The Nucleotide Sequence of Cysteine Transfer Ribonucleic Acid from Baker's Yeast

PRODUCTS OF COMPLETE DIGESTION WITH PANCREATIC RIBONUCLEASE AND RIBONUCLEASE T1

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1. The nucleotide chain of tRNA^{Cys} from baker's yeast was readily split at the anticodon into two large fragments by brief treatment with ribonuclease T1. 2. The whole molecule and the two derived large fragments were completely digested with (a) pancreatic ribonuclease and (b) ribonuclease T1. The fragments present in each of the digests were separated and sequenced by conventional methods. 3. The groups of fragments derived from the two methods of digestion were entirely compatible with each other. 4. The molecule is 75 nucleotides long, but, as isolated, lacks the terminal adenosine and the neighbouring cytidylic acid residue. The minor nucleotides 1-methyladenylic acid, 7-methylguanylic acid, 5-methylcytidylic acid and *N*⁶-(γ -dimethylallyl)adenylic acid (isopentenyladenylic acid) were identified.

The isolation of tRNA^{Cys} from baker's yeast has already been reported in the preceding paper (Holness & Atfield, 1976a).

The present paper describes the separation of the oligonucleotides resulting from complete digestion with pancreatic RNAase* and with RNAase T1 of the whole molecule and of the large fragments obtained by brief treatment with RNAase T1.

The sequences of the individual oligonucleotides were established by conventional methods.

Experimental

Materials

The preparations of the nucleotides adenylic acid, cytidylic acid, guanylic acid and uridylic acid were each the mixture of the 2'- and 3'-isomers as supplied by Calbiochem, Los Angeles, CA, U.S.A. The corresponding nucleosides and 5-methylcytidine and pseudouridine were also obtained from Calbiochem.

Escherichia coli alkaline phosphatase (EC 3.1.3.1) was supplied by Sigma Chemical Co., St. Louis, MO, U.S.A., and pancreatic RNAase (EC 3.1.4.22) by Boehringer und Soehne, Mannheim, Germany; T1 RNAase (EC 3.1.4.8), T2 RNAase (EC 3.1.4.23) and U2 RNAase were supplied by Sankyo Co., Tokyo, Japan; *Crotalus adamanteus* venom phosphodiesterase (EC 3.1.4.1) was supplied by BDH, Poole, Dorset, U.K., and bovine spleen phosphodiesterase

(EC 3.1.4.1) by Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Sephadex LH20 was obtained from Pharmacia, Uppsala, Sweden, and *N*-cyclohexyl-*N'*-[β -morpholinyl-(4-ethyl)carbodi-imide methyl-*p*-toluenesulphonate, Wembley, Middx., U.K.; acrylamide (purified for electrophoresis), bisacrylamide and *NNN'*-tetramethyl-1,2-diaminoethane were from BDH, and 'Stains-All'* was from Eastman-Kodak Co., Rochester, NY, U.S.A.

NaB³H₄ (50 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and 2,5-diphenyloxazole scintillator and toluene, (scintillation-grade) were from BDH. Other materials used are described in the preceding paper (Holness & Atfield, 1976a).

Preparation of half-molecules from tRNA^{Cys}

A solution of tRNA^{Cys} (17 mg) in 3 ml of 0.1M-Tris/HCl buffer, pH 7.5, containing 0.02M-MgCl₂ was incubated for 45 min at 2°C with 0.2 ml of a solution (500 units/ml) of RNAase T1 in 0.1M-Tris/HCl buffer, pH 7.5. The solution was then shaken with 90% (v/v) phenol (3 ml) and the separated phenol layer back-washed with Tris/HCl buffer (1 ml). The phenol layer was discarded and the procedure repeated with a second 3 ml portion of 90% phenol. The combined aqueous layers were

* Abbreviation: 'Stains-All', 1-ethyl-2-[3-[1-ethyl-naphtho(1,2-*d*)thiazolin-2-ylidene]-2-methylpropenyl]-naphtho(1,2-*d*)thiazolium bromide.

* Abbreviation: RNAase, ribonuclease.

extracted with 3×5 ml of diethyl ether to remove residual phenol.

Urea (1 g) was added to the aqueous solution which was then allowed to percolate on to a column of DEAE-cellulose (240 cm×1 cm) equilibrated with 0.25 M-NaCl in 0.02 M-Tris/HCl buffer, pH 7.9, in 7 M-urea. Elution was continued with a linear gradient of 0.25–0.4 M-NaCl in the Tris/HCl/urea buffer (800 ml). Fractions (1.6 ml) were collected at the rate of 2/h.

Fractions containing each of the two half-molecules were combined, and the acceptor and terminal halves of tRNA^{Cys} were precipitated separately by adding 2 vol. of ice-cold ethanol. After being left overnight at 2°C each precipitate was centrifuged, washed with ethanol and dried *in vacuo*.

Purification of the half-molecules on polyacrylamide-bead columns

The respective halves of tRNA^{Cys} prepared above were separated from intact tRNA^{Cys} by chromatography on a column of Bio-Gel P100 polyacrylamide beads as described previously for intact tRNA^{Cys} (Holness & Atfield, 1976a).

Complete digestion with pancreatic RNAase of tRNA^{Cys} and derived half-molecules and separation of the resulting fragments

Intact tRNA^{Cys} (10 mg) or the derived half-molecules (10 mg) were incubated with pancreatic RNAase (1 mg) in 0.1 M-Tris/HCl buffer, pH 7.5, (4.0 ml) containing chloroform (0.02 ml) in a stoppered tube for 4 h at 37°C. Solid urea was then added to the solution to bring the urea concentration to 7 M and this solution was added to a column (80 cm×0.8 cm) of DEAE-cellulose equilibrated with buffer B (0.02 M-Tris/HCl, pH 7.5, in 7 M-urea). Mono- and oligo-nucleotides were then eluted with a linear gradient of 0.0–0.3 M-NaCl in buffer B (500 ml).

Mononucleotides were desalted on activated charcoal (Norit A) by the method of Mandeles & Kammen (1966) and the composition of the dried residue was analysed as described below.

In some early experiments, di-, tri- and tetra-nucleotides were desalted by the method of Madison & Kung (1967) before being applied to a column of DEAE-cellulose equilibrated with buffer C (0.02 M-formic acid in 7 M-urea, pH 3.8). Oligo-nucleotides were then eluted with a linear gradient of 0.0–0.2 M-NaCl in buffer C (200 ml). In later experiments the procedure of Takemura *et al.* (1972) was followed. Column eluates containing these isopliths were diluted with 4 vol. of water and applied to a column of DEAE-cellulose prepared in buffer B. The column was then washed with buffer C until the pH of the eluate was 3.8 when elution was continued with the NaCl gradient described above. Oligonucleo-

tides were recovered from relevant column fractions and kept for further examination.

Digestion of tRNA^{Cys} and the derived half-molecules with RNAase T1

A solution of intact tRNA^{Cys} (20 mg) in 0.02 M-Tris/HCl buffer, pH 7.5, (4 ml) was incubated for 18 h at 37°C with RNAase T1 (500 units). The resulting oligonucleotides were then fractionated on a DEAE-cellulose/7 M-urea column at pH 7.5 as described above. In this case the column length was increased to 70 cm or in some experiments to 150 cm. The total volume of the gradient was increased to 600 ml.

Half-molecules (5 mg of each) were digested with RNAase T1 (400 units) and the resulting oligonucleotides fractionated in a similar way.

Further enzymic digestion of isolated oligonucleotides

Tri- and tetra-nucleotides obtained from pancreatic RNAase digests were hydrolysed by incubating with 0.1 ml of a solution of RNAase T1 (300 units/ml) in 0.02 M-Tris/HCl buffer, pH 7.5, for 3 h at 37°C. The digest (5–15 *E*₂₆₀ units) was chromatographed on a column of DEAE-cellulose (30 cm×0.5 cm) equilibrated with buffer C. Elution was performed with a linear gradient of 0.0–0.2 M-NaCl in buffer C (150 ml).

Oligonucleotides obtained from RNAase T1 digests were hydrolysed by incubating with 0.1 ml of a solution of pancreatic RNAase (1 mg/ml) in 0.02 M-Tris/HCl buffer, pH 7.5, for 2 h at 37°C. The products were fractionated in a similar manner to that described above for the products of pancreatic RNAase digestion of intact tRNA^{Cys}.

Reduction of the terminal half of tRNA^{Cys} with NaB³H₄

The terminal half of tRNA^{Cys} (5.4 mg) was treated with NaB³H₄ as described by Igo-Kemenes & Zachary (1969). The resulting reduced tRNA^{Cys} terminal fragment, after destruction of excess of NaBH₄ and precipitation with ethanol, was dissolved in 1 ml of 0.02 M-Tris/HCl buffer, pH 7.5, and divided into two portions. One was hydrolysed with pancreatic RNAase, the other with RNAase T1, and the products from each experiment were separated by DEAE-cellulose chromatography in 7 M-urea as described above. Samples (0.1 ml) of each fraction were pipetted on to glass-fibre discs (2.1 cm diameter, Whatman) which were then dried under an i.r. lamp. Each disc was placed in a counting vial containing 10 ml of scintillation medium (0.5 g of 2,5-diphenyl-oxazole in 100 ml of toluene) and the ³H content determined in a Tracerlab spectrometer liquid-scintillation counter.

Determination of the composition of mononucleotide mixtures

The dried residues containing mononucleotide mixtures and obtained as described above were

dissolved in 0.25 ml of 0.07 M-sodium acetate buffer, pH 4.15, and applied directly to the DEAE-cellulose column equilibrated with the same buffer for separation of the individual components as described by Holness & Atfield (1971).

For further characterization, mononucleotides were converted into the nucleosides or the free bases and identified by paper chromatography as described by Melchers & Zachau (1964). T.l.c. in the same chromatographic systems but with thin layers of cellulose on aluminium sheet (E. Merck A.G., Darmstadt, Germany) or cellulose MN300 (Machery Nagel and Co., Düren, Germany) on glass plates (20 cm × 10 cm) (prepared in the laboratory) gave similar results.

Determination of the composition of oligonucleotides

Small oligonucleotides also obtained as dried residues from desalting procedures were dissolved in 0.1 ml of a solution of RNAase T2 (25 units/ml) in 0.05 M-sodium acetate buffer, pH 4.6, and incubated for 2 h at 37°C before being applied to the DEAE-cellulose column.

For tRNA^{Cys} and the half-molecules the procedure was as follows. A solution of the oligonucleotide (2 mg) in 0.05 M-sodium acetate buffer, pH 4.6 (0.2 ml), was incubated for 4 h at 37°C with 0.1 ml of a solution of RNAase T2 (250 units/ml) in the same buffer. The resulting mononucleotide mixture was then analysed as described above.

Determination of the composition of oligonucleotides by spectrophotometric methods

The absorption curves between 230 nm and 300 nm of oligonucleotides obtained as solutions in 7 M-urea at pH 3.8 from the DEAE-cellulose columns were recorded in a Unicam SP.800 recording spectrophotometer. In addition the spectral absorption curves of the same oligonucleotides were recorded again when the solutions had been made alkaline by the addition of 15 M-KOH (10 µl/ml). Visual comparison of these spectra with the spectra of mixtures of mononucleotides of known composition prepared in 7 M-urea at pH 3.8 and also at alkaline pH frequently enabled an approximate estimate to be made of the composition of the simpler oligonucleotides.

More recently the spectral-absorption information was recorded on punched paper tape by using a Stogate Data Logger (Stogate Technical Developments Ltd., Billingshurst, Sussex, U.K.) before more detailed computer-based determination of the composition of the oligonucleotides.

Analytical disc electrophoresis

The preparation of gel columns and the location of the components after electrophoresis were based

on the procedures described by Philippsen & Zachau (1972).

Acrylamide (0.82 g) with bisacrylamide (0.08 g) was dissolved in 0.8 ml of a buffer solution containing Tris (366 g), 1 M-HCl (480 ml) and *NNN'*-tetramethyl-1,2-diaminoethane (2.3 ml) in a final volume of 1 litre. The acrylamide solution was made up to 6 ml with 10 M-urea. Ammonium persulphate (5 mg) in 10 M-urea (0.8 ml) was then added to the cooled solution, and six glass tubes (7 cm × 0.5 cm), fitted with silicone rubber caps at the bottom, were filled to within 1 cm of the top with acrylamide solution, which was then overlaid with water to ensure a flat upper gel surface after polymerization. When polymerization was complete the caps could be removed from the tubes which were then suspended in the lower (anode) electrode compartment buffer (5 g of glycine and 1.2 g of Tris/litre). The upper (cathode) electrode compartment was filled with the same buffer, and the nucleotide samples, dissolved in buffer containing 5% (w/v) of sucrose, were carefully layered on to the surfaces of the gels. Electrophoreses were done at about 2 mA/gel and 25 V/cm for about 2 h. Gels were then removed from the tubes by using a silicone rubber plunger and washed once in water for 5 min. The nucleotide zones were located by using 'Stains-All' medium prepared by dissolving 'Stains-All' (20 mg) in formdimethylamide (50 ml) and diluting to 1 litre with water. Each gel was placed in a test tube containing 5 ml of 'Stains-All' medium and kept in the dark for 2 h. The dye solution was then replaced by water and the background pink colour allowed to bleach by exposure to light when nucleotide zones became visible as bluish-purple bands. About 10 µl of a nucleotide solution with E_{260} about 5 applied to a gel was sufficient to yield visible bands.

Miscellaneous procedures

The methods summarized by Brownlee (1972) were adopted for digestion with RNAase U2, partial digestion with spleen phosphodiesterase, for partial digestion with snake venom phosphodiesterase and for the protection of uridylic acid residues in oligonucleotides with *N*-cyclohexyl-*N'*-[β-morpholinyl-(4)-ethyl]carbodi-imide methyl-*p*-toluenesulphonate followed by pancreatic RNAase hydrolysis. The final high-voltage electrophoretic separation was in each case replaced by column chromatography on DEAE-cellulose in 7 M-urea at pH 3.8 or pH 7.5.

Fractionation of nucleoside mixtures on Sephadex LH20

The oligonucleotide believed to contain *N*⁶-(γ-dimethylallyl)adenosine (isopentenyladenosine) was converted into the mixture of its constituent nucleosides which was chromatographed on a

column (20cm×1cm) of Sephadex LH20 with 35% ethanol as solvent (Armstrong *et al.*, 1969). Isopentenyladenosine, which was eluted after the other nucleosides, was concentrated to dryness and examined by mass spectrometry.

Results and Discussion

Brief treatment of tRNA^{Cys} with RNAase T1 split the molecule into two parts described as acceptor half and terminal half and these were separated by chromatography on DEAE-cellulose in 7M-urea (Fig. 1b). It will be demonstrated later that the fragment eluted first was the acceptor half. The terminal half was eluted next at about the same salt concentration required to elute intact tRNA^{Cys} (Fig. 1a) and was in fact frequently contaminated with the intact molecule.

Fig. 2(a) shows that on chromatography on a column of Bio-Gel P100 polyacrylamide beads the

Fig. 1. DEAE-cellulose chromatography of (a) intact tRNA^{Cys} (30mg) and (b) the products of brief RNAase T1 hydrolysis of tRNA^{Cys} (17mg)

The column dimensions were 240cm×1cm and in each case the products were eluted with a linear gradient (800ml) of 0.25–0.4M-NaCl in 0.02M-Tris/HCl, pH7.9, in 7M-urea. The details of the RNAase T1 digestion are given in the Experimental section. ●, E_{260} ; ----, NaCl gradient.

Fig. 2. Comparison of the chromatographic behaviour of intact and half-molecules of tRNA^{Cys} with the mobilities observed during disc electrophoresis

Chromatographic behaviour of (a) the acceptor half of tRNA^{Cys} and (b) the terminal half contaminated with some intact tRNA^{Cys} on a column (90cm×1cm) of Bio-Gel P100 polyacrylamide beads. The eluent was 0.5M-Tris/HCl (pH7.0)/0.01M-MgCl₂/0.4M-NaCl. (c) Positions of the bands, stained with 'Stains-All', after disc electrophoresis of samples of the indicated fractions on polyacrylamide gel as described in the Experimental section. Gel 5 shows the positions of the acceptor and terminal halves resulting from brief treatment with RNAase T1 of a sample from fraction 24 (Fig. 2b) containing intact tRNA^{Cys}.

acceptor half is eluted significantly earlier than the main components which appear when the terminal half is chromatographed under the same conditions. The terminal half is the second main peak of Fig. 2(b); the first main peak is a mixture of intact tRNA^{Cys} and equimolar proportions of the two halves which behaved as the intact molecule under the conditions used. This is demonstrated by disc electrophoresis in urea (Fig. 2c) of the nucleotide material present in these peak fractions. Fraction 24 (Fig. 2b) on electrophoresis (gel 2) showed the presence of intact tRNA^{Cys} with smaller amounts of the two halves

whereas fraction 32 was shown to contain mainly the terminal half (gel 4). Further brief treatment with RNAase T1 of the material in fraction 24 resulted in the hydrolysis of the remaining intact tRNA^{Cys} into the two halves and this was confirmed by electrophoresis of the product (gel 5).

The higher proportion of purine nucleotides in the terminal half with the resultant aromatic binding to the polyacrylamide-gel matrix may account for the delayed elution of the terminal half. The order of elution, acceptor half, intact molecule, terminal half, is thus apparently determined mainly by the purine content rather than by the molecular size although there may be a significant increase in the volume of the acceptor half when it is no longer able to base-pair with the terminal half.

Fig. 3 shows the elution profiles resulting from chromatography on DEAE-cellulose in 7M-urea at pH 7.5 of the oligonucleotide mixtures obtained on

Fig. 3. DEAE-cellulose chromatography of the pancreatic RNAase digests of (a) intact tRNA^{Cys} (10mg), (b) the acceptor half (10mg) and (c) the terminal half (10mg)

In each case the products were eluted with a linear gradient (500ml) of 0.0–0.3M-NaCl in 0.02M-Tris/HCl (pH 7.5) in 7M-urea. —, E_{260} ; ----, NaCl gradient.

Fig. 4. Rechromatography of (a) fraction IIc and (b) fractions IVa and IVb (Fig. 3b) from the acceptor half of tRNA^{Cys} on DEAE-cellulose

In each case the separation was effected with a linear gradient (200ml) of 0.0–0.2M-NaCl in 0.02M-formic acid in 7M-urea at pH 3.8. —, E_{260} ; ----, NaCl gradient.

complete hydrolysis of intact tRNA^{Cys} and the acceptor and terminal halves. In the presence of urea the separation depends predominantly on the net charge and therefore is mainly according to chain length. As shown in Fig. 3(a) there are four main peaks in the profile and these range from mononucleotides (peak I) to tetranucleotides (peak IV).

The mononucleotides present in the fractions corresponding to peak I were identified and estimated quantitatively by chromatography on DEAE-cellulose in acetate buffer at pH 4.15.

The elution pattern is modified for those oligonucleotides containing the more basic methylated purines and methylated pyrimidines. This results in a decrease in the net negative charge and leads to elution from the column earlier than would be expected from a consideration merely of chain length. Thus the oligonucleotides IIa and IIb were identified as trinucleotides which were eluted before the dinucleotides as a result of the presence of bases carrying a greater positive charge than normal. Oligonucleotide IIa on alkaline hydrolysis had given 3'-Up, 2'- and 3'-Gp and the 2'- and 3'-isomers of an unknown nucleotide. This evidence established uridylic acid as the terminal nucleotide. Subsequently,

Fig. 5. *Rechromatography on DEAE-cellulose of (a) fraction IIC, (b) fraction IIIB and (c) fraction IVC (Fig. 3c) from the terminal half of tRNA^{Cys}*

The experimental procedures were as described in the legend to Fig. 4.

RNAase T2 hydrolysis of the trinucleotide and t.l.c. of the free base derived from the third nucleotide identified it as m¹Ap. The identification of guanylic acid as one of the fragments resulting from RNAase T1 digestion of oligonucleotide IIa enabled the sequence to be written as G-m¹A-Up.

RNAase T2 hydrolysis of oligonucleotide IIb gave guanylic acid and an unusual base shown to have the spectral properties identical with those of m⁷Gp. The terminal hUp follows from the method of hydrolysis and the fall in *E*₂₃₀ under alkaline conditions (Molinaro *et al.*, 1968).

The remaining fractions in the profile were complex and were further resolved by chromatography on DEAE-cellulose in urea at pH 3.8 (Figs. 4 and 5). The problem was made easier by examining separately the oligonucleotide mixtures derived from the separate half-molecules. Thus oligonucleotide IIC was further resolved as follows: the dinucleotides IIC-1, IIC-2 and IIC-3 were obtained from the terminal half, whereas IIC-2 was absent

from the corresponding digest of the acceptor half. The sequences of these dinucleotides were determined completely by identification of the mononucleotides resulting from RNAase T2 hydrolysis and were confirmed by the absorption spectra of the dinucleotides recorded at acid pH and at alkaline pH.

The conclusion that the large fragment eluted first in Fig. 1(b) was the acceptor half was supported by the following evidence: nucleotide IIIa which was obtained from this fragment was identified as A-G-Tp by RNAase T1 digestion and it will be shown later that oligonucleotide 5 obtained by RNAase T1 digestion of the same large fragment was C-U-C which must come from the 3' end of tRNA^{Cys}.

The terminal half of the molecule afforded a complex mixture of trinucleotides (Fig. 5). The sequences of nucleotides IIIB-1 and IIIB-2 were established as A-G-Cp and G-G-Cp respectively by RNAase T1 digestion. The 3'-terminal nucleotide of oligonucleotides IIIB-3 and IIIB-4 was identified as hUp by the method of preparation, the fall in *E*₂₃₀ of an alkaline solution of each trinucleotide and by the detection of ³H in these two trinucleotides when they were isolated from the pancreatic RNAase digest of a sample of the terminal half that had been reduced with NaB³H₄.

Table 1. *Fragments produced by pancreatic RNAase digestion of the acceptor and terminal halves of tRNA^{Cys}*

The peak numbers correspond to those shown in Figs. 3-5.

	Peak no.	Mol/mol of tRNA ^{Cys}	Sequence assigned
Acceptor half	I-1	1	m ⁵ Cp
		7	Cp
	I-2	1	ψp
		6	Up
	IIa	1	G-m ¹ A-Up
	IIb	1	G-m ⁷ G-hUp
	IIC-1	1	G-Cp
	IIC-3	1	G-Up
	IIIa	1	A-G-Tp
	IVa	1	G-A-G-Up
Terminal half	IVb-1	1	G-A-G-Cp
	IVb-2	1	A-i ⁶ A-A-ψp
	I-1	1	Cp
	I-2	2	Up
	I-3	1	Gp
	IIC-1	2	G-Cp
	IIC-2	1	A-Up
	IIC-4	1	G-Up
	IIIB-1	2	A-G-Cp
	IIIB-2	1	G-G-Cp
	IIIB-3	1	A-G-hUp
	IIIB-4	1	G-G-hUp
	IVc-1	1	pG-Cp
	IVc-2	1	A-G-A-ψp

Nucleotide IVa was identified as G-A-G-Up by the crucial experiment of partial hydrolysis with spleen phosphodiesterase which gave the trinucleotide A-G-Up.

Determination of the base composition of oligonucleotide IVb indicated that this was a mixture of two tetranucleotides which resisted separation. RNAase T1 hydrolysis gave equimolar quantities of Cp, Gp, A-Gp and a tetranucleotide. Hydrolysis of the latter with RNAase T2 gave ψ p, Ap and i⁶Ap in the ratio 1:2:1. The other component of the mixture of tetranucleotides must be G-A-G-Cp for free Gp was absent from the RNAase T1 digest of this half.

Chromatography of oligonucleotide IVc at pH 3.8 afforded two oligonucleotides. The first had an absorption spectrum identical with G-Cp. From its chromatographic properties and nucleotide composition fragment IVc-1 was given the sequence pG-Cp. Fragment IVc-1 forms the 5'-terminus of the intact molecule and is derived from the pancreatic RNAase digest of the fragment eluted second in Fig. 1(b). This is in agreement with the assignment of acceptor and terminal halves as the fragments eluted first and second respectively in Fig. 1(b). The

Table 2. Fragments produced by RNAase T1 digestion of the acceptor and terminal halves of tRNA^{Cys}

The peak numbers correspond to those shown in Figs. 6-8.

	Peak no.	Mol/mol of tRNA ^{Cys}	Sequence assigned
Acceptor half	5	1	C-U-C
	9-2	1	U-Gp
	9-3	1	C-Gp
	10	2	A-Gp
	11-1	1	U-U-Gp
	13	1	T- ψ -C-Gp
	14-2	1	m ¹ A-U-C-C-U-Gp
	18-1	1	m ⁷ G-hU-m ⁵ C-C-U-U-A-Gp
	20	1	C-A-i ⁶ A-A- ψ -C-U-Gp
Terminal half	7	2	Gp
	9-1	1	hU-Gp
	9-3	2	C-Gp
	11-2	1	hU-A-Gp
	12-1	3	C-A-Gp
	12-2	1	pGp
	14-1	1	C-U-C-Gp
	14-3	1	U-A-U-Gp
	14-4	1	A- ψ -U-Gp

Fig. 6. DEAE-cellulose chromatography of the RNAase T1 digests of (a) intact tRNA^{Cys} (20mg), (b) the acceptor half (5mg) and (c) the terminal half (5mg)

The products were eluted with a linear gradient (600ml) of 0.0-0.3M-NaCl in 0.02M-Tris/HCl (pH 7.5) in 7M-urea. —, E_{260} ; ----, NaCl gradient.

other component of the mixture, namely fragment IVc-2, was split by RNAase T1 into two dinucleotides A-Gp and A- ψ p and the sequence is therefore A-G-A- ψ p.

Table 1 summarizes the distribution between the acceptor and terminal halves of tRNA^{Cys} of the oligonucleotides arising from digestion with pancreatic RNAase.

The elution patterns obtained by chromatographing the mixture of oligonucleotide fragments resulting from RNAase T1 digestion of the intact tRNA^{Cys} and the two half-molecules are shown in Fig. 6. The sequences assigned to the fragments are shown in Table 2 and the supporting evidence was as follows.

Fragment 5 was a trinucleotide which terminated in cytidine and contained in addition one molecule each of Cp and Up. The sequence C-U-C was adopted in preference to the alternative U-C-C, which would not be compatible with the observed number of fragments terminating in Up which were released by pancreatic RNAase hydrolysis of the acceptor half.

Sequences could be assigned immediately to the component fragments of peak 9 and to fragment 10 from the result of base analysis confirmed for the dinucleotide hU-Gp (fragment 9-1) by the result of the experiment which located ³H label among oligonucleotides of the RNAase T1 digest of the NaB³H₄-reduced terminal half.

The two components of peak 11, one of which

came from each half, were identified as hU-A-Gp and U-U-Gp by digestion with pancreatic RNAase. Similar reasoning led to the sequence C-A-Gp for fragment 12-1. Fragment 12-2, separated from 12-1 by chromatography at pH3.8 (Fig. 7), was identified as the terminal nucleotide pGp.

RNAase T2 hydrolysis of fragment 13 indicated the presence of 1 molecule of each of Tp, ψ p, Cp and Gp. Since the sequence must start with Tp, for A-G-Tp was identified among the trinucleotides of the pancreatic RNAase digest, and end in Gp, the

sequence was written T- ψ -C-Gp by analogy with the results of all previous investigations.

The oligonucleotides present in peak 14 were resolved into four components by chromatography at pH3.8 (Fig. 7), fragment 14-2 coming from the acceptor half of the molecule. Incomplete hydrolysis with spleen phosphodiesterase led to the formation of the trinucleotide A-U-Gp from fragment 14-3, and RNAase U2 gave equal proportions of U-Ap and U-Gp confirming the sequence U-A-U-Gp. Snake venom phosphodiesterase hydrolysis of fragment 14-4 yielded the fragments A- ψ p and A- ψ -Up which support the sequence A- ψ -U-Gp.

Partial hydrolysis of fragment 14-1 with spleen phosphodiesterase gave Cp and Up, the dinucleotide C-Gp and traces of the trinucleotide U-C-Gp to establish the structure as C-U-C-Gp.

The fragment 14-2 was a hexanucleotide with the composition (Cp, Cp, Up, Up, m¹Ap)Gp. Experiments with snake venom phosphodiesterase placed Up next to Gp. Since the trinucleotide G-m¹A-Up had been identified among the fragments in the pancreatic RNAase digest the sequence of fragment 14-2 could be written m¹A-U-C-C-U-Gp.

The oligonucleotides present in the fractions forming peak 18 separated into three components when chromatographed at pH3.8 (Fig. 7). Component 18-1 on digestion with pancreatic RNAase gave A-Gp and a mononucleotide mixture. The latter was resolved into m⁵Cp, Cp, Up (2mol) and a nucleotide eluted from the DEAE-cellulose column at pH4.15 between Cp and Up. This unusual nucleotide resembled m⁷Gp, which was normally eluted among the nucleoside components. Here it was present as the dinucleotide m⁷G-hUp whose presence was suggested in the pancreatic RNAase digest trinucleotide G-m⁷G-hUp.

The slowest component, fragment 18-3, had a composition identical with fragment 18-1 but lacked the m⁷Gp-like compound. Component 18-2 was similar to component 18-3 but lacked m⁵Cp. The sequence of component 18-3 was determined after protecting the Up and Gp residues with *N*-cyclohexyl-*N'*-[β -morpholinyl-(4)-ethyl]carbodi-imide methyl-*p*-toluenesulphonate. Hydrolysis with pancreatic RNAase gave a mixture of Cp and m⁵Cp and an oligonucleotide. Removal of the protecting groups led to a rapid hydrolysis with pancreatic RNAase to a mixture of Up and A-Gp in the ratio 2:1. The sequence of this group of compounds could then be written as follows: fragment 18-1, m⁷G-hU-m⁵C-C-U-U-A-Gp, fragment 18-2, C-U-U-A-Gp and fragment 18-3, m⁵C-C-U-U-A-Gp. The order of elution from the DEAE-cellulose column at pH3.8 was modified by the presence of the more basic methylated nucleotides.

Nucleotide 20 was hydrolysed by pancreatic RNAase to give the tetranucleotide A-i⁶A-A- ψ p

Fig. 7. Rechromatography on DEAE-cellulose of (a) fraction 12, (b) fraction 14 and (c) fraction 18 (Fig. 6)

In each case the components were eluted with a linear gradient (200ml) of 0.0–0.2M-NaCl in 0.02M-formic acid in 7M-urea at pH3.8. —, E_{260} ; ----, NaCl gradient.

Fig. 8. Chromatographic separation on the analytical DEAE-cellulose column of the nucleotides obtained by RNAase T2 hydrolysis of (a) the acceptor half (2 mg), and (b) the terminal half (2 mg) of tRNA^{Cys}.

The nucleotides were eluted with 0.07 M-sodium acetate adjusted to pH 4.15 with 2 M-acetic acid.

previously found among fragments in the pancreatic RNAase digest derived from the acceptor half and a mixture of the mononucleotides Cp, Up and Gp in the ratio 2:1:1. Partial hydrolysis with spleen phosphodiesterase placed Cp followed by Ap at the 5' end and partial hydrolysis with snake venom phosphodiesterase established the terminal sequence as C-U-Gp. The sequence of nucleotide 20 is then C-A-i⁶A-A-ψ-C-U-Gp.

Isopentenyladenosine was separated from the mixture of nucleosides derived by RNAase T2 and phosphomonoesterase treatment of fragment 20 as described in the Experimental section. It was shown to be identical by mass spectrometry with an authentic sample kindly provided by Professor C. B. Reese, King's College, London, U.K. Isopentenyladenosine had been previously identified among the nucleosides obtained from a partially purified tRNA^{Cys} of brewer's yeast by Hecht *et al.* (1969).

Examination of the compositions of the two sets of oligonucleotides listed in Tables 1 and 2 shows that they are self-consistent. However, the number of overlaps possible is very few and the longer sequences which can be derived unambiguously are as follows. In the series from the acceptor half,

Table 3. Comparison of the observed and calculated values for the nucleotide composition of tRNA^{Cys} and the acceptor and terminal halves

The values for the composition of the intact molecule and the two half-molecules calculated from the sequences of the fragments obtained on complete pancreatic RNAase and RNAase T1 hydrolyses (Tables 1 and 2) are compared with those determined experimentally by RNAase T2 hydrolysis of the intact molecule and the two halves. The observed composition for tRNA^{Cys} has been taken from Table 3 of the preceding paper (Holness & Atfield, 1976a). Values for the nucleotide extinction coefficients were taken from the published data of Holley *et al.* (1965) and Madison *et al.* (1967). Obs., Observed; Calc., Calculated.

Nucleotide	Percentage composition					
	Intact tRNA ^{Cys}		Acceptor half		Terminal half	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
m ⁷ Gp	2.0	1.5	3.9	2.8	—	—
Cp+m ⁵ Cp+C	16.6	16.8	16.5	17.3	15.7	15.3
m ⁴ Ap	2.2	2.1	1.6	3.3	—	—
Up+Tp+ψp	22.7	22.9	27.2	29.0	17.9	15.0
Ap+i ⁶ Ap	24.0	24.0	23.7	22.6	27.2	26.7
Gp	32.3	32.9	27.3	25.1	39.1	43.1

combination of the oligonucleotides IIb and 18 gives the sequence G-m⁷G-hU-m⁵C-C-U-U-A-Gp, and from oligonucleotides IIIa and 13 the sequence A-G-T-ψ-C-Gp arises. From the terminal half, combination of fragments IVc-2 and 14-4 gives the sequence A-G-A-ψ-U-Gp.

Clearly this degree of overlap is quite insufficient to establish a unique sequence for the tRNA^{Cys} molecule. Further partial hydrolyses permitting the isolation of longer fragments are required and this is described in the following paper (Holness & Atfield, 1976b).

Fig. 8 depicts the Uvicord records for the separation of the mononucleotides obtained by RNAase T2 hydrolysis of the two halves of tRNA^{Cys}. Table 3 compares the percentage composition of the two halves and the entire molecule with the composition calculated from the sequences reported in Tables 1 and 2. The discrepancies between the observed and calculated values for Up, Tp, ψp and Gp in the terminal half may well result from contamination with intact tRNA^{Cys}.

The available information shows that the tRNA^{Cys} molecule contains 75 nucleotides, but, as isolated from baker's yeast, lacks the terminal adenosine. This absence of adenosine has been noted from other tRNA sequences, for example, tRNA^{Ala} (Holley *et al.*, 1965). Also missing entirely or in part is the cytidylic acid residue adjacent to the terminal adenosine and this explains the marked effect of added CTP on the incorporation of cysteine in the conventional assay (Holness & Atfield, 1976a).

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