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The nucleotide sequence of tyrosine tRNA from Bacillus stearothermophilus

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ABSTRACT

The nucleotide sequence of $tRNA^{Tyr}$ from B. stearothermophilus has been determined: pG-G-A-G-G-G-G-s^4U-A-G-C-G-A-A-G-U-Gm-G-C-U-A-A-m^1A-C-G-C-G-G-C-G-G-A-C-U-Q-U-A-ms^2i^6A-A-ψ-C-C-G-C-U-C-C-C-U-U-U-G-G-G-U-U-C-G-G-C-G-G-T-ψ-C-G-A-A-U-C-C-G-U-C-C-C-C-C-U-C-C-A-C-C-A_{OH}. A combination of classical fingerprinting methods, partial nuclease P_1 digestion and two-dimensional homochromatography and a rapid "read off" sequencing gel technique were used to establish the complete nucleotide sequence.

INTRODUCTION

In conjunction with current crystallographic work on the three-dimensional structure of tyrosyl-tRNA synthetase from the thermophile B. stearothermophilus (1) a study was undertaken of the nucleotide sequence of $tRNA^{Tyr}$ from this organism. A knowledge of the primary structure is necessary to facilitate an understanding of the nature of the interaction between a tRNA and its cognate synthetase at the molecular level. Furthermore the sequence homologies revealed by a comparison of $tRNA^{Tyr}$ from B. stearothermophilus and E. coli (2) may provide information about synthetase recognition since they are crosscharged to the same extent by the tyrosyl-tRNA synthetases from both organisms (unpublished results of R.S. Brown and J.R. Rubin).

MATERIALS AND METHODS

General

B. stearothermophilus cells (strain NCIB 8924) and crude tRNA were obtained from Professor B.S. Hartley, Imperial College of Science and Technology, London. Pancreatic RNase and snake venom phosphodiesterase were from Worthington Biochemicals Corp. RNase T_1, T_2 and U_2 (Sankyo) were purchased from Calbiochem. Bacterial alkaline phosphatase and nuclease P_1 were from Sigma Chemical Co., yeast hexokinase from Boehringer Mannheim GmbH and T_4 polynucleotide kinase from Miles Research Products. [^{32}P]orthophosphate and [γ-^{32}P]ATP (1000 Ci/mol) were supplied by the Radiochemical

Centre, Amersham. DEAE-cellulose DE 52 and Whatman DE 81 and 540 paper were obtained from Reeve Angel, Ltd., BD-cellulose from Boehringer, DEAE-Sephadex A50 from Pharmacia Fine Chemicals, AB and RPC5 from Miles Laboratories. Cellulose thin layer plates were from E. Merck and DEAE thin layer plates (Polygram Cel 300) from Macherey-Nagel. Cellulose acetate strips were purchased from Schleicher and Schüll.

Preparation of uniformly ^{32}P-labelled tRNA^{Tyr}

A culture of B. stearothermophilus was prepared at 58°C in LP medium (2) in which 0.4% glucose was replaced by glycerol and 0.1 mCi/ml [^{32}P]orthophosphate was present. Harvested cells were resuspended in 0.2 M sodium acetate (pH 5.0) buffer containing 1% sodium dodecyl sulphate and crude [^{32}P]tRNA was isolated by phenol extraction. Initial separation of tRNA^{Tyr} was achieved by BD-cellulose column chromatography (3). The fraction containing tRNA^{Tyr} eluted from the column before tRNA^{Phe} in the ethanol gradient [0-20% ethanol in 1 M NaCl, 10 mM $MgCl_2$, 1 mM $Na_2S_2O_3$, 50 mM Tris-HCl (pH 7.5)]. The final purification step was by electrophoresis in a 15% polyacrylamide slab gel [acrylamide: N,N'-methylene bisacrylamide (19:1)] containing 7 M urea (4) (see Figure 1). The gel (20 cm x 20 cm) was run at

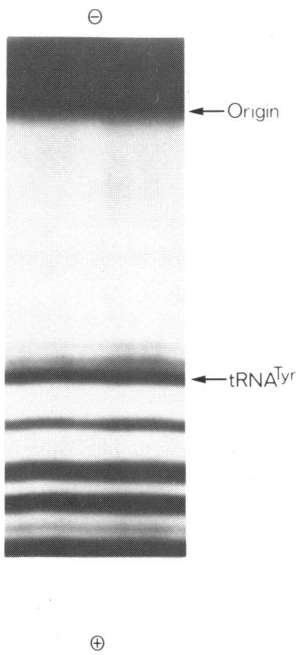

Figure 1. Autoradiogram of the separation of uniformly ^{32}P-labelled tRNA by electrophoresis in a 15% polyacrylamide gel containing 7 M urea and Tris-borate-EDTA buffer (pH 8.3).

200 v until the xylene cyanol FF marker dye had migrated to the bottom. After autoradiography the band containing [^{32}P]tRNA^{Tyr} was cut out of the gel and eluted electrophoretically.

Sequencing techniques

Standard methods (5) were used for pancreatic and T_1 RNase digestion of uniformly ^{32}P-labelled tRNA^{Tyr} and sequence analysis of the resulting oligonucleotides. Oligonucleotides eluted from fingerprints were digested completely with T_2 RNase. The digestion products were analysed by ionophoresis on Whatman 540 paper at pH 3.5 and also by thin layer chromatography (5). Cellulose thin layer plates were developed in the first dimension with isobutyric acid:.880 ammonia:H_2O (66:1:33), and in the second dimension with isopropanol:conc. HCl:H_2O (68:17:15), or 0.1 M sodium phosphate (pH 6.8):ammonium sulphate:n-propanol (100:60g:2) (7). The identity of the modified nucleotide m^1Ap was established by the changes of ionophoretic and chromatographic mobility which were observed on conversion to m^6Ap by treatment with 0.1 M NaOH for 16 hr at 37°C. ms^2i^6Ap was distinguished from i^6Ap by ionophoresis on Whatman DE 81 paper at pH 3.5 (5).

Preparation of 5' ^{32}P-labelled tRNA^{Tyr}

Nonradioactive tRNA^{Tyr} was prepared from crude tRNA obtained from large-scale phenol extraction of *B. stearothermophilus* cells. The crude tRNA sample contained glutinous material which was removed by passage of the solution through a nitrocellulose filter (Millipore HAWP25, 0.45 μ). Purification of tRNA^{Tyr} was achieved by successive chromatography on columns of DEAE-cellulose (Whatman DE 52), BD-cellulose, DEAE-Sephadex A50 (8) and RPC5 (9). The tRNA^{Tyr} thus produced had an amino acid acceptance activity of 1337 μμmol/A260 unit. Final purification of nonradioactive tRNA^{Tyr} for sequence studies was performed by electrophoresis in a 15% polyacrylamide gel containing 7 M urea. After treatment with bacterial alkaline phosphatase for 1 hr at 55°C the 5' terminus of tRNA^{Tyr} was labelled *in vitro* with ^{32}P using T_4 polynucleotide kinase and [γ-^{32}P]ATP as described in (10). 5' ^{32}P-labelled oligonucleotides from complete pancreatic and T_1 RNase digests of tRNA^{Tyr} were also prepared by this method.

Partial P_1 nuclease digestion

The sequences of oligonucleotides from pancreatic and T_1 RNase fingerprints which could not be unambiguously determined by standard methods (5) were established by partial digestion with nuclease P_1 or venom phosphodiesterase of 5' ^{32}P-labelled oligonucleotides (11). Oligonucleotides were separated by two-dimensional homochromatography. The first dimension was

ionophoresis at pH 3.5 on cellulose acetate and then DEAE-cellulose thin layer plates developed with 3% homomixture C at 60°C in the second dimension. Partial digestion of 5' ^{32}P-labelled oligonucleotides with nuclease P_1 (15 ng/ 200 µg RNA) at room temperature (aliquots removed between 2-30 min) was followed by two-dimensional homochromatography or ionophoresis on Whatman DE 81 paper at pH 1.9. Oligonucleotides were also completely digested with nuclease P_1 in 50 mM ammonium acetate (pH 5.3) at 37°C. Digestion products were analysed by thin layer chromatography to identify the nucleotide present at the 5' end of each oligonucleotide.

Characterisation of modified nucleotides

Modified nucleotides present in nonradioactive $tRNA^{Tyr}$ were identified using the following method. 2 µg of $tRNA^{Tyr}$ was partially hydrolysed by treatment with 10 µl of 0.1 M NaOH for 15 min at 20°C. The sample was then neutralised, diluted and dialysed extensively. After lyophilisation, T_4 polynucleotide kinase and [γ-^{32}P]ATP were used (10) to label the 5' ends of oligonucleotides produced by alkali degradation. The 5' ^{32}P-labelled nucleoside phosphates resulting from complete digestion with nuclease P_1 for 30 min at 37°C were characterised by thin layer chromatography (see Figure 2).

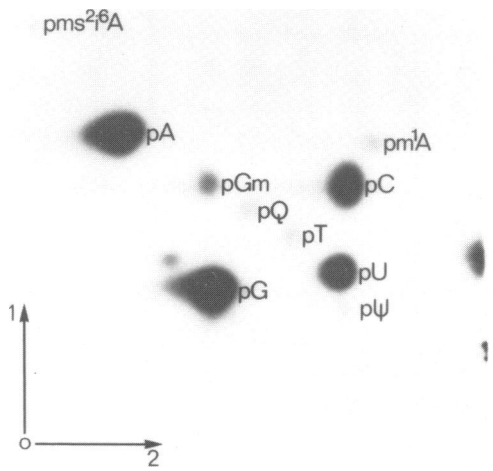

Figure 2. Autoradiogram of a two-dimensional separation by cellulose thin layer chromatography of 5' ^{32}P-labelled nucleoside phosphates derived from B. stearothermophilus $tRNA^{Tyr}$. Solvent 1 was isobutyric acid:.880 NH_3:H_2O (66:1:33) and solvent 2 was 0.1 M sodium phosphate (pH 6.8):$(NH_3)_2SO_4$: n-propanol (100:60g:2).

Direct "read off" sequencing gel

The recently developed rapid sequencing gel method (12,13) was used to produce overlapping tracts of the nucleotide sequence of B. stearothermophilus $tRNA^{Tyr}$. The method is based on the fractionation according to size of the partial digestion products of 5' ^{32}P-labelled RNA obtained with T_1 (G-specific), pancreatic (C + U-specific), U_2 (A-specific) RNases and RNase 1 from Physarum polycephalum (cleaves after G, U and A residues) in adjacent slots of a 20% polyacrylamide gel [acrylamide:N,N'-methylene bisacrylamide (20:0.67)] containing 7 M urea. A ladder of fragments derived from breaks at every phosphodiester bond is generated by hot formamide degradation (100°C for 30 min) of 5' ^{32}P-labelled RNA.

Large fragments required to provide overlapping sequences were prepared by RNase activity present in commercial T_4 polynucleotide kinase and also by specific Pb^{2+} catalysed cleavage of $tRNA^{Tyr}$ as described in (14). Thus $tRNA^{Tyr}$ (1 mg/ml) was treated with 1 mM $Pb(CH_3COO)_2$ in 0.5 M NaCl, 0.07 M Tris-HCl (pH 7.5) at 37°C for 30 min. The reaction was terminated by the addition of 10 mM EDTA and followed by extensive dialysis. Fragments produced by Pb^{2+} cleavage were purified by 15% polyacrylamide gel electrophoresis. After elution from the gel, $tRNA^{Tyr}$ fragments were 5' end-labelled using T_4 polynucleotide kinase and [γ-^{32}P]ATP as described in (10).

Partial digestion of separate aliquots with T_1, pancreatic, U_2 and Phy I RNases under the conditions reported in (13) was followed by sequence analysis in a 20% polyacrylamide denaturing gel.

RESULTS

The fingerprints of the end products from pancreatic and T_1 RNase digestion of uniformly ^{32}P-labelled $tRNA^{Tyr}$ are shown in Figure 3. Molar yields and deduced sequences of the 17 fragments found in the pancreatic RNase fingerprint are presented in Table 1. The results of T_2 and T_1 RNase digestion together with the 5' end group analysis from complete P_1 nuclease digestion of 5' ^{32}P-labelled oligonucleotides are sufficient to determine unambiguously the sequences of all fragments except for p11, p14, p15, p16 and p17. (Table 2) The sequences of these fragments were established by partial digestion with P_1 nuclease and also partial venom phosphodiesterase digestion of 5' ^{32}P-labelled oligonucleotides and two-dimensional homochromatography, examples of which are shown in Figure 4. Fragment p17 was found only in low yield and frequently poorly separated from p14 by ionophoresis on Whatman DE 81 paper in 7% formic acid. These fragments were well resolved by homochromatography on DEAE thin

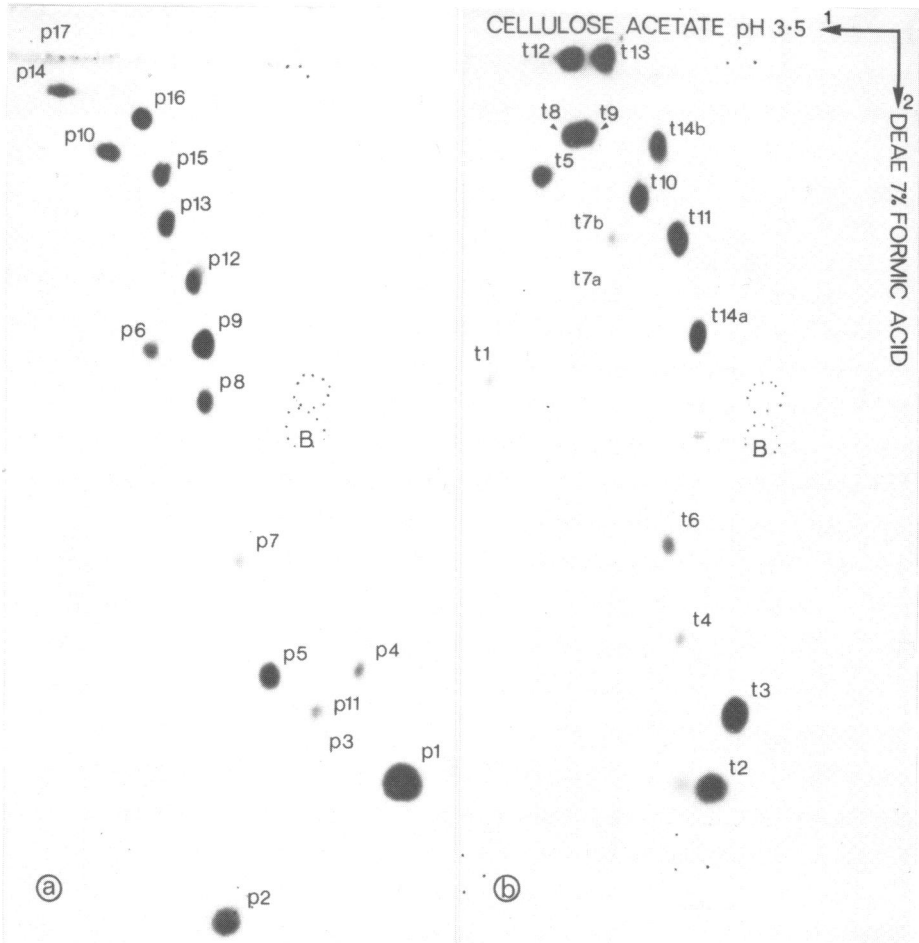

Figure 3. Fingerprint of (a) complete pancreatic RNase digest and (b) complete T_1 RNase digest of tyrosine tRNA. B indicates the position of the blue marker dye (xylene cyanol FF).

layer plates at 60°C. Partial P_1 nuclease digestion of 5' ^{32}P-labelled $tRNA^{Tyr}$ and two-dimensional homochromatography shows the sequence pG-G-A-G-G-G-G (fragment p17) to be located at the 5' end of $tRNA^{Tyr}$.

The deduced sequences and molar yields of the 14 fragments present in the T_1 RNase fingerprint of $tRNA^{Tyr}$ are shown in Table 3. In some preparations of uniformly ^{32}P-labelled $tRNA^{Tyr}$, fragment t14 occurred as a single spot in the T_1 RNase fingerprint with a molar yield of 0.9.

Fragment t7 is found as two spots in the fingerprint, probably the result of the somewhat unstable nature of 4-thiouridine. With the exception of fragments t8, t9, t11, t12, t13 and t14 the sequences of T_1 RNase fragments

Table 1. Pancreatic RNase end products

Fragment number	Sequence	Molar yields	
		Observed	Expected
p1	Cp	13.8	17
p2	Up + ψp	6.3	8 + 1
p3	A-Cp	0.7	1
p4	Q-Up	0.8	1
p5	G-Cp	1.9	2
p6	G-Up	0.8	1
p7	A-G-Cp	0.7	1
p8	Gm-G-Cp	0.9	1
p9	G-G-Cp	1.9	2
p10	G-G-Tp	1.4	1
p11	A-A-m^1A-Cp	0.6	1
p12	C-G-A-Cp	0.9	1
p13	G-A-A-Up	1.0	1
p14	G-G-G-Up	1.3	1
p15	A-ms^2i^6A-A-ψp	1.1	1
p16	G-A-A-G-Up	0.7	1
p17	pG-G-A-G-G-G-G-s^4Up	0.3	1

Table 2. Analysis of pancreatic RNase end products

Fragment number	T_2 RNase products*					T_1 RNase products	5' terminal nucleotide
	Ap	Cp	Gp	Up	Others		
p1		+					
p2				+	ψp (+)		
p3	1.1	1.0				A-Cp	Ap
p4				1.0	Qp 0.5	Q-Up R_{A-Cp} = 1.65	Qp
p5		1.0	0.8			Gp (0.7), Cp (1.0)	Gp
p6			(1)	(1)		Gp (0.7), Up (1.0)	Gp
p7	1.0	1.0	0.8			A-Gp (1.3), Cp (1.0)	Ap
p8		1.0			Gm-Gp 0.8	Gm-Gp (1.4), Cp (1.0)	-
p9		1.0	1.5			Gp (1.3), Cp (1.0)	Gp
p10			(2)		Tp (1)	Gp (1.6), Tp (1.0)	Gp
p11	2.0	1.0			m^1Ap 1.1	A-(A,m^1A)-Cp R_{A-Cp} = 0.36	Ap
p12	1.0	1.0	1.4			Gp (1.7), A-Cp (2.0)	Gp
p13	2.0		1.0	1.0		Gp (1.0), A-A-Up (2.2)	Gp
p14			1.4	1.0			Gp
p15	(2)				ms^2i^6Ap (1) ψp (1)	A-(A,ms^2i^6A)-ψp R_{A-Cp} = 0.04	Ap
p16	1.8		1.4	1.0			Gp
p17	+		+				pGp

* The yields of T_2 RNase products shown in parentheses were estimated visually

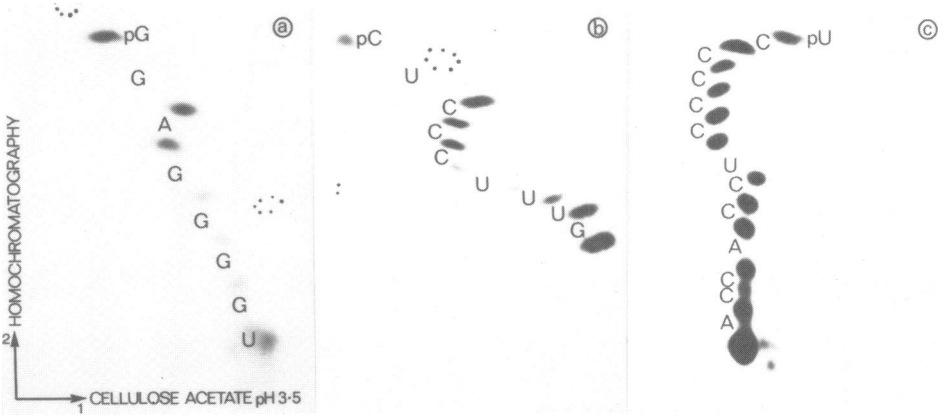

Figure 4. Autoradiogram of the two-dimensional separation of partial P_1 nuclease digestion products from 5' ^{32}P-labelled oligonucleotides. (a) p17, (b) t12, and (c) t14.

can be deduced from the results presented in Table 4. The sequences of fragments t8, t11, t12 and t14 were determined by partial P_1 nuclease and partial venom phosphodiesterase digestion and two-dimensional homochromatography. Fragment t9 was found to be (T,ψ)-C-G and t13 A-C-U(Q-U,A-ms^2i^6A-A-ψ)-C-C-G since modified nucleotides were poorly digested under partial conditions.

The modified nucleosides 1-methyl adenosine, 2-methyl-thio-N^6-isopentenyl adenosine, 2'-O-methyl guanosine, pseudouridine, Q and (ribo)thymidine were found in both uniformly ^{32}P-labelled and nonradioactive $tRNA^{Tyr}$ by two-dimensional thin layer chromatography (Figure 2). The presence of 4-thiouridine in B. stearothermophilus $tRNA^{Tyr}$ was indicated by the UV spectrum at 335 nm although the modified nucleotide was not identified by thin layer chromatography. The identity of m^1Ap was established by its conversion to m^6Ap on treatment with 0.1 M NaOH and the accompanying changes of mobility on thin layer chromatography. Both m^1Ap and Qp were retarded equally (R_{Up} = 0.11) on ionophoresis at pH 3.5 on Whatman 540 paper. ms^2i^6Ap was distinguished from i^6Ap by ionophoresis at pH 3.5 on Whatman DE 81 paper where it ran with R_{Up} = 0.53 (5).

Overlapping tracts of sequence shown in Figures 5 were derived from sequencing gels using 5' ^{32}P-labelled fragments and intact $tRNA^{Tyr}$. It was found that the band separation in sequencing gel patterns is greatest between those partial degradation products which differ in a 3' terminal Gp residue. Bands corresponding to the partial digestion products containing the modified

Table 3. T_1 RNase end products

Fragment number	Sequence	Molar yields	
		Observed	Expected
t1	pGp	0.7	1
t2	Gp + G>	7.7	10
t3	C-Gp	3.5	4
t4	A-Gp	0.8	1
t5	U-Gm-Gp	1.0	1
t6	A-A-Gp	0.9	1
t7a	s^4U-A-Gp	0.2	1
t7b		0.3	
t8	U-U-C-Gp	1.1	1
t9	T-Ψ-C-Gp	1.1	1
t10	A-A-U-C-C-Gp	0.9	1
t11	C-U-A-A-m^1A-C-Gp	0.7	1
t12	C-U-C-C-C-U-U-U-Gp	1.1	1
t13	A-C-U-Q-U-A-ms^2i^6A-A-Ψ-C-C-Gp	0.9	1
t14a	U-C-C-C-C-C-U-C-C-A-C-C-A_{OH}	0.4	1
t14b		0.4	

Table 4. Analysis of T_1 RNase end products

Fragment number	T_2 RNase products*					Pancreatic RNase products	5' terminal nucleotide
	Ap	Cp	Gp	Up	Others		
t1					pGp (+)		
t2			+				
t3		1.0	1.0			Cp (1.1), Gp (1.0)	Cp
t4	1.0		1.0			A-Gp	Ap
t5				(1)	Gm-Gp (2)	Up (1.0), Gm-Gp (1.5)	Up
t6	2.0		1.0			A-A-Gp	Ap
t7a	1.0		1.0	1.1			
t7b	1.1		1.0	1.2		Up (1.0), A-Gp (1.8)	Up
t8		1.1	1.0	2.1		Up (1.5), Cp (1.0), Gp (1.0)	Up
t9		(1)	(1)		Ψp (1), Tp (1)	Tp + Ψp (1.8), Cp (1.2), Gp (1.0)	-
t10	2.2	2.1	1.0	1.1		A-A-Up (1.6), Cp (1.6), Gp (1.0)	Ap
t11	2.1	2.3	1.0	1.4	m^1Ap 1.2	Cp (1.0), Up (0.9)	
						A-(A,m^1A)-Cp (1.8), Gp (1.0)	Cp
t12		3.6	1.0	4.0		Cp (2.1), Up (2.3), Gp (1.0)	Cp
t13	2.9	3.1	1.0	3.1	Qp 0.8	A-Cp (1.3), Up (1.0), Q-Up + Gp (1.2),	
					ms^2i^6Ap 0.6	A-(A,ms^2i^6A)-Ψp (1.7), Cp (1.2)	Ap
t14a	1.0	8.5		2.0		Up (2.1), Cp (4.9), A-Cp (2.0)	Up
t14b						Up (2.1), Cp (3.8), A-Cp (2.0)	-

* The yields of T_2 RNase products shown in parentheses were estimated visually

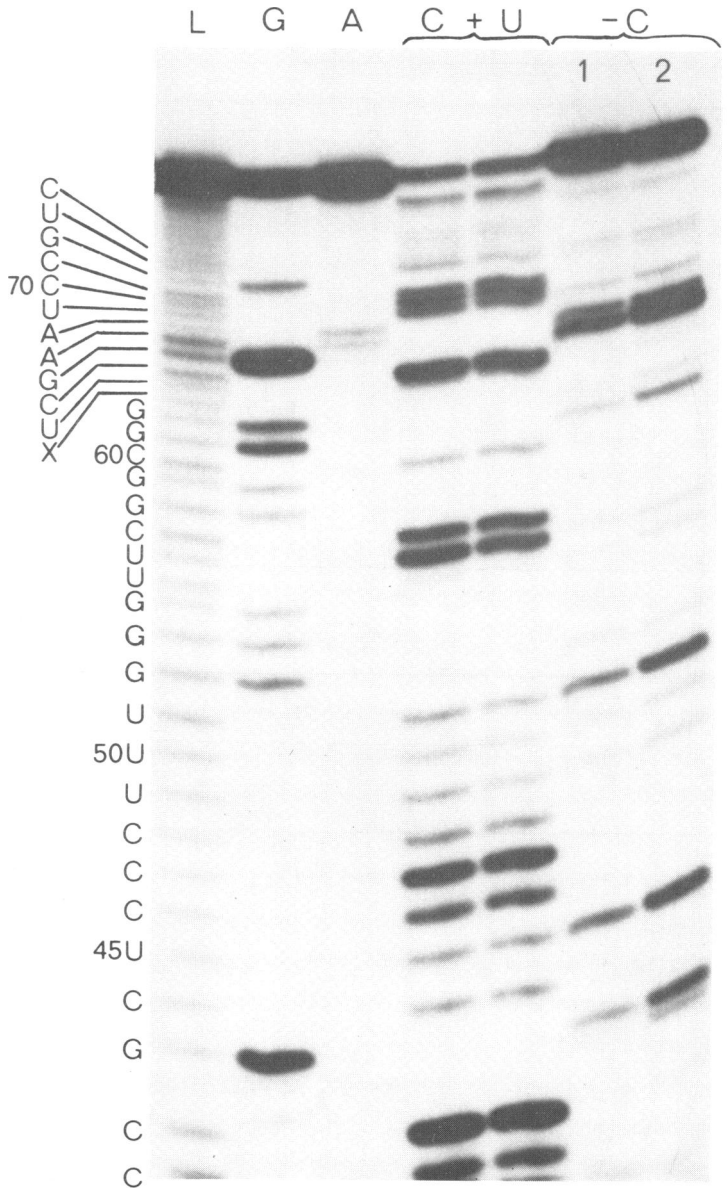

Figure 5a. Autoradiogram of the sequence analysis of a 5' ^{32}P-labelled fragment of $tRNA^{Tyr}$ in a 20% acrylamide-7 M urea sequencing gel as described in (13). L shows the ladder obtained by degradation of the fragment in hot formamide. Partial digestion was carried out under the conditions reported in (13) with RNase T_1 (G), U_2 (A), pancreatic (C + U) and Phy I (-C). Samples run in (-C) slots 1 and 2 were digested with Phy I RNase for 5 and 30 min respectively.

nucleotides Gmp, m^1Ap, Qp and Tp at the 3' end were invariably absent from sequencing gels. Those fragments terminating with ms^2i^6Ap and ψp were present in low yield. Thus the complete nucleotide sequence of $tRNA^{Tyr}$ can be derived from the information shown in Figures 5 except for the identification of the modified nucleotides and the last two residues at the 3' end. These are known, however, from the analysis of the end products of pancreatic and T_1 RNase digestion summarised in Tables 2 and 4.

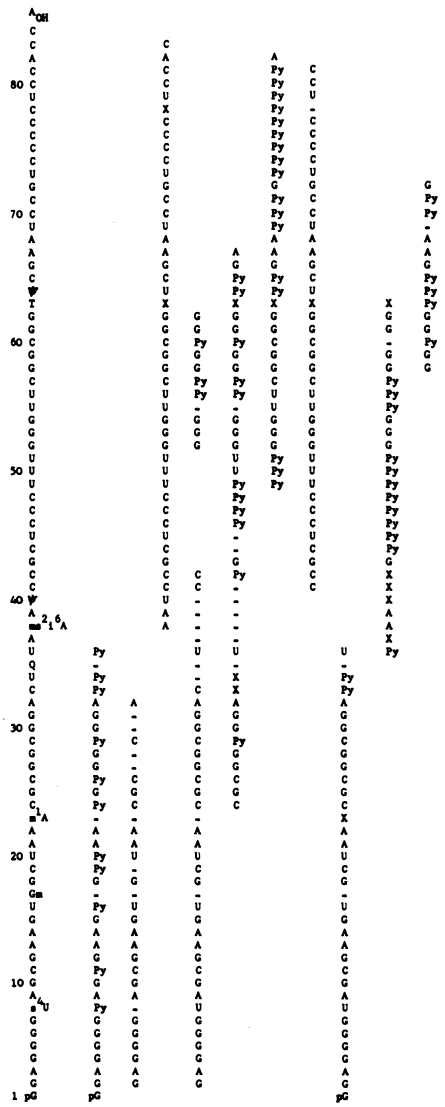

Figure 5b Tracts of nucleotide sequence from $tRNA^{Tyr}$ read off 20% polyacrylamide sequencing gels. X is unknown. - is a missing band.

DISCUSSION

The sequence of $tRNA^{Tyr}$ from B. stearothermophilus was found to be 85 nucleotides in length in common with that of $tRNA^{Tyr}_{su-III}$ from E. coli (2). Modified nucleotides occupy identical positions in both $tRNA^{Tyr}$ sequences with the exception of s^4U9 in E. coli and m^1A23 in B. stearothermophilus (see Figure 6). The presence of m^1A at position 23 as the 3' terminal nucleotide of the D-loop is unusual since it generally is found in the T-ψ-C-G loop in prokaryotic tRNA sequences (18). However, m^1A may also be present in the same position in the D-loop of $tRNA^{Tyr}$ from B. subtilis (G. Keith, personal communication). Recently a methyl transferase has been isolated from B. subtilis (19) which produces m^1A at the equivalent position in the D-loop of yeast $tRNA^{Tyr}$.

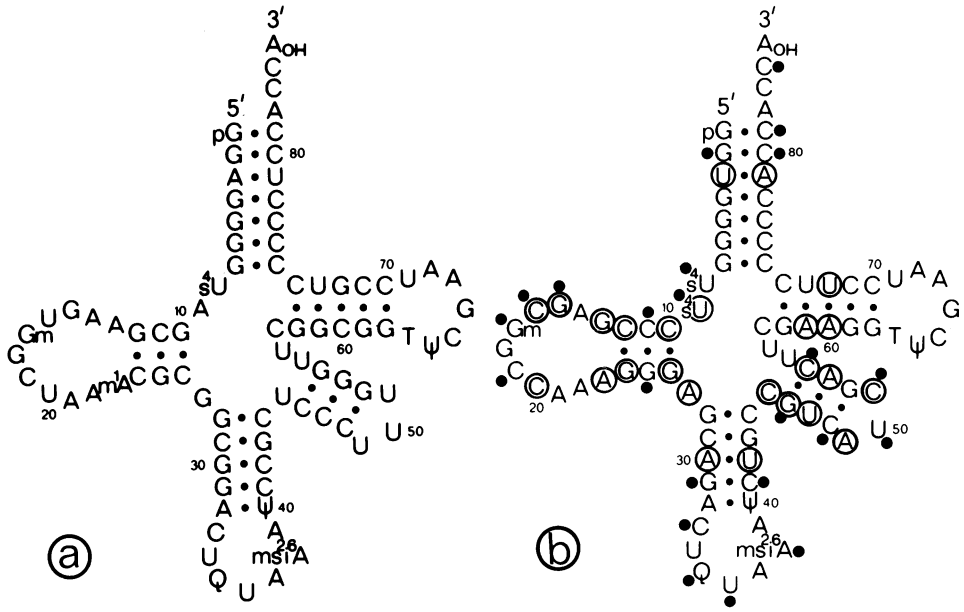

Figure 6. (a) The nucleotide sequence of $tRNA^{Tyr}$ from B. stearothermophilus arranged in the "cloverleaf" model. (b) Sequence comparison with E. coli $tRNA^{Tyr}_{su-III}$ (2). Differences in sequence are shown circled. Modifications in E. coli $tRNA^{Tyr}_{su+III}$ which do not prevent aminoacylation are marked by ● (20,21,22).

The primary structures of $tRNA^{Tyr}$ from B. stearothermophilus and E. coli differ in 25 residues (see Figure 6). Changes of sequence are localised

mainly in the D-stem and the large "extra arm" of the cloverleaf secondary structure. Considerable sequence homology exists in the amino acid acceptor stem, T-ψ-C-G arm and anticodon arm. The hypermodified nucleosides Q and ms^2i^6A are common to the anticodon loops of both $tRNA^{Tyr}$ sequences.

The cloverleaf secondary structure of $tRNA^{Tyr}$ from the thermophile B. stearothermophilus shown in Figure 6 contains an unusually high proportion of G-C base pairs. This is not the case, however, for $tRNA^{Phe}$ (15) and $tRNA_2^{Val}$ (16) from B. stearothermophilus. Furthermore the melting profile of unfractionated tRNA from B. stearothermophilus in 3.3 mM $MgCl_2$ (17) is comparable to that of tRNA from mesophilic microorganisms. These results suggest that tRNA from thermophilic microorganisms such as B. stearothermophilus does not possess an enhanced thermostability.

Many genetic and chemical modification studies have sought to determine the nature of the specific interaction between E. coli $tRNA^{Tyr}$ and its cognate synthetase (20,21,22). Figure 6 shows the nucleotides in E. coli $tRNA^{Tyr}_{su^+III}$ which may be altered either chemically or genetically without preventing enzymatic aminoacylation. Taken together with the differences in sequence between $tRNA^{Tyr}$ from E. coli and B. stearothermophilus it would seem to lead to the conclusion that conserved structural elements rather than specific nucleotide sequences are important in synthetase recognition. The results of a UV cross-linking study (23) suggest that parts of the D-stem, anticodon arm and large extra arm of E. coli $tRNA^{Tyr}$ are in close contact with the synthetase.

Many of the extra tertiary base pairs present in the three-dimensional structure of brewer's yeast $tRNA^{Phe}$ (24,25,26) may also be formed in $tRNA^{Tyr}$ from B. stearothermophilus. In particular the following are possible: s^4U8-A14, G15-C57, Gm17-ψ64, G18-C65, G27-U45 and T63-A67 in a manner analogous to the corresponding tertiary base pairs in yeast $tRNA^{Phe}$. A major difference, however, is the probable absence of tertiary base triplets in those tRNAs which contain a large extra arm (27). Model building suggests the possibility of additional base pairs between A22-U56 and m^1A23-U55 in B. stearothermophilus $tRNA^{Tyr}$ of the reverse Hoogsteen type, examples of which are U8-A14 and T54-m^1A58 in yeast $tRNA^{Phe}$. Thus the helix of the large extra arm may form a continuous stack through A22-U56 and m^1A23-U55 onto the Levitt base pair G15-C57. The probable involvement of residues U55 and U56 in extra tertiary base pairs is further supported by the result that in E. coli $tRNA^{Tyr}_{su^+III}$ these residues are not chemically modified by carbodiimide (28).

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