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Isolation and characterization of an RNA ligase from HeLa cells

(cyclic ends/5′-hydroxyl ends/RNA splicing)

KAREN K. PERKINS*, HENRY FURNEAUX*, AND JERARD HURWITZ*

Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

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ABSTRACT An RNA ligase has been purified from HeLa cells, which catalyzes the intra- and intermolecular ligation of linear RNA substrates possessing 5′-hydroxyl and 2′,3′-cyclic phosphate termini in the presence of ATP or dATP. In this reaction, the 2′,3′-cyclic phosphate is incorporated into a 3′-5′-phosphodiester bond, in agreement with the findings of Filipowicz *et al.* [Filipowicz, W., Konarska, M., Gross, H. J. & Shatkin, A. J. (1983) *Nucleic Acids Res.* 11, 1405–1418]. The activity of the purified enzyme is dependent on the addition of ATP or dATP, a divalent cation (Mg^{2+}), and 5′-hydroxyl, 2′,3′-cyclic phosphate-terminated RNA substrates. No ligation occurs with the substrates $_{OH}(Up)_{\overline{10}}G(3')p$ or $_{OH}(Up)_{\overline{10}}G(2')p$ or with 5′-phosphate, 2′,3′-cyclic phosphate-terminated oligoribonucleotides.

Many eukaryotic RNA molecules are transcribed as large precursors containing intervening sequences flanked by exons. Maturation of the RNA requires removal of the intervening sequences and ligation of the exons. This splicing process has been demonstrated *in vitro* in crude extracts of yeast (1, 2), HeLa cells (3–7), and *Xenopus laevis* (8).

Two purified yeast enzymes have been shown to be sufficient for tRNA splicing (9, 10). A membrane-associated endonuclease cleaved precursor tRNA in the absence of ATP, specifically at the intron-exon junctions, and generated tRNA half-molecules possessing 5′-hydroxyl and 2′,3′-cyclic phosphate termini (9). In the presence of ATP, the half-molecules were joined by a purified yeast RNA ligase (10). An RNA ligase purified from wheat germ was also capable of ligating tRNA half-molecules generated by cleavage of tRNA precursors by the yeast endonuclease (11). Both the wheat germ RNA ligase and the yeast RNA ligase formed 2′-phosphomonoester, 3′-5′-phosphodiester linkages (10, 12, 13).

In contrast, when extracts of HeLa cells or *X. laevis* were used to catalyze the ligation of tRNA half-molecules with 5′-hydroxyl and 2′,3′-cyclic phosphate termini, a 3′-5′-phosphodiester bond was formed (3, 14). Furthermore, it was shown, by using a substrate uniquely labeled at the 3′ terminus, that during ligation the 2′,3′-cyclic phosphate was incorporated into this bond.

To study splicing in more detail, we have purified the RNA ligase activity from extracts of HeLa cells. Here we report the isolation and characterization of this enzyme, using an assay specific for 3′-5′-phosphodiester bond formation.

MATERIALS AND METHODS

Materials. Long-chain poly(U_{10},G), the oligonucleotide $_{OH}(Ap)_9A_{OH}$, RNase T1, RNase T2, polynucleotide phosphorylase, micrococcal nuclease, spleen phosphodiesterase, nuclease P1, and T4 RNA ligase were purchased from P-L Biochemicals. RNase N1 and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase were obtained from Sigma. Proteinase K was from Merck, and bacterial alkaline phosphatase (*Escherichia coli* BAPF), pancreatic RNase and pancreatic DNase were from Worthington. Adenosine 5′-[γ-thio]triphosphate (5′-[γ-thio]ATP) and the β,γ-imido analog of ATP (adenylylimidodiphosphate; AMP-PNP) were purchased from Boehringer Mannheim; vanadyl ribonucleoside was from Bethesda Research Laboratories. T4 polynucleotide kinase (lacking the 3′-phosphatase activity, prepared from the *pseT1* mutant of T4 phage) was obtained from New England Nuclear. *E. coli* RNA polymerase was kindly provided by J. Minden of this institution. All radioisotopes were purchased from Amersham.

Preparation of $_{OH}(Up)_{\overline{10}}[2',3'\text{-}^{32}P]G{>}p$ and $_{OH}(Ap)_9A\text{-}[^{32}P]pCp$. $_{OH}(Up)_{\overline{10}}[2',3'\text{-}^{32}P]G{>}p$ was prepared from $_{OH}(Up)_{\overline{10}}G_{OH}$ by the addition of [5′-^{32}P]pCp with T4 RNA ligase (15) and subsequent cleavage with nuclease N1 to generate 2′,3′-cyclic phosphate termini (16). The following conditions were employed. $_{OH}(Up)_{\overline{10}}G_{OH}$ (average size) was prepared by treating long-chain poly(U_{10},G) first with RNase T1 in 20 mM Tris Cl, pH 7.5/2.5 mM EDTA at 37°C for 60 min and then with bacterial alkaline phosphatase in 20 mM Tris Cl, pH 8.9, at 37°C for 60 min. A reaction mixture (0.05 ml), containing [5′-^{32}P]pCp (2 nmol, 6×10^8 cpm), $_{OH}(Up)_{\overline{10}}G_{OH}$ (1 nmol of ends), 20 mM Hepes (pH 8.0), 10 mM $MgCl_2$/100 μM ATP, and T4 RNA ligase (8 units), was incubated at 25°C for 60 min. After extraction with phenol/$CHCl_3$ (1:1, vol/vol), the reaction was diluted 10-fold with water. The 3′-terminal Cp residue was then removed by the addition of RNase N1 (0.25 unit) and incubation at 37°C for 30 min. The reaction mixture was extracted with phenol/$CHCl_3$ (1:1), alkaline phosphatase (0.28 unit) was added, and the mixture was incubated at 37°C for 60 min and then extracted with phenol/$CHCl_3$ (1:1). $_{OH}(Up)_{\overline{10}}[2',3'\text{-}^{32}P]G{>}p$ and $^{32}PO_4$ were separated on a Sephadex G-25 fine column (22 × 0.8 cm), using 20 mM ammonium acetate as eluant. The radioactive material (300 cpm/fmol) that was excluded from the column was pooled and then analyzed as follows. Treatment with nuclease P1 yielded 95% pG>p and 5% pC, whereas analysis with RNase T2 yielded 95% Gp and 5% Up. These results suggest that a small amount of $_{OH}(Up)_nU_{OH}$ was present in the $_{OH}(Up)_{\overline{10}}G_{OH}$. $_{OH}(Ap)_9A[^{32}P]pCp$ was prepared by the addition of [5′-^{32}P]pCp to $_{OH}(Ap)_9A_{OH}$ with T4 RNA ligase (15), using the ligation conditions described above. This substrate was therefore labeled internally, between the cytosine and adenosine residues.

Preparation of Uniformly Labeled [^{32}P]poly(A)-A>p. Uniformly labeled [^{32}P]poly(A)-A>p was prepared from longer-chain [^{32}P]poly(A) by digestion with pancreatic RNase A as

Abbreviations: Np, any nucleoside 3′-phosphate; pN, any nucleoside 5′-phosphate; N>p, any nucleoside 2′,3′-cyclic phosphate; BSA, bovine serum albumin; PEI, polyethyleneimine.

*Present address: Memorial Sloan–Kettering Cancer Center, Sloan–Kettering Institute, 1275 York Avenue, New York, NY 10021.

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follows. Reaction mixtures (0.2 ml) containing 40 mM Tris Cl (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 0.015 M KCl, [α-^{32}P]ATP (1.3 pmol, $\approx 6 \times 10^5$ cpm), 0.19 A_{260} unit of ϕX174 single-stranded DNA, 25 μg of bovine serum albumin (BSA), and 0.95 unit of *E. coli* RNA polymerase were incubated at 37°C for 2 hr. The reaction mixture then was treated with 100 μg of BSA plus 0.5 ml of 10% trichloroacetic acid and centrifuged at 4°C for 15 min. Approximately 30% of the label was incorporated into acid-insoluble material. The poly(A) was then treated to generate 2′,3′-cyclic phosphate termini, as follows. The pellet was dissolved in 0.1 ml of 0.05 M Tris Cl (pH 8.0) and treated with 0.4 μg of pancreatic RNase A, 0.1 μg of pancreatic DNase, and 1 μl of 1 M $MgCl_2$ for 30 min at 37°C; 24 μg of proteinase K then was added, and the incubation was continued for an additional 20 min. The mixture was then extracted three times with equal volumes of neutralized phenol/$CHCl_3$ (1:1). The phenol/$CHCl_3$ layers were extracted with 100 μl of 0.1 mM NaCl; this wash plus the original aqueous phase were combined, adjusted to 0.15 M NaCl, and treated with four volumes of ethanol. After 12 hr at −20°C, the pellet was collected by centrifugation at 12,000 × *g* for 15 min at 4°C, dried in a Savant Speed-vac and dissolved in 0.5 ml of 0.02 M Hepes, pH 7.5. This solution contained 7.7×10^8 cpm/ml, of which 5.4×10^8 cpm/ml was acid-insoluble.

HeLa RNA Ligase Assays. Three assays were devised to monitor HeLa RNA ligase activity. *Assay 1*: Using the terminally labeled substrate $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p, ligation was assayed by the transfer of the radioactive label from [2′,3′-^{32}P]pG>p to [5′-^{32}P]UMP upon nuclease P1 digestion. Reaction mixtures (0.05 ml), containing $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p (200–800 fmol), 20 mM Tris Cl (pH 7.9), 2 mM dithiothreitol, 4 mM $MgCl_2$, 0.2 mM ATP, 0.02 μg of BSA, and HeLa RNA ligase as indicated, were incubated at 30°C for 15 min. Each reaction mixture then was extracted with phenol/$CHCl_3$ (1:1), precipitated with three volumes of ethanol at −70°C in the presence of 10 μg of carrier tRNA, and centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet, containing the reaction products, was dissolved in 30 mM sodium acetate, pH 6.0, and digested with nuclease P1 (0.1 μg) for 30 min at 37°C. The entire reaction was spotted onto a polyethyleneimine (PEI)-cellulose plate and resolved with solvent A, as described below. One unit of RNA ligase was defined as the activity (at 30°C for 15 min) that produced 1 pmol of [5′-^{32}P]UMP recovered after nuclease P1 digestion of products.

Assay 2: Ligation was assayed by the change in electrophoretic migration of RNA after incubation with HeLa RNA ligase. The substrate used in these reactions was $_{OH}(Ap)_9$-A[^{32}P]pCp. Although this substrate possessed a 3′-phosphate, it was ligated due to the presence of RNA cyclase in the RNA ligase preparation (see below). Reaction mixtures, containing $_{OH}(Ap)_9$A[^{32}P]pCp (1 pmol), 20 mM Tris Cl (pH 7.9), 2 mM dithiothreitol, 4 mM $MgCl_2$, 0.2 mM ATP, 0.02 μg BSA, and HeLa RNA ligase as indicated, were incubated at 30°C for 15 min. Reactions were terminated by the addition of 4 M urea/1.0% sodium dodecyl sulfate/20 mM EDTA/100 mM LiCl, and the reaction mixtures then were extracted with phenol/$CHCl_3$ (1:1). The aqueous phase was ethanol-precipitated at −70°C in the presence of 10 μg of carrier tRNA; the precipitate was suspended in 10 μl of formamide containing 0.01% xylene cyanol FF (C.I. 43535) plus 0.01% bromophenol blue and then was electrophoresed in an 8 M urea/12% polyacrylamide gel. The products were visualized by autoradiography.

Assay 3: Ligation was assayed by the resistance of [^{32}P]-poly(A)-A>p to polynucleotide phosphorylase degradation following incubation with HeLa RNA ligase. Reaction mixtures (25 μl), containing uniformly labeled [^{32}P]poly(A)-A>p (2–3 pmol), 40 mM Tris Cl (pH 7.5), 4 mM $MgCl_2$, 4 mM dithiothreitol, 0.4 mM ATP, and HeLa RNA ligase as indicated, were incubated for 15 min at 30°C. Reactions were terminated by extraction with 40 μl of neutralized phenol/$CHCl_3$ (1:1) plus 0.1 ml of 0.1 M NaCl. After extraction, 0.1 mg of BSA and 0.5 ml of 5% trichloroacetic acid were added to the aqueous phase, and the mixture was centrifuged at 4°C for 15 min. The precipitate was dissolved in 0.135 ml of 0.1 M Tris Cl, pH 8.0/0.4 mM $MgCl_2$/4 mM KCl. The amount of acid-insoluble radioactivity was monitored by Cerenkov radiation. The mixture was then incubated with alkaline phosphatase (0.007 unit) for 5 min at room temperature. At this time, polynucleotide phosphorylase (0.15 unit) and 3 μl of 1 M potassium phosphate (pH 7.5) were added, and the incubation was continued for 45 min at 38°C. The amount of acid-insoluble ^{32}P remaining was then determined. One unit of HeLa RNA ligase produced 1 pmol of acid-insoluble ^{32}P recovered after polynucleotide phosphorylase digestion of products. In this assay, only 20–30% of the uniformly labeled [^{32}P]poly(A)-A>p initially present reacted in the ligase system, as described below.

Purification of HeLa RNA Ligase. A crude cellular extract was prepared from 4×10^9 packed HeLa cells by the method of Weil *et al.* (17). The extract was dialyzed for 60 min against buffer A [40 mM Hepes, pH 7.3/4 mM $MgCl_2$/0.5 mM dithiothreitol/0.12 M KCl/10% (vol/vol) glycerol] and then centrifuged at 12,000 × *g* for 30 min. The supernatant (S-100, 39 ml) was applied to a DEAE-cellulose column (17 × 4 cm) previously equilibrated with buffer A. All of the RNA ligase activity flowed through the column. Fractions containing RNA ligase activity were combined (DEAE-cellulose flow-through, 39 ml) and distributed into 39 Eppendorf centrifuge tubes. The tubes were incubated at 50°C for 60 min, then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants were pooled (heated DEAE-cellulose, 36 ml) and precipitated by the addition of solid ammonium sulfate (0.226 g/ml). The mixture was centrifuged at 12,000 × *g* for 15 min, the supernatant was treated with solid ammonium sulfate (0.153 g/ml), and the suspension was centrifuged at 12,000 × *g* for 15 min. The pellet was dissolved in 6 ml of buffer B [20 mM potassium phosphate, pH 7.5/1 mM dithiothreitol/10% (vol/vol) glycerol] containing 0.02 M NaCl and then dialyzed for 2 hr at 4°C against buffer B. The dialyzed concentrated ammonium sulfate fraction (ammonium sulfate, 40–65%; 6 ml) then was diluted 3-fold with buffer B/0.02 M NaCl and applied to a denatured DNA-cellulose column (5.5 × 2.5 cm) previously equilibrated with buffer B/0.02 M NaCl. The column was washed with 30 ml of the same buffer, and bound material was eluted sequentially with two volumes of buffer B containing 0.1 M NaCl, 0.2 M NaCl, and 1 M NaCl, respectively. The RNA ligase activity bound to the column and was eluted with 0.2 M NaCl (DNA-cellulose fraction, 60 ml). The DNA-cellulose fraction was dialyzed for ≈4 hr at 4°C against 40 mM Hepes, pH 7.3/4 mM $MgCl_2$/0.5 mM dithiothreitol/0.12 M KCl/50% (vol/vol) glycerol and stored at −70°C.

Some DNA-cellulose fractions were further purified by glycerol gradient centrifugation, as follows. A fraction (79 ml) was first precipitated by the addition of solid ammonium sulfate (0.475 g/ml). This suspension was centrifuged at 170,000 × *g* in a Beckman 60 Ti rotor for 15 min. The pellet was resuspended in 3.5 ml of buffer A/0.12 M KCl and dialyzed for 2 hr at 4°C against the same buffer. A portion (0.2 ml) of the concentrated fraction was layered onto a 15–35% (vol/vol) glycerol gradient containing 40 mM Hepes, pH 7.3/4 mM $MgCl_2$/0.5 mM dithiothreitol/0.12 M KCl. Gradients were centrifuged at 45,000 rpm for 21 hr at 4°C in a Beckman SW 41 rotor, and fractions (0.2 ml) were collected from the bottom of the tube.

Analysis of Labeled Reaction Products. PEI-cellulose plates (Merck) were developed in 0.1 M LiCl/1.0 N acetic

acid (solvent A) or 1 M LiCl (solvent B). Unlabeled nucleotide markers were spotted in the same lanes as labeled reaction products. After chromatography, either the PEI-cellulose plates were autoradiographed or the regions corresponding to added markers (visualized under short-wavelength ultraviolet light) were cut out for determination of radioactivity by liquid scintillation spectrometry in Aquasol (New England Nuclear).

RESULTS

Purification of RNA Ligase from HeLa Cells. A summary of the yield and purification of HeLa RNA ligase (isolated using assay 1 as described in *Materials and Methods*) is shown in Table 1. The final preparation of HeLa RNA ligase was purified 70-fold with a recovery of 10%. If the DEAE-cellulose fraction was not heated as described, no RNA ligase activity was detected after ammonium sulfate fractionation or DNA cellulose chromatography. The S-100 fraction was not stable, however, to heating for more than 10 min at 50°C. The DNA-cellulose fraction, which contained 10% (vol/vol) glycerol, was stable for only a few hours at 4°C, unless it was concentrated by dialysis against 50% (vol/vol) glycerol. The 50% glycerol fraction was stable for at least 2 months at −70°C and for at least 2 days at 4°C. The DNA-cellulose fraction contained no significant RNA-independent ATPase activity; it did, however, contain an RNA-dependent ATPase activity that was active in the presence of an excess of both 2′,3′-cyclic phosphate and 3′-hydroxyl, 5′-hydroxyl poly(A). Sedimentation of any of the HeLa RNA ligase fractions, at any stage of the preparation, in a 15–35% glycerol gradient containing buffer A/0.12 M KCl yielded a single peak of activity sedimenting at 7 S, suggesting a native molecular weight of 160,000.

Requirements for Activity of Purified HeLa RNA Ligase. HeLa RNA ligase activity required ATP and a divalent cation (Mg^{2+}) (Table 2). Of the nucleoside triphosphates tested, only dATP substituted for ATP in this reaction. The apparent K_m value for dATP (0.04 μM) was in fact lower than the apparent K_m value for ATP (0.14 μM). ADP substituted poorly for ATP, possibly reflecting the presence of trace amounts of myokinase in the enzyme preparation. Neither the β,γ-imido analog of ATP nor adenosine 5′-[γ-thio]triphosphate substituted for ATP in the reaction.

The ligation of uniformly labeled [^{32}P]poly(A)-A>p was inhibited 72% by pyrophosphate (12 mM), but P_i (40 mM) had no effect (data not shown). NaCl (0.4 M) inhibited the reaction 94%. Vanadylribonucleoside, a ribonuclease inhibitor known to mimic a 2′,3′-cyclic phosphate (18) and which is also a potent inhibitor of RNA cyclase (50% at 1–2 μM, unpublished observations) and RNA splicing [50% at 1 mM (4)], inhibited HeLa RNA ligase 50% at 200 μM.

To determine whether HeLa RNA ligase contained an essential RNA component, we tested the enzyme for activity after incubation with micrococcal nuclease. Pretreatment of the enzyme with micrococcal nuclease in the presence of 0.1 mM $CaCl_2$ for 30 min at 37°C, followed by the addition of 0.1 mM EGTA, was without effect on HeLa ligase activity (assay 1). These results suggest that RNA plays no part, other than its role as a substrate, in the intrinsic ligation reaction. The addition of proteinase K or RNase T2 directly to assays containing [^{32}P]poly(A)-A>p (assay 3) prevented ligation, whereas RNase T1 or pancreatic DNase had no effect.

Table 1. Purification of HeLa RNA ligase

Fraction	Total protein, mg	Total activity, units	Specific activity, units/mg of protein
S-100	2220	444	0.2
DEAE-cellulose	1014	273	0.3
Heated DEAE-cellulose	306	237	0.8
Ammonium sulfate, 40–65%	78	174	2.2
Denatured DNA-cellulose	3	48	14.6

Table 2. Requirements of HeLa RNA ligase

Assay conditions	Activity, fmol/15 min
Experiment 1	
Complete	9.36
$-Mg^{2+}$, +10 mM EDTA	<0.10
Complete, with boiled enzyme	<0.10
−ATP	<0.10
−ATP, +dATP	11.0
−ATP, +GTP, CTP, or UTP	0.67–1.01
Experiment 2	
Complete	5.45
−ATP, +dATP	9.46
−ATP, +ADP	1.50
−ATP, + 5′-[γ-thio]ATP	0.39
−ATP, +AMP-PNP	0.87

The complete assay mixture was as described under *Materials and Methods* (assay 1) and included 165 fmol of $_{OH}(Up)_{\overline{10}}$ [2′,3′-^{32}P]G>p and 0.01 unit of the DNA cellulose fraction. Components were not added (−) or additional components were included (+) as indicated. Nucleoside triphosphate concentrations were 1 μM in experiment 1 and 0.2 μM in experiment 2. 5′-[γ-thio]ATP, adenosine 5′-[γ-thio]triphosphate; AMP-PNP, adenylylimidodiphosphate.

Evidence for 3′-5′-Phosphodiester Bond Formation. The assay used in the purification of HeLa RNA ligase involved nuclease P1 digestion of HeLa RNA ligase products (assay 1, described under *Materials and Methods*; Fig. 1). Sensitivity of the ligated junction to nuclease P1 was proof of 3′-5′-phosphodiester bond formation, indicated by the transfer of the label from the 2′,3′-cyclic phosphate to 5′-UMP, as described below. The identity of 5′-UMP was confirmed using two entirely different chromatographic systems (data not shown).

When $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p was incubated in the absence of HeLa RNA ligase (Fig. 1, lane 1) and digested with nuclease P1, only [2′,3′-^{32}P]pG>p was formed. However, in the presence of the S-100 fraction or the DEAE-cellulose fraction (Fig. 1, lanes 2 and 3), 72% of the $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p was ligated, as determined by the amount of [5′-^{32}P]UMP formed relative to the sum of [2′,3′-^{32}P]G>p and [5′-^{32}P]UMP formed by nuclease P1 digestion.

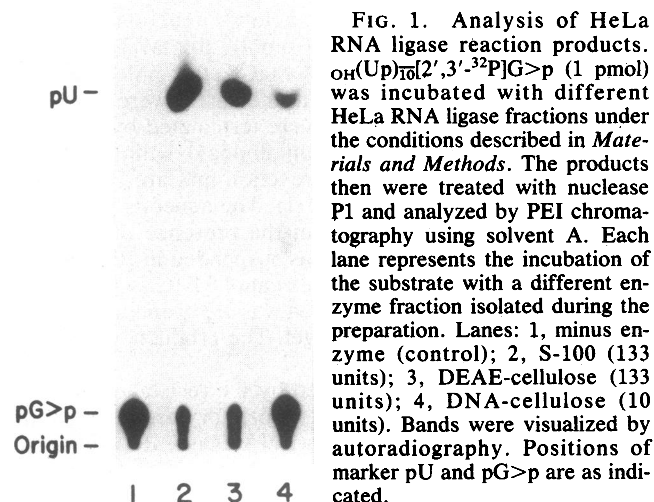

FIG. 1. Analysis of HeLa RNA ligase reaction products. $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p (1 pmol) was incubated with different HeLa RNA ligase fractions under the conditions described in *Materials and Methods*. The products then were treated with nuclease P1 and analyzed by PEI chromatography using solvent A. Each lane represents the incubation of the substrate with a different enzyme fraction isolated during the preparation. Lanes: 1, minus enzyme (control); 2, S-100 (133 units); 3, DEAE-cellulose (133 units); 4, DNA-cellulose (10 units). Bands were visualized by autoradiography. Positions of marker pU and pG>p are as indicated.

Structures of the Reaction Products. To determine whether ligation occurred by an intramolecular or intermolecular reaction, products formed using the substrate $_{OH}(Ap)_9A$-[^{32}P]pCp were separated by polyacrylamide gel electrophoresis (Fig. 2). After incubation with HeLa ligase, all products were treated with alkaline phosphatase to remove any 5′-phosphate ends that were generated by polynucleotide 5′-hydroxyl-kinase (3.8 nmol units/ml) present in the enzyme preparation. The products of incubation of $_{OH}(Ap)_9A$-[^{32}P]pCp in the absence of HeLa ligase are shown in Fig. 2, lane 1. Incubation of this substrate with the DEAE-cellulose fraction (Fig. 2, lane 2) resulted in the formation of two labeled compounds: one migrating more slowly than the starting material and one migrating faster (Fig. 2, lane 2). Treatment with RNase A converted both species to a form that migrated identically to the starting material (Fig. 2, lane 3). Treatment of the material shown in lane 2 with polynucleotide phosphorylase in the presence of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Fig. 2, lane 6) had no effect on the faster migrating compound but removed the slowly migrating species. However, polynucleotide phosphorylase alone (Fig. 2, lane 4) or 2′,3′-cyclic nucleotide 3′-phosphodiesterase alone (Fig. 2, lane 5) had no effect. These results suggested that the phosphorylase-resistant species was a circular molecule and that the polynucleotide phosphorylase/2′,3′-cyclic nucleotide 3′-phosphodiesterase-sensitive band was a linear dimer with a 2′,3′-cyclic terminus that was rendered phosphorylase-sensitive by cleavage of the 2′,3′-cyclic phosphate bond to form a 2′-phosphate-terminated oligoribonucleotide.

The circular product (lane 2) represented 10% (100 fmol) and the linear dimers represented 0.8% (8 fmol) of the input substrate (1 pmol, lane 1).

Substrate Specificity. It was previously reported (14) that, using a crude HeLa cell extract, a 2′-phosphate-terminated RNA molecule was not ligated and that a 3′-phosphate-terminated substrate was ligated only after formation of a 2′,3′-cyclic phosphate terminus by RNA cyclase in the extract (14). These results were confirmed using purified HeLa RNA ligase (DNA-cellulose fraction). $_{OH}(Up)_{\overline{10}}$[2′-^{32}P]Gp was prepared from $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p by quantitative digestion with 2′,3′-cyclic nucleotide 3′-phosphodiesterase. This 2′-phosphate-terminated oligonucleotide was not ligated by HeLa RNA ligase (Fig. 3*a*). $_{OH}(Up)_{\overline{10}}$[3′-^{32}P]Gp, prepared from $_{OH}(Up)_{\overline{10}}$[2′,3′-^{32}P]G>p by quantitative digestion with T1 nuclease, also was not ligated by purified HeLa RNA ligase (Fig. 3*b*). Although the DNA-cellulose fraction used in these experiments contained RNA cyclase activity (enough to convert 2 pmol of 3′-phosphate termini to 2′,3′-cyclic phosphate termini), only the RNA ligase can utilize dATP as well as ATP. RNA cyclase cannot use dATP (unpublished observations); the two activities were therefore distinguished on the basis of their nucleotide specificity. In the presence of dATP, $_{OH}(Up)_{\overline{10}}$[3′-^{32}P]Gp was not ligated (Fig. 3*b*, lane 3), but in the presence of ATP (Fig. 3*b*, lane 2), the 3′-phosphate terminus was cyclized (yielding pG>p after nuclease P1 digestion) and ligated (yielding pU after nuclease P1 digestion).

To examine the ability of HeLa RNA ligase to ligate a 5′-phosphate, 2′,3′-cyclic phosphate-terminated RNA molecule, polynucleotide kinase from the *pse*T1 mutant of phage T4 was added directly to reactions containing uniformly labeled [^{32}P]poly(A)-A>p and either HeLa RNA ligase or wheat germ RNA ligase (which utilizes 5′-phosphate, 2′,3′-cyclic phosphate-terminated oligoribonucleotides as substrates). Only the wheat germ enzyme rendered the substrate resistant to exonucleolytic degradation (data not shown). These results confirmed that HeLa RNA ligase could not ligate RNA molecules with 5′-phosphate termini.

Kinetics of the HeLa RNA Ligase Reaction. The rate and yield of ligation was examined using uniformly labeled [^{32}P]poly(A)-A>p as a substrate (Fig. 4). The reaction ceased within 5 min, at which time only 27% of the added

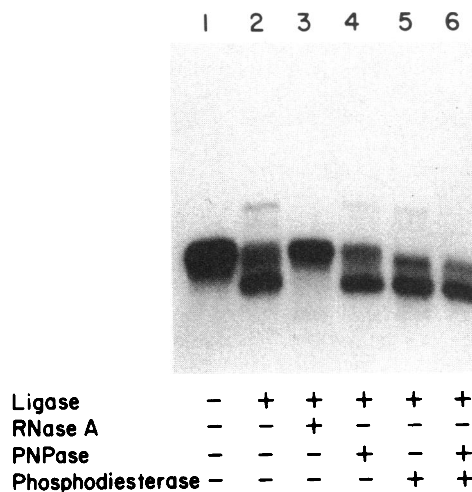

FIG. 2. Identification of reaction products. $_{OH}(Ap)_9A$[^{32}P]pCp (1 pmol) was incubated in the absence (lane 1) or presence (lanes 2–6) of the DEAE-cellulose fraction (133 units). All samples were extracted with phenol/$CHCl_3$ and treated with bacterial alkaline phosphatase (0.6 unit/ml at 37°C for 30 min). Samples in lanes 3–6 also were treated with RNase A (0.2 μg) (lane 3); 2′,3′-cyclic nucleotide 3′-phosphodiesterase (0.05 unit) (lane 4); polynucleotide phosphorylase (PNPase, 0.009 unit) (lane 5); or 2′,3′-cyclic nucleotide 3′-phosphodiesterase (0.05 unit) followed by polynucleotide phosphorylase (0.009 unit) (lane 6). The polynucleotide phosphorylase reaction conditions were 0.4 mM $MgCl_2$/10 mM KCl at 37°C for 30 min. All reactions were terminated by the addition of 50 μg of proteinase K and 0.2% sodium dodecyl sulfate, followed by incubation at 30°C for 15 min. All samples were extracted with phenol/$CHCl_3$ (1:1) and precipitated with ethanol in the presence of 20 μg of carrier tRNA before gel electrophoresis and autoradiography.

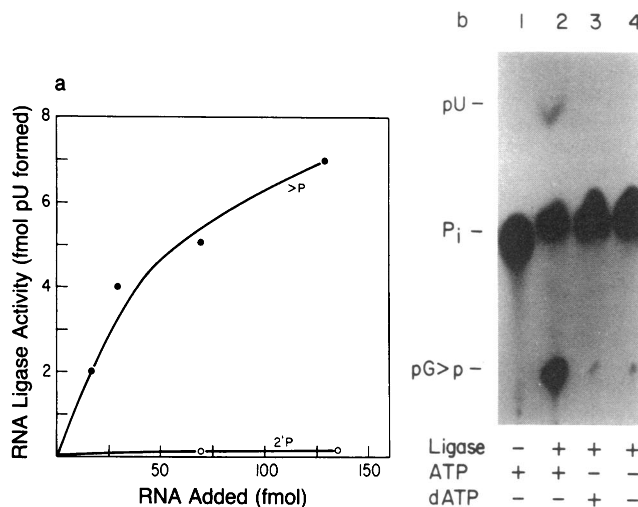

FIG. 3. Substrate specificity of HeLa RNA ligase. (*a*) Comparison of 2′-phosphate- and cyclic phosphate-terminated oligonucleotides $_{OH}(Up)_{\overline{10}}$[2′-^{32}P]Gp (○) or [2′,3′-^{32}P]$_{OH}(Up)_{\overline{10}}$G>p (●) was incubated with a DNA-cellulose fraction [0.005 unit, which had been precipitated with solid ammonium sulfate (0.475 g/ml) and resuspended in buffer A/0.12 M KCl], digested with nuclease P1, and separated on a PEI-cellulose plate with solvent B (see *Materials and Methods*). (*b*) 3′-Phosphate-terminated RNA chains. $_{OH}(Up)_{\overline{10}}$[3′-^{32}P]Gp was incubated with a DNA-cellulose fraction (0.005 unit, precipitated and resuspended as in *a*) under the conditions described in *Materials and Methods*, except that ATP or dATP was present at 0.2 μM. The products were digested with nuclease P1 and chromatographed on PEI-cellulose plates, using solvent A.

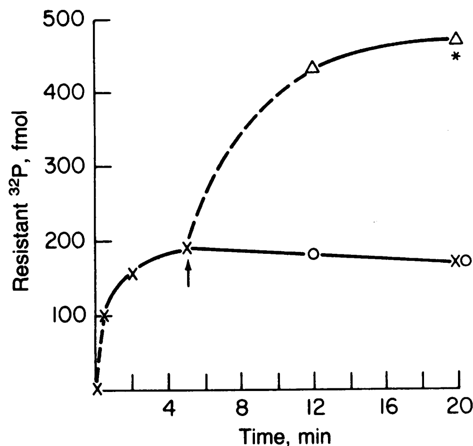

FIG. 4. Kinetics of the ligation reaction. Reaction mixtures (30 μl), containing uniformly labeled [^{32}P]poly(A)-A>p (0.73 pmol), 10 μl of HeLa RNA ligase (118 units/ml, ammonium sulfate 40–65% fraction), 0.5 mM ATP, and other ingredients as described in *Materials and Methods*, were incubated at 30°C for 5 min; at the time indicated by the arrow, some reaction mixtures were supplemented with an additional 2.19 pmol of uniformly labeled [^{32}P]poly(A)-A>p (△) or with 1.2 units of HeLa RNA ligase (○), and the incubation was continued. The amount of polynucleotide phosphorylase-resistant material generated was measured as described in *Materials and Methods*. Point indicated by the × at 20 min denotes reaction mixtures that were not further supplemented. Asterisk represents reaction mixtures to which 2.92 pmol of [^{32}P]poly(A)-A>p was added at time zero.

substrate had been rendered polynucleotide phosphorylase-resistant. Addition of more ligase at this time had no effect. However, the addition of more [^{32}P]poly(A)-A>p caused an immediate resumption of activity, indicating that the reaction had ceased due to the quantitative utilization of active substrate. The reason that <30% of the substrate was active is not known.

DISCUSSION

We have isolated an RNA ligase from HeLa cell extracts, which ligates RNA molecules with 5′-hydroxyl and 2′,3′-cyclic phosphate termini in a reaction requiring ATP (or dATP) and a divalent cation. Characterization of the purified enzyme showed that $_{OH}$(Ap)$_9$ApCp, in the presence of RNA cyclase, or $_{OH}$(Up)$_{\overline{10}}$G>p were substrates for the HeLa RNA ligase, whereas $_{OH}$(Up)$_{\overline{10}}$G(3′)p; $_{OH}$(Up)$_{\overline{10}}$G(2′)p; and 5′-phosphate, 2′,3′-cyclic phosphate-terminated oligoribonucleotides were not.

We also have shown that partially purified HeLa RNA ligase will carry out intermolecular ligation. Intermolecular joining of tRNA half-molecules and accurate excision-ligation of tRNA precursors was demonstrated in S-100-type extracts of HeLa cells (3, 19). In the reactions described here, intermolecular ligation of oligoribonucleotides occurred under conditions optimal for intramolecular ligation, although with a lower efficiency. In contrast, the intermolecular reaction catalyzed by T4 RNA ligase must be induced by suitably juxtaposing the substrate termini—for example, by using as substrates hydrogen-bonded tRNA half-molecules (14) or by using high concentrations of substrate termini (20).

The role of ATP in the HeLa RNA ligase reaction is unknown. However, many enzymes that catalyze phosphodiester bond formation use ATP to activate substrate termini for subsequent bond formation. For example, T4 RNA ligase forms an enzyme-AMP complex as an intermediate in the ligation reaction. The adenylyl group is then transferred from the enzyme to a 5′-phosphorylated substrate, forming a 5′,5′-anhydride linkage (21).

HeLa RNA cyclase, which converts 3′-phosphate ends of RNA chains to 2′,3′-cyclic phosphate termini, requires ATP to activate the 3′ terminus via an adenylylated intermediate prior to the formation of the 2′,3′-cyclic phosphate bond. It was recently shown, by using 2′-*O*-methyl,3′-phosphate- and 2′-deoxy,3′-phosphate-terminated oligoribonucleotides, that 3′-adenylylated oligoribonucleotide intermediates accumulated (W. Filipowicz and A. Shatkin, personal communication). It is possible that ATP activates termini in reactions catalyzed by HeLa RNA ligase and that cyclase and ligase intermediates are identical.

The precise function of this HeLa ligase *in vivo* is not clear. It is possible that this ligase plays a role in RNA splicing, since extracts of HeLa cells ligated tRNA half-molecules (3, 7). A role for this HeLa RNA ligase in splicing is supported by the fact that the intermolecular ligation occurs so readily with synthetic oligoribonucleotides. However, it is unlikely that this HeLa RNA ligase plays a role in mRNA splicing because it was recently shown in an *in vitro* adenovirus mRNA-splicing system that the phosphate forming the bond between the two exons was derived from the 5′ terminus of the donor exon (22). It also was shown in a different *in vitro* mRNA-splicing system that the exon, prior to ligation, contained a 3′-hydroxyl terminus (23). Further studies will determine whether any mRNA splicing intermediates exist that possess 5′-hydroxyl,2′,3′-cyclic phosphate termini prior to ligation.

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