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Cyclization of RNA 3′-terminal phosphate by cyclase from HeLa cells proceeds via formation of N(3′)pp(5′)A activated intermediate

(RNA activation/splicing/RNA processing/2′,3′-cyclic phosphate)

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ABSTRACT **RNA 3′-terminal phosphate cyclase has been partially purified from HeLa cells. In the presence of ATP and Mg^{2+}, cyclase preparations catalyze conversion of RNA 3′-terminal phosphate to the 2′,3′-cyclic phosphodiester. The mechanism of 3′-phosphate cyclization was studied with oligoribonucleotides containing terminal 2′-deoxy- or 2′-*O*-methylribose. Incubation of these substrates with cyclase and ATP results in formation of the corresponding activated 3′-terminal structures, dN(3′)pp(5′)A and N^m(3′)pp(5′)A. It is proposed that an intermediate step in cyclization is transfer of the adenylyl group from ATP to the 3′ phosphate of RNA. Rapid attack of the adjacent 2′-OH normally follows, resulting in elimination of AMP and formation of the cyclic phosphodiester. Cyclase preparations can be covalently labeled with [α-^{32}P]ATP, suggesting that an earlier step in the cyclization reaction involves formation of an adenylylated enzyme intermediate.**

RNA 3′-terminal phosphate cyclase, an enzyme that catalyzes conversion of a 3′-phosphate group to the 2′,3′-cyclic phosphodiester at the 3′ end of RNA, has been identified previously in extract of HeLa cells (1). Although the exact function of this enzyme remains unknown, it is likely that the cyclase plays a role in RNA splicing. For example, two distinct RNA ligases involved in tRNA processing in plant (2–8) and animal (1, 9, 10) cell extracts both apparently require substrates with 2′,3′-cyclic phosphate ends (1–4, 6–9). In the case of yeast, it has been demonstrated that cleavage of tRNA precursors by purified splicing endonuclease generates tRNA 5′ halves with cyclic phosphodiester termini (11). The 5′-half molecules that accumulate during pre-tRNA processing in HeLa cell extract also contain 2′,3′-cyclic phosphate termini (9). However, it is not known whether they also are made by a splicing endonuclease or secondarily by the action of the RNA 3′-terminal phosphate cyclase. Since no processes other than RNA ligation are presently known to require a terminal cyclic phosphate (for reviews, see refs. 12 and 13), it is tempting to speculate that a role of cyclase is to generate and/or maintain cyclic structures at the ends of ligation substrates. The presence of cyclase activity in extracts of *Xenopus* oocyte nuclei is consistent with this possibility (1).

Here we report on partial purification of the RNA 3′-terminal phosphate cyclase from HeLa cells. We demonstrate that transfer of the adenylyl group from ATP to the 3′-phosphate of RNA, resulting in formation of an N(3′)pp(5′)A structure, is an intermediate step in the cyclization reaction.

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Materials and Methods

Materials. 2′-*O*-Methylcytidine 3′,5′-bisphosphate (pC^mp) was kindly provided by O. Uhlenbeck (University of Illinois). Sources of enzymes and chemicals have been specified (1–3). [γ-^{32}P]ATP and [α-^{32}P]ATP (≈3000 Ci/mmol; 1 Ci = 37 GBq) were from Amersham. [5′-^{32}P]pCp and [5′-^{32}P]pdCp were prepared from the corresponding nucleoside 3′-monophosphates with T4 polynucleotide kinase and [γ-^{32}P]ATP (14); for [5′-^{32}P]pC^mp, a mixture of pC^m and C^mp, obtained by limited digestion of pC^mp with alkaline phosphatase followed by cellulose TLC in solvent A (see below), was used as acceptor.

Purification Procedure. A cell-free 100,000 × *g* extract was prepared from 15 × 10^9 HeLa S3 cells that had grown to a density of 5 × 10^5 cells per ml in suspension culture (15). After dialysis of the extract against buffer A [20 mM Hepes/KOH, pH 7.6/0.5 mM dithiothreitol/0.1 mM EDTA/10% (vol/vol) glycerol] containing 50 mM NaCl, 55 ml of packed, wet DE-52 cellulose was added and the suspension was stirred for 3 hr. Protein not retained was collected by filtration, and the cellulose cake was washed with an additional 50 ml of the buffer. The combined filtrates were applied to heparin-Sepharose, and proteins were eluted with a linear gradient of 0.05–0.45 M NaCl in buffer A. Fractions containing cyclase activity were combined as pools *a* and *b* (see Fig. 1*A*), and protein was precipitated by addition of $(NH_4)_2SO_4$ to 75% saturation. Protein pellets were collected by ultracentrifugation, dissolved in buffer A containing 0.1 M NaCl, and dialyzed against the same. The retentates were fractionated on Sephacryl S-200 (Fig. 1 *B* and *C*). Fractions containing cyclase activity were pooled and stored in aliquots at −70°C. All purification steps were done at 0–4°C.

Preparation of ^{32}P-Labeled $(Ap)_nA\overset{*}{p}$. $(Ap)_nA_{OH}$ (average chain length, n = 30) was obtained by incubation of 0.5 mg of poly(A) (Miles) in 0.1 ml of 50 mM NaOH for 3 min at 80°C. After acid treatment to open the terminal 2′,3′-cyclic phosphates, the hydrolysate was digested with calf intestine phosphatase (16) and purified by Sephadex G-25 gel filtration. A 5-μg portion of this material was used as acceptor for T4 RNA ligase-catalyzed ligation of [5′-^{32}P]pCp ($\overset{*}{p}$Cp) (14). The resulting $(Ap)_nA\overset{*}{p}Cp$ was converted to $(Ap)_nA\overset{*}{p}$ by treatment with phosphatase, followed by periodate oxidation and β-elimination as described (17). The $(Ap)_nA\overset{*}{p}$ was purified by G-25 gel filtration after addition of 5 μg of tRNA carrier. Analysis of the product (120–400 Ci/mmol, 45–150 cpm/fmol) by nuclease P1, RNase T2, and phosphatase

Abbreviation: $\overset{*}{p}$, ^{32}P-labeled phosphate group.

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digestions indicated that the extent of β-elimination was >90%.

Preparation of 2′-Deoxy and 2′-*O*-Methyl Substrates. $(Ap)_n\overset{*}{p}dCp$ and $(Ap)_nA\overset{*}{p}C^mp$ (150–250 Ci/mmol) were prepared by T4 RNA ligase-catalyzed ligation of [5′-^{32}P]pdCp and [5′-^{32}P]pC^{m}p to $(Ap)_nA_{OH}$ (1, 18) and purified as above. Nonradioactive $(Ap)_n$ApdCp and $(Ap)_n$ApC^{m}p were prepared in the same way but purified without carrier tRNA.

RNA 3′-Terminal Phosphate Cyclase Assay. Reaction mixtures (10 μl) containing 0.1–0.2 pmol of $(Ap)_nA\overset{*}{p}$, 20 mM Hepes/KOH, pH 7.6, 0.1 M NaCl, 2 mM Mg acetate, 80 μM spermidine, 1 mM dithiothreitol, 0.1 mM EDTA, 0.3 mM ATP, and enzyme (30–80 ng of Sephacryl S-200 fraction *a* unless indicated otherwise) were incubated at 25°C for 20 min (or 10 min for cyclase specific activity determinations). Reactions were terminated by addition of 1 μl of 10% $NaDodSO_4$ and 1 unit of phosphatase followed by incubation for 30 min at 37°C. The amount of phosphatase-resistant material was determined either by precipitation with trichloroacetic acid or by retention on activated charcoal. One unit of cyclase renders 1 pmol of ^{32}P-labeled termini resistant to phosphatase in 10 min at 25°C.

Activation of 2′-Deoxy and 2′-*O*-Methyl Substrates. $(Ap)_n\overset{*}{A}pdCp$ and $(Ap)_n\overset{*}{A}pC^mp$ were incubated with cyclase and ATP (or the indicated nucleotide) under conditions identical to those used with $(Ap)_nA\overset{*}{p}$. After incubation, samples were extracted with phenol, and oligonucleotides were recovered by ethanol-precipitation with tRNA carrier. Nonradioactive $(Ap)_n$ApdCp and $(Ap)_n$ApC^{m}p were activated—i.e., adenylylated at the 3′ end—under similar conditions except that reaction mixtures (25 μl) contained 5 pmol of substrate and 3 μM [α-^{32}P]ATP (500 Ci/mmol). Labeled oligonucleotides were recovered by phenol extraction and Sephadex G-25 gel filtration. Excluded material (≈100,000 cpm) was concentrated by ethanol-precipitation and digested with nuclease P1. After TLC in solvent A, radioactive material was recovered from the cellulose plate and used for further analysis. Adenylylation of the 3′ end of $(Ap)_n\overset{*}{A}pC^mp$ in the presence of ATP and T4 RNA ligase was as described (19).

Incorporation of [α-^{32}P]ATP into Protein. Incubations were as described for the cyclase assays except that oligonucleotide substrate was omitted and [α-^{32}P]ATP was 20 μM (50 Ci/mmol). Products were analyzed by electrophoresis in 15% polyacrylamide/$NaDodSO_4$ gels (20) and autoradiography.

Analytical Procedures. Conditions for the various enzyme digestions as indicated have been described (1–3). The following solvents were used for cellulose and PEI-cellulose TLC: A, isobutyric acid/NH_4OH/H_2O (pH 4.3; 577:38:385, vol/vol); B, saturated $(NH_4)_2SO_4$/1 M Na acetate/isopropanol (80:18:2); C, 0.25 M KH_2PO_4. Two-dimensional electrophoresis/homochromatography was as described (21). Nuclease P1-resistant products were analyzed for net charge by DEAE-Sephadex chromatography (22).

RESULTS

RNA 3′-terminal phosphate cyclase was partially purified from HeLa cell extract by ion-exchange chromatography and gel filtration. Two peaks of cyclase activity, *a* and *b*, consistently were eluted from heparin-Sepharose at ≈0.26 and 0.33 M NaCl (Fig. 1*A*). This result was apparently not due to an inhibitor eluted between the peaks because addition of fractions 49 and 51 to pools *a* and *b* did not diminish cyclase activity. Sephacryl S-200 gel filtration of the pooled fractions yielded cyclase preparations *a* (Fig. 1*B*) and *b* (Fig. 1*C*), each ≈100-fold purified as compared to the starting post-ribosomal supernatant sample (Table 1). Fractions *a* and *b* were eluted from Sephacryl S-200 in identical positions near marker chymotrypsinogen (not shown), suggesting that the native cyclase activities have similar M_rs of ≈25,000; the basis for their separation on heparin-Sepharose is unknown.

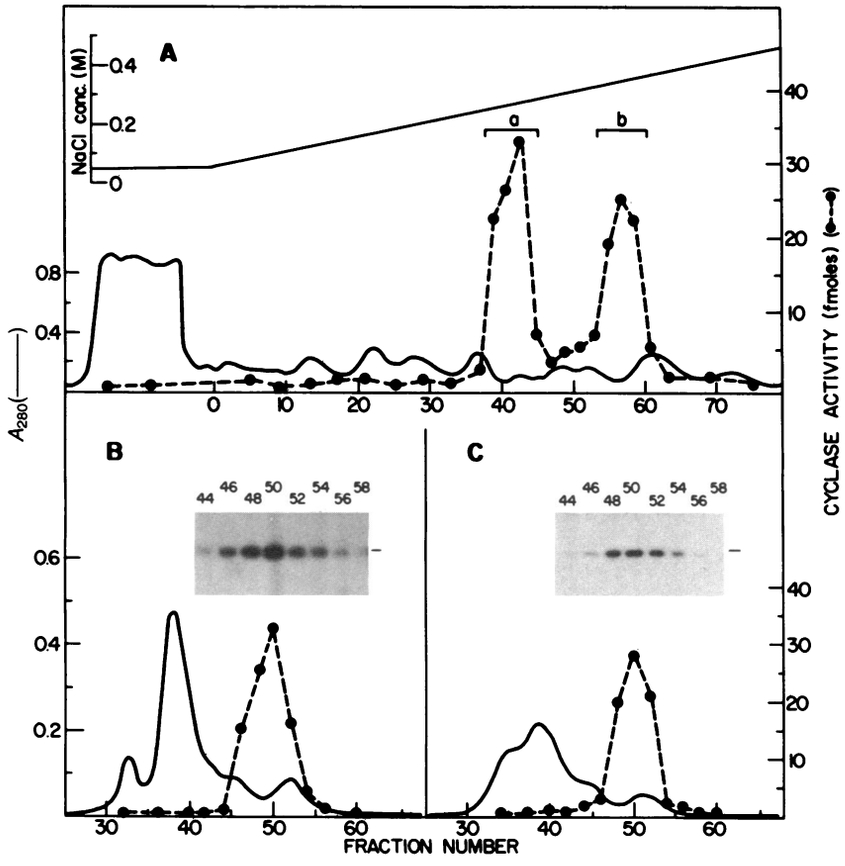

FIG. 1. Purification of cyclase. Volumes assayed for cyclase activity were 1.5 μl (*A*), 0.5 μl (*B*), and 2 μl (*C*). Cyclase activity is expressed as fmol of 3′-termini rendered resistant to alkaline phosphatase in 20 min at 25°C. (*A*) DEAE-cellulose purified protein (Table 1) was applied to heparin-Sepharose (Pharmacia, 1.5 × 20 cm column) equilibrated with buffer A containing 0.1 M NaCl. After washing with 50 ml of buffer, proteins were eluted with 300 ml of a NaCl linear gradient made in buffer A. Fractions of 5 ml collected at 15 ml/hr were combined to give pools *a* and *b*. (*B* and *C*) After $(NH_4)_2SO_4$ precipitation, pools *a* (*B*) and *b* (*C*) were applied to Sephacryl S-200 (Pharmacia, 1.5 × 86 cm column) equilibrated with buffer A plus 0.1 M NaCl. Fractions of 1.85 ml were collected at 7.4 ml/hr. (*Insets*) Protein radiolabeling by [α-^{32}P]ATP. Samples (5 μl) of fractions were incubated with [α-^{32}P]ATP, and proteins were fractionated on 15% polyacrylamide gels relative to several M_r markers. Markers at right correspond to M_r 40,000. Fraction numbers are given above lanes. Autoradiographs of relevant regions of gels are shown.

Table 1. Partial purification of RNA 3′-terminal phosphate cyclase

Fraction	Volume, ml	Total protein, mg	Total activity, units	Specific activity, units/mg of protein
Post-ribosomal supernatant	89.5	280	801	2.86
DEAE-cellulose	135	92.5	897	9.7
Heparin-Sepharose				
a	47	10.3	665	64.4
b	47	12.9	199	15.5
75% $(NH_4)_2SO_4$				
a	2.0	9.3	607	65.7
b	1.9	8.8	156	17.8
Sephacryl S-200				
a	13	0.98	234	239
b	13	0.24	87	361

As reported previously for a crude cyclase preparation (1), ATP stimulated cyclization of the 3′-phosphate of $(Ap)_nA\overset{*}{p}$ 10- to 20-fold (Table 2). The α,β- and β,γ-methylene (or β,γ-imino, not shown) analogs did not substitute for ATP, and 1.2 mM P_i and PP_i were without marked effect. Omission of Mg^{2+} diminished the reaction by 95% (not shown). Other ribonucleoside triphosphates and ADP but not dATP partially substituted for ATP (Table 3). However, trypsin pretreatment substantially decreased the level of cyclization obtained in their presence without affecting the ATP-dependent reaction. Since cyclase is resistant to proteases (1), the activity of the other nucleotides probably resulted largely (but not entirely, see below) from contaminating, trypsin-sensitive enzymes (e.g., nucleoside diphosphate kinase, myokinase and/or others).

An ATP hydrolysis requirement for conversion of RNA 3′-terminal phosphate to the 2′,3′-cyclic form suggested that cyclization proceeds via an activated intermediate structure—N(3′)pp(5′)A, analogous to the A(5′)pp(5′)N (activated 5′-phosphate) formed at the terminus of the donor RNA molecule in T4 RNA ligase-catalyzed reactions (reviewed in ref. 23). Hinton *et al.* (19) have observed that in the absence of natural, 5′-phosphorylated substrate, T4 RNA ligase can activate 3′-phosphate-terminated oligodeoxyribonucleotides at a low rate. However, it was anticipated that intermediate RNA molecules containing N(3′)pp(5′)A ends would be difficult to demonstrate, because of rapid elimination of AMP and formation of the cyclic phosphate (19, 24). Consequently, $(Ap)_nA\overset{*}{p}dCp$ and $(Ap)_nA\overset{*}{p}C^mp$, oligonucleotides containing terminal 2′-deoxy- and 2′-*O*-methylribose, respectively, were tested with cyclase and

Table 2. Requirements of cyclase fractions *a* and *b*

Additions	Activity, fmol/20 min *a*	*b*
None	2.0	0.7
ATP	16.4	10.1
AMPCPP	1.1	0.6
AMPPCP	1.3	0.8
ATP + PP_i	18.1	10.1
ATP + P_i	14.9	9.6

a, 10 ng of fraction 50 protein, Fig. 1*B*; *b*, 5 ng of fraction 50 protein, Fig. 1*C*. Note that the specific activities are correspondingly ~3-fold greater than obtained with pooled fractions *a* and *b*, Table 1. Concentrations: 0.3 mM nucleotides, 1.2 mM P_i and PP_i. AMPCPP and AMPPCP, α,β- and β,γ-methylene analogs of ATP.

Table 3. Nucleotide requirements of RNA 3′-phosphate cyclase

Nucleotide	Activity, fmol/20 min Control	Trypsin-treated
None	1.50	0.71
ATP	30.15	28.43
GTP	4.85	1.75
CTP	6.20	1.20
UTP	5.02	1.29
dATP	1.70	0.97
ADP	12.62	1.48

A 1-μg sample of the Sephacryl S-200 pool *a* cyclase preparation was preincubated for 20 min at 37°C with 2 μg of trypsin (Worthington) in buffer A containing 0.1 M NaCl. Aliquots (1 μl, 80 ng of protein) then were used in 10-μl cyclase reactions containing the indicated nucleotides (0.3 mM).

ATP. After incubation the oligonucleotides were digested with nuclease P1, and products were analyzed by TLC. $(Ap)_nA\overset{*}{p}C^mp$ and $(Ap)_nA\overset{*}{p}dCp$ incubated in the absence of ATP yielded $\overset{*}{p}C^m$ and $\overset{*}{p}dC$, respectively (Fig. 2, lanes 6 and 9), indicative of unreacted substrates. $(Ap)_nA\overset{*}{p}C^mp$ incubated in the presence of 0.3 mM ATP yielded a novel, more slowly migrating spot (lane 1); it was not formed when ATP was replaced with β,γ- or α,β-methylene analogs or with 1 mM dATP or GTP (lanes 7, 8, 2, and 5, respectively). The ATP-dependent spot also was not obtained in the presence of 1 mM UTP or CTP, although lower levels of other products (identified below) were formed (lanes 3 and 4). Analysis of products formed from $(Ap)_nA\overset{*}{p}dCp$ in the presence of ATP or other nucleotides gave results identical to those obtained with 2′-*O*-methylated substrate (Fig. 2, lane 10; and data not shown). Incubation of either substrate with Sephacryl fraction *b* and ATP yielded nuclease P1-resistant products identical to those formed with fraction *a* as tested by cellulose TLC (not shown). Modification of substrates in the presence of other nucleotides was not studied with fraction *b*.

Chromatographic mobilities of nuclease P1-resistant products formed in the presence of ATP (Fig. 2, lanes 1 and 10) are consistent with the structures $\overset{*}{p}C^m(3')pp(5')A$ and $\overset{*}{p}dC(3')pp(5')A$. To verify these structures, the slowly mi-

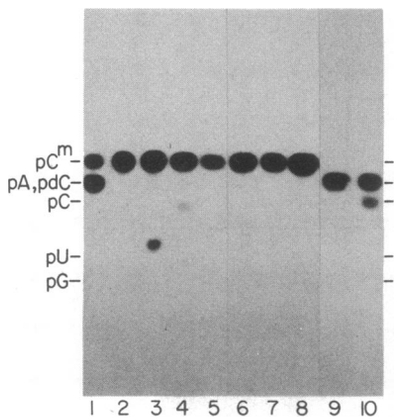

FIG. 2. Activation of 2′-*O*-methyl and 2′-deoxy substrates. $(Ap)_nA\overset{*}{p}C^mp$ (lanes 1–8) or $(Ap)_nA\overset{*}{p}dCp$ (lanes 9 and 10) were incubated with cyclase and the following nucleotides: 0.3 mM ATP (lanes 1 and 10), 1 mM dATP (lane 2), 1 mM UTP (lane 3), 1 mM CTP (lane 4), 1 mM GTP (lane 5), none (lanes 6 and 9), 0.3 mM adenosine 5′-[β,γ-methylene]triphosphate (lane 7), and 0.3 mM adenosine 5′-[α,β-methylene]triphosphate (lane 8). After phenol extraction and ethanol-precipitation, radioactive oligonucleotides were digested with nuclease P1, and products were analyzed by TLC on cellulose plates in solvent A followed by autoradiography.

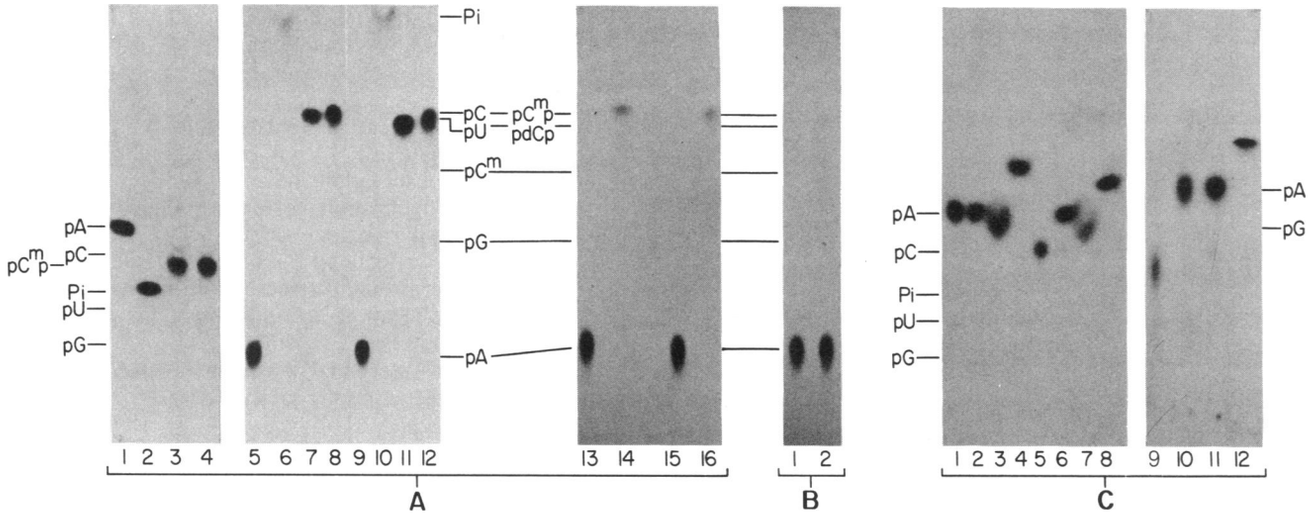

FIG. 3. Analysis of the structure of nuclease P1-resistant activation products. (*A*) After incubation of $(Ap)_nA\overset{*}{p}C^mp$ (lanes 1–8, 13, and 14) or $(Ap)_nA\overset{*}{p}dCp$ (lanes 9–12, 15, and 16) with cyclase and ATP, nuclease P1-resistant material isolated as in Fig. 2 was treated with calf intestine phosphatase (lanes 2, 6, and 10), tobacco acid pyrophosphatase (lanes 3, 7, and 11), snake venom phosphodiesterase (lanes 4, 8, and 12), or periodate followed by β-elimination (lanes 14 and 16). Control, untreated samples are shown in lanes 1, 5, 9, 13, and 15. Cellulose TLC was in solvent A (lanes 1–4) or solvent B (lanes 5–16). (*B*) $(Ap)_nA\overset{*}{p}C^mp$ was incubated with T4 RNA ligase and ATP for 72 hr at 17°C (19), and products were digested with nuclease P1 and subjected to cellulose TLC in solvent A. The slowly migrating spot [7% of input cpm, mobility identical to nuclease P1-resistant products of activation of $(Ap)_nA\overset{*}{p}C^mp$ with cyclase] was isolated and subjected to cellulose TLC in solvent B (lane 1) together with the corresponding activated product obtained by reaction of $(Ap)_nA\overset{*}{p}C^mp$ with cyclase (lane 2). (*C*) Unlabeled $(Ap)_nApC^mp$ (lanes 1–4 and 9–12) or $(Ap)_nApdCp$ (lanes 5–8) was incubated with cyclase and [α-^{32}P]ATP, and nuclease P1-resistant material, isolated as described in *Materials and Methods*, was subjected to digestion with snake venom phosphodiesterase (lanes 2, 6, and 10), tobacco acid pyrophosphatase (lanes 3, 7, and 11), or calf intestine phosphatase (lanes 4, 8, and 12). Untreated samples are in lanes 1, 5, and 9. TLC was on cellulose plates in solvent A (lanes 1–8) or on PEI-cellulose plates in solvent C. Trailing in lanes 3 and 7 is due to glycerol in the enzyme preparation.

grating nuclease P1-resistant compounds were isolated from TLC plates and characterized further. Digestion of presumptive $\overset{*}{p}C^mppA$ with calf intestine phosphatase yielded radioactive P_i (Fig. 3*A*, lanes 2 and 6), and treatment with tobacco acid pyrophosphatase (lanes 3 and 7) or snake venom phosphodiesterase (lanes 4 and 8) generated material comigrating with pC^mp. Analysis of activated 2′-deoxy product gave analogous results (Fig. 3*A*, lanes 9–12). Both activated compounds had a net charge close to −4 that was decreased 1 unit by β-elimination, in agreement with conversion of $\overset{*}{p}C^mppA$ and $\overset{*}{p}dCppA$ (−4) to $\overset{*}{p}C^mpp$ and $\overset{*}{p}dCpp$ (−5), respectively (data not shown). Products of β-elimination of presumptive $\overset{*}{p}C^mppA$ and $\overset{*}{p}dCppA$ were also analyzed by cellulose TLC. They migrated as expected for $\overset{*}{p}C^mpp$ and $\overset{*}{p}dCpp$; i.e., slower in solvent A and slightly faster in solvent B relative to pC^mp (Fig. 3*A*, lanes 13–16; and data not shown). In addition, $(Ap)_nA\overset{*}{p}C^mp$ was incubated with T4 RNA ligase and ATP under conditions expected to generate the modified oligonucleotide, $(Ap)_nA\overset{*}{p}C^m(3')pp(5')A$ (19). Nuclease P1 digestion of the reaction product yielded a compound that comigrated with material generated in the cyclase-catalyzed reaction (Fig. 3*B*).

Unlabeled $(Ap)_nApC^mp$ and $(Ap)_nApdCp$ were also incubated with cyclase in the presence of [α-^{32}P]ATP, and the ^{32}P-labeled oligonucleotide products were analyzed. Digestion of each oligonucleotide with nuclease P1 yielded a single radioactive spot that comigrated with the respective nuclease P1-resistant compound obtained after activation of $(Ap)_nA\overset{*}{p}C^mp$ and $(Ap)_nA\overset{*}{p}dCp$ (not shown). These spots were identified as $pC^mp\overset{*}{p}A$ and $pdCp\overset{*}{p}A$ as follows. Treatment with snake venom phosphodiesterase (Fig. 3*C*, lanes 2, 6, and 10) or tobacco acid pyrophosphatase (lanes 3, 7, and 11) yielded $\overset{*}{p}A$. Digestion with phosphatase did not release radioactive P_i but changed the mobility consistent with the expected products $C^mp\overset{*}{p}A$ (lanes 4 and 12) and $dCp\overset{*}{p}A$ (lane 8). The results support the conclusion that $(Ap)_nApC^mp$ and $(Ap)_nApdCp$ are activated in the presence of cyclase and ATP to yield the 3′-terminal N(3′)pp(5′)A structures and indicate that ATP is a donor of the terminal adenylyl group in the activation reaction.

An intermediate step during activation of 5′-terminal phosphate by T4 RNA ligase is the formation of a covalent AMP-enzyme complex from which the adenylyl group is subsequently transferred to the RNA terminus to yield an A(5′)pp(5′)N structure (reviewed in ref. 23). To test if a similar AMP-protein intermediate can be formed by the 3′-phosphate activating enzyme, aliquots of Sephacryl S-200 fractions that contained cyclase activity were incubated with [α-^{32}P]ATP, and proteins were analyzed by NaDodSO$_4$/PAGE. For both cyclase fractions *a* and *b*, a single radiolabeled polypeptide of apparent $M_r \approx 40{,}000$ was obtained (*Insets*, Fig. 1 *B* and *C*). In each case the intensity of labeling coincided exactly with the profile of cyclase activity, strongly suggesting that the adenylylated protein corresponds to the RNA 3′-terminal phosphate cyclase. Presumably, changes in conformation account for the difference in apparent M_r of native enzyme (≈25,000) compared to that of denatured, presumptive adenylylated cyclase (≈40,000).

The activation products of $(Ap)_nA\overset{*}{p}dCp$ obtained with ATP, CTP, and UTP also were analyzed. Radiolabeled dinucleotides released by nuclease P1 digestion were subjected to two-dimensional electrophoresis/homochromatography before and after digestion with snake venom phosphodiesterase. In each case phosphodiesterase treatment yielded radioactive $\overset{*}{p}dCp$, and the mobility shifts were diagnostic for removal of pA (not shown), pC (Fig. 4*A*), and pU (Fig. 4*B*), respectively. It is therefore likely that the partially purified cyclase preparation can use CTP and UTP, although less efficiently than ATP, for activation of 2′-deoxy and 2′-*O*-methyl substrates.

DISCUSSION

We have partially purified RNA 3′-terminal phosphate cyclase from HeLa cells, using as a convenient assay the

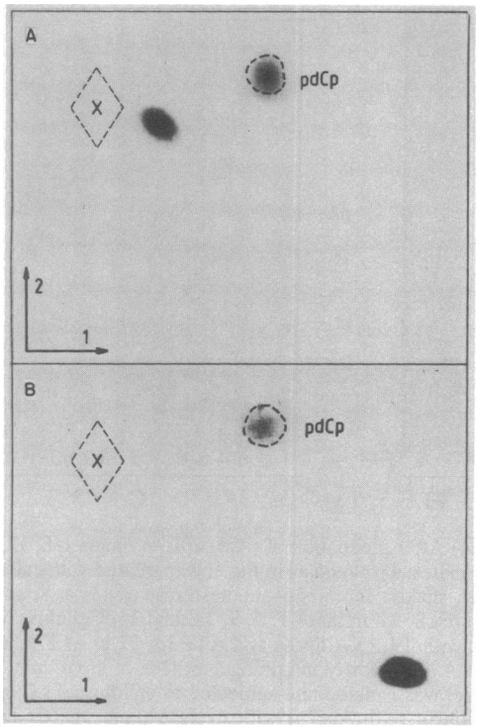

FIG. 4. Two-dimensional analysis of activated dinucleotides. $(Ap)_n\overset{*}{A}pdCp$ was incubated with cyclase and 1 mM CTP (*A*) or UTP (*B*). Aliquots of nuclease P1-resistant, activated dinucleotides isolated from cellulose TLC plates as in Fig. 2 were digested with snake venom phosphodiesterase, combined with an undigested aliquot, and subjected to high-voltage electrophoresis (dimension 1) and homochromatography (21) with 50 mM homomix (dimension 2). Xylene cyanol (X) migrated 8 and 22 cm in dimensions 1 and 2, respectively. Broken circle indicates position of $\overset{*}{p}dCp$ marker on a parallel plate.

conversion of 3′-^{32}P-labeled substrate to material resistant to alkaline phosphatase. Two enzyme preparations were obtained as a result of an unexplained separation of cyclase activity into distinct peaks during chromatography on heparin-Sepharose. Each required ATP and Mg^{2+} as cofactors for cyclization of RNA 3′-terminal phosphate. Cyclase activity in each preparation was eluted in a position corresponding to $M_r \approx 25{,}000$ during gel filtration on Sephacryl S-200, and after incubation with [α-^{32}P]ATP each yielded a single radiolabeled polypeptide of apparent M_r 40,000 by NaDodSO$_4$/PAGE. A M_r 40,000 adenylylated polypeptide also has been obtained with highly purified HeLa cell cyclase (D. Reinberg, J. Arenas, and J. Hurwitz, personal communication). The discrepancy between the molecular weights estimated by gel filtration and NaDodSO$_4$/PAGE implies that native cyclase may be a very compact protein, a suggestion supported by the relative resistance of the enzyme to proteases (1).

The mechanism of 3′-phosphate cyclization was studied with oligonucleotides containing terminal 2′-deoxy- or 2′-*O*-methylribose. Incubation of these substrates with cyclase and ATP resulted in the formation of activated 3′-terminal structures corresponding to dN(3′)pp(5′)A and N^m(3′)pp(5′)A, respectively. Adenylylation of the RNA 3′-terminus was confirmed by incorporation into these structures of radioactive pA from [α-^{32}P]ATP. Together with the observation that protein fractions containing cyclase activity can be covalently labeled with [α-^{32}P]ATP, these results suggest the following reaction mechanism for RNA 3′-terminal phosphate cyclase: (*i*) formation of an enzyme-AMP intermediate; (*ii*) transfer of the adenylyl group from protein to the RNA 3′-terminal phosphate to form the N(3′)pp(5′)A structure; and (*iii*) rapid attack of the adjacent 2′-hydroxyl, resulting in elimination of AMP and formation of the 2′,3′-cyclic phosphodiester. Further characterization of the [α-^{32}P]ATP-labeled protein and direct demonstration that an adenylyl group can be transferred from protein to the 3′-terminal phosphate in RNA will be required to prove this mechanism. Two observations have not yet been reconciled with the proposed cyclization mechanism—inactivity of β,γ-methylene and -imino analogs as substitutes for ATP and lack of an inhibitory effect by PP_i on the cyclization reaction. Possibly the former is due to structural differences between the analogs and ATP or to a requirement for β,γ-linkage hydrolysis during enzyme activation.

Activation of the 3′-terminal phosphate in RNA by cyclase is very similar to the reactions catalyzed by T4 (reviewed in ref. 23) and eukaryotic RNA ligases (4, 7) which produce A(5′)pp(5′)N structures at the 5′ end of RNA. With all these enzymes, transfer of the adenylyl group from ATP to RNA occurs via formation of a covalent AMP-protein intermediate (4, 23). T4 RNA ligase can also activate the 3′-phosphorylated ends of oligodeoxynucleotides (19) by reactions that closely resemble those catalyzed by the RNA 3′-terminal phosphate cyclase from HeLa cells. With the HeLa cyclase preparations, low levels of cytidylylation and uridylylation of substrates containing terminal 2′-deoxy- or 2′-*O*-methylribose have also been observed. However, further studies will be necessary to determine whether these reactions are catalyzed by cyclase or by other HeLa cell proteins.

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