Joining of 3′-Modified Oligonucleotides by T4 RNA Ligase. Synthesis of a Heptadecanucleotide Corresponding to the Bases 61–77 from *Escherichia coli* $tRNA^{fMet}$†

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ABSTRACT Chemically synthesized fragments corresponding to the 3′ end of $tRNA^{fMet}$ from *Escherichia coli* were joined by T4-induced RNA ligase to yield a heptadecanucleotide (bases 61–77). The 3′ terminus of C-C-A was modified by introduction of the ethoxymethylidene group to prevent intra- and intermolecular self-joining reactions at the 3′ end. The terminal trimer was phosphorylated using polynucleotide kinase and joined to C-A-A with RNA ligase. The hexamer [C-A-A-C-C-A(ethoxymethylidene)] corresponding to bases 72–77 was obtained in a yield of 60%. An undecanucleotide (bases 61–71) which had been synthesized in a yield of 34% by similar enzymatic joining of U-C-C-G-G to pC-C-C-C-C-G was allowed to react with the 5′-phosphorylated hexamer (bases 72–77) using an excess of RNA ligase to yield the heptadecanucleotide U-C-C-G-G-C-C-C-C-C-G-C-A-A-C-C-A (bases 61–77). The product was identified by homochromatography and nearest neighbor analysis.

A method to synthesize RNA molecules such as tRNA has become available with the discovery of RNA ligase from T4-infected *Escherichia coli* (Silber et al., 1972). To date, chemically synthesized natural RNA fragments have been limited in size to nine nucleotides (Ohtsuka et al., 1973; Neilson & Werstiuk, 1974). T4-induced RNA ligase catalyzed joining of shorter RNAs (Kaufman & Littauer, 1974; Kaufmann et al., 1974; Walker et al., 1975; Kaufmann & Kallenback, 1975; Ohtsuka et al., 1976; Sninsky et al., 1976; Uhlenbeck & Cameron, 1977; Ohtsuka et al., 1977a) and DNA (Snopek et al., 1976; Sugino et al., 1977) has been demonstrated by several investigators. We have previously synthesized chemically C-C-A (Ohtsuka et al., 1971, 1977b), C-C-Ap (Ohtsuka et al., 1970), C-A-A (Ohtsuka et al., 1977b), C-C-C-C-C-G and U-C-C-G-G (Markham et al., 1977) which are sequences occurring in the 3′ terminus of $tRNA^{fMet}$ from *E. coli* (Dube et al., 1968, 1969; Dube & Marcker, 1969). C-A-A was joined to the 3′-phosphorylated trinucleotide, *pC-C-A,[1] with RNA ligase to yield C-A-A-C-C-Ap and several ribotriplets were also joined to *pC-C-A without self-polymerization of the 5′-phosphorylated trinucleotide (Ohtsuka et al., 1977a). In order to elongate the 3′-terminal oligonucleotide chain in the 5′ direction by repeated phosphorylation and ligation, the 3′ terminus has to be blocked to prevent self-polymerization and self-cyclization of 5′-phosphorylated polynucleotides, since the intramolecular cyclization reaction has been shown to be preferred to the intermolecular reaction in oligonucleotides with chain lengths longer than 8 (Kaufmann et al., 1974). In the present paper we describe a number of approaches for modification of the 3′-terminal nucleoside and joining of synthetic oligonucleotides corresponding to the 3′-fragments from *E. coli* $tRNA^{fMet}$ to yield a heptadecanucleotide, U-C-C-G-G-C-C-C-C-C-G-C-A-A-C-C-A (Figure 1).

Materials and Methods

C-C-A>p (**1**). C-C-Ap (3 μmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (10 μmol) were dissolved in water (0.3 mL). The solution was adjusted to pH 5.5 with 0.1 N HCl and kept at 16 °C for 5 h. The product was isolated by paper chromatography in solvent A (R_f 0.39). The reaction was essentially quantitative.

C-G-A(Em) (*3*). The trimer was synthesized by condensation of bzC(Bz)-ibG(Bz)p (0.07 mmol; Ohtsuka et al., in preparation) with 2′,3′-ethoxymethylideneadenosine (1.1 mmol; Chladek et al., 1960) using DCC (1.4 mmol) in pyridine (2 mL) for 4 days. After aqueous pyridine treatment, the filtered solution was concentrated and precipitated with ether from its solution in pyridine, and the precipitate was treated with 15 N methanolic ammonia at room temperature for 20 h. Ammonia was removed by evaporation and the residue was dissolved in 50% aqueous ethanol (50 mL). The crystallized nucleoside was removed by filtration and the filtrate was applied to a column (3 × 47.5 cm) of DEAE-cellulose. Elution was performed with a linear gradient of 0–0.2 M triethylammonium bicarbonate (4 L in 50% ethanol) and 15.5-mL fractions were collected every 13 min. The product was eluted at a salt concentration of 0.08–0.09 M and identified by paper chromatography (R_f 0.46 in solvent A) and paper electrophoresis (R_m 0.59). The appropriate fractions were combined and evaporated, and C-G-A(Em) was separated from contaminating C-G>p (R_m 0.80) by paper electrophoresis or paper chromatography in solvent A. The isolated yield was 72 A_{260} units, 2.5 μmol.

C-C-A(Em) (**5**). The trimer was synthesized by a similar

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[1] Abbreviations used: *pC-C-A, [5′-^{32}P]pC-C-A; bzC(Bz)ibG(Bz)p, *N*,2′-*O*-dibenzoylcytidylyl-(3′–5′)-*N*-isobutyryl-*O*-benzoylguanosine 3′-phosphate; Em, ethoxymethylidene; DCC, dicyclohexylcarbodiimide; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; ADP-Sepharose, adenosine 2′,5′-diphosphate–Sepharose; DTT, dithiothreitol; BSA, bovine serum albumin.

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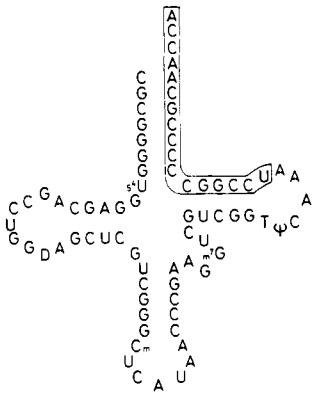

FIGURE 1: The 3′-heptadecanucleotide from *E. coli* $tRNA^{fMet}$

approach to that outlined above except that the dinucleotide bzC(Bz)bzC(Bz)p (6.2 μmol; Ohtsuka et al., 1970) was activated with TPS (12.4 μmol) and treated with 2′,3′-ethoxymethylideneadenosine (12.3 μmol; Chladek et al., 1960) for 5 h. Aqueous pyridine (50%, 3 mL) and diisopropylethylamine (25 μmol) were added with cooling. The nucleotides were extracted with butan-1-ol (10 mL), washed with water, rendered anhydrous by evaporation of added pyridine, deacylated by treatment with 15 N methanolic ammonia, and separated by paper chromatography in solvent A. The product (R_f 0.37) was homogeneous on paper electrophoresis (R_m 0.5) and the yield was 0.42 μmol.

Other Oligonucleotides. The oligonucleotides were synthesized chemically (see text) except for 5′-phosphorylation. The phosphorylation with polynucleotide kinase was performed as described previously (Ohtsuka et al., 1976).

Enzymes. T4 RNA ligase was purified according to a procedure of Cranston et al. (1974) using a previously described modification and affinity chromatography on ADP-Sepharose (Sugiura et al., in preparation). The other enzymes were also obtained as previously described (Ohtsuka et al., 1976) except for nuclease P1 which was obtained from Yamasa Shoyu Co.

RNA Ligase Reaction. Unless otherwise stated, 50 mM Hepes-NaOH (pH 8.3), 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP, and 0.5–2 μg of BSA were used in 10 μL at 37 °C.

Paper Chromatography, Electrophoresis and Other Methods. Paper chromatography was performed by the descending technique using solvent systems: (A) propan-1-ol:concentrated ammonia:water (6:1:3, v/v); (B) 0.1 M phosphate (pH 6.8):ammonium sulfate:propan-1-ol (100:60:2, v/w/v); (C) 1 M ammonium acetate (pH 7, saturated with boric acid):ethanol (7:3, v/v). Paper electrophoresis was performed at 900 V/40 cm using 0.05 M triethylammonium bicarbonate (pH 7.5). Ion-exchange chromatography on DEAE-cellulose was performed with Whatman DE-23 and triethylammonium bicarbonate. Homochromatography (Brownlee & Sanger, 1969) was performed using Homomix I–V (Jay et al., 1974).

Nuclease P1 Partial Digestion. *pC-A-A-C-C-A>p (24.2 pmol, 32 000 cpm) was digested in the presence of oligo(dpT) (0.38 A_{260}, 3.5 nmol) with nuclease P1 (1 ng) in 40 mM ammonium acetate, pH 5.0 (10 μL) at 31 °C. Two-microliter aliquots were applied to homochromatography after 2, 5, 10, 20, and 30 min on the same spot.

Venom Phosphodiesterase Partial Digestion. *pC-A-A-C-C-A(Em) (5.65 pmol, 6000 cpm) was incubated with venom phosphodiesterase (50 μg) in 50 mM triethylammonium bicarbonate (10 μL) at 30 °C. Two-microliter aliquots were applied to homochromatography after 2, 5, 15, 30, and 60 min.

SCHEME I

C-C-Ap → C-C-A>p → *pC-C-A>p

1 2

(Bz)bzC(Bz)-ibG(Bz)p + bzA(Em)

1. DCC, 2. NH_3 → C-G-A(Em) → *pC-G-A(Em)

3 4

(Bz)bzC(Bz)-bzC(Bz)p + A(Em)

1. TPS, 2. NH_3 → C-C-A(Em) → *pC-C-A(Em)

5 6

C-C-A + 6 → C-A-A*pC-C-A(Em) → *pC-A-A*pC-C-A(Em)

7 8

Acid Hydrolysis of the Terminal Cyclic Phosphate and Dephosphorylation. C-C-C-C-C-G*pC-A-A-C-C-A>p (1.3–1.6 pmol, 2000 cpm) was treated with dilute hydrochloric acid (pH 1, 3 μL) at 37 °C for 30 min and added to 0.1 M Tris-HCl (pH 9.0, 5 μL). The mixture was treated with *E. coli* alkaline phosphatase (13 μunits) at 37 °C for 40 min.

Results

Preparation of Trinucleotides with Modified 3′ End and Their Joining by RNA Ligase. Previously we have shown that the 2′-modified trinucleotide pC-C-A(o-nitrobenzyl) served as a donor but could not be an acceptor molecule in the RNA ligase reaction, indicating the *o*-nitrobenzyl group as a possible blocking group against the self-polymerization (Ohtsuka et al., 1977a). However, the photo-labile *o*-nitrobenzyl derivatives require complete shielding from light during all the reactions and isolation. The 3′-phosphorylated trimer pC-C-Ap has also been used as a donor molecule. 3′-Dephosphorylation (Cameron & Uhlenbeck, 1977) during kination would decrease yields prohibitively in multi-step elongation in the 5′ direction. The recent isolation of the 3′-phosphatase free kinase may overcome this problem (Cameron et al., 1978). If the 3′-phosphatase activity is specific, cyclic 2′,3′-phosphates of oligonucleotides may not be sensitive to this undesirable dephosphorylation. C-C-A>p (**1**) was prepared by treatment of C-C-Ap with a water soluble carbodiimide as shown in Scheme I and subjected to phosphorylation with [γ-^{32}P]ATP using polynucleotide kinase (Richardson, 1965). The 5′-phosphorylated product **2** was isolated by DEAE-cellulose chromatography in a yield of 49%. Certain decomposed side products [the opened 2′(3′)-phosphomonoester and the 3′-dephosphorylated trimer] were detected during the column chromatography. The trimer **2** was used as a donor in joining to C-A-A by RNA ligase. Conditions for the ligase reaction are summarized in Table I and the joined product C-A-A*pC-C-A>p was isolated by chromatography on DEAE-cellulose in a yield of 52%. The hexamer was characterized by homochromatography (Figure 2), nearest neighbor analysis with RNase M, and by digestion with RNase A plus phosphatase. Further characterization of the product by partial digestion with nuclease P1 was performed after labeling the 5′ terminus with polynucleotide kinase (Figure 2). *pC-A-A-C-C-A>p was joined to C-C-C-C-C-G using the conditions shown in Table I. The product was isolated by chromatography on an RPC-5 column as shown in Figure 3. Compounds from peaks were identified by digestion with RNase A plus phos-

TABLE I: Summary of RNA Ligase Reactions.

acceptor	(nmol)	donor	(nmol)	RNA ligase (unit)	vol (μL)	time (min)	yield (%)
CAA	5.8	*pCCA>p	4.3	2.4	50	60	52
CAA	8.4	*pCCA(Em)	2.2	0.94	40	60	60
CCCCCG	1.1	*pCAACCA>p	0.3	0.47	10	300	8.8
CCCCCG	1.4	*pCAACCA(Em)	0.3	0.47	10	60	16
UCCGG	10.8	*pCCCCCG	2.7	3.72	80	60	34
UCCGG	0.4	*pCCCCCG	0.1	0.23	5	60	65[a]
UCCGGCCCCCG	0.4	*pCAACCA(Em)	0.1	0.42	6	24 hr	5

[a] Observed in homochromatography.

TABLE II: Identification of Peaks in Figure 3.

peak	nearest neighbor analysis paper chromatography solvent B R_f	cpm	digestion with RNase A plus phosphatase paper electrophoresis R_m	cpm	identification
1	0.30 A(5′)*pCp	2171	0.82 A(5′)*pC	2282	Ap(5′)*pC-A-A-C-C-A
2	0.30 A(5′)*pCp	1278	0.82 A(5′)*pC	1793	Ap(5′)*pC-A-A-C-C-A>p
3	0.30 G*p	2057	0.40 G*pC	1725	C-C-C-C-C-G*p-C-A-A-C-C-A
4	0.30 G*p	1300	0.40 G*pC	1280	C-C-C-C-C-G*pC-A-A-C-C-A>p

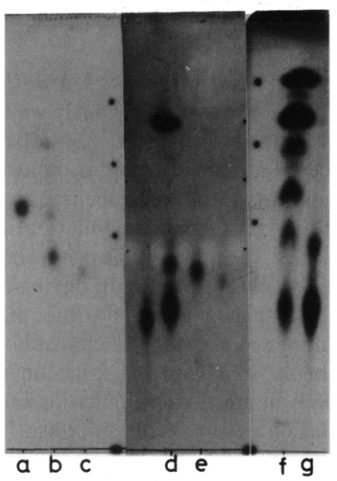

FIGURE 2: Homochromatography in the joining of *pC-C-A>p to C-A-A using Homomix V. (a) *pC-C-A>p; (b) the reaction mixture; (c) C-A-A*pC-C-Ap; (d) kination of C-A-A-C-C-A>p; (e) C-A-A*pC-C-A>p; (f) nuclease p_1 partial digestion of *pC-A-A-C-C-A>p; (g) *pC-A-A-C-C-A>p.

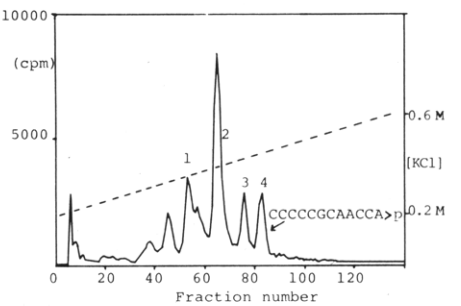

FIGURE 3: Chromatography of the products in the ligation of *pC-A-A-C-C-A>p to C-C-C-C-C-G on a column (0.7 × 27.5 cm) of RPC-5. Elution was performed using a linear gradient of KCl (0.1–0.6 M, total 200 mL) in 10 mM Tris-HCl (pH 7.5). Fractions of 1.47 mL were collected every 2 min. Identification: (peak 1) A(5′)p*pC-A-A-C-C-A; peak 2) A(5′)p*pC-A-A-C-C-Ap; (peak 3) C-C-C-C-C-G*pC-A-C-C-A; (peak 4) C-C-C-C-C-G*pC-A-A-C-C-A>p.

phatase. The result is summarized in Table II. Nearest neighbor analysis agreed with the above data. Homochromatograms of compounds from each peak are shown in Figure 4. The desired product (in peak 4) showed phosphatase sensitivity only after acidic treatment and the compound from peak 3 was insensitive to phosphatase at any stage. The isolated yield of the dodecanucleotide with the terminal cyclic phosphate (in peak 4) was 8.8% and the 3′-dephosphorylated product (in peak 3) was obtained in almost the same yield. This product presumably arises from enzymatic removal of the cyclic 2′,3′-phosphate during ligation. A large amount of the adenylated intermediate (in peak 2) accumulated in this case presumably due to the presence of an excess (17-fold) of ATP.

To improve the stability of the 3′-terminal blocking group for donor oligonucleotides, the ethoxymethylidene derivative of adenosine was introduced at the 3′ termini of two trimers. As shown in Scheme I, C-G-A(Em) was synthesized by condensation of the fully acylated dinucleotide with *N*-benzoyl-2′,3′-ethoxymethylideneadenosine using dicyclohexylcarbodiimide (DCC) as the condensing reagent. The trimer was phosphorylated with polynucleotide kinase and joined to U-U-U by treatment with RNA ligase (data not shown). The product U-U-U*pC-G-A(Em) was isolated by DEAE-cellulose column chromatography in a yield of 28%.

C-C-A(Em) was synthesized by condensation of fully benzoylated C-Cp with 2′,3′-ethoxymethylideneadenosine using triisopropylbenzenesulfonyl chloride (TPS) as the activating reagent and then phosphorylated enzymatically (Figure 5). Joining of *pC-C-A(Em) to C-A-A was performed as summarized in Table I. The elution profiles of the joined products (**7**) and subsequent phosphorylated hexanucleotide (**8**) in chromatography on DEAE-cellulose column are shown in Figure 5. The product was identified by homochromatography (Figure 6) after partial digestion with venom phosphodiesterase. The yield of the ligation and subsequent phosphorylation were 60 and 70%, respectively. The hexanucleotide *pC-A-A-C-C-A(Em) was utilized as the donor molecule in the reaction with C-C-C-C-C-G (Table I). The joined product was isolated by chromatography on an RPC-5 column in a yield of 16% (Figure 7). The dodecanucleotide was charac-

TABLE III: Identification of Peaks in Figure 7.

	nearest neighbor analysis paper chromatography solvent B R_f			digestion with RNase A plus phosphatase paper electrophoresis R_m			
peak	0.13 A*p	0.32 G*p or A(5′)p*pC	0.73 *pCp	0.40 G*pC	0.66 A-A*pC	0.82 A(5′)p*pC	identification
2	450	407	0	0	693	608	Ap(5′)*pC-A-A*pC-C-A(Em)
3	1061	970	0	1606	1926	212	C-C-C-C-C-G*pC-A-A*pC-C-A(Em)

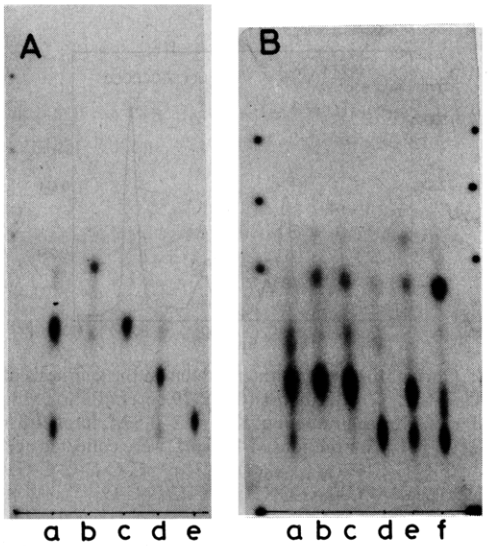

FIGURE 4: Homochromatography in the joining of *pC-A-A-C-C-A>p to C-C-C-C-C-G (Figure 3) using Homomix V. [A] (a) The reaction mixture; (b) peak 1; (c) peak 2; (d) peak 3; (e) peak 4. [B] (a) peak 3; (b) peak 3 after acid hydrolysis of the terminal phosphate followed by phosphatase treatment; (c) peak 3 after phosphatase treatment; (d) peak 4; (e) peak 4 after acid hydrolysis of the terminal cyclic phosphate followed by phosphatase treatment; (f) peak 4 after phosphatase treatment.

terized by homochromatography and by enzymatic hydrolyses (Table III).

The removal of the ethoxymethylidene group of the hexanucleotide *pC-A-A-C-C-A(Em) was performed by treatment with hydrochloric acid at pH 1 and 0 °C for 16 h and deprotection was confirmed by paper chromatography in solvent C.

Synthesis of the Heptadecanucleotide U-C-C-G-G-C-C-C-C-C-G-C-A-A-C-C-A(Em) (**12**). The 3′-blocked hexanucleotide (**8**) was then used in the synthesis of the heptadecanucleotide (**12**) as shown in Scheme II. Since the hexanucleotide *pC-C-C-C-C-G (**10**) was found not to be polymerized by incubation with an excess of RNA ligase and thus was able to serve as a donor molecule without blocking at the 3′ end, **10** was joined to the pentanucleotide **9** to yield the undecanucleotide **11**. Conditions for these ligase reactions are summarized in Table I and the elution profile of the undecanucleotide **11** in RPC-5 chromatography is shown in Figure 8. The compounds were identified by homochromatography. Although

SCHEME II

U-C-C-G-G + *pC-C-C-C-C-G → U-C-C-G-G*pC-C-C-C-C-G

9 **10** **11**

$\xrightarrow{8}$ U-C-C-G-G*pC-C-C-C-C-G*pC-A-A*pC-C-A(Em)

12

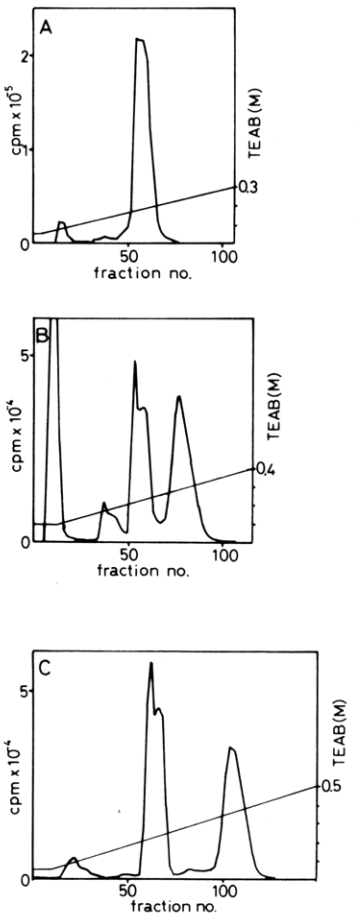

FIGURE 5: Chromatography of the products in the synthesis of *pC-A-A*pC-C-A(Em) on a column (0.6 × 12.5 cm) of DEAE-cellulose (bicarbonate). (A) Elution of *pC-C-A(Em) with a linear gradient (0.05–0.3 M, total 160 mL). ATP was eluted at the same place as the trinucleotide. (B) Elution of C-A-A*pC-C-A(Em) with a linear gradient (0.1–0.4 M, total 160 mL). The product was obtained in the last peak. (C) Elution of *pC-A-A*pC-C-A(Em) with a linear gradient (0.05–0.5 M, total 220 mL). The product was obtained in the last peak.

the homochromatogram of the reaction mixture showed 60–65% conversion, the isolated yield of **11** was 34%. The product **11** was characterized by nearest neighbor analysis and by digestion with RNase A plus phosphatase (Table IV). For further characterization, **11** was phosphorylated enzymically and subjected to partial digestion with venom phosphodiesterase followed by homochromatography. The undecanucleotide **11** was then joined to the 3′-blocked hexanucleotide **8** obtained above. After 24-h incubation with RNA ligase, the heptadecanucleotide **12** was detected in homochromatography (Figure 9). The yield of **12** was estimated by counting the radioactivity on the chromatogram and found to be 5% from **8**. The product was isolated by chromatography on an RPC-5 column and characterized by nearest neighbor analysis with

TABLE IV: Identification of Peaks in Figure 8.

	nearest neighbor analysis paper chromatography solvent B R_f			digestion with RNase A plus phosphatase paper electrophoresis R_m				
peak	0.32 G*p or A(5′)p*pCp	0.67 C*p	0.73 *pCp	0.40 G*pC	0.66 G-G*pC	0.82 A(5′)p*pC	1.67 *P_i	
1	1093	0	1768					*pC-C-C-C-C-G
2	2423	0	852	471	250	5689	861	Ap(5′)*p-C-C-C-C-C-G
3	3219	0	0	1135	2779	443		unidentified
4	2890	0	0	0	4005	148		U-C-C-G-G*pC-C-C-C-C-G

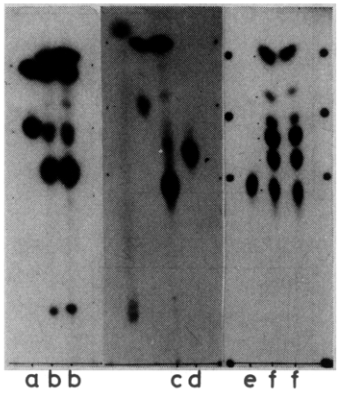

FIGURE 6: Homochromatography of compounds in the joining of *pC-A-A-C-C-A(Em) (Figure 5) to C-C-C-C-C-G using Homomix III. (a) *pC-C-A(Em) and [γ-^{32}P]ATP from Figure 5A; (b) the ligation mixture of *pC-C-A(Em) and C-A-A; (c) the kination mixture of C-A-A*pC-C-A(Em); (d) C-A-A*pC-C-A(Em) from Figure 5B; (e) *pC-A-A*pC-C-A(Em) from Figure 5C; (f) *pC-A-A*pC-C-A(Em) after venom phosphodiesterase partial digestion.

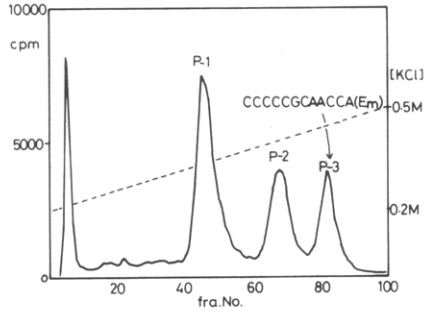

FIGURE 7: Chromatography of the products in the synthesis of C-C-C-C-C-G*pC-A-A*pC-C-A(Em) on a column (0.7 × 29 cm) of RPC-5. Elution was performed with a linear gradient of KCl (0.2–0.6 M, total 200 mL) in 10 mM Tris-HCl, pH 7.5. Fractions of 1.56 mL were collected every 3 min. (Peak 1) *pC-A-A-*pC-C-A(Em); (peak 2) A(5′)p*pC-C-A(Em); (peak 3) C-C-C-C-C-G*pC-A-A-C-C-A(Em).

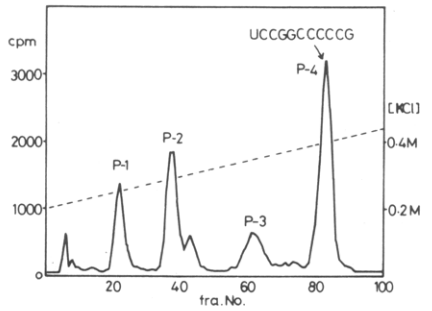

FIGURE 8: Chromatography of the products in the synthesis of U-C-C-G-G*pC-C-C-C-C-G on a column (0.7 × 29 cm) of RPC-5. Elution was performed with a linear gradient of KCl (0.2–0.5 M, total 200 mL) in 10 mM Tris-HCl, pH 7.5. Fractions of 1.56 mL were collected every 3 min. (Peak 1) *pC-C-C-C-C-G; (peak 2) A(5′)p*pC-C-C-C-C-G; (peak 3) unidentified; (peak 4) U-C-C-G-G*pC-C-C-C-C-G.

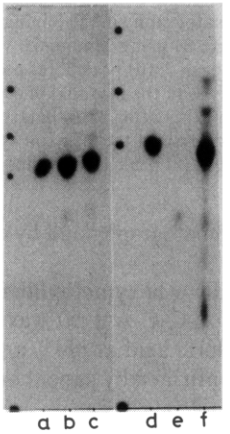

FIGURE 9: Homochromatography of compounds in the joining of *pC-A-A-C-C-A(Em) and U-C-C-G-G*pC-C-C-C-C-G. (a and d) *pC-A-A*pC-C-A(Em); (b) the reaction mixture (1 h); (c) the reaction mixture (3 h); (f) the reaction mixture (24 h). The lowest spot corresponded to the heptadecanucleotide; (e) U-C-C-G-G*pC-C-C-C-C-G.

RNase M and digestion with RNase A plus phosphatase (Figure 10). Since the specific activity of ^{32}P in **11** was 1/70th of the terminal and internal ^{32}P activities in **8**, the radioactive spots from **8** (A*p and G*p) were detected on the chromatogram (Figure 10A). In Figure 10B the radioactive compounds corresponding to a dinucleoside monophosphate and a trinucleoside diphosphate were assigned as G*pC and A-A*pC.

Discussion

In RNA ligase reactions, blocking of the 3′ end of 5′-phosphorylated oligonucleotides is required in order to prevent self-polymerization of the donor molecules. Intramolecular cyclization is the more probable side reaction when the donor has a chain length longer than eight nucleotides (Kaufmann et al., 1974). This paper illustrates two approaches to blocking of the 3′ terminus of ribooligonucleotides. As shown in Scheme I, chemically synthesized C-C-A>p (**1**) and C-C-A(Em) (**5**) were phosphorylated enzymatically and then used as donor molecules in joining reaction to C-A-A with RNA ligase. These hexanucleotides were then further joined similarly to another acceptor oligonucleotide C-C-C-C-C-G. The dodecanucleotides were isolated by chromatography on RPC-5 as shown in Figures 3 and 7. Although the last peaks in both chromatograms contained the desired product, in the case of C-A-A*pC-C-A>p (Figure 3) a part of the product lost the terminal phosphate residue (peak 3 in Figure 3). This indicates that the cyclic phosphate is not stable enough as a blocking group under these particular conditions. The 2′,3′-ethoxymethylidene de-

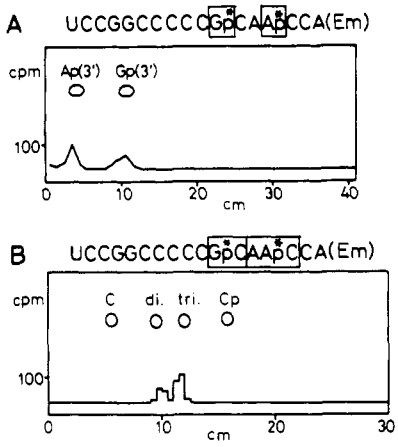

FIGURE 10: Analyses of the heptadecanucleotide. (A) Nearest neighbor analysis by RNase M digestion using paper chromatography in solvent B. (B) Paper electrophoresis after RNase A plus phosphatase treatment.

rivative of the dodecanucleotide (obtained in peak 3, Figure 7) was phosphorylated with polynucleotide kinase and subjected to the ligase reaction. No detectable cyclization occurred as determined by nearest neighbor analysis (data not shown). This confirmed the presence of the terminal blocking group in the dodecanucleotide *pC-C-C-C-C-G-C-A-A-C-C-A(Em). The 2′,3′-ethoxymethylidene group seems therefore preferable to the cyclic phosphate as a terminal blocking group and the hexamer having the 2′,3′-ethoxymethylidene group (**8**) was used for the synthesis of the heptadecanucleotide **12**. As shown in Figure 7 the yield in the joining of **8** to C-C-C-C-C-G was not high and a large amount of the adenylated intermediate (peak 2 in Figure 7) accumulated. A similar phenomenon was observed in the joining reaction of *pC-A-A-C-C-A>p to C-C-C-C-C-G (Figure 3). It seemed therefore that C-C-C-C-C-G must be a poor acceptor. To overcome this difficulty, the hexanucleotide was joined to the next pentamer U-C-C-G-G (**9**) before connection to **8** as shown in Scheme II. The reaction proceeded well as indicated in Figure 9 and *pC-C-C-C-C-G was thus proved to be an excellent donor molecule. It was also confirmed that *pC-C-C-C-C-G was not self-polymerized in the conditions used. An oligonucleotide which only acts as a poor acceptor has less tendency for self-polymerization and can therefore be a more efficient donor molecule. This is consistent with our previous experiments which showed U-U-U-U to be a poor acceptor of oligoadenylates but a good donor after 5′-phosphorylation (Ohtsuka et al., 1976). It may be the case that pyrimidine oligonucleotides are recognized less easily as acceptors by RNA ligase especially when oligonucleotides such as oligoadenylates with significantly higher affinities for the enzyme are used as donor molecules. However, the short pyrimidine oligonucleotides C-C-C and U-U-U served as reasonably efficient acceptors when *pC-C-A was used as a donor (Ohtsuka et al., 1977a).

The undecanucleotide U-C-C-G-G*pC-C-C-C-C-G (**11**) was then used as an acceptor in a joining reaction with *pC-A-A-*pC-C-A(Em) (**8**). The reaction was rather slow and the heptadecanucleotide (**12**) was only obtained after 24 h in a yield of 5%. The elongated pyrimidine-rich acceptor oligonucleotide (**11**) did not show significantly better reaction properties than C-C-C-C-C-G when the adenine rich donor (**8**) was participating. Higher concentrations of substrates and the enzyme would presumably improve joining reaction yields even though there seem to be base sequence preferences in these RNA ligase reactions.

The present paper has described the joining of chemically synthesized tRNA fragments to yield the heptadecanucleotide corresponding to the 3′ end (61-77) of $tRNA^{fMet}$ from *E. coli* (Figure 1), using the 2′,3′-ethoxyethylidene group as a blocking group for the 3′ terminus.

The 2′,3′-ethoxymethylidene group was also shown to be useful in the synthesis of an mRNA model compound U-U-U-C-G-A(Em). Elongation of this type of oligomer in the 5′ direction by repeated kination and ligation of triplets suggests itself as a general method for obtaining synthetic mRNAs.

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