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Chemical Studies on Amino Acid Acceptor Ribonucleic Acids. VIII. Degradation of Purified Alanine *Escherichia coli* B Transfer Ribonucleic Acid by Pancreatic Ribonuclease*

Claudio G. Alvino, Lynne Remington, and Vernon M. Ingram

ABSTRACT: The mono- and oligonucleotides in total pancreatic ribonuclease digests of purified *Escherichia coli* B alanine transfer ribonucleic acid peak 1 were separated on DEAE-cellulose columns in 7 M urea. Certain oligonucleotides were further separated by paper chromatography or by DEAE-Sephadex A-25 column chromatography. Their sequences and the properties of a new minor base were studied by alkaline hydrolysis and by digestion with ribonuclease T_1-takadiastase, snake venom diesterase, or micrococcal nuclease.

The nucleotide sequences of several yeast tRNAs are now known through the studies of Holley and his coworkers (1965) on alanine tRNA, of Madison *et al.* (1966) on tyrosine tRNA, of Zachau *et al.* (1966) on the sequences of two serine tRNAs, and of RajBhandary *et al.* (1966, 1967) on phenylalanine tRNA. It would be of interest to compare these nucleotide sequences from one organism, yeast, with those of a tRNA with the same amino acid acceptor activity from a different species. Alanine $tRNA_1$[1] from *Escherichia coli* B was chosen because Goldstein *et al.* (1964) had shown that it could be purified easily by countercurrent distribution and because so much is already known about the structure–function relationships in the corresponding yeast tRNA. The present report describes the study of some of the oligonucleotides produced by complete digestion with pancreatic RNase from *E. coli* B alanine $tRNA_1$.

Materials and Methods

Purification of E. coli B Alanine Acceptor RNA. Mixed amino acid acceptor RNA was purchased from General Biochemicals (Chagrin Falls, Ohio). Countercurrent distribution (Figure 1a,b) was carried out as described by Goldstein *et al.* (1964) in a 200-tube automatic apparatus (E. C. Apparatus Co., Swarthmore, Pa.). The countercurrent distribution runs were performed at a controlled temperature of 24°. Batches of 0.7–1.0 g of mixed acceptor RNA were distributed at one time. The RNA was recovered from the fractions, five tubes together, and the amino acid acceptor activity was determined by the method of Ingram and Pierce (1962). The countercurrent distribution method gives a major (I) and minor peak (II) (Ala-$tRNA_1$ and Ala-$tRNA_2$, respectively). The structural work reported in this paper has all been on the major peak (Ala-$tRNA_1$), corresponding to fractions 14–18 in Figure 1b. The purity of the RNA used was estimated to be about 80% from its acceptor activity.

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[1] Abbreviations used are: $tRNA_1$, transfer ribonucleic acid peak 1; p indicates a phosphate group; OH is used to indicate a free 2 (3′)-hydroxyl end group; P-RNase, pancreatic ribonuclease; T_1 or T_1-RNase, ribonuclease T_1-takadiastase.

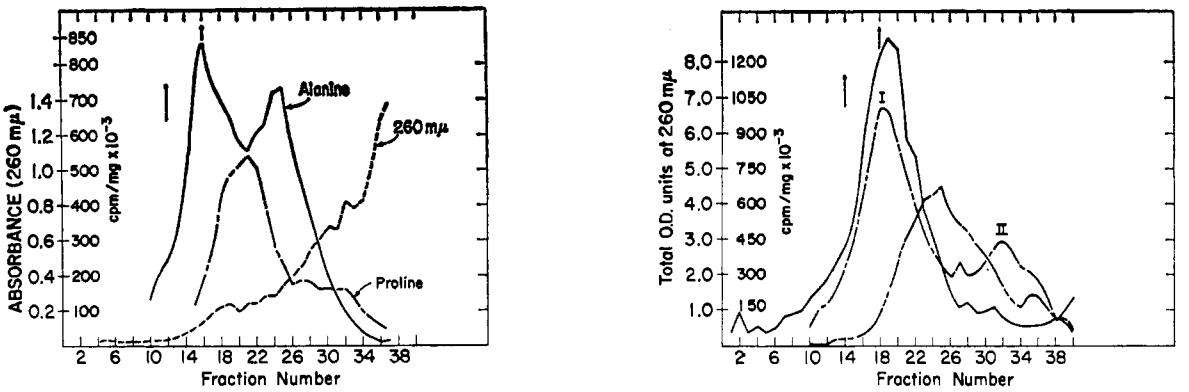


FIGURE 1: Countercurrent distribution studies. Left: (a) 360 transfer of 750 mg of *E. coli* B tRNA. (- - - - - -) Absorbance at 260 mμ; (———) alanine acceptor activity; (— — —) proline acceptor activity. Right: (b) 1000 transfer of alanine acceptor fractions corresponding to 12–16 (Figure 1a) from pooled 360 transfer distributions. Fractions 14–18 were used for enzymatic degradation. (- - - -) Absorbance at 260 mμ; (——) alanine acceptor activity; (— — —) proline acceptor activity.

Amino acid activating enzyme was prepared from *E. coli* B according to the procedure of Berg and Muench (1966). DEAE-cellulose was purchased from the Brown Co., Berlin, N. H. Dowex 50W-X4 was obtained from Dow Chemical Co., Midland, Mich., and Amberlite IRC-50 from Rohm and Haas, Ltd., Philadelphia, Pa. Pancreatic ribonuclease (Bovine) was supplied by Mann Research Laboratories, Inc., New York, N. Y., and snake venom phosphodiesterase (type II) by Sigma Chemical Co., St. Louis, Mo. Alkaline phosphomonoesterase from *E. coli*, ribonuclease T_1, and micrococcal nuclease (*Staphylococcus aureus*) were purchased from the Worthington Biochemical Corp., Freehold, N. J. Tris-HCl buffer was prepared by titrating a solution of Tris of the stated molarity to the desired pH with HCl. Beckman Desicote was obtained from Beckman Instruments, Inc., Fullerton, Calif., and DEAE-Sephadex A-25 from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Optical density measurements were made using a Cary Model 14 spectrophotometer. Urea was purchased from the J. T. Baker Chemical Co., Phillipsburg, N. J.

Enzyme Incubations. The digestion of the tRNA with pancreatic RNase is described in a later section. Ribonuclease T_1 digestions were carried out by adding to the dried eluate of an oligonucleotide 100 μl of 0.02 M ammonium acetate (pH 7.6) and 5 μl of enzyme (50 units). Incubation was for 2 hr at 37° in a sealed-glass capillary. Snake venom diesterase incubations (10 μl containing 25 μg) were performed in 100 μl of 0.1 M ammonium acetate (pH 8.0) for 4 hr at 37° unless otherwise stated. *E. coli* alkaline phosphatase (2 μl containing 2 μg) digestions were in 0.02 M ammonium acetate (pH 7.8) for 1 hr at 37°. Partial digestion with micrococcal nuclease (Zamir *et al.*, 1965) was carried out by dissolving the oligonucleotide in 25 μl of H_2O and adding 5 μl of 1 M Tris-HCl buffer at pH 8.0, 5 μl of 0.1 M $CaCl_2$, and 2 μl of micrococcal nuclease (5700 viscosity units/ml). Incubation was for 15 min at 37° in a sealed-glass capillary.

Fingerprinting Procedures. Method D. The fingerprinting of oligonucleotides from further digestion of material in some of the peaks in Figure 2 was carried out according to method D of Armstrong *et al.* (1964) using Whatman No. 3MM paper.

Nucleotide Composition of Fractions. DEAE-cellulose, with fines removed with water, was suspended in 2 M ammonium acetate and washed several times with water. Appropriate fractions from the column chromatography were pooled, diluted 1:5 with water, and desalted on DEAE-cellulose columns (0.9 × 5 cm) which had been packed under pressure (5 lb). The ultraviolet-absorbing material was eluted with small aliquots of 2 M ammonium acetate under pressure (1 lb). The eluates were diluted with water and lyophilized for 24–30 hr in a vacuum desiccator to remove all traces of ammonium acetate. The lyophilized residues were dissolved in small amounts of water. Aliquots were hydrolyzed with 0.25 ml of 0.3 M KOH for 18 hr at 37°. They were next desalted on Amberlite IRC-50 according to the procedure of Bell *et al.* (1964). The dried hydrolysates were examined by electrophoresis at pH 3.0 in 20% acetic acid–NH_3 (Armstrong *et al.*, 1964). The total amount of oligonucleotide originally present was calculated from the absorbance at 260 mμ of the unhydrolyzed oligonucleotide by using the sum of the extinction coefficients of the individual nucleotides.

Results

Products of Digestion with Pancreatic RNase. To a solution of 8.7 mg (about 208 optical density units at

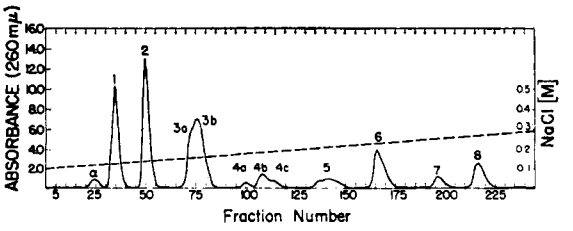


FIGURE 2: Chromatography of a pancreatic digest of purified *E. coli* B tRNA on DEAE-cellulose in 7 M urea–0.01 M Tris-HCl (pH 7.55). Column dimensions, 120 × 0.7 cm; fraction volume, 1.7 ml; flow rate, 10 ml/hr.

TABLE I: Mononucleotides from Total Pancreatic Ribonuclease Digest.

Mononucleotide	Peak No.[a] (Figure 2)	Yield (moles/mole of G-G-T)[b]	Identified by	Yield (mμmoles)	Comment
Adenosine	α	0.76	Electrophoretic position and spectrum	20.0	Impurities detected during electrophoresis
Cp + C>	1	12.5	Electrophoretic position and spectrum	330.0	
Up + U> + hUp	1	4.6[c]	Electrophoretic position and spectrum	120.0	Separated from ψp by fingerprinting procedure method D (Armstrong *et al.*, 1964)
hUp	1	1.0		26.0	Converted into β-alanine (Magrath and Shaw, 1967)
ψp	1	0.7	Electrophoretic position and spectrum	18.0	Low because of partial destruction by ultraviolet light on electrophoresis paper

[a] Peak α was desalted on Dowex 50W-X4 (ammonium) according to the procedure of Bell *et al.* (1964). Peak 1 was desalted on DEAE-cellulose (see Materials and Methods). [b] Yield of GpGpTp was 26.4 mμmoles. [c] This yield does not include hU which is independently analyzed as shown on the next line.

260 mμ) of *E. coli* B alanine $tRNA_1$ in 1.2 ml of 0.02 M Tris-HCl (pH 7.55), 0.1 ml (0.26 mg) of pancreatic RNase in the same buffer and 0.05 ml of chloroform were added. After 24 hr at 38°, the digestion products were made 7 M with respect to urea, 3 ml of 7 M urea–0.01 M Tris-HCl (pH 7.55)–0.1 M NaCl was added, and the mixture of oligonucleotides was fractionated on a DEAE-cellulose column, essentially according to Tomlinson and Tener (1962).

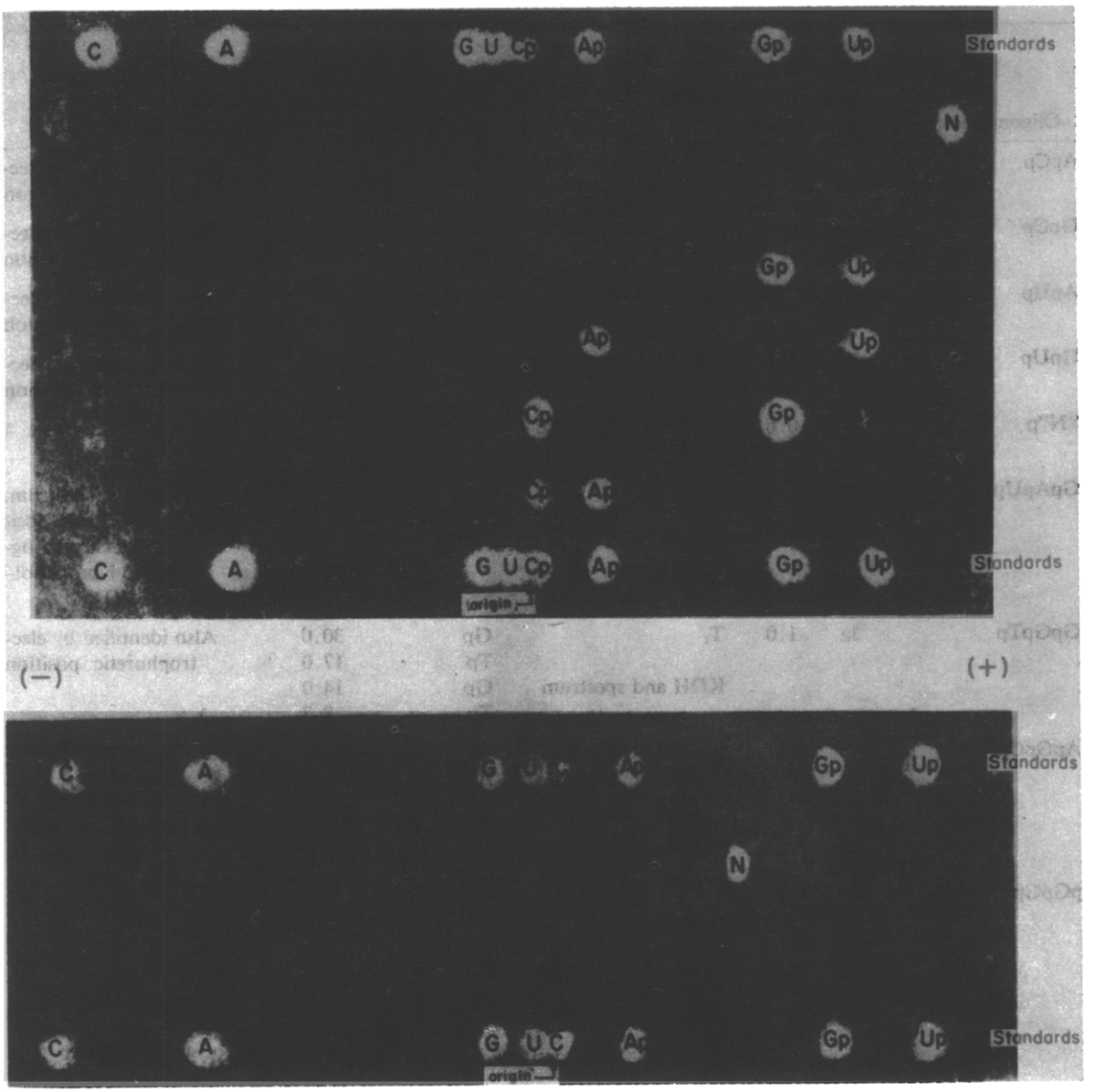
DEAE-cellulose (Brown Co., Berlin, N. H., 40, 0.93 mequiv/g with fines removed by suspension in water) was washed with 0.5 M NaOH, water, 0.5 M HCl, water, the maximum concentration of the elution gradient, and finally with water; the exchanger was stored in water. Before use, the DEAE-cellulose was washed several times with water and a suspension of DEAE-cellulose in 7 M urea, 0.01 M Tris-HCl (pH 7.55), and 0.1 M NaCl was used to pack a column 0.7 × 120 cm (siliconized with Desicote), allowing the DEAE-cellulose to settle by gravity. The column was washed overnight with 250 ml of the lowest concentration of the elution gradient. The solution of the total pancreatic RNase digest was loaded onto the column, the last traces of RNA solution were transferred with 7 M urea–0.01 M Tris-HCl (pH 7.55) and the column was eluted with a linear gradient of NaCl formed from 400 ml each of 0.1 and 0.4 M NaCl in 7 M urea–0.01 M Tris-HCl (pH 7.55) (Figure 2). In Table I are listed the compounds found in peaks α and 1, respectively, after desalting and electrophoresis at pH 3.0. The presence of hUp among the listed mononucleotides found in peak 1 was detected by the procedure of Magrath and Shaw (1967). The reaction product from the Up-hUP mixture and from authentic hUP was desalted on Amberlite IRC-50 (Bell *et al.*, 1964) and then examined by electrophoresis at pH 1.9 using standards of β-alanine at different concentrations. The spots were developed in an 0.6% solution of ninhydrin in acetone, dried, and heated at 60° for 20 min (Dreyer, 1960). The stained spots were eluted in 1.5 ml of 80% ethanol and the absorbance was measured at 550 mμ.

In Table II are listed the oligonucleotides belonging to the peaks represented in Figure 2.

Peak 2. The dinucleotides listed were found by electrophoresis at pH 2.7 or 3.0 (Armstrong *et al.*, 1964), by descending chromatography in 1-propanol–NH_3–H_2O, or by DEAE-cellulose chromatography in 7 M urea–0.01 M Tris-HCl (pH 7.55). The unusual nucleotide "N" is notable. Because of its unchanged electrophoretic mobility before and after KOH hydrolysis (Figure 3a), it is either a mononucleotide with an additional negative charge or an alkali-resistant dinucleotide. After treatment with alkaline and snake venom diesterase phosphatase its mobility, which should now be that of a mononucleoside, decreases, but not enough to make this similar to one of the four common nucleosides (Figure 3b). Nucleotide "N" has a spectrum reminiscent of cytosine at pH 7.0, but there is virtually no shift in the absorbance between pH 2 and 7 (Figure 3c). Apparently this is a new nucleotide whose nature seems to differ from that described by Ofengand (1966). This nucleotide "N" has

TABLE II: Oligonucleotides from Total Pancreatic Ribonuclease Digest.

Oligonucleotides	Peak (Figure 2)	Yield (moles/mole of G-G-T)	Structure Determined by	Product	Yield (mμmoles)	Comment
ApCp	2	2.0	KOH and spectrum	Ap	12.0	Also identified by electrophoretic position
				Cp	12.7	
GpCp	2	5.0	KOH and spectrum	Gp	72.0	Also identified by electrophoretic position
				Cp	75.7	
ApUp	2	1.0	KOH and spectrum	Ap	19.3	Also identified by electrophoretic position
				Up	17.0	
GpUp	2	0.7	KOH and spectrum	Gp	8.8	Also identified by electrophoretic position
				Up	8.3	
"N"p	2	0.6	KOH, APase, snake venom diesterase	"N"p	16.3 (as C)	For details, see text
GpApUp	3a	1.1	T_1	Gp	12.4	Identified by spectrum, electrophoretic position, and chromatography in 1-propanol–NH_3–H_2O
				ApUp	12.4	
GpGpTp	3a	1.0	T_1	Gp	30.0	Also identified by electrophoretic position
				Tp	17.0	
			KOH and spectrum	Gp	14.0	
				Tp	8.7	
ApGpCp	3b	3.8	T_1 and spectrum	Cp	42.0	
				Ap·Gp	40.0	
			KOH and spectrum	Ap	11.8	Also identified by electrophoretic position
				Cp	12.8	
				Gp	12.4	
pGpGpUp	4b	0.8	T_1, KOH, and spectrum	pGp	10.4	Purified from peaks 4a and 4c by fingerprinting (method D; Armstrong *et al.*, 1964). Low because additional separation step on paper may have caused partial destruction by ultraviolet light
				Gp	11.7	
				Up	5.0	
Two hexanucleotides	6	2	APase and snake venom diesterase	G_{OH}	4.70	Identified by spectrum and electrophoretic position
				A_{OH}	4.65	
				pA	7.75	
				pG	26.4	
				pU	6.50	
Gp[(GpGp), (ApGpAp)]GpCp	8	1	KOH	Ap	11.2	Identified by electrophoretic position and spectrum
				Cp	5.2	
				Gp	26.7	
			T_1	Gp	9.4	Identified by electrophoretic position and spectrum
				Ap·Gp	6.2	
				Cp	4.7	
			APase and snake venom diesterase	G_{OH}	3.0	Identified by electrophoretic position and spectrum
				pG	12.0	
				pA	6.0	
				pC	3.2	
			Micrococcal Nuclease	Gp·Cp	4.2	Major fragments
				Ap·Gp·Ap	9.6	



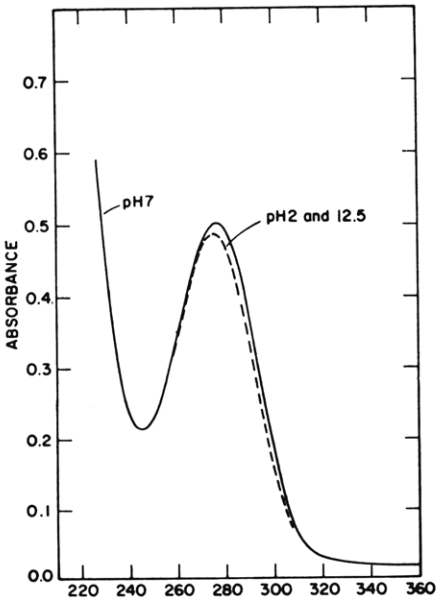


FIGURE 3: Mobility and spectra of nucleotide "N". (a, Upper) Mobility in electrophoresis at pH 3 (20% acetic acid, NH_3) of an alkaline hydrolysate of the various dinucleotides found in peak 2. (b, Lower) Mobility after treatment with alkaline phosphatase followed by digestion with snake venom diesterase. (c, Left) Spectra in 0.1 M ammonium acetate at pH 12.5 and in 0.1 M HCl, respectively.

been found in a T_1 fragment with the sequence CpUpUp"Np"Gp (C. G. Alvino and V. M. Ingram, unpublished data).

Peaks 3a and 3b. The trinucleotides found in these peaks have been separated either by chromatography on DEAE-Sephadex A-25 in 7 M urea–0.01 M Tris-HCl (pH 7.55) or by fingerprinting (method D; Armstrong *et al.*, 1964). Both methods led also to the separation of traces of minor oligonucleotides.

Peak 4b. This peak yielded one major oligonucleotide (pGpGpUp) by the fingerprinting procedure (method D; Armstrong *et al.*, 1964). Since complete digestion with RNase yielded pGp, this oligonucleotide produced by pancreatic RNase must represent the 5′-terminal sequence of alanine $tRNA_1$. The very small peaks 4a and 4c are contaminants which are not included in our molecule because of their trace amounts.

Peaks 5a, 5b, and 7. These peaks contain small amounts of penta- and heptanucleotides, respectively, representing probably contaminating tRNA molecules.

Peak 6. Because of the position in the separation and the high yield of this peak (about 25 optical density units at 260 mμ), it was suspected to contain two hexanucleotides. The finding of both guanosine and adenosine after snake venom diesterase digestion supports this idea. Work is continuing on these oligonucleotides, but so far a separation has not been achieved.

Peak 8. The complete sequence of this oligonucleotide has not yet been established, but attempts to elucidate it by means of both partial snake venom diesterase digestion (15 min at room temperature) and limited micrococcal nuclease digestion (see Materials and Methods) have been made. The incomplete overlapping of the fragments produced either by snake venom diesterase or by micrococcal nuclease does not allow us to describe the correct sequence. However it was possible to isolate from the fragments produced by micrococcal nuclease a trinucleotide (ApGpAp) and a dinucleotide (GpCp). These findings led us to speculate that the two ApGp residues are sequential in this octanucleotide, and that GpCp represents its end. It is remarkable that an octanucleotide of similar composition (G-G-G-A-G-A-G-U-) has been found in the yeast alanine tRNA (Holley *et al.*, 1965) and in the yeast phenylalanine tRNA (RajBhandary *et al.*, 1967).

TABLE III: Total Pancreatic Digest of Alanine $tRNA_1$.

Table of Oligonucleotides	Per Molecule	
	E. coli B[a]	Yeast
Adenosine	1	
Cytidine		1
Cp + C>	12.5	13
Up + U>	4.6	6
hUp	1	
ψp	0.7	1
"N"[b]	0.7	
ApCp	2	1
ApUp	1	
GpCp	5	2
GpUp	0.7	4
MeIpψp		1
DiMeGpCp		1
MeGpGpCp		1
ApGpCp	3.7	1
GpApUp	1.1	1
ApGpUp		1
pGpGpUp	0.8	
IpGpCp		1
GpGpTp	1	1
GpGphUp		1
GpGpApCp		1
Hexanucleotide	2	
GpGpGpApGpGpUp		1
Gp[(GpGp), (ApGpAp)]GpCp	1	
Total number of residues	81 approximately	77

[a] Total KOH hydrolysis confirms that the molecule contains only 1 equiv each of adenosine, pGp, Tp, ψp, and "N." [b] "N" is the new nucleotide described in the present paper.

Discussion

The results indicate clearly the differences which exist between *E. coli* B and yeast alanine tRNA (Table III). Our experiments, described in this paper, together with structural studies on the relevant T_1 oligonucleotide (C. G. Alvino and V. M. Ingram, in preparation) indicate that the unique ribothymidine and pseudouridine residues are found in the same sequence G-T-ψ-C-G- which seems to be a common feature of most tRNA molecules. Inosine is absent in alanine tRNA from *E. coli*, but it is well known that total *E. coli* tRNA is deficient in this base.

During the present work special attention was placed on obtaining quantitative results by means of triplicate analysis. The high yield obtained for all oligonucleotides indicate that the starting preparations are reasonably homogeneous with respect to nucleotide sequences. Information from the corresponding T_1 oligonucleotides will help to determine the remaining sequences in the present work. Particularly interesting is the finding of a new nucleotide ("N") and its occurrence in our molecule has been confirmed by the identification of a T_1-RNase fragment containing it.

Acknowledgments

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Purification and Properties of Nuclear and Cytoplasmic "Deoxyribonucleic Acid like" Ribonucleic Acid from Ehrlich Ascites Cells*

W. K. Roberts and V. D. Quinlivan

ABSTRACT: Newly synthesized ribonucleic acid was extracted from the nuclear and cytoplasmic fractions of Ehrlich ascites cells. These ribonucleic acids were fractionated further by chromatography on methylated albumin kieselguhr columns. Methods were developed whereby the ribonucleic acid rich in adenylic acid and uridylic acid ("deoxyribonucleic acid like" ribonucleic acid) from the nucleus and cytoplasm could be isolated from methylated albumin kieselguhr columns in a highly purified form. The cytoplasmic deoxyribonucleic acid like ribonucleic acid was found to differ significantly from the nuclear deoxyribonucleic acid like ribonucleic acid in both sedimentation properties and in base composition. This provides additional evidence that within animal cells there exists a species of ribonucleic acid which is restricted to the nucleus.

The nuclei of animal cells have been shown to contain species of RNA which are heterogeneous in size and which are characterized by having a base composition high in adenylic and uridylic acid. This ndRNA,[1] therefore, is distinct from the rRNA, rpRNA (Scherrer *et al.*, 1963), tRNA, and 5S RNA (Knight and Darnell, 1967) which are also found in the nucleus. The ndRNA represents a large fraction of the rapidly labeled RNA in the cell and can be isolated from nuclei in amounts which are readily detectable by optical density measurements (Muramatsu *et al.*, 1966).

Several types of experiments have indicated that a sizable fraction of this ndRNA cannot be a precursor to cdRNA (see Discussion). This means that in animal cells there exists a species of RNA which is restricted to the nucleus, which may be of considerable importance to the cells, but whose function is presently unknown. Many experiments designed to help elucidate the function of the ndRNA would become feasible if a simple method could be found for the quantitative isolation of ndRNA free from contamination. This would enable chemical and biological studies to be made on the isolated RNA, as well as permit investigations concerning the effects of hormones, antigens, and other stimuli and stresses on the ndRNA in the appropriate target cells.

Several procedures have been used for the isolation of ndRNA. These include nuclear fractionation (Soeiro *et al.*, 1966; Willems *et al.*, 1968) and nuclear fractionation in combination with methylated albumin kieselguhr chromatography (Muramatsu *et al.*, 1966). Of the various methods of RNA fractionation which are presently available, chromatography on methylated albumin kieselguhr columns is potentially one of the most useful, since the procedure is convenient, the recovery can be made quantitative, and large amounts of RNA can be fractionated.

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[1] Abbreviations used in this paper: rpRNA, ribosomal precursor RNA; cdRNA, cytoplasmic "DNA-like" RNA; ndRNA, nuclear "DNA-like" RNA.