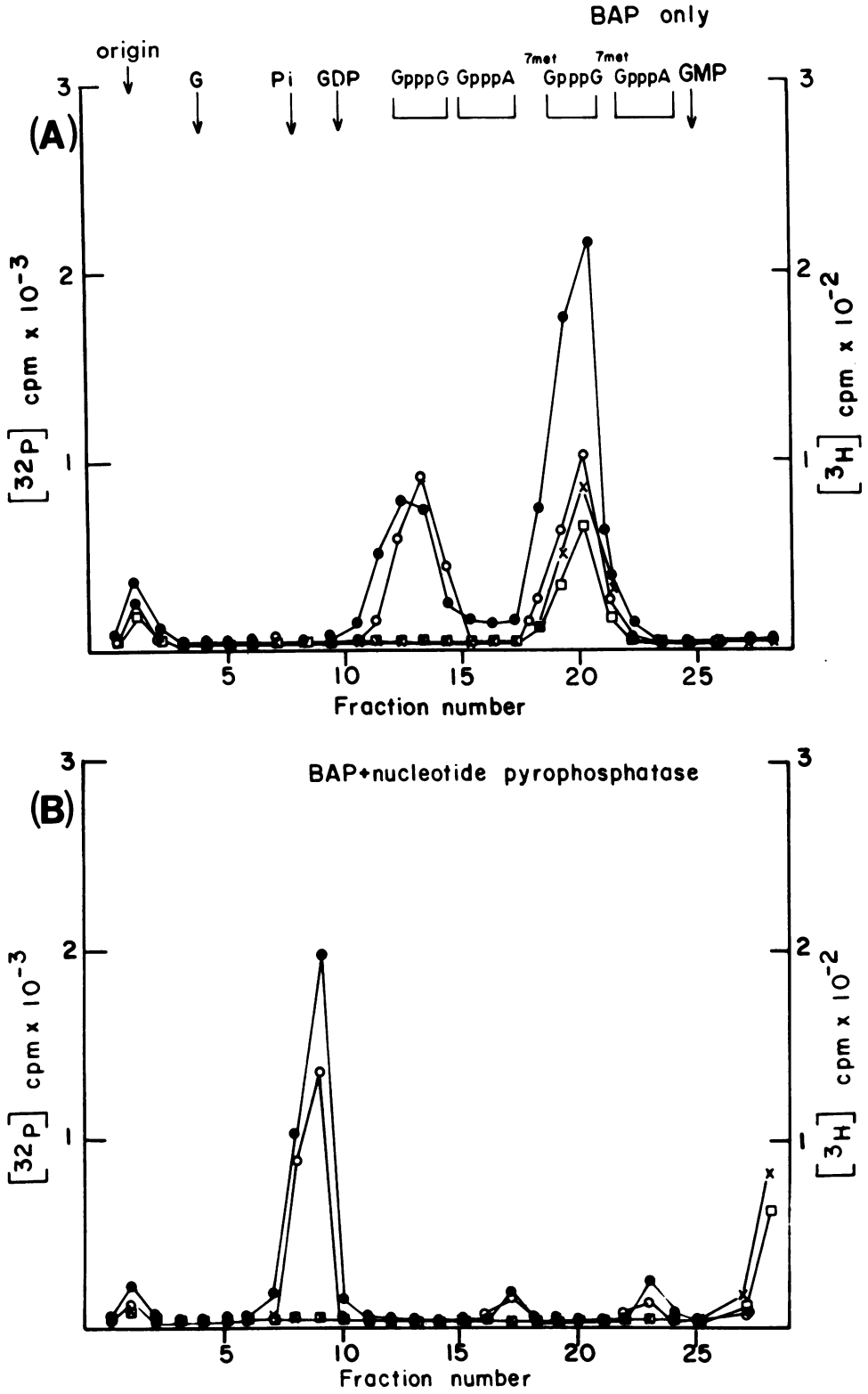

FIG. 7. RNA capping by activated human rotavirus particles. Reaction mixtures (250 μl) containing 60 mM Tris-hydrochloride, 10 mM $MgCl_2$, 100 mM NaCl, 1.5 mM each ATP, CTP, and UTP, 150 μM ^{3}H-labeled Adomet (SA, 1,850 cpm/pmol) and 50 μM [^{32}P]GTP (SA, 4,000 cpm/pmol) were incubated with 20 μg of heat-activated virus or with a similar amount of EDTA-activated virus for 45 min at 45°C. The reaction was heated by centrifugation, and the supernatant was succesively phenol extracted, ethanol precipitated, suspended in buffer, and treated with nucleases T2 and P1 as described in the text. After a second phenol extraction and desalting by passage through a Sephadex G-10 column, the material was lyophilized and the pellet was suspended in 50 μl of 0.05 mM Tris-hydrochloride buffer (pH 8) and divided into two aliquots. The first was treated with BAP (7 U) for 60 min at 37°C. The second aliquot was digested by a combination of BAP and nucleotide pyrophosphatase (100 μg) and incubated for 60 min at 37°C in the presence of 2 mM $MgCl_2$. The samples were then deproteinized by phenol extraction. The supernatant was lyophilized and, after being dissolved in 20 μl of water, was spotted on a polyethyleneimine-cellulose plate and developed with 1.2 M LiCl. When the solvent reached the top of the plate, it was dried and cut into 0.5-cm strips. Radioactivity on the strip was determined by counting in a toluene-based scintillation fluid in a liquid scintillation counter, using the previously described settings that allowed simultaneous detection of ^{32}P and ^{3}H. (A) ^{32}P (●) and ^{3}H (×) radioactivity was measured in BAP-digested RNA synthesized by heat-treated human rotavirus; a similar experiment with transcripts obtained from an EDTA-treated virus mixture is also shown: ○, ^{32}P; □, ^{3}H. (B) The result of a simultaneous RNA digestion with both BAP and nucleotide pyrophosphatase is shown. Symbols are the same as in A.

presence of a guanilyl transferase and an associated methyl transferase activity in single-shelled human rotavirus particles.

DISCUSSION

Human rotavirus particles contain an associated RNA polymerase activity (23, 29) which is able in vitro to transcribe all of the RNA segments after the virus has been activated by incubation with EDTA or a heat shock treatment. EDTA removes the viral outer protein shell (3), but the heat shock does not seem to modify the overall structure of the particle (25). Since no major differences were observed in the activity and in the products synthesized under each condition explored for virus activated by either procedure, the outer shell protein seems not to be directly involved in the transcription process.

The fact that no radioactivity could be detected in association with the virion strongly suggests that transcription is a conservative process rather than a strand displacement reaction, which is possible considering the nature of the template and the transcript (Table 2).

In several eucaryotic and viral systems, ATP seems to play an important role in transcription, besides its requirement for the polymerization itself (9, 17, 27). In vaccinia virus, for example, ATP seems to be required to allow the RNA polymerase to proceed through the template (an activity similar to one described for the Rep protein of *Escherichia coli* during bacterial DNA replication) (26). The reaction seems to be associated with the elongation of newly synthesized RNA molecules. In other viruses such as VSV and CPV, however, ATP is mainly related to the initiation of the transcripts (11). This particular requirement for ATP is reflected in its high K_m for the polymerization reaction, which diminishes with the addition of Adomet (9). In the cases of VSV and CPV, preincubation of the virus with ATP allows transcription in the presence of the β-γ-imido analog, suggesting that ATP is required for some reaction associated with the initiation of RNA synthesis (9, 27). In vaccinia virus, the results are different because preincubation does not overcome the need for ATP during in vitro transcription, suggesting that it is associated with the elongation of RNA chains and that the nucleotide is continually required during transcription (22). The results for human rotavirus described in the present communication suggest that this virus also has a specific requirement for a hydrolyzable form of ATP, which is not overcome by preincubation of the virus with this or the other nucleotides. The requirement for ATP seems to be similar to those observed in vaccinia virus (22). As we reported previously, in human rotavirus all of the nucleoside triphosphates have a similar K_m, except for that of GTP, which is ca. five times higher. In this system, Adomet stimulates transcription up to two to three times (Fig. 2) in heat- and EDTA-treated viruses. In this case, transcription is not dependent on Adomet addition, but it is progressively enhanced by this methyl donor as the ribonucleoside concentration goes up (Fig. 3). The overall effect of Adomet addition is the lowering of the apparent K_m for all of the ribonucleoside triphosphates, including GTP (Fig. 5). The effect of Adomet, in turn, depends on its concentration and may be inhibitory at high values, depending on the level of ribonucleoside triphosphate tested (Fig. 3). One of the interesting features of the Adomet effect is that it does not modify the specific requirement for ATP. Moreover, β-γ-imido analogs of CTP, GTP, or UTP do support transcription, which is also stimulated by the addition of Adomet (Fig. 4). These results strongly suggest that the ATP requirement is not associated with the effect of Adomet and that the addition of this compound modifies the characteristics of the transcription reaction, making it more efficient or increasing the affinity of the system for the substrates. The replacement of AdoHcy for Adomet in an ongoing reaction overcomes its stimulatory effect, suggesting that Adomet does not induce any permanent modification in the transcription system, and that it is continually needed to stimulate polymerization (Fig. 2). A similar observation has been made with eucaryotic RNA polymerase II (17).

The fate of methyl groups incorporated into acid-insoluble material was investigated with the acid of ^{3}H-labeled Adomet (Table 2). Incorporation of methyl groups into acid-insoluble material is proportional to the amount of RNA synthesized, and it is inhibited by the replacement of ATP for the analog, regardless of whether virus was preincubated with cold Adomet (Table 2). In addition, methyl groups were incorporated into transcripts that hybridized to all of the genome RNA segments (Fig. 6), the electrophoretic migration pattern being very similar to that of hybrids formed from transcripts synthesized under standard conditions (Fig. 1).

These results led us to investigate the possible presence of a 5′ cap structure by means of a method previously described for vaccinia virus capped RNA (15, 21). The presence of two types of cap, one of them methylated, was detected in the analyzed RNA (Fig. 7). To show that these products, which comigrated with the standard cap structures GpppG and 7meGpppG were in fact so, their sensitivity to nucleotidyl pyrophosphatase were assayed. The complete sensitivity to the enzyme in combination with BAP strongly suggests the presence of such cap structures, and, based on the results shown in Fig. 6, it can be assumed that all of the RNA segment transcripts have a similarly capped 5′ end. The high K_m for GTP may be explained because it corresponded to the nucleotide involved in the cap and the following nucleotide (Fig. 7).

As a consequence of the results presented above, the stimulatory effect of Adomet on rotavirus transcription may now be explained in terms of the ability of the virus to form and methylate cap structures at the 5′ end of transcripts, assuming that the formation of methylated caps in nascent RNA molecules early commits transcription towards the elongation fraction, causing the decrease in K_m values for the ribonucleoside triphosphates. Our findings also imply that rotavirus particles may contain several enzymes for use in the formation of caps (e.g., a guanilyl transferase) and for other as yet unknown functions, at least one of them related to the continuous need of the system for ATP, which seems to be required for the elongation phase of transcription. Further efforts to investigate the reactions associated with RNA polymerization in viruses or eucaryotic cells will bring a better understanding of the transcription process in these systems, which clearly differs from its counterpart in procaryotes, in which transcription is carried out by RNA polymerase alone and no associated activity is required.

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