The Enzymatic Conversion of 3'-Phosphate Terminated RNA Chains to 2',3'-Cyclic Phosphate Derivatives*

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The enzyme, RNA cyclase, has been purified from cell-free extracts of HeLa cells approximately 6000-fold. The enzyme catalyzes the conversion of 3'-phosphate ends of RNA chains to the 2',3'-cyclic phosphate derivative in the presence of ATP or adenosine 5'-(γ-thio)triphosphate (ATPγS) and Mg++. The formation of 1 mol of 2',3'-cyclic phosphate ends is associated with the disappearance of 1 mol of 3'-phosphate termini and the hydrolysis of 1 mol of ATPγS to AMP and thiophosphate. No other nucleotides could substitute for ATP or ATPγS in the reaction.

The reaction catalyzed by RNA cyclase was not reversible and exchange reactions between [32P]pyrophosphate and ATP were not detected. However, an enzyme-AMP intermediate could be identified that was hydrolyzed by the addition of inorganic pyrophosphate or 3'-phosphate terminated RNA chains but not by 3'-OH terminated chains or inorganic phosphate.

3'-[32P]dGp* could be converted to a form that yielded (pG)2Pp'-U, after degradation with nuclease P1, by the addition of wheat germ RNA ligase, 5'-hydroxylpolynucleotide kinase, RNA cyclase, and ATP. This indicates that the RNA cyclase had catalyzed the formation of the 2',3'-cyclic phosphate derivative, the kinase had phosphorylated the 5'-hydroxyl end of the RNA, and the wheat germ RNA ligase had catalyzed the formation of a 3',5'-phosphodiester linkage concomitant with the conversion of the 2',3'-cyclic end to a 2'-phosphate terminated residue.

The coding regions of many eukaryotic genes are interrupted by noncoding regions called intervening sequences or introns (1, 2). Structural studies of several RNA precursor molecules have provided evidence that split genes are transcribed as collinear precursor molecules from which the intervening sequences are excised by a cleavage-ligation reaction, a process known as splicing (1-6). Elucidation of the biochemical steps in the splicing of RNA is of fundamental importance for understanding of regulation of gene expression. In vitro systems that accurately splice mRNA (7-13), tRNA (14-16), and rRNA (17) have been developed. The nature of the endonucleases and RNA ligases involved in these processes has only recently been addressed. Konarska et al. (18, 19) discovered that crude extracts of wheat germ contains an RNA ligase activity that requires an RNA substrate with 2',3'-cyclic phosphate and 5'-phosphate termini, and that ligation of the ends produces an unusual 2'-phosphomonoester-3',5'-phosphodiester linkage. The general importance of this reaction has been underscored by the observation that an RNA ligase that catalyzes a similar reaction is involved in the in vitro splicing of yeast precursor tRNA (20). In yeast, endonucleolytic cleavage of the tRNA precursor produces a 2',3'-cyclic phosphate on the 5' exon and a 5'-hydroxyl on the 3' exon (20). The 5'-hydroxyl group is then phosphorylated by transfer of the γ-phosphate from ATP, and it is thought that this end is further activated by transfer of AMP using a second molecule of ATP (20). Ligation of the two exons results in the formation of a 2'-phosphomonoester, 3',5'-phosphodiester bond. The phosphate forming the phosphodiester bond originates from the γ-phosphate residue of ATP, and the 2'-phosphate is derived from the 2',3'-cyclic phosphate derivative.

A different situation occurs with tRNA splicing in animal cells (3, 21, 22). Analysis of the junction oligonucleotides isolated from tRNA spliced in extracts of HeLa cells demonstrated that the 3'-terminal phosphate from the 5' half of the tRNA molecule is incorporated into a normal 3',5'-phosphodiester linkage. Filipowicz, Shatkin and co-workers (21, 23) have shown that a ribonuclease T1-generated oligonucleotide from tobacco mosaic virus (called ω-RNA) is also an effective substrate for detecting ligase activity in HeLa cell extracts, and as in tRNA ligation, the ends are joined by a 3',5'-phosphodiester bond. Studies with different substrates indicated that the phosphate residue in the newly formed phosphodiester linkage was derived from a terminal 2',3'-cyclic phosphate residue. Furthermore, they showed that HeLa cell extracts could circularize ω-RNA containing a 3'-terminal phosphomonoester group. In this case, the ligation reaction was preceded by an ATP-dependent conversion of the 3'-phosphate end to the 2',3'-cyclic form (23). These newly discovered reactions have underscored the importance of enzymes involved in altering the termini of RNA chains. In this

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and adsorbable material was measured as described under "Materials and Methods," contained 500 fmol (as molecules) of (Up),Gp* (140 cpm/fmol) and varying amounts of extract (14.8 mg of protein/ml). Norit-adsorbable material was measured as described under "Materials and Methods." Closed circles indicate reactions which contained 1 mM ATP and open circles indicated reactions without ATP.

report, the purification and characterization of the reaction catalyzed by 3'-phosphate RNA cyclase isolated from HeLa cells are described.

MATERIALS AND METHODS\(^1,2\)

RESULTS

RNA Cyclase Activity in Crude Extracts of HeLa Cells—Filipowicz et al. (23) discovered that HeLa cell extracts contain an activity that converts 3'-phosphate terminated RNA chains to the 2',3'-cyclic phosphate derivative. When 3'-32P-labeled RNA was incubated with varying amounts of cell-free extract of HeLa cells, prepared as previously described (28), and then subjected to the action of nuclease P1, a 32P-labeled product was recovered as Norit-adsorbable material. The formation of this product, as previously reported (23), was ATP-dependent (Fig. 1). The nature of the P1 nuclease-resistant material was further investigated by digesting the products formed from (Up),Gp* with RNase A followed by thin layer chromatography analysis. These results (Fig. 2A) indicated that 93% of the 32P label co-migrated with G>p and 7% co-migrated with 2'-GMP, 3'-GMP, and a faster migrating spot. The formation of the 2'-phosphate terminated product was expected since it was determined that extracts of HeLa cells contain an ATP-independent activity that hydrolyzes 2',3'-cyclic phosphate derivatives to the 2'-ester. When AUG>Gp* was incubated with extracts of HeLa cells in the absence of ATP, followed by treatment with RNase A and analyzed by thin layer chromatography, the product formed was 2'-GMP and no 3'-GMP was detected (Fig. 2B). The same result was obtained with 2',3',5'-P-cyclic mononucleotides or (Up),Gp* (data not shown).

Purification of HeLa Cell RNA Cyclase—The procedure developed for the isolation of RNA cyclase activity is summarized in Table I. The enzyme was purified from the cytosolic fraction (S100) although nuclear extracts contained an equal amount of activity.

FIG. 1. RNA cyclase activity in crude extracts of HeLa cells. Reaction mixtures (30 µl), as described under "Materials and Methods," contained 500 fmol (as molecules) of (Up),Gp* (140 cpm/fmol) and varying amounts of extract (14.8 mg of protein/ml). Norit-adsorbable material was measured as described under "Materials and Methods." Closed circles denote reactions which contained 1 mM ATP and open circles indicated reactions without ATP.

FIG. 2. Analysis of reaction products formed with HeLa cell extracts (A) and phosphodiesterase activity in HeLa cell extracts (B). A, reaction mixtures (30 µl), as described in the legend to Fig. 1 with (Up),Gp*, contained 15 µg of protein. Reactions were stopped by extraction with an equal volume of phenol-chloroform. The aqueous phase was adjusted to 0.1 M Tris-HCl buffer, pH 8.0 (100 µl), and 1 µg of RNase A was added. After 30 min of incubation at 37°C, 25 µl of Norit (10% suspension) and 1 ml of 1% trichloroacetic acid were added. The Norit was recovered by centrifugation and adsorbed material was eluted with 2% NH4OH in 60% ethanol. Samples were concentrated in a vacuum speed centrifuge and resuspended in 5 µl of 0.05 M Tris-HCl buffer, pH 8.0. Products were separated on cellulose plates using Solvent A. Unlabeled markers were run in the same lanes, and their positions are noted in the center of the autoradiogram. B, reaction mixtures (30 µl) were as described in the legend to Fig. 1. In this experiment, AUG>Gp* (500 fmol) was incubated with HeLa cell crude extract (15 µg of protein) in the absence of ATP. Reactions were stopped and processed as described above. Samples treated with RNase T1 received 1 unit of enzyme, and incubations were carried out as described for RNase A. Products were separated on polyethyleneimine-cellulose TLC plates using Solvent A.

1 The "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3040, cite the authors, and include a money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; ATPγS, adenosine 5'-[(γ-thio)triphosphate; AMP-PNP, adenosine 5'-[(β,γ-imido)triphosphate; AMP-PCP, adenosine 5'-[(β,γ-methylene)triphosphate; AMP-CPP, adenosine 5'-[(α,β-methylene)triphosphate; Mops, 3-(N-morpholino)propanesulfonic acid; BALP, bacterial alkaline phosphatase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEl, polyethyleneimine.
dialysis, insoluble material was removed by centrifugation at 14,000 rpm for 25 min, and the supernatant (115 ml) was applied to a 90-ml DEAE-cellulose column (12 x 3 cm) equilibrated with Buffer B containing 0.05 M NaCl. Variable amounts, but no more than 30% of the RNA cyclase activity, were recovered in the flow-through fraction. The activity retained by the column was eluted with 3 volumes of Buffer C containing 0.2 M NaCl. The enzyme recovered in this fraction (43 ml) was dialyzed against 4 liters of buffer containing 20 mM Hepes buffer, pH 7.3, 1 mM dithiothreitol, 10^{-4} M phenylmethylsulfonyl fluoride, 10% glycerol (Buffer C), containing 0.05 M NaCl for 3 h and applied to a 65-ml poly(A)-agarose column (9 x 3 cm) equilibrated with the same buffer; the flow-through was collected and passed through the column again to ensure maximum retention of the enzyme activity. The column was then washed with 3 volumes of Buffer C plus 0.05 M NaCl. The activity was eluted with a linear gradient (500 ml) of 0.1-0.8 M NaCl in Buffer C. The cyclase activity was recovered as a single peak eluting at 0.5 M NaCl. This fraction was concentrated 10-fold by dialysis against 1.5 liters of 27% polyethylene glycol (20,000) in 0.1 M NaCl, 20 mM Hepes buffer, pH 7.3, 1 mM dithiothreitol, 0.01% Nonidet P-40, 10^{-4} M phenylmethylsulfonyl fluoride, and 10% glycerol overnight and then dialyzed against 1 liter of Buffer B containing 0.05 M NaCl for 3 h. The material (15 ml) was applied to a 1.5-ml heparin-Ultrigel column (2.5 x 0.8 cm) equilibrated with the same buffer, and the flow-through was collected and passed through the column again. The column was then washed with 10 ml of Buffer B containing 0.05 M NaCl and eluted stepwise with 6 ml of 0.25 M NaCl and 6 ml of 0.55 M NaCl in buffer B; 62, 5, and 8% of the activity was recovered in the flow-through fraction, the 0.25 M NaCl fraction, and the 0.55 M NaCl fraction, respectively. In some cases almost all of the activity was recovered in the 0.25 M NaCl fraction. When an aliquot of the flow-through (after being frozen at -70 °C for 1 week) was loaded onto a new heparin-Ultrigel column under the same conditions described above, the same three fractions were obtained (data not shown).

The enzyme isolated by the above procedure was purified 6000-fold with a 25% recovery. The enzyme sedimented as a single peak (2.6 S) in glycerol gradients containing 0.1 M NaCl, suggesting a molecular weight of 40,000 (Fig. 3).

SDS-polyacrylamide gel analysis of glycerol gradient fractions followed by silver staining yielded four major bands, one of which migrated with the labeled RNA cyclase-AMP complex (as described below). The heparin-Ultrigel fraction was stable for at least 3 months at -70 °C but lost 50% of its activity after 3 days at 4 °C. This fraction contained no detectable myokinase, 2'-3'-cyclic phosphodiesterase, phosphatase, RNase, or RNA ligase (using (Up),,pG>p substrate) activities; however, it contained detectable ATPase (ATP → ADP + P) and 5'-hydroxyxypolyynucleotide kinase. The ATPase contaminant that hydrolyzed ATP to ADP and P, was partially removed by glycerol gradient centrifugation as shown in Fig. 3.

The heparin-Ultrigel fractions contained an activity that hydrolyzed small amounts of ATP and ATPγS to AMP and PP, or thionophosphate in the absence of added 3'-phosphate terminated RNA under the conditions specified in Fig. 10A, lane 4 (see also Table IV). It is unclear whether this activity is intrinsic to the cyclase activity or whether it is due to trace amounts of 3'-phosphate terminated RNA in the enzyme preparation or a contaminant. The activity that hydrolyzed ATPγS co-sedimented with cyclase activity on glycerol gradient centrifugation.

Characterization of Products and Requirements of Purified RNA Cyclase—With the purified enzyme, the product formed from 3'-phosphate terminated oligonucleotides was shown to be the cyclic derivative. For this purpose, AUp* was incubated with the enzyme plus ATP or ATPγS and then treated with RNase A and bacterial alkaline phosphatase. The product was subjected to thin layer chromatography, and the only labeled material detected co-migrated with Gp·p and P·p (Fig. 4). The latter product was derived from unreacted 3'-[32P]AUp. This reaction was heat-labile, required Mg^{2+} (K_{M} 2.5 mm), and was N-ethylmaleimide-sensitive (Table II): Mn^{2+} could not replace Mg^{2+} in the reaction (data not presented). Sodium pyrophosphate and sodium phosphate inhibited the
reaction; 50% inhibition was obtained at concentrations of 3 and 30 mM, respectively. Cyclase activity was inhibited 50% by 0.4 M NaCl. Vanadyl-ribonucleoside complex inhibited RNA cyclase activity 50% at concentrations of 1–2 μM (Fig. 5). The effect of this inhibitor could be overcome by the addition of more enzyme. Thus, for example, under the conditions described in the legend to Fig. 5, 1.5 μM vanadyl-ribonucleoside complex inhibited the reaction 52%; the addition of a 3-fold higher concentration of AUGp or ATP to the inhibited reaction had no effect, whereas the addition of more cyclase (0.45 unit) stimulated the cyclase activity 4-fold (data not presented).

The purified enzyme showed an absolute requirement for ATP. No other commonly occurring ribonucleotide or deoxyribonucleotide substituted for ATP (Table III). As previously reported (23), the α, β- or β,γ-methylene analogues of ATP did not substitute for ATP nor did the β,γ-imido derivatives. ADP, AMP, and the β,γ-methylene and β,γ-imido ATP derivatives inhibited the RNA cyclase reactions only at relatively high concentrations. ATPγS was 3-fold more effective for RNA cyclase than ATP (Table III and Fig. 6). The Kₘ values for ATP and ATPγS were 6 and 2 μM, respectively. The same cyclic phosphate product was produced in reactions containing ATPγS as those containing ATP (Fig. 4).

In contrast to the reaction observed with (Up)₃G-3'-p, (Up)₃G-2'-p was totally inactive (Fig. 7); the 2'-phosphate terminated RNA did not inhibit the cyclase reaction with the 3'-phosphate derivative (data not shown). The activity of RNA cyclase with different guanine 3'-phosphate terminated polynucleotide chains was also examined (Fig. 7). (Up)pGp* was cyclized at a faster rate than tRNApGp* or AUGp*. All nucleoside bisphosphate derivatives tested were inactive.

The nucleotide specificity at the 3' end of RNA chains was also investigated. For this purpose, 3'-OH,5'-OH tRNA₉fmet was ligated with 3'-[¹³⁵P]PCp* or 3'-[²³P]PCp* or 3'-[¹³⁵P]PCp*
TABLE III
Nucleotide requirement for RNA cyclase activity
Reactions (20 μl) contained 20 mM Hepes buffer, pH 7.9, 5 mM MgCl₂, 3 mM dithiothreitol, 50 μM ATP (where indicated), 1 pmol of AUGp*, and 0.15 unit of cyclase in Experiment 1 and 0.1 unit in Experiments 2 and 3 (heparin-agarose flow-through). Incubation was for 30 min at 30 °C, and reactions were halted and processed as described under "Materials and Methods."

| Experiment | Additions | Nuclease P1-resistant material (fmol) |
|------------|-----------|-------------------------------------|
| 1          | Complete  | 132                                 |
|            | Omit ATP, add GTP or UTP or CTP (1 mM) | <1                                  |
|            | Omit ATP, add dATP or GdTP or dCTP or dTTP (1 mM) | <1                                  |
| 2          | Complete  | 103                                 |
|            | Omit ATP  | <1                                  |
|            | Omit ATP, add ADP (1 mM) | 13                                  |
|            | Omit ATP, add AMP (1 mM) | <1                                  |
|            | Complete plus ADP (1 mM) | 49                                  |
|            | Complete plus AMP (1 mM) | 34                                  |
| 3          | Complete  | 94                                  |
|            | Omit ATP, add AMP-PCP (0.1 mM) | 3                                   |
|            | Omit ATP, add AMP-CPP (0.1 mM) | <1                                  |
|            | Omit ATP, add AMP-PNP (0.1 mM) | <1                                  |
|            | Omit ATP, add ATP₇S (0.1 mM) | 197                                 |
|            | Complete plus AMP-PCP (0.1 mM) | 65                                  |
|            | Complete plus AMP-PNP (0.1 mM) | 49                                  |
|            | Complete plus ATP₇S (0.1 mM) | 166                                 |

and incubated with RNA cyclase. No marked differences in the rate of utilization of these substrates were detected.

Stoichiometry of the RNA Cyclase Reaction and Reaction Products—In the presence of excess ATP or ATP₇S and limiting amounts of AUGp*, the latter compound could be quantitatively converted to the 2',3'-cyclic product (Fig. 8). Similar quantitative reactions were observed with (Up)_₃Gp* and tRNApGp*.

In contrast to the rapid reaction in the presence of excess ATP or ATP₇S, the reaction proceeded more slowly in the presence of limiting concentrations of ATP₇S. The most highly purified preparations of RNA cyclase hydrolyzed ATP in the absence of RNA while other less purified fractions contained RNA- and DNA-dependent ATPase activities as well. However, when ATP₇S was used, these reactions were either not detected or markedly reduced. For these reasons,
the fate of the adenine nucleotide in the reaction using ATPγS was examined. In the presence of excess AUGp* and a limiting amount of ATPγS, the reaction proceeded until the synthesis of the cyclic derivative reached an amount that approximated the input of ATPγS (Fig. 9). At this point (at 180 min), the reaction ceased, and the addition of more enzyme had no effect while the addition of more ATPγS caused an immediate resumption of cyclosome activity (Fig. 9). Similar experiments were carried out using concentrations of ATPγS which were twice and one-half of that described in the legend to Fig. 9. In each case, the yield of cyclic derivative formed approached twice and one-half of that described in the legend to Fig. 9. When [cI-$^{35}$S]ATPγS was used in place of [cI-$^{35}$S]ATP, but not in the presence of [γ-$^{35}$P]- or [β,γ-$^{35}$P]- ATP, suggesting that the complex was formed by transfer of AMP from ATP to the enzyme with the concomitant release of thiophosphate was observed. When [α-$^{32}$P]ATPγS was used in place of [32P]ATPγS, [32P]AMP was produced in the presence of AUGp and no [32P]ADP could be detected (Fig. 10B). ATPγS hydrolysis in excess of what occurred in the absence of added trinucleotide was not detected when AUGp was replaced with AUG-3'-OH.

The solvent system used for thin layer chromatography on polyethyleneimine-cellulose plates (Solvent D) was capable of separating the products and starting materials. The amount of cyclic ends formed (measured as pA>p after nuclease P1 digestion), tRNAAp* remaining (measured as 30P after P1 nuclease digestion), $[^{35}$S]ATPγS remaining, and $[^{35}$S]thiophosphate produced could be quantified (Table IV). As shown, for each mol of cyclic derivative formed, 1 equivalent of thiophosphate was formed concomitant with the disappearance of 1 equivalent of ATPγS and tRNAAp*. Similar experiments to those shown in Table IV were carried out using either lower or higher concentrations of substrates, as well as with AUG-3'-p* in place of tRNAAp*. In all cases, the products formed (on a molar basis) were equivalent to the disappearance of the starting substrates.

Studies on the Formation of Intermediates in the RNA Cyclase Reaction—All attempts to reverse the RNA cyclase reaction have failed. Incubation of cyclic derivatives and RNA cyclase in the presence of ATP, AMP and PPi, or ADP and P1 did not lead to the production of detectable 3'-phosphate ended chains. In addition, no exchange reactions could be detected when RNA cyclase was incubated with $[^{35}$P]PPi and ATP, ATP and $[^{32}$P]P, or $[^{32}$P]AMP in the presence or absence of cyclic derivatives (data not shown).

When RNA cyclase fractions were incubated with [α-$^{32}$P]ATP in the absence of added RNA, treated with SDS, and analyzed by electrophoresis on polyacrylamide gels containing SDS, a labeled protein band was observed (Fig. 11). The ability to form this complex and RNA cyclase activity co-sedimented through glycerol gradients (as shown in Fig. 2). Formation of $[^{32}$P-labeled enzyme-adenylate complex with the purified RNA cyclase fractions occurred in the presence of [α-$^{32}$P]ATP, but not in the presence of [γ-$^{35}$P]- or [β,γ-$^{35}$P]- ATP, suggesting that the complex was formed by transfer of AMP from ATP to the enzyme with the concomitant release...
TABLE IV
Stoichiometry of the RNA cyclase reaction

| Additions          | [32P]Thio-pyrophosphate | [32P]ATP+S | pA2p | RNA-AMP (as 32P) |
|--------------------|--------------------------|------------|------|-----------------|
| Complete           | 476                      | 310        | 369  | 605             |
| Omit tRNA-AMP*     | 50                       | 701        |      |                 |
| Omit ATP+S         | 17                       | 737        | <1   | 987             |
| Omit enzyme        |                          |            | <1   | 980             |
| Overall differences| +426                     | -427       | +369 | -375            |

Fig. 11. Formation of 32P-labeled adenine nucleotide-RNA cyclase complex. A reaction mixture (0.26 ml) contained 20 mM Hepes buffer, pH 7.9, 5 mM MgCl2, 1 mM dithiothreitol, [α-32P]ATP (48 pmol, 410 Ci/mmol), and 10 units of purified RNA cyclase (glycerol gradient of heparin-Ultrogel, 250 mM eluate). After 5 min at room temperature the sample was divided into 20-μl aliquots and received 2 μl of the following additions: a, no additions (21.7); b, 1 mM AMP (22.7); c, 1 mM ADP (19.9); d, 1 mM ATP (3.4); e, 10% SDS followed by 1 mM ATP (18.6); f, 0.3 mM NaOH as described by Gumport and Lehman (39); g, 0.3 mM (as molecules) poly(A)-3′-OH (23.5); h, 0.3 mM (as molecules) poly(A)-3′-p (0.8); i, 10% SDS followed by 0.3 mM Na2HPO4 (21.2); j, 0.1 M sodium pyrophosphate (0.9); k, 10% SDS followed by 0.1 M sodium pyrophosphate (18.3); l, 7 units/ml of bacterial alkaline phosphatase (25.9); m, 0.5 mg/ml of proteinase K (not determined). The numbers in parentheses indicate fmol of 32P-labeled protein-AMP complex material, determined as described under "Materials and Methods."

of pyrophosphate. The requirements for complex formation were the same as those observed for cyclase activity. Formation of the complex was also observed with [α-32P]thio-ATP. In contrast, when RNA cyclase was incubated with [α-32P]dATP or other [α-32P]dNTPs or -rNTPs, no labeled complex was detected. The association between the 32P moiety and the enzyme appears to be covalent, since the labeled protein was stable to precipitation with 10% trichloroacetic acid at 4°C and to boiling in 1% SDS prior to electrophoresis.

Isolation of the complex free of unreacted ATP by Sephadex G-25 (fine) filtration was attempted in order to determine whether the complex was a functional intermediate in the RNA-cyclase reaction. However, no labeled complex could be isolated by these techniques under nondenaturing conditions, suggesting that this complex was unstable. In contrast, when gel filtration was performed in the presence of 0.1% SDS, the complex could be recovered in a stable (but inactive) form and free of unreacted ATP (data not shown). In order to correlate complex formation with RNA cyclase activity, the adenylated enzyme complex was formed in a first reaction, and the mixture was subsequently incubated in a second reaction supplemented with RNA as well as other reagents. As shown in Fig. 11 the complex was not detected when the second incubation occurred in presence of (Ap)12-3′-p. This effect was not observed when (Ap)12-3′-OH was used. The addition of excess ATP also caused the disappearance of the adenylated enzyme complex, suggesting an exchange reaction. Sodium pyrophosphate but not sodium phosphate caused disappearance of the adenylated enzyme complex. Incubation with AMP, ADP, NaCl, or, bacterial alkaline phosphatase did not alter the complex significantly.

The stability of the RNA cyclase-AMP complex was examined. The 40,000-dalton cyclase-AMP complex was unaffected by heating for 1 min at 100°C in H2O or in 0.1 N NaOH but was quantitatively hydrolyzed by heating in 0.1 N HCl. Incubation of the complex for 2 min at 37°C with NH4OH at pH 4.75 as described by Gumport and Lehman (39) also quantitatively hydrolyzed the complex; similar treatment with NH4OH at neutral pH or sodium acetate at pH 4.75 did not alter the amount of cyclase-AMP complex. This lability is identical to that noted for a phosphomide linkage, probably involving the ε-amino group of a lysine and the phosphate moiety of AMP. When the complex (isolated free of unreacted ATP in the presence of 0.1% SDS by gel filtration on Sephadex G-25 (fine)) was hydrolyzed by heating at 100°C for 3 min in 0.1 M HCl, the only labeled product that could be detected by thin layer chromatography on polyethyleneimine-cellulose plates was AMP (data not shown).

Coupling of RNA Cyclase with RNA Ligase Isolated from Wheat Germ—Previous studies (29–31) have shown that wheat germ RNA ligase catalyzes an ATP-dependent ligation of RNA substrates containing 2′,3′-cyclic phosphate and 5′-phosphate termini. Ligation of the ends produces an unusual 2′-phosphomonoester, 3′-5′-phosphodiester linkage in which the 5′-phosphate is esterified to the 3′-hydroxyl group of the terminal residue, with the concomitant displacement of the phosphate residue from 2′,3′-cyclic end to the 2′-position. In addition, the wheat germ RNA ligase contains two other activities. One is a phosphodiesterase that hydrolyzes 2′,3′-cyclic phosphate ends to the corresponding 2′ ester. The other activity intrinsic to the wheat germ RNA ligase is a 5′-hydroxyl polynucleotide kinase.

(Upp)30Gp* was incubated with RNA cyclase, purified wheat germ RNA ligase, and the T4 5′-hydroxyl polynucleotide kinase (Pse T* mutant, lacking the 3′-phosphatase activity (32)). The reaction mixture were then treated with nuclease P1 and bacterial alkaline phosphate followed by polyethyleneimine-cellulose thin layer chromatography as shown in Fig. 12. The dinucleotide (pG2P) was observed after

>4

4. L. Pick and J. Hurwitz, unpublished results.
treatment with nuclease P1, consistent with the known action of the RNA ligase (Fig. 12, lane 7). In addition, pG-2'-p* was produced by the action of the 2',3'-cyclic phosphodiesterase on the cyclic ends produced by the RNA cyclase. The reaction was absolutely dependent upon the RNA cyclase (lanes 5 and 6) and the wheat germ RNA ligase (lanes 3 and 4). The addition of T4 5'-polynucleotide kinase (Pse T- mutant) did not affect the reaction indicating that the kinase activity present in the wheat germ RNA ligase was ample enough to support the reaction. Furthermore, lanes 2 and 3 in Fig. 12 showed that the RNA cyclase quantitatively converted the 3'-phosphate terminus to the 2',3'-cyclic derivative, which is required for the action of the wheat germ RNA ligase.

A summary of the reactions catalyzed by the enzymes used and the expected products is also presented in Fig. 12.

**DISCUSSION**

In this paper we have described the purification and properties of HeLa cell RNA cyclase. In the presence of Mg²⁺, ATP, and an oligonucleotide bearing a 3'-phosphate end, the enzyme catalyzes the formation of the 2',3'-cyclic phosphate derivative. The reaction results in the hydrolysis of ATP or ATP-P₅S to AMP. The stoichiometry of the reaction indicates that for each mol of 3'-phosphate terminus cyclized, 1 mol of 3'-phosphate terminus disappearance and 1 mol of AMP was formed. Preliminary data indicate that in a first step the enzyme hydrolyzes ATP to form an enzyme-AMP covalent complex with the release of PP₁ (an intermediate also seen with DNA ligase (36) and T4 RNA ligases (33, 37)). In the presence of a 3'-phosphate terminated RNA, the enzyme catalyzes the cyclization of the terminus with the release of AMP.

Experiments carried out by Filipowicz and Shatkin⁵ indicated that if the 2'-position of a RNA terminus is blocked (either as 2'-OCH₃ or 2'-deoxy), the 3'-phosphate end is adenylated by the cyclase. These results, coupled with the observations reported here, suggest that the reactions catalyzed by the cyclase are the following:

(a) Enzyme + ATP → Enzyme-AMP + PP₁
(b) RNA-Xₚ + Enzyme-AMP → RNA-XₚpA-Enzyme
(c) RNA-XₚpA-Enzyme → RNA-Xₚ + Enzyme + AMP

Overall Reaction:
RNA-Xₚ + ATP → RNA-Xₚ + AMP + PP₁

The inability to detect any exchange reactions suggest that the cyclization reaction must be extremely rapid in contrast to the formation of the enzyme-AMP intermediate. It is puzzling that no exchange reaction was detected between PP₁ and ATP. The significance of this finding remains to be explained.

The importance of RNA molecules bearing 2',3'-cyclic phosphate termini has been accentuated with the discovery of eukaryotic RNA ligases. These enzymes require 2',3'-cyclic ends and differ in their requirement for either a 5'-phosphate

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⁵ W. Filipowicz and A. J. Shatkin, personal communication.
or 5'-hydroxyl end. Wheat germ (18-20, 29), chlamydomonas (23, 38), and yeast (31) RNA ligases require a 5'-phosphate end; the HeLa RNA ligase requires a 5'-hydroxyl group (21, 22). In contrast, the T4 RNA ligase requires the presence of 5'-phosphate and 3'-hydroxyl ends (33).

Cyclic ends on RNA chains can be generated by a number of different mechanisms. One way is with T4 RNA ligase (41) and another way is with the yeast endonuclease associated with tRNA splicing (20, 34). In this case the endonucleolytic cleavage of the precursor tRNA produces a 2',3'-cyclic phosphate end on the 5' exon and a 5'-hydroxyl end on the 3' exon (34). In addition to the yeast endonuclease, a number of RNases (35) catalyze the formation of 2',3'-cyclic ends. Another method for generating cyclic ends is with HeLa RNA cyclase described here. The physiological role of this enzyme is unknown at present; nevertheless, indirect evidence suggests that RNA cyclase may be involved in HeLa cell tRNA splicing (20,34). In this case the endonucleolytic cleavage of the precursor tRNA produces a 2',3'-cyclic phosphate end; the HeLa RNA ligase requires a 5'-hydroxyl group on the 3' end. This list includes HeLa cell RNA ligase, T4 RNA ligase, wheat germ RNA ligase, and cell-free extracts capable of splicing exogenously added precursor RNA (11, 12). In all cases 10^-4 M and higher concentrations of vanadyl-ribonucleoside were required to inhibit the reactions. In contrast, 10^-6 M vanadyl-ribonucleoside markedly reduced cyclase activity. Thus, this inhibitor can be used to prevent RNA cyclase activity in relatively crude extracts.

Unequivocal evidence supporting the role of RNA cyclase in any type of RNA splicing must await the purification and characterization of the cyclase. The enzyme described here provides a highly sensitive reagent for quantitatively generating 2',3'-cyclic ends and should be proved useful in assessing the role of the RNA end in processing systems.

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Supplement to: The enzymatic conversion of 3'-phosphate terminated RNA chains to 2',3'-cyclic phosphate derivatives by Danny Reinesberg, Jamie Aizenman and Jared Markowitz

MATERIALS AND METHODS

Materials

HOPS buffer: 50 mM Hepes buffer, pH 7.0, 0.5 mM MgCl2, 100 mM KCl, 10% (v/v) glycerol.

Sephadex G-50 (fine) (17.8 g) was dissolved in 100 ml of 30% ethanol, 70% water, 10% acetic acid and repurified with ethanol. The progress of the reaction was halted by extraction with phenol-chloroform. The reaction mixture (100 µl) containing 32P-labeled RNA was added to 0.1 ml reaction buffer, 100 µM ATP (25 µCi), and 1 ml of bacterial alkaline phosphatase. Incubation for 1 h at 37°C was followed by extraction with 1 ml of chloroform. The reaction was terminated by extraction with equal volumes of phenol-chloroform (1:1). The samples were extracted with phenol-chloroform, ethanol precipitated and resuspended in 30 mM Tris-Cl buffer, pH 7.5, 1 mM DTT. 32P-labeled RNA was a final 100 µCi per reaction. The reaction was run for 1 h at 37°C. The reaction mixture was extracted with a layer of 10% Triton X-100, 0.1% sodium dodecyl sulfate, followed by chloroform. The reaction mixture was added to a formamide gel (20% acrylamide), 30% sucrose, 50 µM EDTA, 3% formaldehyde) and heated to 65°C for 5 min. The samples were loaded onto a 6% polyacrylamide gel for electrophoresis. The gel was dried and exposed to X-ray film. The autoradiograms were scanned with a densitometer. The relative content of 32P-labeled RNA was determined by densitometry.

Preparation of 32P-labeled RNA

32P-labeled RNA was prepared by treatment of the corresponding 3'-phosphate terminated RNA chains with bacterial alkaline phosphatase. The reaction mixture (100 µl) containing 32P-labeled RNA was added to 0.1 ml reaction buffer, 100 µM ATP (25 µCi), and 1 ml of bacterial alkaline phosphatase. Incubation for 1 h at 37°C was followed by extraction with 1 ml of chloroform. The reaction was terminated by extraction with equal volumes of phenol-chloroform (1:1). The samples were extracted with phenol-chloroform, ethanol precipitated and resuspended in 30 mM Tris-Cl buffer, pH 7.5, 1 mM DTT.

Analysis of RNA synthesis

RNA synthesis in E. coli was monitored by the labeling of newly synthesized RNA with 32P-labeled ATP. E. coli strain HB101 was grown in M9 minimal medium containing 32P-labeled ATP (30 µCi/ml). The reaction was stopped by boiling for 5 min and the samples were analyzed by electrophoresis on a 15% polyacrylamide gel. The RNA was extracted from the gel and radioactivity was determined by scintillation counting.

Protein

Protein content was determined by the method of Bradford (1976) using the reagent obtained from Bio-Rad.

RNA Cyclase Effect on 3'-Phosphate Terminated RNA Chains

The enzymatic conversion of 3'-phosphate terminated RNA chains to 2',3'-cyclic phosphate derivatives was achieved by Danny Reinesberg, Jamie Aizenman and Jared Markowitz.