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Biotin and fluorescent labeling of RNA using T4 RNA ligase

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ABSTRACT

Biotin, fluorescein, and tetramethylrhodamine derivatives of P^1-(6-aminohex-1-yl)-P^2-(5'-adenosine) pyrophosphate were synthesized and used as substrates with T4 RNA ligase. In the absence of ATP, the non-adenylyl portion of these substrates is transferred to the 3'-hydroxyl of an RNA acceptor to form a phosphodiester bond and the AMP portion is released. E. coli and D. melanogaster 5S RNA, yeast $tRNA^{Phe}$, $(Ap)_3C$, and $(Ap)_3A$ serve as acceptors with yields of products varying from 50 to 100%. Biotin-labeled oligonucleotides are bound selectively and quantitatively to avidin-agarose and may be eluted with 6 M guanidine hydrochloride, pH 2.5. Fluorescein and tetramethylrhodamine-labeled oligonucleotides are highly fluorescent and show no quenching due to attachment to the acceptor. The diverse structures of the appended groups and of the chain lengths and compositions of the acceptor RNAs show that T4 RNA ligase will be a useful modification reagent for the addition of various functional groups to the 3'-terminus of RNA molecules.

INTRODUCTION

Several methods have been used to add both nucleotide and other derivatives to the 3'-terminus of RNA. A common technique involves the periodate oxidation of the 2',3'-cis hydroxyls of the terminal ribose and a subsequent coupling of the desired addendum to the aldehyde groups. This method has been used to introduce various functional groups onto RNA; e.g., biotin or fluorescent dyes for use as in situ hybridization probes (1,2), a fluorophore or antigenic determinant to probe the location and accessibility of the 3'-end of rRNAs in E. coli ribosomes (3,4), and a photoaffinity label to trace the mRNA binding region of E. coli ribosomes (5). Although the periodate method provides a general means to chemically modify the 3'-end of RNA, there is no analogous enzymatic methodology. Such an enzymatic method would provide a gentle, highly specific, and facile way to modify RNA.

We have been examining bacteriophage T4 RNA ligase as a means of accomplishing this goal. This versatile enzyme catalyzes a variety of reactions; the intra- and inter-molecular joining of RNA or DNA, the

addition of single mononucleotides to RNA or DNA, and the addition of non-nucleotide compounds to the 3'-hydroxyl of RNA in an ATP-independent reaction (6,7). The latter reaction is based upon using analogues of one of the covalent intermediates of the reaction mechanism (Ado-5'PP5'-RNA) as substrates. For example, we have shown that a number of β-substituted ADP derivatives of the form Ado-5'PP-X will transfer P-X to the 3'-hydroxyl of an RNA acceptor to form a 3'-phosphodiester bond and release 5'-AMP (8).

In this paper we describe the chemical synthesis of four new ADP derivatives and give reaction conditions that allow them to be used as substrates with T4 RNA ligase. They are used to modify the 3'-terminus of oligoribonucleotides, yeast $tRNA^{Phe}$ and 5S RNA (*D. melanogaster* and *E. coli*) by the addition of a primary amino group, a biotin molecule, or fluorescent dyes.

MATERIALS

$(Ap)_2A$, $(Ap)_3A$, cytidine 3',5'-bisphosphate (pCp), N^6-(6-aminohex-1-yl)-adenosine 3',5'-bisphosphate (pA-HA-p), and 5'-AMP were purchased from PL Biochemicals. RNase A (type 1-A, bovine pancreas), D-biotin, 1,1'-carbonyldiimidazole (CDI), avidin (1 unit binds 4 nmoles of biotin), dithiothreitol (DTT), and bovine serum albumin (BSA) were obtained from Sigma. N-hydroxysuccinimide (NHS) was purchased from Aldrich. D-[carbonyl-^{14}C]biotin was obtained from Amersham. Tetramethylrhodamine isothiocyanate (TMRITC) isomer R and mixed isomers (R + G) were purchased from Curtin-Matheson and Research Organics, Inc., respectively. Yeast $tRNA^{Phe}$ was obtained from Boehringer-Mannheim. *D. melanogaster* Canton S. and *E. coli* AB 257 5S RNA were gifts from D. M. Steffensen and N. Pace, respectively. Avidin bound to agarose was obtained from Vector Laboratories. ATP[γ-^{32}P] was purchased from New England Nuclear or prepared by the method of Johnson and Walseth (9). Analytical grade dimethylformamide (DMF), dimethylsulfoxide (DMSO), triethylamine, and $POCl_3$ were redistilled before use. Cellulose thin layer plates were obtained from Eastman Kodak. Silica gel thin layer plates were obtained from Merck or Brinkman. Woelm 32-63 silica for flash chromatography and fluorescein isothiocyanate (FITC) isomer I were gifts from W. Mangel. P^1-(6-aminohex-1-yl)-P^2-(5'-adenosine) pyrophosphate (ADP-HA) was prepared by the methods of Barker et al. (10,11) and Trayer et al. (12). Biotinyl-N-hydroxysuccinimide ester (NHS-biotin) was prepared by the procedures described by Bayer and Wilchek (13). NHS-[carbonyl-^{14}C]biotin was prepared as described by Jasiewicz et al. (14).

T4 RNA ligase was purified as described (15). PseT 1 polynucleotide kinase (16) was a gift from D. Soltis and O. C. Uhlenbeck. Bacterial alkaline phosphatase (BAP), grade C, and venom phosphodiesterase (VPD) were purchased from Worthington. RNase U_2 and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were obtained from Calbiochem.

METHODS

Thin layer and paper chromatography.

The solvents used for thin layer and paper chromatography were: I, 2-propanol:1.3 M sodium acetate, pH 5 :: 7:3; II, methanol:dichloromethane :: 15:85; III, methanol:dichloromethane::2:8; IV, ethanol:1 M triethylammonium bicarbonate (TEABC) pH 7.5 :: 5:2; V, methanol:0.5 M NaCl :: 1:1; VI, 1-propanol:conc. $NH_4OH:H_2O$ :: 55:10:35; VII, 1.89 grams of NH_4CO_2H and 1.23 ml of HCO_2H per 100 ml; and VIII, 1 M ammonium acetate:ethanol :: 7:3.

Electrophoresis.

High voltage paper electrophoresis on Whatman 3MM paper was performed in 50 mM TEABC, pH 8, 2 mM EDTA and 5% 2-propanol at 50 V/cm for 3 hours using a Savant Model FP-30A flat bed apparatus. Denaturing polyacrylamide gel electrophoresis was as described by Maxam and Gilbert (17) for 20% and 8% gels.

Synthesis of P^1-(N-biotinyl-6-aminohex-1-yl)-P^2-(5'-adenosine) pyrophosphate (ADP-HA-biotin).

ADP-HA, 2.73 µmol, and 13.2 µmol of NHS-biotin were dissolved in 0.42 ml 20 mM sodium borate, pH 9, 60% DMF. After 5 hours at room temperature in the dark, the mixture was applied to a DEAE-Sephadex A-25 column (0.9 x 15 cm) and eluted with a 200 ml gradient of 0.1 to 1 M TEABC, pH 7.5. The last peak of material absorbing light at 260 nm was pooled and repeatedly dried under vacuum with methanol and stored at -20°C in H_2O. The R_f values by cellulose thin layer chromatography in solvent I were 0.18, 0.48, and 0.82 for ADP-HA, ADP-HA-biotin, and biotin, respectively. Phosphate analysis and UV spectroscopy revealed 2.08 mol of organic phosphate per mol of compound. A_M(259 nm) = 15,400 $M^{-1}cm^{-1}$. Yield: 2.5 µmol, 92%. N^6-(N-biotinyl-6-aminohex-1-yl)-adenosine 3',5'-bisphosphate (pA-HA-biotin-p) was synthesized from pA-HA-p and NHS-biotin as described for ADP-HA-biotin.

Synthesis of P^1-(N-[carbonyl-^{14}C]-biotinyl-6-aminohex-1-yl)-P^2-(5'-adenosine) pyrophosphate (ADP-HA-[^{14}C]-biotin).

NHS-[carbonyl-^{14}C]-biotin (55 mCi/mmol), 455 nmol, and 910 nmol of ADP-

HA were dissolved in 35 µl of 60 mM sodium borate, pH 9, 70% DMF. After 5 hours at room temperature in the dark, the reaction mixture was spotted on Whatman 3MM paper and subjected to high voltage paper electrophoresis. The radiolabeled product [R_m = 0.61 (relative to picric acid)] migrated faster than ADP-HA (R_m = 0.34). The ADP-HA-[^{14}C]biotin was eluted from the electropherogram with H_2O and stored at -20°C. Cellulose thin layer chromatography in solvent I showed that >95% of the radioactivity comigrated with a ADP-HA-biotin marker. Yield: 40% by radioactivity.

Synthesis of P^1-(N-5-fluorescein-6-thioureidohex-1-yl)-P^2-(5'-adenosine) pyrophosphate (ADP-HA-Fl).

ADP-HA, 400 nmol, and 2.6 µmol of FITC were dissolved in 0.1 ml of 50 mM $NaHCO_3$, pH 9, 50% DMSO. After 3 hours at room temperature in the dark, the reaction mixture was applied to Whatman 3MM paper and subjected to high voltage paper electrophoresis. The ADP-HA-Fl [R_m = 0.95 (relative to picric acid)] migrated faster than FITC (R_m = 0.71). The ADP-HA-Fl region of the paper was washed with 100% ethanol, eluted with H_2O, and the solution stored at -20°C. A_M (491 nm) = 90,200 $M^{-1}cm^{-1}$. Yield: 177 nmol, 44%.

Synthesis of P^1-(N-5-tetramethylrhodamine-6-thioureidohex-1-yl)-P^2-(5'-adenosine) pyrophosphate (ADP-HA-TMR).

Synthesis of N-5-tetramethylrhodamine-6-thioureidohexan-1-ol (HA-TMR).

TMRITC, isomer R, 111 µmol, was dissolved in 2 ml of anhydrous pyridine and added to 5 ml of pyridine containing 338 µmol of 6-aminohexan-1-ol (HA). After 3 hours at room temperature, the reaction mixture was dried under vacuum and redissolved in 3 ml of DMF. The HA-TMR was precipitated with 10 volumes of 2 N HCl at 0°C overnight and centrifuged at 25,000 x *g*. for 25 min. at -5°C. The pellet was redissolved in 3 ml of methanol and precipitated with 10 volumes of diethyl ether. The precipitate was again centrifuged for 20 min. and redissolved in methanol:dichloromethane (10:90,v/v).

The HA-TMR was purified by silica flash chromatography (18) using successive methanol:dichloromethane steps: 1) (10:90,v/v); 2) (15:85,v/v); and 3) (30:70,v/v). The HA-TMR was precipitated with 2 N HCl twice and with diethyl ether as described. Yields: 17.6 mg, 27.7 µmol, 25%. Silica thin layer chromatography (Merck) in solvent II gave R_f values of 0.20 and 0.40 for HA-TMR and TMRITC, respectively. Field atomic bombardment mass spectral analysis showed the expected molecular ions: 562 (M^+-2H-3Cl), 563 (M^+-H-3Cl), and 564 (M^+-3Cl).

Synthesis of O-Phosphoryl-N-5-tetramethylrhodamine-6-thioureidohexan-1-ol (P-HA-TMR).

HA-TMR, 7.4 µmol, was dissolved in 4.5 ml trimethylphosphate and cooled to 0°C in an ice bath. $POCl_3$, 1.06 mmol, was added with stirring while keeping the temperature between -5°C and 0°C. The reaction was monitored by thin layer chromatography on silica (Brinkman) in solvent III. After 1.5 hours, the reaction was terminated by slowly adding 4 grams of chopped ice and stirring at 0°C for 30 min. Conc. NH_4OH (0.3 ml) was added to neutralize the mixture, the solution was stirred at 0°C for 5 min, extracted four times with equal volumes of diethyl ether, and concentrated to 7.5 ml under vacuum. The P-HA-TMR was diluted with water (400 ml) to reduce the conductivity and the solution applied to a Whatman DE-52 column (0.9 x 7 cm) that had been equilibrated with 20 mM TEABC, pH 7.5. The column was washed with 2 column volumes of 20 mM TEABC, 10 volumes of 100 mM TEABC, and 10 volumes of 150 mM TEABC. The P-HA-TMR was eluted with 250 mM TEABC and coevaporated several times with 100% ethanol. Methanol was added (1 ml) to the dry red residue and the product was precipitated with 10 volumes of diethyl ether at 0°C. The P-HA-TMR was further purified on a DEAE-cellulose (Sigma, coarse) column (1 x 35 cm) as described (19), except using 5, 10, and 15 mM ammonium acetate washes. Yield: 3.2 µmol, 43%. Thin layer chromatography on silica (Merck) in solvent IV gave R_f values of 0.24 and 0.42 for P-HA-TMR and HA-TMR, respectively.

Synthesis of P^1-(N-5-tetramethylrhodamine-6-thioureidohex-1-yl)-P^2-(5'-adenosine) pyrophosphate (ADP-HA-TMR).

P-HA-TMR, 3.2 µmol, was dissolved in 1 ml of anhydrous DMF and 160 µmol of CDI were added. The reaction was monitored by thin layer chromatography on silica (Merck) in solvent IV. After 12 hours at room temperature in the dark, 196 µmol of anhydrous methanol were added. After 20 min at room temperature, 280 µmol of 5'-AMP (tri-n-butylammonium salt) in 2 ml anhydrous DMF were added. The reaction was monitored by thin layer chromatography on C18 plates (Whatman) in solvent V. After 18 hours at room temperature in the dark, the reaction was diluted into 5 ml of methanol: dichloromethane:H_2O :: 3:3:1 and applied to a DEAE-cellulose (Sigma, coarse) column (1 x 35 cm) as described (19), except the elution used 10, 30, and 40 mM ammonium acetate washes. The ADP-HA-TMR was further purified by reverse phase HPLC using a Beckman Ultrasphere C8 column (0.46 x 25 cm). The sample was applied to the column in methanol:H_2O :: 20:80 (v/v) at 1.5 ml/min at 40°C and eluted isocratically using the same solvent. The

pooled fractions were dried under vacuum, resuspended in H_2O, and stored at -20°C. Yield: 1.31 µmol, 41%. A_M(550 nm) = 66,000 $M^{-1}cm^{-1}$ as determined by organic phosphate analysis. Thin layer chromatography on silica (Merck) in solvent V gave R_f values of 0.05, 0.15, and 0.40 for HA-TMR, P-HA-TMR, and ADP-HA-TMR, respectively. Attempts to synthesize ADP-HA-TMR by the method described above for the synthesis of ADP-HA-Fl were inconsistently successful and gave poor yields (3-40%) of impure product.

Preparation of nucleotides and oligonucleotides.

The oligoribonucleotide [Cyd-^{3}H](Ap)$_3$C (12 Ci/mmol) was synthesized as previously described (20). The donor [5'-^{32}P]pCp was synthesized as described by Hinton and Gumport (21). The ^{32}P-labeled acceptor A(pA)$_2$[3'→5'-^{32}P]pC was synthesized in a 50 µl mixture containing 50 mM HEPES, pH 8.3, 20 mM $MgCl_2$, 10 µg/ml BSA, 3.3 mM DTT, 6 µM ATP, 3 µM [5'-^{32}P]pCp, 300 µM (Ap)$_2$A and 3 µM RNA ligase. After 2 hours at 37°C, the product was isolated by paper chromatography on prewashed Whatman 3MM paper (22) in solvent VI and eluted with H_2O. The tetramer was dephosphorylated in a 50 µl mixture containing 50 mM HEPES, pH 8.3, 20 mM $MgCl_2$, 10 µg/ml BSA, 3.3 mM DTT and 20 µg/ml BAP. After 2 hours at 37°C, the A(pA)$_2$[3'→5'-^{32}P]pC was isolated by paper chromatography as described above and stored at -20°C in 50% ethanol. The unlabeled acceptor A(pA)$_2$pC was prepared exactly as was the labeled tetramer except in a 0.3 ml reaction mixture with 2 mM ATP, 2 mM (Ap)$_2$A and 4 mM pCp.

Characterization of substrates.

The nucleoside pyrophosphates were analyzed by: 1) the determination of their total and free phosphate content as described by Chen et al. (23) using the ashing procedure of Ames and Dubin (24) and 2) by hydrolysis with VPD (70 µg/ml) in 20 mM HEPES, pH 8.3, and 10 mM $MgCl_2$ for 1 hour at 37°C followed by BAP treatment (90 µg/ml) under the same conditions. The digested mixtures were analyzed by thin layer chromatography on cellulose in solvent I or C18 reverse phase plates in solvent V. Thin layer plates were developed with a molybdate spray for phosphates (25), a ninhydrin spray for primary amines and a potassium iodoplatinate spray (10% KI:5% H_2PtCl_6:H_2O::45:5:50) for biotin (26). Adenine and fluorescent compounds were detected with short and long wavelength UV light, respectively.

Assays of the joining reactions.

Table 1 shows the reaction conditions that were used for each type of acceptor RNA tested. The acceptor RNA and Ado-5'PP-X were dried under vacuum in 1.5 ml siliconized Eppendorf tubes. The remaining components were

TABLE 1. T4 RNA ligase reaction conditions with RNA substrates.

Oligomers	tRNA	5S RNA
50-330 μM Acceptor[a]	11 μM Acceptor[a]	5-20 μM Acceptor[a]
250-670 μM ADP-HA-X[b]	30-340 μM ADP-HA-X[b]	50-200 μM ADP-HA-X[b]
Buffer Mix, pH 8.3[c]	Buffer Mix, pH 7.5[c]	Buffer Mix, pH 8.0[c]
10 μg/ml BSA	10 μg/ml BSA	0-30% (v/v) DMSO
0-10% (v/v) DMSO	0-10% (v/v) DMSO	4-9 μM RNA ligase
3 μM RNA ligase	3-6 μM RNA ligase	5-10 μl[d]
10-30 μl[d]	15 μl[d]	5°[e]
17° or 37°[e]	5° or 17°[e]	

[a]All concentrations are in terms of molecules
[b]X: biotin, fluorescein, or tetramethylrhodamine
[c]Buffer Mix: 50 mM HEPES, 3.3 mM DTT, and 20 mM $MgCl_2$
[d]Volume of reaction
[e]Incubation temperatures

added in a single volume and the reaction started by adding enzyme.

Aliquots of the oligonucleotide reactions were applied directly to Whatman DE 81 paper and developed by descending chromatography for 6 hours in solvent VII or to Whatman No. 1 paper and analogously developed for 24 hours in solvent VIII. The ^{32}P-labeled chromatograms were scanned for radioactivity in a Packard 701B strip scanner and quantified by Cerenkov counting. The ^{3}H- and ^{14}C-labeled chromatograms were cut into one centimeter strips and quantified by liquid scintillation counting. The reactions yields were calculated as the ratio of radioactivity in the product region of the chromatogram to the total radioactivity present and were based upon the limiting substrate present in the reaction mixture.

The yeast $tRNA^{Phe}$ and 5S RNA reactions using ADP-HA-[^{14}C]biotin were assayed by trichloroacetic acid precipitation and filtration on nitrocellulose or by polyacrylamide gel electrophoresis. The ^{14}C-labeled product was quantified by liquid scintillation counting. When using the fluorescent donor ADP-HA-TMR, the reactions were assayed by polyacrylamide gel electrophoresis followed by microdensitometry of the photographic negative of the ethidium bromide stained gel (27).

Characterization of products.

The oligonucleotide products were analyzed by hydrolysis with RNase A (60 μg/ml) or, in addition, BAP (60 μg/ml) at 37°C for 2 hours. The

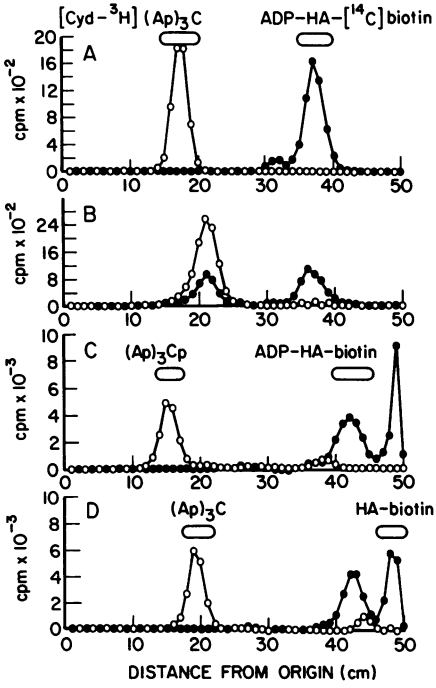

Figure 1. Characterization of the T4 RNA ligase reaction between $(Ap)_3C$ and ADP-HA-biotin. Incubation was at 17° with 3 μM RNA ligase in 30 μl as described in Table 1. After hydrolysis with RNase A or RNaseA plus BAP the reaction was analyzed by descending paper chromatography in solvent VIII for 24 hr. as described in Methods. Panel A: control of [Cyd-^{3}H]$(Ap)_3C$ (0.33 mM, 48 Ci/mol) and ADP-HA-[carbonyl-^{14}C]biotin (0.67 mM, 440 mCi/mol) before incubation; Panel B: complete reaction after incubation for 6 hours; and panels C and D: complete reaction after 6 hours treated with RNase A (C) or RNase A plus BAP (D). ^{14}C: solid circle and ^{3}H: open circle.

samples, along with marker compounds, were analyzed by Whatman DE 81 paper chromatography in solvent VII. For reactions using ADP-HA, the chromatograms were treated with a ninhydrin spray. For reactions using ADP-HA-biotin the chromatograms were treated with an iodoplatinate spray or monitored for [^{14}C]biotin. ADP-HA-TMR was detected by viewing under long wavelength UV light. The yeast $tRNA^{Phe}$ and 5S RNA products were monitored for [^{14}C]biotin or for fluorescently labeled gel bands. Quantum yield determinations were as described by Melhado et al. (37).

RESULTS

Oligomer reactions.

The new Ado-5'PP-X compounds served as substrates in the ATP-independent RNA ligase reaction. The nonadenylyl group (P-X) was added to the 3'-hydroxyl of $(Ap)_3C$ releasing 5'-AMP. When a reaction mixture containing [Cyd-^{3}H]$(Ap)_3C$ and ADP-HA-[carbonyl-^{14}C]biotin was incubated with RNA ligase and analyzed by descending paper chromatography, the results shown in Figure 1 were obtained. The ^{3}H-label originally present as $(Ap)_3C$ (Fig. 1A) was converted into a single ^{3}H- and ^{14}C-labeled product migrating

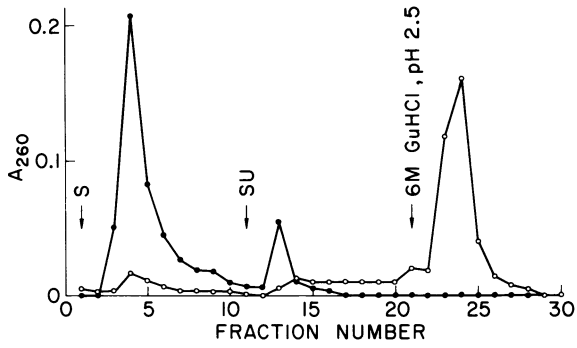

Figure 2. Affinity chromatography of $(Ap)_3Cp$-HA (solid circle) and $(Ap)_3Cp$-HA-biotin (open circle) on an immobilized avidin-agarose column. $(Ap)_3C$-HA-biotin was synthesized as described in Fig. 1 except that $(Ap)_3C$ was 1.7 mM and ADP-HA-biotin was 5.0 mM. The compound was isolated from a paper chromatogram developed in solvent VIII. Each modified $(Ap)_3C$ was applied separately to the 0.8 ml column and washed successively with 0.01 M NaH_2PO_4, pH 7.0, 1 M NaCl (S); 0.01 M NaH_2PO_4, pH 7.0, 1 M NaCl, 2 M urea (SU); and 6 M guanidine hydrochloride (GuHCl), pH 2.5. Fractions of 1 ml were collected at a flow rate of 2 ml/hr.

faster than $(Ap)_3C$ (yield = 96%, Fig. 1B). Treatment of the double labeled product with RNase A resulted in the formation of a ^3H-labeled product comigrating with $(Ap)_3Cp$ and a ^{14}C-labeled product migrating at the solvent front, i.e., faster than ADP-HA-biotin (Fig. 1C). A double treatment with RNase A and BAP yielded a ^3H-labeled product comigrating with $(Ap)_3C$ and a rapidly migrating ^{14}C-labeled product (Fig. 1D). HA-biotin migrates at the solvent front. When reactions containing nmol amounts of $(Ap)_3C$ and ADP-HA-biotin were run, the expected product 5'-AMP could be identified by paper chromatography. These findings confirm that a 3'-phosphodiester bond linked the oligomer and biotin residue in the product oligoribonucleotide and that 5'-AMP was formed. Since the $^3H/^{14}C$ ratio in the product shows a 1:1 ratio of $(Ap)_3C$ to biotin, the structure of the product is $(Ap)_3Cp$-HA-biotin. The products of the reactions of ADP-HA and ADP-HA-TMR with [Cyd-^3H]$(Ap)_3C$ were analyzed similarly (data not shown). These analyses showed: 1) the oligoribonucleotide product using ADP-HA was ninhydrin positive and ^3H-labeled, 2) the product with ADP-HA-TMR was fluorescent and ^3H-labeled, and 3) treatments with RNase A and RNase A plus BAP yielded ^3H-label comigrating with $(Ap)_3Cp$ and $(Ap)_3C$, respectively. These results confirm the respective reaction products as $(Ap)_3Cp$-HA and $(Ap)_3Cp$-HA-TMR.

We have also added hexylamine and hexylaminobiotin to $(Ap)_3C$ using pA-HA-p and pA-HA-biotin-p in the ATP-dependent RNA ligase reaction in which a

nucleoside 3',5'-bisphosphate is added to an oligoribonucleotide (29). In contrast to the ATP-independent reaction the products of these reactions contained an added Ado residue as well as the named addenda. The reaction with pA-HA-biotin-p proceeded to approximately the same extent as did the ATP-independent reaction that added p-HA-biotin but at a more rapid rate.

Biotin labeled $(Ap)_3C$ Binding to Avidin.

Figure 2 shows the results of an experiment designed to test whether the biotin covalently linked to $(Ap)_3C$ is capable of efficient binding to avidin. $(Ap)_3Cp$-HA-biotin and $(Ap)_3Cp$-HA were synthesized using T4 RNA ligase, purified by paper chromatography and passed over immobilized avidin agarose as described previously (1). Figure 2 shows that biotin labeled $(Ap)_3C$ is selectively bound to the column under high salt and high urea conditions which disrupt ionic and hydrophobic interactions, respectively. Biotin labeled $(Ap)_3C$ was eluted with 6 M guanidine hydrochloride, pH 2.5 which disrupts the biotin-avidin interaction. The control, $(Ap)_3Cp$-HA, washed through the column under high salt and high urea conditions. These results show that the biotin covalently linked to the 3'-terminus of RNA via a hexamethylene linker binds avidin tightly and that the biotin is solely responsible for binding to agarose-bound avidin.

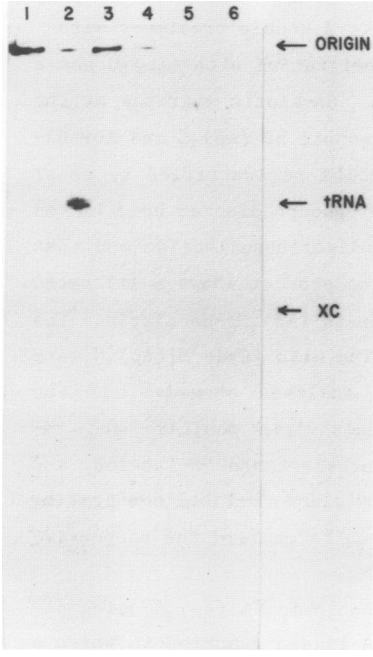

Figure 3. T4 RNA ligase reactions with yeast $tRNA^{Phe}$ and ADP-HA-[carbonyl-^{14}C]biotin analyzed by fluorography of 20% polyacrylamide gel. The reactions were incubated at 17° for 10 hr. with 32 µM ADP-HA-biotin and 6 µM RNA ligase in 15 µl as described in Table 1. Avidin (0.25 unit) was added prior to analysis by electrophoresis. Lane 1, complete reaction treated with avidin; Lane 2, complete reaction; Lane 3, reaction incubated without T4 RNA ligase and treated with avidin; Lane 4, reaction incubated without T4 RNA ligase; Lane 5, reaction incubated without T4 RNA ligase and ADP-HA-biotin and treated with avidin; and Lane 6, reaction incubated without T4 RNA ligase and ADP-HA-biotin. XC represents xylene cyanol.

Yeast $tRNA^{Phe}$ and 5S RNA reactions.

The ATP-independent RNA ligase reaction with ADP-HA-[carbonyl-^{14}C]biotin and ADP-HA-TMR was also used to label the 3'-terminus of yeast $tRNA^{Phe}$ and 5S RNA from D. melanogaster and E. coli. Analysis of the reactions by polyacrylamide gel electrophoresis revealed biotin and tetramethylrhodamine were incorporated into the RNA. Figure 3 shows a fluorogram of polyacrylamide gel electrophoretic separations of yeast $tRNA^{Phe}$ and ADP-HA-[carbonyl-^{14}C]biotin reactions. Incubation of the tRNA and the substituted pyrophosphate with T4 RNA ligase yields a ^{14}C-labeled band comigrating with tRNA (Lane 2). Addition of avidin to the reaction immediately prior to electrophoresis prevented the ^{14}C-labeled tRNA from entering the gel (Lane 1). This result shows that all the biotin labeled tRNA is capable of binding to avidin. Incubation of the tRNA and ADP-HA-biotin without T4 RNA ligase results in no incorporation of label into tRNA (Lane 3). Assaying the biotin addition to yeast $tRNA^{Phe}$ by TCA precipitation and filtration through nitrocellulose gave yields of 52%

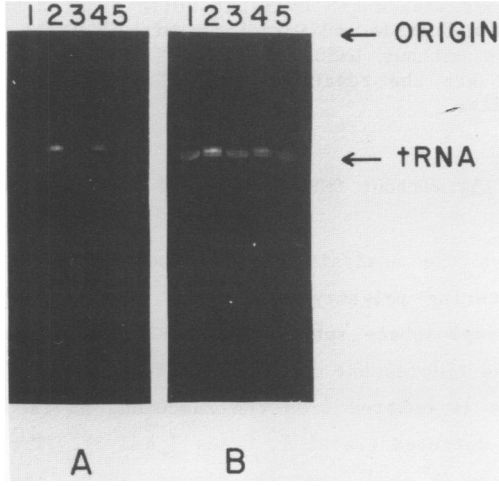

Figure 4. T4 RNA ligase reactions with yeast $tRNA^{Phe}$ and ADP-HA-TMR (isomer R or G) analyzed by 20% polyacrylamide gel electrophoresis. The reactions were performed as described in Fig. 3 except that incubation was for 15 hr. and 340 µM ADP-HA-TMR was used. Panel A shows the gel irradiated with short wavelength UV light while panel B shows the same gel stained with ethidium bromide and irradiated with UV light. Lane 1, yeast $tRNA^{Phe}$ control; Lane 2, reaction with ADP-HA-TMR (isomer R) and T4 RNA ligase; Lane 3, same as lane 2 except without T4 RNA ligase; Lane 4 reaction with ADP-HA-TMR (isomer G) and T4 RNA ligase; and Lane 5, same as lane 4 except without T4 RNA ligase.

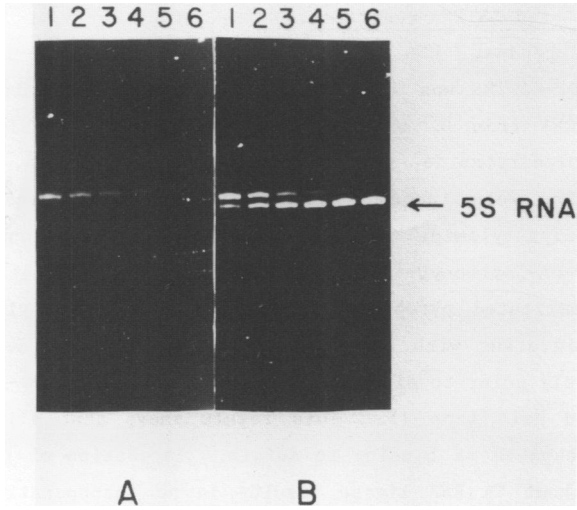

Figure 5. T4 RNA ligase reactions with D. melanogaster 5S RNA and ADP-HA-TMR, isomer R analyzed by 8% polyacrylamide gel electrophoresis. The reactions were performed as described in Table 1 with 20 μM 5S RNA, 200 μM ADP-HA-TMR, and 4 μM RNA ligase in 10 μl volumes. Panel A shows the gel irradiated with short wavelength UV light while panel B shows the same gel stained with ethidium bromide and irradiated with UV light. Lanes 6, 5, and 4 are the reaction without DMSO after 2, 4, and 8 hours, respectively. Lanes 3, 2, and 1 are the reaction with 10% (v/v) DMSO after 2, 4, and 8 hours, respectively.

with 10% DMSO and 15% without DMSO and showed a pH optimum of 7.5 for the reaction.

Figure 4 shows the analysis of reactions of ADP-HA-TMR with yeast $tRNA^{Phe}$ on a denaturing polyacrylamide gel. The incubation of the tRNA and fluorescent pyrophosphate substrate with T4 RNA ligase results in the incorporation of the fluorescent dye into the tRNA (Panel A, Lanes 2 and 4). When T4 RNA ligase is omitted from the reaction, no incorporation of the fluorescent dye is detected (Panel A, Lanes 3 and 5). The ethidium bromide stained gel in Figure 4B shows that the fluorescently labeled tRNA migrates more slowly than unlabeled tRNA and is resolved as a distinct band. The yield in this reaction was 50%. The absence of any other ethidium bromide stained RNA shows that little degradation of the tRNA occurs under these reaction conditions.

A similar analysis of D. melanogaster 5S RNA reactions with ADP-HA-TMR in the presence and absence of 10% DMSO is shown in Figure 5. A fluorescent band (Panel A) migrating slower than 5S RNA (Panel B) can be detected upon

TABLE 2. ATP-Independent T4 RNA Ligase Reactions

No.	Acceptor	ADP Derivative	DMSO (%,v/v)	Time (hr)	Yield (%)
1.	$(Ap)_3C$	ADP-HA	10	2	82
2.	$(Ap)_3C$	ADP-HA-biotin	10	2	92
3.	$(Ap)_3C$	ADP-HA-TMR	10	1	93
4.	$(Ap)_3A$	ADP-HA-Fl	10	6	100[b]
5.	$(Ap)_3A$	ADP-HA-TMR	10	6	100[b]
6.	yeast $tRNA^{Phe}$	ADP-HA-biotin	10	10	52
7.	yeast $tRNA^{Phe}$	ADP-HA-TMR	10	15	50
8.	*E. coli* 5S RNA	ADP-HA-biotin	10	14	86
			20	14	76
9.	*E. coli* 5S RNA	ADP-HA-TMR	20	2	75
			20	8	100
10.	*D. m.*[a] 5S RNA	ADP-HA-biotin	10	10	58
			10	20	70
11.	*D. m.*[a] 5S RNA	ADP-HA-TMR	10	8	68
			20	8	90
			30	8	70

All reactions were as described in Table 1 using 4 μM RNA ligase except that in reaction No. 9. 9 μM enzyme was used.
[a] *D. m.*: *Drosophila melanogaster*
[b] Using excess acceptor over ADP derivative and basing the yield upon the ADP-derivative consumed.

UV irradiation of the gel. The reaction without DMSO (Lanes 4, 5, and 6) shows only a 15% yield of product after 8 hours. However, in the presence of 10% DMSO (Lanes 1, 2, and 3) the velocity and yield of product increases significantly (68% after 8 hours). Prolonged incubation (>12 hours) in the presence of 20% DMSO results in the complete reaction of *D. melanogaster* 5S RNA (data not shown). Figure 5 also demonstrates the absence of degradation products arising from nucleases.

The yield of the ATP-independent RNA ligase reactions using various acceptors and Ado-5'PP-X derivatives is shown in Table II. Greater than 80% yields were obtained in 1 to 6 hours using oligoribonucleotide acceptors. With larger RNA acceptors the lowest yields observed were with yeast $tRNA^{Phe}$ (50%), while greater than 70% yields were achieved with the

5S RNA acceptors. Longer reaction times are probably required for the larger RNA acceptors because their concentration is lower than can be obtained with oligoribonucleotides. The same yield of TMR-derivatized E. coli 5S RNA can be obtained with one-half the amount of enzyme shown in Table II if the reaction is run for twice the time indicated (data not shown). We did not observe that the addition of BAP (34) stimulated the reactions under our conditions.

Fluorescence of $(Ap)_3A$ labeled with fluorescein and tetramethylrhodamine.

A fluorescent group attached to RNA may have different fluorescence properties than does the unattached compound (28,38). In order to determine if the fluorescence characteristics of fluorescein and tetramethylrhodamine were affected because of their attachment to RNA, we determined the quantum yields of $(Ap)_3Ap$-HA-Fl and $(Ap)_3Ap$-HA-TMR before and after digestion with RNase U_2. Hydrolysis of the oligonucleotide would disrupt any ordered or stacked structures that might affect the fluorescence of the dyes. The quantum yield of both fluorescein (Q = 0.77) and tetramethylrhodamine (Q = 0.43) remained identical after the RNase treatment. Paper chromatography before and after RNase treatment showed complete digestion of the fluorescently labeled oligonucleotides. In addition, there were no changes in the λ_{max} of emission of either dye after RNase digestion suggesting that no dye-protein interactions were complicating interpretations of the quantum yield measurements.

DISCUSSION

The discovery by England et al. (8) that T4 RNA ligase uses disubstituted pyrophosphates with the general structure Ado-5'PP-X as substrates led us to synthesize several such compounds in order to modify the 3'-ends of RNA molecules by the attachment of useful non-nucleotide groups. We have incorporated an alkane primary amine, biotin, and two fluorophores onto RNA. Generally, the Ado-5'PP-X reaction component was used in five- to ten-fold molar excess over the RNA. The pH optimum of the reaction varied with the type of RNA acceptor used (Table 1). To obtain good yields with the high molecular weight RNA acceptors the inclusion of 10 to 20% DMSO in the reactions was particularly important. The optimal amount depended upon the particular acceptor with 10% being best for E. coli 5S RNA and 20% for the Drosophila 5S RNA. The stimulation of several types of RNA ligase reactions by DMSO is a widespread observation (20,29-31) but the mechanism is unknown. It may have a direct effect upon the enzyme as well as affecting the

structure of the RNA acceptors (32). The temperature of the reaction also markedly affects the yield. Only the reaction with ADP-HA gave product when reacted for 2 hr. with $(Ap)_3C$ at 37°C. Decreasing the temperature to 17°C resulted in good yields with all the ADP derivatives in 6 hr. Although comparable yields were obtained at 5°C and 17°C with yeast $tRNA^{Phe}$, the reactions were run at the lower temperature to minimize the activity of RNases.

Studies on the products of the addition of formycin to the 3'-end of $tRNA^{Phe}$ (28) and of the attachment of the dansyl group to the 5'-end of $tRNA^{fMet}$ (38) showed significant changes in the fluorescence properties of the dyes. The quantum yields of both $tRNA^{Phe}$-formycin and the oligonucleotide CpApCpC-formycin were similar, suggesting that stacking interactions between the formycin and the adjacent nucleosides were the only cause of the quenching (28). We find no change in the quantum yield of the either fluorescein or tetramethylrhodamine when it is attached to $(Ap)_3A$. The conformational flexibility provided by the hexamethylene spacer between the RNA terminus and the fluorophore may account for our results.

Fluorescent or biotin labeled RNAs may be useful as probes to detect complementary DNA sequences that have been immobilized on nitrocellulose. Preliminary experiments using TMR-labeled D. melanogaster 5S RNA as a hybridization probe to detect 5S DNA sequences on a Southern blot indicate that from 80 to 110 fmol of 5S genes can be readily detected. When the RNA is labeled with biotin and detection is by means of steptavidin and biotinylated horseradish peroxidase (39) the sensitivity is approximately four-fold better (40). By using the methods recently described by Leary et al. (41) for biotin labeled DNA, the sensitivity of detection of the hybridized labeled-RNAs should be considerably better than we have seen. These techniques offer a potentially attractive alternative to methods requiring radioactively labeled RNA and the ability to form the requisite tagged RNAs by the methods we have described may facilitate their development.

In addition to the addenda we have described here, Hecht and colleagues (33) attached amino acids or amino acid derivatives to the 3'-hydroxyl of tRNA using RNA ligase and Ado-5'PP5'-Ado bearing a blocked amino acid on the 3'-hydroxyl group of one of the adenosines. After reaction with RNA ligase, deblocking of the amino acid function resulted in an aminoacylated tRNA carrying the desired amino acid or its analogue. Profy et al. (34) have added 2'(3')-O-DL-alanyl inosinic acid to $I(pI)_4$ using a β-substituted ADP.

Similarly Ohtsuka et al. (30) incorporated ribose and base analogues of adenosine onto ApCpC as a model of their attachment to tRNAs.

There is at least one general limitation to the kinds of groups that can be added to RNA by this reaction of RNA ligase. When the β-substituent of the ADP derivative is a good leaving group it is likely to be expelled from the RNA product by the attack of the adjacent 2'-hydroxyl upon the phosphorus of the newly formed phosphodiester bond. For example, when Ado-5'PPl-(4-nitrophenol) was reacted with $(Ap)_3C$ and RNA ligase the products were the 2',3'-cyclic phosphate-terminated oligomer $(Ap)_3C>p$ and the 4-nitrophenolate anion (35). The ADP derivatives of 4-methoxyphenol, 4-methylumbelliferone, 5'-GMP, l-glucose, and fluorine all reacted similarly to give the identical cyclic phosphate-terminated oligomer. These results confirm the expectation that electron withdrawing groups cannot be stably attached to the 3'-phosphate of RNA. However, Ohtsuka et al. (36) have demonstrated that the 2-nitrobenzyl group of Ado-5'PPl-(2-nitrophenylmethanol) is stably added to an oligoribonucleotide. This result indicates that a methylene group between the 3'-phosphate of the RNA product and the electron withdrawing group is likely to be sufficient to prevent expulsion. The chemical reactivity of the addendum should therefore be considered in the light of the above findings when a substrate is designed for use in the ATP-independent RNA ligase reaction.

The major advantage of modifying RNA with Ado-5'PP-X compounds and RNA ligase rather than by direct chemical methods derives from the highly specific nature of enzyme catalyzed reactions. The required organic chemistry is carried out at the level of the ADP derivative and this product can by highly purified before it is brought into contact with the RNA. The group is joined to the RNA in a single step with the enzyme and, if the enzyme preparation is free of contaminating activities, there will be no other chemical changes in the RNA which might alter its properties. In general, the remarkable lack of specificity of RNA ligase for the P-X group of Ado-5'PP-X substrates will allow many different molecular structures to be added to RNAs.

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