The Use of Nucleoside Phosphotransferase and (32P)p-Nitrophenyl Phosphate in the Determination of the 5'-Linked Termini of Ribosomal RNA

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Abstract. A method for the identification of the 5'-linked termini of ribosomal RNA is described. The method involves the phosphorylation of the nucleosides released from the 5'-linked termini after hydrolysis of the ribonucleic acid chain with alkali. The radioactive 5'-nucleotide derivatives are formed by a nucleoside phosphotransferase mediated phosphoryl transfer from (32P)p-nitroph enyl phosphate to the nucleosides. The sensitivity of the method allows the use of small amounts of ribosomal RNA.

Phosphorylation of the terminal nucleosides released from unfractionated cauliflower ribosomal RNA yielded radioactive adenylic acid and uridylic acid primarily, with the amount of adenylic acid being approximately twice that of uridylic acid.

The existence of a group of enzymes, nucleoside phosphotransferases, which catalyze the transfer of phosphate from such donors as p-nitroph enyl phosphate, phenyl phosphate and nucleotides to nucleosides has been documented (2,3,16). Since the artificial substrate p-nitroph enyl phosphate could serve as a donor, and it appeared that radioactive p-nitroph enyl phosphate could be synthesized, it was felt that the enzymatic synthesis of radioactive nucleotides utilizing (32P)p-nitroph enyl phosphate and the enzyme could prove to be useful in the identification of nucleosides.

In studies on the terminal groups of ribosomal RNA it is either necessary to have large quantities of RNA available or to utilize techniques capable of detecting small amounts of the terminal groups. Often it is not feasible to obtain large quantities of material and consequently the latter method must be pursued. This paper then reports the isolation and partial purification of nucleoside phosphotransferase from carrot leaves, the synthesis of (32P)p-nitroph enyl phosphate, and the use of these reagents as a new method for the identification and detection of small quantities of nucleosides, especially termini released by alkaline hydrolysis of ribosomal RNA molecules. We have tested the efficacy and the validity of the method by utilizing it in the identification of the 5'-linked termini (free 2',3'-hydroxyl termini) of cauliflower ribosomal RNA because these end groups have been investigated fairly extensively in this laboratory by several methods (6). The major disadvantages of the methods currently in use are that (a) they require exceedingly large quantities of RNA which involves manipulations that are bulky and laborious, and (b) the techniques for detecting nucleic acid derivatives are not very sensitive although some progress has been made by the use of labeling techniques.

For a discussion concerning the formation, structure, and function of ribosomal RNA the reader is referred to the reviews of Madison (12) and Osawa (16). The various aspects of determining the nucleic acid sequences and termini also have been recently reviewed by RajBhandary and Stuart (17). Several papers have been published dealing with the identification of the 5'-linked termini of ribosomal RNA (7,9,10,11,13,15). From the studies on these termini, the results most consistently obtained are that there is a preferential termination of ribosomal RNA by adenosine and uridine. Exceptions to the rule are the investigations on wheat germ ribosomal RNA (9,11) from whence equal amounts of each of the 4 nucleosides were isolated. Recently, evidence was presented from this laboratory which indicated that adenosine and uridine are the predominant 5'-linked termini of wheat germ ribosomal RNA as well as ribosomal RNA from 4 other higher plants and a fungus (6).

Materials and Methods

Chromatographic solvents employed were: (A) Isopropanol (7) ; concentrated NH4OH (1) : water (2) ; (B) 75% ethanol-10% saturated (NH4)2SO4 impregnated paper ; (C) t-Butanol (70) :88% formic acid (15) : water (15) ; and (D) 0.1 m sodium phosphate pH 6.8 (1000 ml) : (NH4)2SO4 (600 g) :
n-propanol (20 ml). All chromatograms were run in the descending manner at room temperature on Whatman No. 1 chromatographic paper.

Radioactivity of samples of chromatogram sections, electrophoretogram sections and of samples spotted on filter paper was determined in a Packard 3003 Tri-Carb scintillation spectrometer with 15 ml of toluene containing 1,4-bis-(4-methyl-5-phenyl-oxazolyl)-benzene (0.3 g/l) and 2.5-diphenyloxazole (5 g/l).

Unless indicated otherwise, all aspects of the preparation of nucleoside phosphotransferase were performed at 0 to 5°. Protein concentration was determined on the basis of the absorbance at 260 and 280 m\(\mu\) (20).

A crude enzyme preparation was obtained from leaves of field grown carrot plants (Daucus carota L., variety sativa DC.) with the first steps of the procedure being essentially those of Tunis and Chargaff (18). Four pounds of carrot leaves were homogenized in a Waring Blender with 1 liter of the 0.1 M sodium acetate buffer at pH 5.1 by successive re-homogenizations and filtrations through cheesecloth. The fraction precipitating between 30 and 70 % by volume of cold acetone (\(-20^\circ\)) was collected by centrifugation and dissolved in a minimum amount of distilled water. The fraction precipitating between 40 and 80 % of saturation of ammonium sulfate was collected by centrifugation. The pellet was dissolved in distilled water, dialyzed to remove residual ammonium sulfate, and lyophilized to dryness. At this stage it was designated crude nucleoside phosphotransferase and was stored in the lyophilized state at \(-20^\circ\) until further use.

After solubilization in the appropriate buffer the crude nucleoside phosphotransferase preparation was subjected to further purification by chromatography on DEAE-cellulose (Sigma Chemical Company, St. Louis, Missouri) which had been thoroughly washed with 1 N HCl, 1 N NaOH, and water. Following equilibration of the DEAE-cellulose with a 0.01 M tris, 0.005 M magnesium acetate buffer at pH 7.5 it was packed from a thin slurry into a 21 \(\times\) 1.5 cm column under a positive pressure of approximately 10 lbs/in\(^2\) nitrogen. The preparation (180 mg protein) was added to and eluted from the column with a linear NaCl gradient (see Fig. 2). After dialysis and lyophilization the major peak of transferase activity was dissolved in the pH 5.7 buffer mentioned below and was added to a second DEAE-cellulose column. The column was equilibrated with solution of 0.05 M malic, 0.025 M acetic, and 0.05 M maleic acid at pH 5.7. Elution of the column was accomplished with a linear pH gradient with 500 ml of the pH 5.7 buffer in the mixing chamber and 500 ml of the same buffer at pH 1.8 in the reservoir. The volume of the fractions collected was 10 ml. The fractions exhibiting high transferase activity and low phosphotransferase activity were dialyzed against distilled water, freeze-dried, and resuspended in a final volume of 5 ml of distilled water.
react for another 30 min with occasional mixing. After extracting the aqueous phase with three 8 ml portions of chloroform to remove the unreacted p-nitrophenol, it was banded on 2 large chromatographic sheets (46 × 57 cm) and chromatographed in the descending manner in solvent system A. The UV absorbing band (RF = 0.58) was eluted from the paper with water and evaporated under reduced pressure. Yield was determined on the basis of absorbance at 285 mμ in 0.1 N HCl.

Identification of the 5'-linked Terminal Nucleotides. The isolation of ribosomal RNA from cauliflower (Brassica oleracea L., variety botrytis L.), hydrolysis of the RNA, and the separation of the nucleosides from the hydrolysis mixture was according to Halloin et al. (6). Cauliflower ribosomal RNA prepared in this manner yielded ratios of absorbance at 260 mμ to that at 280 mμ of 2.0 or greater. When centrifuged in a sucrose density gradient it gave the typical bimodal distribution for ribosomal RNA and no evidence of the presence of low molecular weight RNA species. The nucleosides obtained from the 5'-linked termini of ribosomal RNA were incubated with (32P)p-nitrophenyl phosphate, an aqueous solution of the enzyme, and sodium acetate buffer. The amounts of each used are given in the Results.

Separation of the labeled nucleotides was performed in either of 2 manners. For low levels of radioactivity the radioactive mixture was separated by electrophoresis according to the procedure of Chandra and Varner (5) with the concentration of pyridine reduced to 0.34 ml/600 ml. When higher levels of radioactivity were used it was necessary to separate the components on paper chromatograms since overlapping of the radioactive components was observed on the electrophoretograms. In this case the incubation mixture was banded on the chromatogram along the respective carrier 5'-nucleotides and developed in solvent system C for 3 days. After the development of the chromatogram the slower moving nucleotides which were separated from the faster moving (32P)H2PO4 and (32P)p-nitrophenyl phosphate, were eluted from the paper with water. In some cases 5'-UMP was sufficiently separated from the other 3 nucleotides so that it could be eluted separately. The water was removed by evaporation under reduced pressure. The radioactive nucleotide residue was dissolved in a small amount of water and spotted on a second chromatogram and developed in a second chromatographic system.

Fig. 1. Time course of formation of 5'-UMP by crude nucleoside phosphotransferase. Present in the incubation mixtures were 0.2 ml p-nitrophenyl phosphate (18 μmoles/0.1 ml), 0.5 ml uridine (2 μmoles/0.1 ml), 1 ml purified enzyme preparation, and 0.3 ml of 0.1 M sodium acetate, pH 5.1. The mixtures were incubated at 37° and at time intervals indicated equal volumes of 10% trichloroacetic acid were added. After neutralization and dilution such that the salt concentration did not exceed 0.02 M they were added to 4 × 1 cm quaternary amine resin columns (Cl- form). The nucleosides were first eluted with water and the UMP synthesized was eluted with 0.005 N HCl. Yield was determined on the basis of absorbance at 260 mμ.

Results

Purification of Nucleoside Phosphotransferase. The enzymatic preparation obtained after the acetone and ammonium sulfate fractionations contained a considerable amount of transferase and phosphatase activity as evidenced by the rapid synthesis of 5'-UMP followed by the hydrolysis of the compound (Fig. 1). The NaCl gradient elution of this crude nucleoside phosphotransferase preparation from the DEAE-cellulose column yielded an elution profile shown in Fig. 2. Although separation of phosphatase and transferase activities was not achieved by this first DEAE-cellulose column a large portion of the “polyphenolic” compounds was removed. Further purification of the enzyme was achieved by the elution of the phosphatase and transferase activity from a second DEAE-cellulose column with a pH gradient. The elution profile (Fig. 3) shows that most of the phosphatase activity was eluted from the column rapidly and that the transferase activity, accompanied by some phosphatase activity, was eluted later.

Preparation of (32P)p-nitrophenyl Phosphate. In a representative experiment, the yield of (32P)p-nitrophenyl phosphate synthesized was 0.11 nmol (33.7 mg) as determined by absorbance at 285 mμ. This is 40.5% of the theoretical based on the amount of PCl3 added. Radioactivity of the product was
Chromatography of the radioactive product in solvent system B yielded essentially one radioactive area corresponding to the single UV absorbing area on the paper which migrated at the same rate as

![Graph](image1)

**FIG. 2.** Sodium chloride gradient elution of the crude nucleoside phosphotransferase preparation from a DEAE-cellulose column (21 x 1.5 cm). Illustrated are phosphatase activity as measured by \( p \)-nitrophenol liberated •—•—•—•—•—•—•—, relative protein concentration (absorbance at 280 m\( \mu \)) ○—○—○, and \( \mu \)moles of 5'-UMP synthesized by the nucleoside phosphotransferase reaction ∆—∆—∆—∆—∆. Enzymatic assays were performed as described in the Materials and Methods. The 500 ml gradient (0-1 \( M \) NaCl) was buffered by 0.01 \( M \) tris and 0.005 \( M \) magnesium acetate at pH 7.5. The gradient was prepared by placing 250 ml of buffer in the mixing chamber and 250 ml of buffered 1 \( M \) NaCl in the reservoir. The volume of the fractions collected was 10 ml.

![Graph](image2)

**FIG. 3.** Elution of phosphatase and transferase activities from a DEAE-cellulose column with a pH gradient. The linear pH gradient ○—○—○ was established with 500 ml of 0.05 \( M \) malic acid, 0.05 \( M \) maleic acid, and 0.025 \( M \) acetic acid at pH 5.7 in the mixing chamber and 500 ml of the same buffer but at pH 1.8 in the reservoir. Ten ml fractions were collected from the column (23 x 1.5 cm) with the phosphatase activity measured by \( p \)-nitrophenol release from \( p \)-nitrophenyl phosphate •—•—•—•—•—•—•—•—•— and the transferase activity measured by UMP synthesized ∆—∆—∆—∆. Enzymatic assays were performed as described in the Materials and Methods.

![Graph](image3)

**FIG. 4.** Chromatography of the purified \((^{32}P)p\)-nitrophenyl phosphate in solvent system B by the descending method. The UV absorbing area of \( p \)-nitrophenyl phosphate and the distribution of the radioactivity on the chromatographic sheet are illustrated.

![Graph](image4)

**FIG. 5.** Partial hydrolysis of \((^{32}P)p\)-nitrophenyl phosphate (boiling in 6 \( N \) HCl for 10 min). Separation of the hydrolysis mixture was achieved by chromatography in solvent system B. Illustrated is radioactivity in the slower moving inorganic phosphate and unhidrolyzed \( p \)-nitrophenyl phosphate plus the UV absorbing areas of the phosphate ester and \( p \)-nitrophenol.

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commercially available (Sigma) p-nitrophenyl phosphate (Fig. 4). Partial hydrolysis (boiling in 6 N HCl for 10 min) and chromatography as above, yielded 2 areas of radioactivity, one corresponding to phosphoric acid and the other to the originally synthesized product. Two areas of UV absorbance were also observed, one corresponding to p-nitrophenol and one to the product (Fig. 5). The UV absorbance spectrum was identical to that of commercially available p-nitrophenyl phosphate and that reported previously (8).

Hydrolysis of the synthesized product by both an acid and an alkaline phosphatase preparation yielded equivalent amounts of p-nitrophenol released as compared to the hydrolysis of commercial p-nitrophenyl phosphate (Table I). The acid phosphatase was prepared from sodium hypochlorite treated lettuce seeds (Lactuca sativa L., variety capitata L.), which had imbibed water overnight. The seeds were homogenized in a blender with cold 0.1% NaCl and the supernatant after centrifugation served as the source of the enzyme. The alkaline phosphatase preparation was from calf mucosa (Sigma).

**Table I. Hydrolysis of the Synthesized (32P)p-Nitrophenyl Phosphate and Commercially Purchased p-Nitrophenyl Phosphate by Alkaline and Acid Phosphatases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>p-Nitrophenol phosphate</th>
<th>p-Nitrophenol released</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>commercial</td>
<td></td>
<td>0.064</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>synthesized</td>
<td></td>
<td>0.064</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>commercial</td>
<td></td>
<td>0.033</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>synthesized</td>
<td></td>
<td>0.032</td>
</tr>
</tbody>
</table>

Enzymatic Synthesis of Nucleotides. By utilizing the purified nucleoside phosphotransferase preparation, unlabeled p-nitrophenyl phosphate, and the respective nucleosides it was possible to determine which nucleotide isomer was enzymatically synthesized. This was accomplished by chromatographing a portion of the incubation mixture in solvent system B where the 2'-3' nucleotides travel faster than the 5'-nucleotides. By a comparison to standard nucleotides it was demonstrated that the 5'-nucleotides were the only derivatives synthesized.

Since some phosphatase activity was still present in the purified nucleoside phosphotransferase preparation, the hydrolysis reaction was performed also in the absence of enzyme. The incubation for 10 min at 37° released a small amount of radioactivity, which was not resolved from the labeled product on the chromatogram. The aliquots were spotted on a paper chromatogram, thoroughly dried with a heat gun, and to these areas was added carrier UMP. The chromatogram was developed in solvent system B and the radioactivity in the UMP areas counted (see Materials and Methods).

Fig. 6. Time course of phosphorylation of uridine. The incubation mixture consisted of 0.1 ml purified nucleoside phosphotransferase preparation, 0.025 ml (32P) p-nitrophenyl phosphate (0.53 µmoles/0.1 ml) and 0.05 ml uridine (5 µmole/0.01 ml) in 1 ml sodium acetate pH 5.1. The mixture was incubated at 37° and 20 µl aliquots withdrawn at the time intervals indicated. The aliquots were spotted on a paper chromatogram, thoroughly dried with a heat gun, and to these areas was added carrier UMP. The chromatogram was developed in solvent system B and the radioactivity in the UMP areas counted (see Materials and Methods).
where spectrophotometry was utilized to estimate the nucleosides after separation by paper chromatography. The presence of the much smaller quantities of cytidine and guanosine was not observed in the earlier studies and the fact that they were noted here could be attributable to the increased sensitivity of detection of the present method.

Fig. 7. Distribution of radioactivity on chromatogram of nucleotides resulting from phosphorylation of nucleoside fraction from 10 mg cauliflower RNA with \( (^{32}P)\)-p-nitrophenyl phosphate and nucleoside phosphotransferase. The positions of UV absorbing carrier nucleotides are illustrated above the histogram. Nucleotides were chromatographed in solvent system C, eluted and rechromatographed in system B for 23 hr.

Table II. The Effect of Increasing Substrate Concentration on the Incorporation of Radioactivity Into a Mixture of Nucleotides by Nucleoside Phosphotransferase

<table>
<thead>
<tr>
<th>Conc of ( (^{32}P))-p-nitrophenyl phosphate</th>
<th>AMP</th>
<th>CMP</th>
<th>GMP</th>
<th>UMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu \text{moles} )</td>
<td>( % \text{ mole cpm} )</td>
<td>( % \text{ mole cpm} )</td>
<td>( % \text{ mole cpm} )</td>
<td>( % \text{ mole cpm} )</td>
</tr>
<tr>
<td>0.53</td>
<td>304</td>
<td>11</td>
<td>23</td>
<td>372</td>
</tr>
<tr>
<td>1.06</td>
<td>630</td>
<td>23</td>
<td>23</td>
<td>873</td>
</tr>
<tr>
<td>2.11</td>
<td>1002</td>
<td>37</td>
<td>30</td>
<td>737</td>
</tr>
</tbody>
</table>

Discussion

In the present studies nucleoside phosphotransferase was isolated from carrot leaves because this tissue proved to be a much richer source than roots from which it had been previously isolated (4, 18, 19). Until a homogenous enzyme preparation is obtained or phosphatase and transferase activities can be separated, it will not be possible to determine whether the enzyme possesses only transferase activity, or if both phosphatase and transferase activities are exhibited by a single enzyme.

For the synthesis of the \( (^{32}P)\)-p-nitrophenyl phosphate it was necessary to modify Axelrod's (1) method since micro-quantities of reactants could not be refluxed and distilled without suffering tremendous losses. In addition, in our hands, the major radioactive product obtained by his procedure behaved chromatographically like an impurity found in trace amounts in commercial \( p \)-nitrophenyl phosphate. This compound was not identified, but its spectrum was similar but not identical with that of \( p \)-nitrophenyl phosphate. Interestingly, the material was hydrolyzed by acid and alkaline phosphatases but did not serve as a phosphate donor for the transferase reaction. In the procedure described here, the methodology for the synthesis of the radioactive derivative is considerably simplified, the yield of product is substantial, and the product is highly radioactive. In conjunction with the nucleoside phosphotransferase enzyme, it is conceivable that radioactive phosphate ester derivatives could be synthesized which hitherto have not been available. The intermediate, \( (^{32}P)\)POCl\(_2\), could also prove to be useful since it readily reacts with hydroxyl groups and therefore might be advantageous in organic synthesis.

On the basis of the results obtained and those mentioned previously (6) it is evident that adenosine and uridine are the major terminal 5'-linked nucleosides of cauliflower ribosomal RNA. Thus the correspondence of the results helps to validate the method. The major advantage of the procedure is...
the increased sensitivity in detection of the nucleosides after formation of labeled derivatives. Additionally, only one derivative, the 5'-phosphate ester, is produced. The enzyme may be prepared easily, is stable in the frozen state and appears to be stable indefinitely after lyophilization. The labeled substrate is also easily prepared and is available from commercial sources.

In view of the ability of the enzyme to affect net synthesis of nucleotides it may be potentially useful in the preparation of analogues of mononucleotides for studies on RNA synthesis. We would recommend, however, that the enzyme be purified further for such studies as well as for other investigations of a quantitative nature where greater yields are desired.

**Literature Cited**