Purification of Wheat Germ RNA Ligase

I. CHARACTERIZATION OF A LIGASE-ASSOCIATED 5'-HYDROXYL POLYNUCLEOTIDE KINASE ACTIVITY*

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An RNA ligase that catalyzes the formation of a 2'-phosphomonoester-3',5'-phosphodiester bond in the presence of ATP and Mg²⁺ was purified approximately 6000-fold from raw wheat germ. A 5'-hydroxyl polynucleotide kinase activity copurified with RNA ligase through all chromatographic steps. Both activities co-sedimented upon glycerol gradient centrifugation even in the presence of high salt and urea. RNA ligase and kinase activities sedimented as a single peak on glycerol gradients with a sedimentation coefficient of 6.2 S. The purified polynucleotide kinase activity required dithiothreitol and a divalent cation for activity and was inhibited by pyrophosphate and by ADP. The kinase phosphorylated a variety of 5'-hydroxyl-terminated polynucleotide chains including some that were substrates for the RNA ligase (e.g., 2',3'-cyclic phosphate-terminated poly(A)) and others that were not ligase substrates (e.g., DNA or RNA containing 3'-hydroxyl termini). RNA molecules containing either 5'-hydroxyl or 5'-phosphate and 2',3'-cyclic or 2'-phosphate termini were substrates for the purified RNA ligase activity. The rate of ligation of 5'-hydroxyl-terminated RNA chains was greater than that of 5'-phosphate-terminated molecules, suggesting that an interaction between the wheat germ kinase and ligase activities occurs during the course of ligation.

The excision of intervening sequences and subsequent joining or ligation of exons, RNA splicing, is a process required for the formation of many mature RNA species in eukaryotes. In vitro systems have been developed to study RNA splicing with the aim of identifying and characterizing individual components involved in splicing reactions.

Among the first eukaryotic enzymes to be identified that were suspected to play a role in RNA splicing were the RNA ligases from yeast (1) and from wheat (2, 3). The yeast RNA ligase that requires ATP for its action. Highly purified preparations of yeast tRNA ligase contain a 2',3'-cyclic phosphorydiesterase activity and a 5'-hydroxyl polynucleotide kinase activity. It thus seems likely that the ligase, cyclic phosphorydiesterase, and kinase activities are intrinsic to a single protein species (6).

Wheat germ RNA ligase was originally discovered by Filipowicz and co-workers (2, 3) who identified an activity in crude extracts of wheat germ that circularized RNA molecules. The unusual feature of the ligation reaction catalyzed by the wheat germ activity was that a 2'-phosphomonoester-3',5'-phosphodiester bond was formed at the ligated junction. The phosphate in the 3',5'-phosphodiester bond originated from a phosphate at the 5' terminus of the RNA substrate while the phosphate in the 2'-position originated from a 2',3'-cyclic phosphate on the 3'-end of the substrate molecule. RNA containing 2',3'-cyclic and either 5'-hydroxyl or 5'-phosphate termini were substrates for the ligase activity in crude extracts. 5'-Hydroxyl-terminated RNA chains supported ligation because of the presence of a polynucleotide kinase activity in the extracts that generated 5'-phosphate termini. Interestingly, the yeast tRNA ligase also joins RNA termini containing 2',3'-cyclic and 5'-hydroxyl ends to form this unusual 2'-phosphomonoester-3',5'-phosphodiester bond. Half-tRNA molecules containing these termini are the preferred substrates for the yeast enzyme; the ligation of synthetic substrates by purified tRNA ligase was 10³-fold less efficient than the ligation of tRNA halves (6). On the other hand, wheat germ RNA ligase is highly active on a variety of substrates including tRNA halves generated by the yeast tRNA endonuclease (7, 8), viroid RNA (9), and several synthetic substrates that contain the appropriate termini (Refs. 8 and 10 and see below).

Since wheat germ RNA ligase was active on synthetic substrates that could be easily prepared and since wheat germ could be obtained in large quantities, it was an attractive choice for the preparation of a eukaryotic RNA ligase that could be subjected to biochemical analysis. Thus, a rapid assay for the purification of RNA ligase using a synthetic poly(A) substrate containing 5'-phosphate and 2',3'-cyclic phosphate termini was devised (10). Using this assay, the enzyme was partially purified and was shown to be associated with a 2',3'-cyclic phosphorydiesterase activity that hydrolyzed the cyclic bond to generate a 2'-phosphate terminus. The enzyme was also partially purified by Abelson and co-workers...
who used tRNA halves generated by the yeast tRNA endonuclease containing 5'-hydroxyl and 2',3'-cyclic phosphate termini as substrate (7). In this case, a 2',3'-cyclic phosphodiester as well as a 5'-hydroxyl polynucleotide kinase activity was associated with the RNA ligase.

A new protocol for the extensive purification of wheat germ RNA ligase is presented here. The association of the ligase with a 5'-hydroxyl polynucleotide kinase activity and the biochemical properties of this ligase-associated kinase activity have been examined. An analysis of the mechanism of ligation by the purified RNA ligase is presented in the accompanying paper.

**Experimental Procedures**

**Preparation of Substrates**—Substrates for the assay of RNA ligase, [5'-32P]p(A)p(A)p and [2',3'-32P](C)pGp containing either 5'-hydroxyl or 2',3'-phosphate termini, were prepared as described previously (10). The substrate used for the assay of 5'-hydroxyl polynucleotide kinase was 5'-hydroxyl-terminated poly(A) prepared by digestion of 4 mg of poly(A) with 0.1 mg of pancreatic RNAse as previously described (10). 5'-Hydroxyl-3'-phosphate-terminated poly(A), poly(C), and poly(I) with an average chain length of 50 nucleotides were prepared by digestion of 0.5 mg of the long chain polynucleotide with 0.4 unit of micrococcal nuclease in reaction mixtures (0.1 ml) containing 10 mM Tris-HCl, pH 7.8, and 2 mM CaCl₂ for 30 min at 37 °C. 5'-Hydroxyl-3'-hydroxyl-terminated poly(A) was prepared by treatment of 0.1 mg of 3'-phosphate-terminated poly(A) (0.07 unit of bacterial alkaline phosphatase in reaction mixtures (0.05 ml) containing 50 mM Tris-HCl, pH 7.9, for 60 min at 65 °C). 5'-Hydroxyl terminated salmon sperm DNA and 5'-hydroxyl-terminated poly(GA) were prepared using bacterial alkaline phosphatase, as above. The concentration of 5'-hydroxyl termini in these preparations was determined with T4 polynucleotide kinase.

**Assay of RNA Ligase**—RNA ligase was assayed as previously described (10) in reaction mixtures (0.05 ml) containing 2.5 pmol of 5'-32P-labeled terminal (30-60 cpm/pmol), 20 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM DTT, 1 mM ATP, and enzyme. One unit of RNA ligase activity rendered 1 pmol of 5'-32P-labeled terminal resistant to bacterial alkaline phosphatase after 30 min at 37 °C.

**Assay of Kinase**—Kinase activity was measured by the incorporation of 32P from [γ-32P]ATP into an acid-insoluble form. Reaction mixtures (0.05 ml) containing 20 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM DTT, 50 pmol of 5'-hydroxyl termini of poly(A) (average chain length of 70), 0.1 mM ATP (1500-4000 cpm/pmol), and enzymes were incubated at 37 °C for 15 min. The mixture was diluted to 0.2 ml with distilled water and extracted with an equal volume of phenol/chloroform (1). Wheat germ albumin (0.5 ml) and 0.05 ml of cold 10% trichloroacetic acid were added to the aqueous phase. After 7 min at 0 °C, the insoluble material was collected by centrifugation and resuspended in 0.2 ml of 0.1 M Tris-HCl, pH 7.9. Cold 5% trichloroacetic acid (5 ml) and 0.1 ml of 0.1 M sodium pyrophosphate were added, and acid-insoluble material was collected on glass fiber filters. The filters were washed with cold 1% trichloroacetic acid and ethanol, dried, and counted using Ecolonflor as the scintillation fluid. One unit of kinase activity rendered 1 pmol of 32P acid insoluble in 15 min at 37 °C.

Since crude extracts incorporated a substantial amount of 32P into an acid-insoluble form in the absence of RNA, assays of the first three fractions (crude extract/DEAE-cellulose) routinely included a proteinase K digestion prior to the phenol/chloroform extraction. With more purified fractions, no incorporation was detected in the absence of added RNA.

**Carboxypeptidase Y Treatment**—Reaction mixtures (0.1 ml) containing 50 mM imidazole buffer, pH 6.7, 1 mM DTT, 5 mM EDTA, 0.5 mM ATP, and 40 μg of wheat germ RNA ligase (ATP-agarose fraction) were incubated in the presence or absence of 2.5 μg of carboxypeptidase Y at 25 °C. In order to minimize the action of the protease during the assay, aliquots were assayed for ligase and kinase activities using a 10-min incubation at 37 °C.

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1 The abbreviations used are: DTT, dithiothreitol; NEM, N-ethylmaleimide; ATP, S, adenosine 5'-3'-thiotriphosphate; PMSF, phenylmethylsulfonyl fluoride; PEI, polyethyleneimine; EGT, ethylenebis(oxyethylenenitrite); tetraacetic acid.

**Protease Inhibitors**—Protease inhibitors were added to all buffers just before use. The following mixture is referred to in the text as protease inhibitor mix: 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM benzamidine, 0.2 μg/ml aprotinin, 10 μg/ml bacitracin, 0.2 μg/ml leupeptin, 0.1 μg/ml antipain, and 0.01 mM PMSF. PMSF was stored as a 100 mM stock solution in isopropyl alcohol; all other reagents were prepared with distilled water.

**Protein Determination**—Protein concentration was determined using the Bio-Rad dye reagent with bovine γ-globulin as standard, according to the method of Bradford (11).

**Materials**—Raw wheat germ was a gift from Pillsbury Mills. Long chain polynucleotides, polyoxynucleotides, αd(Ad)₃α₃dU₃, nucleotides P1, micrococcal nuclease, RNase T2, and T4 RNA ligase were purchased from P-L Biochemicals. αd(Cp)₃γOH was the kind gift of Dr. O. Uhlenbeck (University of Illinois). Bacterial alkaline phosphatase (Escherichia coli BAPF), snake venom phosphodiesterase, and pancreatic DNase were purchased from Worthington, RNase N1, carboxypeptidase Y, benzamidine, aprotinin, bacitracin, PMSF, and carboxypeptidase Y were from Sigma. Leupeptin and antipain were a kind gift of Dr. R. Abeles (Brandeis University). T4 polynucleotide kinase (from the pse T1 mutant lacking the 3'-phosphatase activity) and Ecolonflor were purchased from New England Nuclear. All radioisotopes were from Amersham Corp. Polyethyleneimine-cellulose thin layer sheets (PEI-F) were from Merck.

**Results**

**Purification of RNA Ligase**

All operations were carried out at 0-4 °C.

**Step 1. Crude Extract**—Wheat Germ (25 g) was suspended in 100 ml of extraction buffer (50 mM Tris-HCl, pH 7.9, 5 mM DTT, 200 mM ammonium sulfate and protease inhibitor mix at 10 times the concentration described under "Experimental Procedures"). After 20 min at 4 °C, protease inhibitor mix, again at 10 times the concentration described under "Experimental Procedures" was added, and the suspension was homogenized in a Waring blender two times at low speed and two times at high speed, each for 10 s at 2-min intervals. The suspension was then centrifuged at 100,000 × g for 30 min. The supernatant was removed and pellets were resuspended in 50 ml of extraction buffer and centrifuged as above. The supernatants were combined and recentrifuged as described above. The final supernatant was then filtered through sterile cheesecloth (crude extract, 125 ml).

**Step 2. Polymin P**—Material precipitated at 0.1% Polymin P was removed by adding 1.25 ml of 10% Polymin P to the crude extract. After stirring slowly for 5 min, the extract was centrifuged at 10,000 × g for 10 min and the supernatant collected (Polymin P, 123 ml).

Resilable assay of the crude extract of RNA ligase activity required the addition of large amounts of unlabeled poly(A) chains (approximately 100 pmol of 5'-OH termini). After treatment with Polymin P, the addition of this large amount of poly(A) was no longer required, and the activity of RNA ligase increased 5-10 fold (see Table 1).

**Step 3. DEAE-cellulose**—The Polymin P fraction was diluted to a conductivity equivalent to that of 0.2 M NaCl and loaded (0.5 column volumes/h) onto a 500-ml DE52 column (6 × 19 cm) previously equilibrated with 0.2 M NaCl in Buffer A (25 mM Tris-HCl, pH 7.9, 2 mM DTT, 10% glycerol, 0.01% Nonidet P-40). The column was eluted with 2 liters of the same buffer containing protease inhibitor mix. RNA ligase activity was recovered in 1.4 liters (DEAE-cellulose).

**Step 4. Bio-Rex**—The DEAE-cellulose fraction was diluted with an equal volume of Buffer A containing protease inhibitor mix and loaded (2 column volumes/h) onto a 125-ml Bio-Rex 70 column (3.5 × 12 cm), previously equilibrated with 0.1 M NaCl in Buffer A. The column was washed with 375 ml of the same buffer containing protease inhibitor mix and eluted with a 650 ml linear gradient of 0.1-0.7 M NaCl in Buffer A.
containing protease inhibitor mix. The peak fractions, eluting between 0.2 and 0.275 M NaCl, were pooled (Bio-Rex, 84 ml) and diluted with Buffer A containing protease inhibitor mix to a concentration of 0.1 M NaCl.

RNA ligase activity was quantitatively bound to Bix-Rex when the Polymin P fraction was loaded onto the column at concentrations as high as 50 mg of protein/ml of resin, at pH 7.9. Under these conditions, more than 60% of the total protein flowed through the column. The use of Bio-Rex at an early step of the purification procedure allowed for the concentration of the DEAE-cellulose fraction (2.8 liters loaded onto the column was eluted in 84 ml), as well as a 5-fold purification of RNA ligase activity with a good yield (see Table I).

**Step 5. Phosphocellulose**—The diluted Bio-Rex fraction was loaded (2 column volumes/h) onto a 50-ml phosphocellulose column (2.7 x 9.0 cm) previously equilibrated with 0.1 M NaCl in Buffer A. The column was washed with 200 ml of the same buffer containing protease inhibitor mix and eluted with a 300-ml linear gradient of 0.1-1 M NaCl in Buffer A containing protease inhibitor mix. Peak fractions eluting between 0.275 and 0.375 M NaCl were pooled (phosphocellulose, 44 ml) and diluted with Buffer A plus protease inhibitor mix to a final concentration of 0.2 M NaCl.

**Step 6. Hydroxylapatite**—The phosphocellulose fraction was loaded (0.6 column volume/h) onto a 10-ml Bio-Gel HTP column (1.6 x 6.5 cm) previously equilibrated with 0.2 M NaCl in Buffer A. The column was washed successively with 40 ml of 0.2 M NaCl in Buffer A plus protease inhibitor mix, 40 ml of 1 M NaCl in Buffer A plus protease inhibitor mix, and 40 ml of 1 M NaCl in Buffer B (10 mM sodium phosphate buffer, pH 7.9, 2 mM DTT, 10% glycerol, 0.01% Nonidet P-40) containing protease inhibitor mix. RNA ligase activity eluted with Buffer B was immediately dialyzed against 1 liter of 0.5 M NaCl in Buffer A plus protease inhibitor mix (hydroxylapatite, 33 ml).

**Step 7. ATP-Agarose**—One-eighth of the hydroxylapatite fraction was further dialyzed against 0.05 M NaCl in Buffer A plus protease inhibitor mix, loaded (1 column volume/h) onto a 1-ml ATP-agarose column (0.8 x 0.8 cm); previously equilibrated with 0.05 M NaCl in Buffer A, and washed with 4 ml of the same buffer plus protease inhibitor mix. The column was eluted with a 5-ml linear gradient of 0.05-1 M NaCl in Buffer A plus protease inhibitor mix, and peak fractions, eluting at approximately 0.55 M NaCl, were pooled (ATP-agarose, 1.1 ml). A preparation in which the entire hydroxylapatite fraction was loaded onto an ATP-agarose column gave results identical to those described above.

**Step 8. Glycerol Gradient**—A portion (0.2 ml) of the ATP-agarose fraction was layered onto a 5-ml linear gradient of 10-36% glycerol in Buffer A containing 0.5 M NaCl plus protease inhibitor mix. The gradient was centrifuged at 48,000 rpm for 24 h in a Beckman SW 50.1 rotor. Twelve drop fractions were collected from the bottom of the tube, and 67% of the recovered RNA ligase activity was found in the three peak fractions that were pooled (10-30% glycerol gradient, 0.5 ml).

The procedure described above resulted in a 6000-fold purification of RNA ligase. During the course of the purification, RNA ligase was assayed and fractions were pooled accordingly. 5'-Hydroxyl polynucleotide kinase activity was monitored at each step. A summary of the purification procedure and activities of RNA ligase and kinase (discussed below) is presented in Table I.

**Properties of the Purified Enzyme**—RNA ligase sedimented as a single peak with a sedimentation coefficient of 6.2 S in a 15–35% glycerol gradient in Buffer A containing 0.5 M NaCl and protease inhibitor mix, indicating a native molecular weight of approximately 100 kDa. Analysis of glycerol gradient fractions by polyacrylamide gel electrophoresis, followed by silver staining, revealed the presence of a doublet protein of approximately 110 kDa that comigrated precisely with the peak of RNA ligase activity (data not shown). No other visible protein bands comigrated with ligase activity. Assuming that both of the bands of the doublet represent RNA ligase, the glycerol gradient fraction was estimated to be approximately 70% pure.

**Stability of Enzyme Preparation**—RNA ligase activity in the ATP-agarose fraction was stable for at least 1 year when stored at ~70°C. The activity was unaffected by at least three freeze-thaw cycles and was 68% active after storage at 4°C for 14 days. Enzyme fractions were routinely stored in 0.5 M NaCl in Buffer A containing protease inhibitor mix. Ligase activity was unstable when stored at low ionic strength (greater than 90% of the activity of the glycerol gradient fraction was lost after storage at 4°C in 0.5 M NaCl in Buffer A for 12 h).

**Absence of Interfering Enzyme Activities**—RNA ligase fractions were assayed for the presence of ATPase, RNase, 5'-phosphatase, 3'-phosphatase, RNA cyclase, pyrophosphatase, and DNA ligase. The most purified fractions contained significant RNA-independent ATPase activity that hydrolyzed ATP to ADP (10 units of the ATP-agarose fraction and 2.5 units of the glycerol gradient fraction yielded 12 and 3 pmol of ADP, respectively, after incubation for 30 min at 37°C). No hydrolysis of ATP to AMP (<0.1 pmol) was detected in the absence of RNA by either fraction. A possible relationship between the RNA ligase activity and the RNA-independent ATPase activity was not further investigated. The ATP-agarose fraction contained no detectable RNase activity (<0.4 pmol of nucleotide of 32P-labeled β-globin RNA solubilized per unit of RNA ligase after 30 min at 37°C) and no detectable 5'-phosphatase (<5 fmol of 32P from 5'-32P-labeled poly(A) solubilized per 2 units of RNA ligase after 30 min at 37°C) in the absence or presence of ATP. There was no detectable 3'-phosphatase (discussed below) or pyrophosphatase activity (<0.1 nmol of P released from [32P]PP/6 units of RNA ligase after 60 min at 37°C) in the presence or absence of ATP or RNA. No RNA cyclase activity was detected either in crude fractions (<10 fmol/0.2 unit of crude extract at 37°C after 30 min) or in purified preparations (<10 fmol/2 units of glycerol gradient fraction after 30 min at 37°C). The glycerol gradient fraction contained no detectable DNA ligase activity (<0.01 nmol of 5'-phosphate termini of oligo(dA) ligated per 2 units of glycerol gradient fraction at 30 min at 30°C).

| Fraction       | Protein Concentration | Activity | Specific Activity | Protein Concentration | Activity | Specific Activity |
|----------------|-----------------------|----------|------------------|-----------------------|----------|------------------|
| Crude extract  | 4900                  | 37,000   | 7.6              | 1,575,000             | 321      |
| Polymin P      | ND                    | 422,000  | 0.0              | 1507,000              |          |
| DEAE-cellulose | 3700                  | 491,000  | 133              | 1,136,000             | 307      |
| Bio-Rex        | 350                   | 220,000  | 0.6              | 605,000               | 1730     |
| Phosphocellulose| 48                    | 138,000  | 0.2              | 418,000               | 8710     |
| Hydroxylapatite| 14                    | 82,000   | 0.5              | 172,000               | 12,300   |
| ATP-agarose    | 1.7                   | 22,000   | 1.2              | 45,000                | 26,500   |
| Glycerol gradient| 0.24               | 11,000   | 0.9              | 21,000                | 87,500   |

*ND = Not determined.
Copurification of RNA Ligase and Kinase

Crude extracts of wheat germ contain 5'-hydroxyl polynucleotide kinase activity (2). This kinase activity, monitored by the RNA-dependent incorporation of 32P from [γ-32P]ATP into an acid-insoluble form as described under "Experimental Procedures" was followed during the purification of RNA ligase. The assay used to measure RNA ligase activity did not depend upon the presence of a 5'-hydroxyl polynucleotide kinase activity since the substrate used was poly(A) that had been phosphorylated with T4 polynucleotide kinase. Thus, ligase and kinase activities could be monitored independently of each other.

As shown in Table I, the kinase activity detected in the crude extract copurified with RNA ligase activity in the Polymer P and DEAE-cellulose steps. Approximately one-half of the kinase activity passed through the Bio-Rex 70 column, free of RNA ligase activity. This fraction may include a different enzymatic activity capable of phosphorylating termini of RNA chains. The remaining kinase activity, however, eluted from the Bio-Rex column coincidently with the peak of RNA ligase activity. During all subsequent stages of purification, RNA ligase and 5'-hydroxyl polynucleotide kinase activities cochromatographed. Representative column profiles of the activities eluted from phosphocellulose and ATP-agarose are shown in Fig. 1; although the activity peaks were clearly separated from the protein peaks, ligase and kinase activities comigrated. Ligase and kinase activities also coeluted from a Sephacryl S-300 column and cosedimented on a 15-35% glycerol gradient run in the presence of 0.5 M NaCl. Neither activity was stable to centrifugation in the presence of 0.05 M NaCl. In a further attempt to dissociate the activities that catalyze ligation and phosphorylation, portions of either the hydroxylapatite or ATP-agarose fractions were adjusted to 2 M urea and 0.5 M NaCl (in Buffer A containing protease inhibitor mix) and layered onto 10-30% glycerol gradients in the same buffer. As shown in Fig. 2, the activities cosedimented even in this denaturing environment.

The ratio of RNA ligase to kinase activity remained relatively constant during the purification after the Bio-Rex step (see Table I). The recovery of RNA ligase from the Bio-Rex fraction through the glycerol gradient fraction was 5% while the recovery of kinase activity was 3%. After the separation of the two kinase activities on Bio-Rex, no kinase activity was detected in fractions lacking RNA ligase, and no fraction was obtained that lacked kinase activity but contained RNA ligase.

The heat stability of purified ligase and kinase activities was examined. Incubation at 56 °C for 2 min completely inactivated both the ligase and kinase activities, whereas both were stable at 30 °C for at least 2 h. When fractions were incubated at 45 °C, both activities were inactivated at the same rate (Fig. 3).

Concerted Reaction between Kinase and Ligase—Although the substrate used for assay of RNA ligase during the course of purification contained a 5'-phosphate end, RNA ligase was also active on substrates containing 5'-hydroxyl termini since they were phosphorylated by the intrinsic kinase activity. In order to compare the ligation of 5'-hydroxyl- and 5'-phosphate-terminated substrates, [2',3'-32P] (CP)1, G > p containing either 5'-hydroxyl or 5'-phosphate ends were synthesized. As shown in Fig. 4, the rate of ligation of RNA molecules containing 5'-hydroxyl termini was 5-6-fold greater than the rate of ligation of substrates containing 5'-phosphate ends. The original 5'-hydroxyl-terminated substrate and the same substrate phosphorylated by T4 kinase and then dephosphorylated with bacterial alkaline phosphatase all gave the same type of results. This striking difference suggests that the 5'-hydroxyl polynucleotide kinase activity plays an important role in the ligation reaction.

Fig. 1. Cochromatography of RNA ligase and 5'-hydroxyl polynucleotide kinase. Chromatography on phosphocellulose (panel A) and ATP-agarose (panel B) were carried out as described in the text. Ligase ( ) and kinase ( ) activities, assayed as described under "Experimental Procedures," are presented as pmol/μl of fraction/30 min and pmol/μl of fraction/10 min, respectively.
FIG. 2. Urea glycerol gradient centrifugation of ligase and kinase activities. The ATP-agarose fraction (0.05 ml) was adjusted to 2 M urea in Buffer A containing 0.5 M NaCl and protease inhibitor mix. The sample was layered onto a 5-ml linear gradient of 10-30% glycerol in the same buffer and centrifuged at 48,000 rpm for 24 h in a Beckman SW 50.1 rotor at 4 °C. Twelve drop fractions were collected from the bottom of the tube, and aliquots were assayed. The ligase (●—●) and kinase (○—○) activities are expressed as described in the legend to Fig. 1.

FIG. 3. Heat inactivation of ligase and kinase activities. The ATP-agarose fraction (0.035 ml) was incubated at 45 °C, and aliquots were removed at the times indicated. Assays of ligase (●—●) and kinase (○—○) activities were as described under “Experimental Procedures.”

can be selectively inactivated under conditions that have little effect on RNA ligase activity. This suggests that the active sites of the kinase and ligase are situated on different parts of the enzyme.

Characterization of the Kinase Activity

Requirements for 5'-Hydroxyl Polynucleotide Kinase Activity—The requirements of the purified 5'-hydroxyl polynucleotide kinase activity are shown in Table II. Individual requirements are discussed in more detail below. Incorporation of 32P into an acid-insoluble form was completely dependent upon the presence of 5'-hydroxyl-terminated polynucleotide chains and a divalent cation (see below). ATPyS, which supports the ligation reaction (data not shown), did not substitute for ATP in the phosphorylation of 5'-hydroxyl termini. Omission of DTT resulted in a 95% decrease of kinase activity, as well as RNA ligase activity (data not shown). Both activities were sensitive to treatment with NEM. Preincubation of the enzyme fraction with 0.1 mM NEM (the lowest concentration tested) resulted in complete inactivation of both enzymatic activities.

Effect of pH—5'-Hydroxyl polynucleotide kinase activity showed a broad alkaline pH optimum around 7.9 (data not shown) as did the RNA ligase activity (10). The kinase remained 90% active when assayed at pH 8.9; however, activity decreased by about 40% when the kinase was assayed at pH 5.9.

Divalent Cation Specificity—As shown in Table III, a divalent cation was absolutely required for 5'-hydroxyl polynucleotide kinase activity. Mg2+ supported the highest rate of
TABLE II
Requirements of 5'-hydroxyl polynucleotide activity

The complete system was as described under "Experimental Procedures" for 30 min at 25 °C, and an aliquot was removed and assayed.

| Experiment | Additions | Kinase activity (pmol/15 min) |
|------------|-----------|------------------------------|
| 1          | Complete  | 3.20                         |
|            | Omit 5'-OH poly(A) | <0.10                      |
|            | Omit MgCl₂ | <0.10                      |
|            | Omit MgCl₂, add 10 mM EDTA | <0.10                      |
| 2          | Complete  | 7.58                         |
|            | Omit ATP, add 10 μM ATPγS | 0.17                      |
|            | Omit ATP, add 50 μM ATPγS | 0.15                      |
| 3          | Complete  | 4.13                         |
|            | Omit DTT | 0.20                         |
|            | Omit DTT, add NEM | <0.10                      |
| 4          | Preincubation: |                           |
|            | 2.0 mM DTT | 1.07                        |
|            | 2.0 mM DTT plus 1 mM NEM | 0.93                      |
|            | 0.1 mM NEM | <0.10                       |
|            | 1.0 mM NEM | <0.10                       |

TABLE III
Influence of cations on kinase activity

5'-Hydroxyl polynucleotide kinase activity was assayed as described under "Experimental Procedures," in the presence of 0.1 μg of wheat germ kinase (ATP-agarose fraction). MnCl₂ (1 mM), CaCl₂ (5 mM), or ZnSO₄ (1 mM) were substituted for or combined with MgCl₂ (6 mM) as indicated.

| Divalent cation added | Kinase activity (pmol/15 min) |
|----------------------|------------------------------|
| None                 | <0.10                        |
| Mg²⁺                 | 2.54                         |
| Mn²⁺                 | 1.55                         |
| Mn²⁺ + Mg²⁺          | 3.96                         |
| Ca²⁺                 | 1.70                         |
| Ca²⁺ + Mg²⁺          | 4.16                         |
| Zn²⁺                 | <0.10                        |
| Zn²⁺ + Mg²⁺          | <0.11                        |

kinase activity; the S₀1/₂ value for Mg²⁺ was 8 × 10⁻⁴ M. Concentrations of MgCl₂ as high as 20 mM were not inhibitory. At saturating concentrations, Mn²⁺ (1 mM) and Ca²⁺ (5 mM) each supported approximately 25% of the kinase activity detected in the presence of Mg²⁺; neither was inhibitory in combination with MgCl₂. Zinc was inhibitory either alone or in combination with Mg²⁺, as was noted for the HeLa cell RNA kinase (12).

Effects of Anions and Nucleotides—As shown in Fig. 6, 5'-hydroxyl polynucleotide kinase activity was inhibited by low levels of sodium pyrophosphate, as is the polynucleotide kinase isolated from T2 phage-infected E. coli (13). Kinase activity was inhibited 65% when 0.5 mM sodium pyrophosphate was added to the reaction. Inorganic phosphate was only slightly inhibitory; at a concentration of 20 mM, kinase activity was inhibited 54%. ATP, at a concentration of 0.5 mM, inhibited kinase activity 61%; at concentrations of 10 mM ATP or higher, there was no detectable kinase activity. The ability of ADP to support a reversal of the kinase reaction was not tested. 5'-AMP inhibited the kinase activity 48% at a concentration of 20 mM, the highest concentration tested.

Characterization of the Reaction Product—In order to analyze the product formed by the wheat germ kinase activity, it was necessary to use a substrate that could not be circularized by the intrinsic RNA ligase activity. For this purpose, ou(1)A₃O₄H was incubated with [γ-²³P]ATP under standard reaction conditions in the presence of either T₄ polynucleotide kinase or wheat germ kinase. The reaction products were separated from labeled ATP by electrophoresis through a 15% polyacrylamide, 50% urea gel. With either kinase, a single product was identified by autoradiography and excised from the gel. As shown in Fig. 7, lane 2, in both panels, treatment of either product with nuclease P₁, followed by separation of the products by PEI-cellulose TLC, yielded exclusively [5'-²³P]AMP. Treatment of either product with bacterial alkaline phosphatase (lane 3, both panels) yielded material that comigrated with authentic pAp while treatment with bacterial alkaline phosphatase (lane 4, both panels) produced material that migrated as labeled P₁. These results demonstrated that the purified wheat germ kinase activity catalyzed the transfer of a phosphate moiety from the γ position of ATP to the 5'-hydroxyl end of an RNA chain.

Effect of the 3'-End of RNA Chains on the Activity of 5'-Hydroxyl Polynucleotide Kinase—Since the 5'-hydroxyl polynucleotide kinase is associated with an RNA ligase that requires 2',3'-cyclic or 3'-phosphate ends for its activity, the effect of different 3' termini on the activity of the 5'-hydroxyl polynucleotide kinase was investigated. 5'-Hydroxyl-terminated poly(A) containing either 3'-phosphate, 3'-hydroxyl, or 2',3'-cyclic phosphate termini was incubated with wheat germ kinase and [γ-²³P]ATP, as described in the legend to Fig. 8. No difference was noted in the rates of phosphorylation of these three substrates by the purified wheat germ kinase activity.

The fate of a 3'-phosphate group at the end of an RNA chain was examined using [3'-²³P]10(Cp)₁₄,Gp as substrate. No significant release of P₁ was detected (<4% released/0.3 unit of ATP-agarose fraction after 60 min at 37 °C) as determined by quantitation of P₁, following separation of products by PEI-cellulose TLC. Furthermore, no cyclization of the 3'-phosphate terminus was detected and no 2'-phosphate termini were produced during the incubation (data not shown).

Phosphate Donor Requirement—The effect of ATP on the kinase reaction was investigated. As shown in Fig. 9, the Kₘ value for ATP was 18 μM. No other phosphate donors were tested in the reaction.

Phosphate Acceptor Specificity—Various polynucleotides were tested for their ability to act as phosphate acceptors in the kinase reaction. As shown in Table IV, 5'-hydroxyl-terminated poly(A), poly(C), and poly(I) were all phosphorylated by the wheat germ enzyme with poly(C) being the most active substrate. The wheat germ kinase phosphorylated long chain molecules of either RNA or DNA. 5'-Hydroxyl-termi-
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A. Kinase

B. T4 Kinase

Fig. 7. Characterization of the kinase reaction product. Reaction mixtures (0.05 ml) containing 100 pmol of 5'-hydroxyl termini of poly(A), 10 mM [γ-32P]ATP, and other components as described under "Experimental Procedures" were incubated either in the presence of 2 μg of wheat germ kinase (ATP-agarose fraction) or in the presence of 10 units of T4 polynucleotide kinase for 60 min at 37 °C. Reaction mixtures were extracted twice with equal volumes of phenol/chloroform (1:1), once with chloroform, and were ethanol precipitated in the presence of 20 μg of carrier tRNA. Pellets were resuspended and electrophoresed through a 15% acrylamide (acrylamide/bisacrylamide, 30:0.8, w/w), 50% urea gel in TBE buffer, pH 8.3 (100 mM Tris base, 100 mM boric acid, 1 mM EDTA). Following autoradiography, products were excised from gel slices and treated as follows: A, product of the T4 polynucleotide kinase; B, product of the wheat germ kinase. In both panels: lane 1, undigested eluted material; lane 2, nuclease P1 digestion (0.04 μg of nuclease P1 in 100 mM sodium acetate, pH 5.0, for 60 min at 37 °C); lane 3, RNase T2 digestion (0.1 unit of RNase T2 in 100 mM sodium acetate, pH 5.0, for 60 min at 37 °C); lane 4, bacterial alkaline phosphatase (BALP) treatment (0.14 unit of bacterial alkaline phosphatase in 100 mM Tris-HCl, pH 7.9 for 60 min at 37 °C). Reaction mixtures were extracted once with phenol/chloroform (1:1) and subjected to PEI-cellulose TLC using 0.5 M LiCl, 1 N acetic acid as the solvent.

nated salmon sperm DNA, native or heat denatured, supported the incorporation of similar amounts of 32P into an acid-insoluble form. In order to compare the relative efficiencies of RNA and DNA as substrates, 5'-hydroxyl-terminated poly(dA) was prepared and compared to 5'-hydroxyl-terminated poly(rA) in the kinase reaction. As shown in Fig. 10, for both substrates kinase activity was dependent upon the concentration of 5'-hydroxyl termini added. The Km values for poly(rA) and poly(dA) were 0.05 and 0.08 μM, respectively. At saturating levels, the incorporation of 32P into an acid-insoluble form supported by poly(dA) was about 50% of that observed when poly(rA) was used as substrate. The product of the reaction with poly(dA) was largely resistant to hydrolysis by alkali (80%), as was the product of the same poly(dA) phosphorylated with T4 polynucleotide kinase. The 32P incorporated into poly(dA) by the wheat germ enzyme was completely sensitive to treatment with bacterial alkaline phosphatase, and combined digestion with pancreatic DNase and snake venom phosphodiesterase liberated exclusively 5'-dAMP (data not shown).

The ability of short RNA chains and mononucleotides to act as substrates for the wheat germ kinase was tested by monitoring the adsorption of reaction products to Norit following acid hydrolysis of [γ-32P]ATP, as previously described (13). As shown in Table V, the wheat germ kinase phospho-

Fig. 8. Effect of 3' terminus on 5'-hydroxyl polynucleotide kinase activity. Reaction mixtures (0.05 ml) containing 5 pmol of 5'-hydroxyl-terminated poly(A) with 2',3'-cyclic phosphate (O——C), 3'-phosphate (O——O), or 5'-hydroxyl (Δ——Δ) ends were incubated with 0.2 μg of wheat germ kinase (ATP-agarose fraction) at 37 °C for the indicated times.

Fig. 9. The effect of ATP concentration on kinase activity. 5'-Hydroxyl polynucleotide kinase activity was assayed as described under "Experimental Procedures" except that the concentration of ATP was varied as indicated.

| Experiment | Substrate added | Kinase activity (pmol/15 min) |
|------------|----------------|-------------------------------|
| 1          | None           | <0.10                         |
|            | 5'-OH poly(A)  | 1.67                          |
|            | 5'-OH poly(C)  | 2.23                          |
|            | 5'-OH poly(I)  | 1.04                          |
| 2          | None           | <0.10                         |
|            | 5'-OH poly(A)  | 1.30                          |
|            | 5'-OH poly(dA) | 0.57                          |
|            | 5'-OH salmon sperm DNA | 0.85                  |
|            | 5'-OH salmon sperm DNA; heat denatured | 0.94                |
purified oligoribonucleotides as short as a tetranucleotide, while phosphorylation of a dinucleoside monophosphate (ApA) was not detected. Furthermore, no phosphorylation of 2',3'- or 2'(3')-cyclic monophosphate mononucleotides was observed.

Quantitative Phosphorylation of 5'-Hydroxyl Termini—The rate and yield of phosphorylation of 5'-hydroxyl termini in the presence of a limiting number of poly(A) chains was examined. As shown in Fig. 11, the kinase reaction proceeded until the 5'-hydroxyl termini had been quantitatively phosphorylated. At this point, addition of enzyme had no effect on the reaction, while addition of more 5'-hydroxyl-terminated poly(A) resulted in an immediate resumption of phosphorylation at the same rate as that seen in the early phase of the reaction.

DISCUSSION

RNA ligase was purified approximately 6000-fold from crude extracts of raw wheat germ using a new purification procedure. The ligase activity sedimented as a single peak on glycerol gradients with a sedimentation coefficient of 6.2 S, corresponding to a protein doublet of about 110 kDa that represented approximately 70% of the protein detected by silver staining of polyacrylamide gels. A covalent enzyme-AMP complex formed by the wheat germ RNA ligase, that was detected by incubation of the enzyme in the presence of [α-32P]ATP and separation of the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see accompanying paper), comigrated with the doublet protein band detected by silver staining. The ratio of the two silver-staining species varied slightly from preparation to preparation, although both bands were always present. Furthermore, the amount of labeling of these bands with [α-32P]ATP (yielding the enzyme-adenylate complex) correlated with the intensity of their silver stain. Since the sedimentation coefficient of RNA ligase indicated a protein of about 100 kDa and since both of the silver-stained bands bind AMP, both of these proteins are presumed to contain RNA ligase activity. The migration of wheat germ RNA ligase as a doublet on polyacrylamide gels may be due to proteolytic cleavage or to another type of modification of the protein. The purification procedure presented here differs from those previously reported in several respects. Crude extracts prepared according to this protocol had a 40-50-fold higher specific activity of RNA ligase than that reported previously (10). A 5'-hydroxyl polynucleotide kinase activity was detected that comigrated with RNA ligase through all stages of purification and was not separated from RNA ligase by glycerol gradient centrifugation in the presence of high salt and urea. This association of the ligase and kinase activity was not observed with the enzyme preparations described earlier (10). However, an association of these activities from wheat germ was described by Abelson and co-workers (7) who used rRNA halves containing 5'-hydroxyl ends to assay for ligase activity. In this case, a 5'-hydroxyl polynucleotide kinase activity was mandatory (i.e., each wheat germ fraction) for ligation to proceed. In the scheme presented here, there was no requirement for an association of the kinase and the ligase; RNA ligase was assayed using a 5'-phosphate-ended substrate, and kinase activity was monitored independently.

Perhaps the most important new feature of the purification procedure presented here was the use of protease inhibitors.

**TABLE V**

| Substrate added | Kinase activity (pmol/30 min) |
|-----------------|-------------------------------|
| 5'-OH poly(A)   | 6.3                           |
| oh(Ap)ApA      | 3.0                           |
| oh(Ap)ApA      | 3.3                           |
| oh(Ap)ApA      | 4.5                           |
| oh(Ap)ApA      | 3.6                           |
| Ap3'           | <0.1                          |
| C3'            | <0.1                          |
| C3'            | <0.1                          |
| C6'            | <0.1                          |
| C6'            | <0.1                          |

**Fig. 10.** Comparison of the effect of the concentration of 5'-hydroxyl poly(rA) and poly(dA) termini on kinase activity. Kinase activity was assayed as described under "Experimental Procedures" except that the concentrations of 5'-hydroxyl termini of poly(rA) (○—○) and poly(dA) (○——○) were as indicated.

**Fig. 11.** Quantitative phosphorylation of 5'-hydroxyl termini. Individual reaction mixtures (0.05 ml) were incubated with 7 pmol of 5'-hydroxyl termini of poly(A) and 0.4 µg of wheat germ kinase (ATP-agarose fraction) as described under "Experimental Procedures." At the indicated times, aliquots were removed and precipitated with 5% trichloroacetic acid as described. At 30 min, one-third of the aliquots received no further addition (○—○), one-third received an additional 0.4 µg of wheat germ kinase (△—△), and one-third received an additional 7 pmol of 5'-hydroxyl termini of poly(A) (□——□). All aliquots were removed at 35, 40, 50, and 60 min and treated as above.
in buffers throughout all stages of purification. Although it is generally accepted that proteolysis can be a problem in the purification of proteins from animal tissues and cells, the existence of proteases in plants has received less attention. Higher plants are known to contain a variety of classes of proteases, including serine, sulfhydryl, and lysosomal-like endopeptidases as well as amino- and carboxyl-terminal exopeptidases (for review, see Ref. 14). The role of at least some of these enzymes in vivo is to break down storage proteins during the early development of seeds. Proteases present in seedlings are mobilized early in germination or are synthesized later during germination in response to hormonal signals to provide nutrients for the growing embryo. Proteases present in the wheat embryo are, therefore, likely to be activated upon hydration of the raw wheat germ during the first steps of purification. In light of the observed sensitivity of the wheat germ 5'-hydroxyl polynucleotide kinase activity to carboxypeptidase Y (see Fig. 5), it is particularly interesting that protease inhibitors and PMSF were used as protease inhibitors in the procedure described by Abelson and co-workers (6) in which kinase activity was retained throughout purification.

Indirect evidence for proteolysis occurring in wheat germ extracts was obtained by monitoring the formation of labeled covalent enzyme-adenylate complex (mentioned above) in various enzyme preparations. The 110-kDa enzyme-AMP doublet was detected in wheat germ extracts prepared in the absence or presence of protease inhibitors. However, in the absence of protease inhibitors, up to seven additional protein species of lower apparent molecular weight were detected as well. These protein bands were present at various intensities in different ligase preparations, and no single band consistently copurified with RNA ligase activity. Our experience, therefore, suggests that RNA ligase can readily suffer proteolytic attack during its extraction from raw wheat germ. It is, therefore, likely that our earlier preparations of wheat germ RNA ligase that contained no detectable kinase activity (10) had been attacked by proteases.

Purified RNA ligase circularized RNA molecules that contained either 5'-phosphate or 5'-hydroxyl termini (see Figs. 4 and 5). While RNA chains containing either type of 5'-end could be quantitatively ligated, the rate of ligation of 5'-hydroxyl-terminated RNA was 5–6-fold greater than that of 5'-phosphate-terminated substrates. This result indicates that RNA molecules that are phosphorylated by the wheat germ kinase activity are preferentially ligated by the wheat germ enzyme, suggesting that the kinase and the ligase interact during the ligation event possibly in a concerted fashion. RNA and DNA kinase activities have been observed and characterized from several prokaryotic (17) and eukaryotic (18) sources. None of these has been reported to be associated with a ligase activity, and the physiological role of these kinases has remained obscure. Polynucleotide kinase activities, originally identified in extracts of T2- and T4-infected E. coli, have been purified and thoroughly characterized. These kinases are active on both RNA and DNA as well as on substrates as short as 3'-mononucleotides. The T4 kinase contains intrinsic 3'-phosphatase and 2',3'-cyclic phosphodiesterase activities (19) and can generate 3'-hydroxyl termini from 2',3'-cyclic phosphate-terminated RNA or 3'-phosphate-terminated polynucleotide chains. The eukaryotic kinases which have been studied are more limited in their activities. The kinases isolated from rat liver (20) and calf thymus (21) show strong or absolute preference for 5'-hydroxyl-terminated DNA substrates while the kinase purified from HeLa cell nuclei (12) shows a strong preference for 5'-hydroxyl-terminated RNA chains. Also, in contrast to the T4-encoded kinase, these kinases lack 3'-phosphatase or phosphodiesterase activities.

The 5'-hydroxyl polynucleotide kinase activity from wheat germ shares properties of both the prokaryotic and eukaryotic enzymes. Both RNA and DNA are substrates for the wheat germ enzyme, although RNA chains are preferred (Fig. 10). The wheat germ enzyme catalyzes the phosphorylation of oligoribonucleotides as short as four in length, but not dinucleotide monophosphates or mononucleotides (Table V). No 3'-phosphatase activity was associated with the purified kinase; under conditions sufficient for quantitative phosphorylation of 5'-hydroxyl termini the 3' termini remained intact. A 5'-hydroxyl polynucleotide kinase activity is associated with both the wheat germ and yeast (5, 6) RNA ligases suggesting that these kinases play a role in RNA-processing reaction(s) in vivo. The fact that the wheat germ kinase is also active on nonligatable substrates (e.g. DNA or 3'-phosphate- or 3'-hydroxyl-terminated RNA) may indicate other physiological functions for this enzyme as well.

Raw wheat germ is inexpensive and readily available, making it an attractive source for the preparation of large quantities of purified enzyme. Purified wheat germ 5'-hydroxyl polynucleotide kinase can quantitatively phosphorylate 5'-hydroxyl termini of RNA chains, and in the presence of 2'-phosphate or 2',3'-cyclic phosphate termini, ligation can proceed concomitantly. The purified RNA ligase and kinase activities from wheat germ will, therefore, be useful reagents for the phosphorylation of 5'-hydroxyl-terminated polynucleotide chains, for the detection of RNA molecules containing 2'-phosphate or 2',3'-cyclic phosphate termini, and for the joining and/or circularization of these RNA molecules.

Acknowledgments—We thank Thomas Schmidt-Glenewinkel for critical reading of the manuscript.

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