Addition of mononucleotides to oligoribonucleotide acceptors with T4 RNA ligase
(oligonucleotide synthesis/minimal substrate/5'-adenylylated pCp)

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ABSTRACT RNA ligase induced by bacteriophage T4 catalyzed the addition of nucleoside 5',3'-diphosphates to oligoribonucleotide acceptors in the presence of ATP. The reactions proceeded in equimolar concentrations of donors and acceptors. Several oligonucleotides of defined sequence were synthesized by this method in yields of 28–88%. The enzyme required the phosphate ester at the 3' position of the donor molecule, nucleoside 5',2'-diphosphates being unable to serve as donors. Thymidine 5',3'-diphosphate was active as a donor for the enzyme. The ligation product, (Ap)2ApCp, was also obtained from the reaction of (Ap)2A and 5'-adenylylated cytidine 5',3'-diphosphate (A5'pp5'Cp) with RNA ligase in the absence of ATP. These results show that the minimal substrate for RNA ligase is a nucleoside 5',3'-diphosphate.

Bacteriophage T4-induced RNA ligase catalyzes the ATP-dependent, intramolecular and intermolecular joining between the terminal 5'-phosphate and 3'-hydroxyl group of RNA (1–9). Single-stranded DNA is also joined by this enzyme (10, 11). Recently, T4 RNA ligase has been reported to be the product of gene 63 of T4 DNA, which promotes the formation of the tail fiber onto the base plate (12). However, the physiological role of the joining activity in the cell still remains to be revealed.

In spite of the obscurity of its physiological role, RNA ligase has been regarded as a powerful tool for the in vitro synthesis of oligonucleotides of defined sequence; several oligonucleotides have been synthesized by using this enzyme (4–9).

Although this enzyme has been known to catalyze the joining of poly- or oligonucleotides (1–11), the present paper shows that mononucleoside 5',3'-diphosphates, including a deoxyribonucleotide, can be attached to the acceptor oligoribonucleotides. These are the shortest substrates for RNA ligase, and this reaction is useful for stepwise synthesis of oligonucleotides of defined sequence.

MATERIALS AND METHODS

Materials. Oligonucleotides, (Ap)2A, (Ap)4A, and (Up)3U were purchased from Boehringer/Mannheim GmbH. Adenosine 5',3'-diphosphate and adenosine 5',2'-diphosphate were from Sigma Chemical Co. and thymidine 5',3'-diphosphate was from Worthington Biochemical Co. Cytidine 5',3'-(or 2')-diphosphate, guanosine 5',3'(2')-diphosphate, and uridine 5',3'- (2')-diphosphate were synthesized chemically by the method of Yoshikawa et al. (13). Snake venom phosphodiesterase was purchased from Worthington Biochemical Co. Nuclease P1 and poly(C) were from Yamasa Shoyu Co. RNase T2 was obtained from Sankyo Co. and nuclease SW (silkworm nuclease) was from Seikagaku Kogyo Co. Bacterial alkaline phosphatase was prepared from Escherichia coli A19 cells by the method of Garen and Levinthal (14).

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Purification of RNA Ligase. RNA ligase was prepared from E. coli A19 infected with T4 em N82XE1140 and purified by the procedure of Last and Anderson (5) except that the ATP/ inorganic [32P]pyrophosphate exchange assay (2) was used during the purification process. The reaction mixture [67 mM Tris-HCl, pH 7.6/6.7 mM MgCl2/1 mM dithiothreitol/0.13 mM ATP/0.13 mM sodium [32P]pyrophosphate (2.1 × 104 cpm/mmol)/20 μl of enzyme solution in a total volume of 60 μl] was incubated at 37° for 20 min, and Norit-adsorbable radioactivity was determined by the method of Huang (15). RNA ligase used in these experiments was the step 7 enzyme purified through the second DEAE-cellulose column (5) (specific activity, 1071 units/mg of protein). One unit of activity is defined as the amount catalyzing the conversion of 1 nmol of [32P]-pyrophosphate to a Norit-adsorbable form in 20 min under the reaction condition described. The step 7 enzyme was concentrated and dialyzed against 20 mM Tris-HCl, pH 7.5/0.1 mM EDTA/10 mM 2-mercaptoethanol/0.1 M KCl/50% glycerol and stored at −20°. The preparation contained no detectable DNA ligase activity; DNA ligase activity was assayed by the method of Dugaiczky et al. (16).

Chromatographic Methods. The solvent systems for chromatography were as follows: solvent A, isobutyric acid/water/concentrated NH4OH, 10:5:0.15 (vol/vol); solvent B, ethanol/1 M ammonium acetate, pH 5.5, 50:50 (vol/vol); solvent C, linear gradient, 0.1−0.7 M NH4HCO3 in 200 ml of water; solvent D, linear gradient, 0.1−0.85 M NH4HCO3 in 200 ml of water. Thin-layer chromatography was done on Merck DC-plastic roll cellulose with solvent A or B. DEAE-Sephadex A-25 column chromatography of the reaction mixture was performed at room temperature as follows. The sample was applied to a column (0.5 × 25 cm) that had been previously equilibrated with 0.1 M NH4HCO3. The column was washed with 10 ml of 0.1 M NH4HCO3 and then eluted with solvent C or D at a flow rate of about 25 ml/hr. Fractions of 2 ml were collected and the absorbance at 260 nm was measured.

Characterization of Oligonucleotide Products. The structures of products were determined by analysis involving enzymatic hydrolysis.

(i) Degradation with nuclease P1. The reaction mixture (10 μl) containing 0.5−1 A260 unit of oligonucleotide, 1 μg of nuclease P1, and 0.1 M acetate buffer (pH 5.3) was incubated at 50° for 1 hr.

(ii) Degradation with snake venom phosphodiesterase. The reaction mixture (10 μl) containing 0.5−1 A260 unit of oligonucleotide, 50 μg of snake venom phosphodiesterase, and 0.05 M Tris-HCl (pH 8.5) was incubated at 37° for 3 hr.

(iii) Degradation with RNase T2. The reaction mixture (10 μl) containing 0.5−1 A260 unit of oligonucleotide, 1 unit of

Abbreviation: A5'pp5'Cp, 5'-adenylylated cytidine 5',3'-diphosphate.

pCp hydroxyl Acceptors
bonucleotide
phodiesterase:
was
tected,
sparated by DEAE-Sephadex
allowed
mg
40
the

taining 0.05 M
by
(A5'pp5'Cp). The dinucleotide
at
of
ml
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mension
The
chromatography
The
valently
The
370
at
reaction mixture of RNA
analyzed by
لت
A5'pp5'CpC

The absorbance
nm,
absorption,
The

Fraction number
Absorbance at 260 nm


(inc) Degradation with RNase T2 after dephosphorylation. The 3'-phosphate groups of oligonucleotides were removed by passage through a column of E. coli alkaline phosphatase covalently bound to Sepharose 4B in 33 mM NH4HCO3 (pH 8.5). The eluted sample (0.5–1 A260 unit) was evaporated and further treated with RNase T2 as described in (43).

The digests were separated by two-dimensional thin-layer chromatography on Merck DC-plastic cellulose as described (17). The plate was developed with solvent A in the first dimension and with solvent B in the second dimension. Spots were located by their UV absorption, cut out, and extracted with 0.6 ml of 0.01 M HCl. The absorbance of the extract was measured at 260 nm and 280 nm, and the ratio of components was determined.

Synthesis of 5'-Adenylylated Cytidine 5',3'-Diphosphate (A5'pp5'Cp). The dinucleotide pCpCp was prepared by the complete degradation of poly(C) with nuclease SW as described by Muki et al. (18). For this reaction, a mixture (0.4 ml) containing 0.05 M sodium carbonate (pH 10.3), 0.1 M NaCl, 2 mM MgCl2, 3.7 mg of poly(C), and 400 units of nuclease SW was incubated at 37°C for 2 hr. Then the mixture was boiled for 5 min to stop the reaction and chromatographed on a DEAE-Sephadex column with solvent C. The peak of pCpCp (27 A260 units) was collected and lyophilized. A5'pp5'CpCp was synthesized by the procedure of Snisky et al. (17). pCpCp (10 A260 units) was dissolved in 100 μl of 0.1 M AMP/0.1 M MgCl2 (pH 5.0) and 40 mg of N-cyclohexyl-N'-β-(4-methylmorpholinium) ethylcarbodiimide p-toluene sulfonate was added. The mixture was allowed to stand for 2 hr. The A5'pp5'CpCp synthesized was separated by DEAE-Sephadex column chromatography, collected, and lyophilized (3 A260 units). A5'pp5'Cp was prepared by the degradation of A5'pp5'CpCp with RNase A. The yield was 1.97 A260 units of A5'pp5'Cp by this method. The preparation was analyzed by hydrolysis with snake venom phosphodiesterase: pA and pCp were obtained with a molar ratio of 1:1.

RESULTS

Addition of Nucleoside 5',3'-Diphosphates to Oligoribonucleotide Acceptors with RNA Ligase. RNA ligase catalyzed the formation of a phosphodiester bond between the 3'-hydroxyl group of (Ap)3A (acceptor) and the 5'-phosphate of pCp (donor). The 200-μl reaction mixture [50 mM Tris-HCl, pH 7.9/20 mM MgCl2/5 mM dithiothreitol/2.5 mM ATP/1 mM pCp (the mixture of 5',3'- and 5',2'-diphosphates in a ratio of 1:1)/0.5 mM (Ap)3A/65 μg of RNA ligase] was incubated at 37°C for 4 hr. The mixture was diluted 5-fold with water, applied to a DEAE-Sephadex A-25 column, and chromatographed with solvent C (Fig. 1).

Peak IV was identified by enzymatic analysis to be the intermolecular ligation product (Ap)3ApCp as follows. The oligonucleotide was cleaved with snake venom phosphodiesterase, yielding A, pA, and pCp, which showed that the 5' end of the oligomer was A with a free hydroxyl terminus and the 3' end was pCp. Treatment with nuclease P1, produced A, pA, and pC in the ratio 1:0.01:0.98/0.92. Thus, the oligonucleotide of peak IV was identified as (Ap)3ApCp. The molar yield of (Ap)3ApCp from (Ap)3A was 68% [yield calculated as described (17)]. Nuclease P1 specifically hydrolyzes 3'-phosphate groups; 2'-phosphate groups are not hydrolyzed under the conditions used in this experiment (19). Thus, it was verified that the terminal phosphate of the oligonucleotide was present only at the 3'-position and not at 2'-position, proving that RNA ligase used exclusively cytidine 5',3'-diphosphate but not 5',2'-diphosphate as a donor. To confirm this specificity, this reaction was conducted with adenosine 5',2'-diphosphate and adenosine 5',3'-diphosphate. The reaction conditions were as described above, except that 0.5 mM adenosine 5',2'-diphosphate or adenosine 5',3'-diphosphate was used instead of 1 mM cytidine 5',3'(2')-diphosphate. From (Ap)3A and adenosine 5',3'-diphosphate, (Ap)3ApApAp was obtained in a yield of 34%, whereas no detectable ligation product was obtained from (Ap)3A and adenosine 5',2'-diphosphate, verifying that the RNA ligase could not utilize a nucleoside 5',2'-diphosphate as a donor.

Several oligonucleotides were synthesized by this mononucleotide-joining reaction with RNA ligase (Table 1). The 200-μl reaction mixture contained 50 mM Tris-HCl buffer (pH 7.9), 20 mM MgCl2, 5 mM dithiothreitol, 2.5 mM ATP, 65 μg of RNA ligase, 0.5 mM acceptor oligonucleotide [(Ap)3A, (Ap)3A, or (Up)3U], and donor pNp. The concentration of pNp (donor) in the reaction mixture was 0.5 mM for adenosine 5',3'-diphosphate (pAp) and thymidine 5',3'-diphosphate (pTp) or 1 mM for pCp, pUp, and pCp, the latter three nucleotides being 1:1 mixtures of 5',3'- and 5',2'-diphosphates. Therefore, the reactions were carried out with equimolar concentration of acceptors and donors. The mixtures were incubated at 37°C for 4 hr and analyzed as described for (Ap)3ApCp (Table 1). The yields were calculated as described (17).

The deoxyribonucleotide pTp also served as a donor for RNA ligase. The yield (41%) of (Ap)3ApTp from (Ap)3A and pTp was comparable to that of other oligonucleotide products (Table 1).

| Table 1. Joining of mononucleotides with T4 RNA ligase |
|---|---|---|---|---|
| Acceptor | Donor | Product | Molar yield, % | Solvent* |
| (Up)3U | pCp | (Up)3UpCp | 49 | D |
| (Up)3U | pAp | — | 0 | C |

* The solvent used for the separation of products from the reaction mixture by DEAE-Sephardex column chromatography; see Materials and Methods.
**Table 2. Analysis of ligated products by enzymatic hydrolyses**

<table>
<thead>
<tr>
<th>Product</th>
<th>Enzyme*</th>
<th>Products of hydrolysis</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A, pA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ap)2ApGp</td>
<td>V</td>
<td>A, pA, pGp</td>
<td>1.00:1.70:0.84</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A, pA, pG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ap)2ApCp</td>
<td>V</td>
<td>A, pA, pCp</td>
<td>1.00:1.88:0.92</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A, pA, pC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ap)2ApUp</td>
<td>V</td>
<td>A, pA, pUp</td>
<td>1.00:2.14:0.93</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A, pA, pU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A, pA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A, pA, pT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Up)3UpCp</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Up, Cp</td>
<td>4.00:0.93</td>
</tr>
<tr>
<td>BAP → T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Up, C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>U, pU, pC</td>
<td></td>
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</tbody>
</table>

Ligated products were analyzed by enzymatic hydrolyses as described in Materials and Methods.

* BAP, E. coli alkaline phosphatase; P<sub>1</sub>, nuclease P<sub>1</sub>; V, snake venom phosphodiesterase; T<sub>2</sub>, RNase T<sub>2</sub>; BAP → T<sub>2</sub>, RNase T<sub>2</sub> after dephosphorylation with BAP.

No ligation product was detected from (Up)3U and pAp, although (Up)3UpCp was synthesized in a yield of 49% from (Up)3U and pCp.

Table 2 shows the characterization of ligated products, as analyzed by enzymatic hydrolysis. The results were consistent with the postulated formula.

**Synthesis of (Ap)2ApCp from (Ap)2A and A5'pp5'5'Cp in the Absence of ATP.** The presence of ATP in the reaction mixture was indispensable for the synthesis of (Ap)2ApCp from (Ap)2A and pCp with RNA ligase, as well as for the ligation of RNA by this enzyme. The omission of ATP from the reaction mixture completely prevented the formation of (Ap)2ApCp. To confirm the similarity of the mechanism of mononucleotide addition with the joining mechanism of oligonucleotides by this enzyme previously reported (6, 7, 11), A5'pp5'5'Cp, which is the expected reaction intermediate, was synthesized and incubated with (Ap)2A in the absence of ATP. The 100-μl reaction mixture [50 mM Tris-HCl, pH 7.9/20 mM MgCl<sub>2</sub>/5 mM dithiothreitol/0.5 mM (Ap)2A, 1.12 A<sub>260</sub> units of A5'pp5'5'Cp/33 μg of RNA ligase] was incubated at 37° for 3 hr and analyzed by DEAE-Sephadex A-25 column chromatography with solvent C. (Ap)2ApCp was synthesized from (Ap)2A and A5'pp5'5'Cp in a yield of 33% without the addition of ATP. The product was identified as (Ap)2ApCp by enzymatic analysis. This confirmed that the adenylated donor, A5'pp5'5'Cp, was the reaction intermediate.

**DISCUSSION**

This paper shows that the bacteriophage T4-induced RNA ligase can catalyze the joining of nucleoside 5'-3'-diphosphates to oligoribonucleotide acceptors and that its mechanism of joining is the same as that of the joining of oligonucleotide. Kaufmann and Kallenbach (3) reported that a dinucleoside diphosphate, pApA, was a minimal 3'-hydroxyl recognition site of RNA ligase; however, the minimal 5'-phosphate recognition site was unclear. We show here that the minimal 5'-phosphate recognition site of RNA ligase was a nucleoside 5',3'-diphosphate. The RNA ligase was unable to catalyze the joining of nucleoside 5'-monophosphates or nucleoside 5',2'-diphosphates to acceptor molecules. Therefore, the phosphate ester at the 3'-position of the donor molecules is indispensable for the ligation reaction. It is noteworthy that the 3'-phosphate is required also in the case of T4 polynucleotide kinase. Polynucleotide kinase cannot phosphorylate the 5'-hydroxyl of adenosine or 2'-AMP, but 3'-AMP can serve as the substrate (20). This 3'-phosphate serves two functions in the mononucleotide joining reaction with RNA ligase; one is recognition of RNA ligase as described above and the other is protection against further additions of donor molecules.

The pyrimidine oligonucleotide (Up)3U was a good acceptor for joining with pCp by RNA ligase. The ligated product (Up)3 UpCp was obtained in a yield of 49%, whereas no ligation product was obtained from (Up)3U and pAp (Table 1). From the results in Table 1, it is conceivable that pyrimidine oligonucleotides serve as acceptors for only pyrimidine donors, but purine oligonucleotides serve for both pyrimidine and purine donors. A similar phenomenon was also reported in the case of the joining of oligonucleotides. Ohtsuka et al. (6) reported that the ligation products of pyrimidine acceptors [(Up)3U and (Cp)3C] and a purine donor [pAp(Ap)3A] were obtained in low yields (0–6%) even when a great excess of acceptor was used. On the other hand, the (Cp)3C acceptor and the pCpCpA donor were efficiently ligated in a yield of 71% (9). Sinsky et al. (7) also reported that the (Up)3C acceptor and the pAp(Ap)3A donor were ligated in the equimolar reaction in a yield of only 5%. Under the reaction conditions so far reported, the specificity for homo-oligomers may be simple. However, the specificity for hetero-oligomers should be further studied.

Although these substrate specificities do exist, the mononucleotide joining reaction reported here offers a useful method for the stepwise synthesis of oligonucleotides of defined sequence. The dephosphorylation of the first ligation product with phosphomonoesterase forms the acceptor for the second ligation reaction. Similar methods for stepwise synthesis of oligonucleotides using polynucleotide phosphorylase have been developed in various laboratories, including ours (17, 21–26); however, the method using RNA ligase may be useful for mononucleotide additions to longer polynucleotides because RNA ligase has no degrading activity for polynucleotide acceptors such as phosphorylase by polynucleotide phosphorylase (26, 27).

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