Structure and processing of yeast precursor tRNAs containing intervening sequences

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We have isolated a precursor of yeast tRNA^{Tyr} and shown that it contains an intervening sequence identical to that found in the gene for tRNA^{Tyr}. The conformation of pre-tRNA^{Tyr} is similar to that of mature tRNA^{Tyr} except for the anticodon loop. The loop is sensitive to endonucleolytic cleavage by S_1 nuclease near to the ends of the intervening sequence. This pre-tRNA is functionally inactive as it cannot be aminoacylated and the anticodon is not accessible for hydrogen bonding. A crude nuclear extract from yeast contains an excision-ligase activity which will process pre-tRNA^{Tyr} into mature tRNA^{Tyr}.

ANALYSIS of the genes coding for mouse[1] and rabbit globin[2], ovalbumin[3] and immunoglobulin[4] have resulted in the unexpected finding that these DNAs contain additional sequences which are not used in encoding the final gene product. Further evidence that genes are not necessarily co-linear with their gene products derives from experiments showing that several viral mRNAs contain sequences complementary to non-adjacent regions of their genomes[5–9]. We have also previously shown that, immediately to the 3′ side of their anticodon triplets, the yeast genes coding for tyrosine and phenylalanine tRNAs[10,11] contain a base pair tract, called an intervening sequence, that is not present in the mature tRNA.

There are eight unlinked genetic loci for tRNA^{Tyr} in yeast[12] and the nucleotide sequence of three have been determined[10]. One of these was identified and analysed as coding for the suppressor tRNA associated with the genetic locus *SUP* 4 (ref. 10). All three genes contained a 14 base pair intervening sequence, ATTTAYCACTACGA (Y = pyrimidine). Two genes (called G and C in plasmid pYT-G and pYT-C, respectively) had a C, and one gene (called A in plasmid pYT-A) had a T at the position indicated by Y. Analysis of the *SUP* 4 locus for tRNA^{Tyr} demonstrated that the intervening sequence was present in an 'active' gene, as this mutation confers a dominant phenotype on *SUP* 4–0 strains and the mutant gene contained the intervening sequence[10].

There are at least 10 unlinked tRNA^{Phe} genes in a tetraploid yeast strain[13]. The nucleotide sequence of four alleles has been determined to contain an intervening 18 or 19 base pair sequence, AAAAACTTCGGTCAAGTTA or AATACTTCGGTCAAGTTA (ref. 11). Thus, the intervening sequences in these two different tRNA genes have no obvious sequence homology or structure, yet both occur in a similar position in the tRNAs.

These findings indicate that the biosynthetic pathway for some if not all tRNAs and mRNAs must involve elimination of the intervening sequence. Various possibilities for the removal of the intervening sequence include DNA splicing, RNA polymerase jumping, or cleavage and ligation of RNA precursors. We report here evidence for the latter mechanism, as direct nucleotide sequence analysis indicates that one isolated RNA precursor to yeast tRNA^{Tyr} contains the intervening sequence. Using crude nuclei preparations from yeast, pre-tRNA^{Tyr} and several other precursor tRNAs have been processed into tRNA^{Tyr} and 4S size molecules, respectively. Evidence is presented that pre-tRNA^{Tyr} has an overall conformation similar to that of mature tRNA^{Tyr}, except in the anticodon stem. In contrast to mature tRNA, it cannot be aminoacylated. Evidence has been obtained by Knapp *et al.*[14] for the presence of intervening sequences in several yeast tRNA precursors and for their removal *in vitro* by yeast extracts. The existence of such evidence was communicated to us by John Abelson during our own investigations. Our results confirm and extend their observations.

Isolation of a precursor to tRNA^{Tyr} which contains the intervening sequence

The presence of intervening sequences in yeast tRNA genes suggested that yeast tRNA may be derived from a precursor containing the intervening sequence. Evidence has already been obtained for the transcription of intervening sequences in the case of the 15S precursor to globin mRNA (ref. 15).

To determine conditions which maximise synthesis of precursor RNAs we compared the amount of 4.5S ^{32}P-labelled RNA synthesised in a 15-min pulse at 37 °C in either wild-type yeast, mutant *ts*136 (refs 16, 17) which accumulates larger size RNAs at 37 °C (ref. 18, 47, and A. Hopper and F. Banks, personal communication), or in a cell-free extract of nuclei prepared from *ts*136 cells. The RNA prepared from mutant *ts*136 cells labelled *in vivo* synthesised the largest amount of 4.5S RNA (data not shown). We demonstrated that this extract contained a precursor for tRNA^{Tyr} by hybridisation to the DNA insert from clone pYT-G which contains the gene for tRNA^{tyr} in a 1.2 kilobase yeast *Eco*RI DNA fragment[10]. The hybridised RNA was isolated by chromatography on Agarose 5M (data not shown; RNA isolated as in ref. 19). RNA sequence analysis[20] of the hybridised RNA indicated that it was a precursor of tRNA^{Tyr}. This method, however, was not applicable to isolation and preparative quantities of pre-tRNA^{Tyr}.

Preparative separation and isolation of pre-tRNA^{Tyr} was achieved by polyacrylamide gel electrophoresis (Fig. 1). Comparison of the profile of separation of RNA isolated from wild type (Fig. 1*a*) and *ts*136 (Fig. 1*b*) yeast strains shows a significant increase in the number of species in the region of the gel between 5S RNA and tRNA in the mutant strain. Four of these species have been identified as precursors of tRNAs for tyrosine, phenylalanine, tryptophan and serine (see also ref. 14). The tyrosine and phenylalanine precursors were identified by hybridisation of the RNA eluted from the gel to filters containing cloned DNA specific for the tRNA^{Tyr} or tRNA^{Phe}

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genes, respectively. The tryptophan and serine precursors were identified by direct RNA sequence analysis (ref. 14 and M. J. Etcheverry, D. S. Colby and C. Guthrie, personal communication). As pre-tRNA^{Tyr} occurs in high yield and migrates in a relatively uncrowded region of the gel, it can be isolated in high purity by extraction from the 10% first dimension gel (right hand panel, Fig. 1*b*).

Pre-tRNA^{Tyr} isolated from the gel was further characterised by T_1 ribonuclease digestion and two-dimensional oligonucleotide separation (Fig. 2). The separation method used was electrophoresis on cellulose acetate paper at *p*H 3.5 in the first dimension and homochromatography on DEAE, thin layer chromatography plates, in the second dimension[20,21]. The partial sequence of each of the 18 T_1 oligonucleotides was determined after scraping and elution from the chromatography plates, by subsequent digestion with pancreatic RNase (RNase A) and separation on DEAE paper at *p*H 3.5 (ref. 22). A summary of these results is presented in Table 1. The sequence of oligonucleotides nos 1, 2, 3, 5, 6, 7, 8, 11 and 13 are uniquely determined from their RNase A digestion products. These sequences were confirmed and the remaining T_1 oligonucleotides identified by comparison of their positions on the fingerprint and their RNase A digestion products with the known sequences of tRNA^{Tyr} (ref. 23) and/or its gene[10]. Additional support for these assignments was obtained by the relative migration of the T_1 products on cellulose acetate followed by DEAE paper in 7% formic acid in the second dimension (data not shown; compare with ref. 24), and by localisation of each T_1 product to the 5′ or 3′ 'halves' of the molecule after S_1 nuclease digestion (see below, Fig. 7). The position of each T_1 product in the sequence is shown in Fig. 3.

Three oligonucleotides (15, 16, 17) occur in the precursor which are not present in the mature tRNA. These must arise from the intervening sequence,

intervening sequence
U
...GΨAAUUUACCACUACGAAAΨCUUGA...
T_1 oligonucleotides 16(C), 17(U) 15

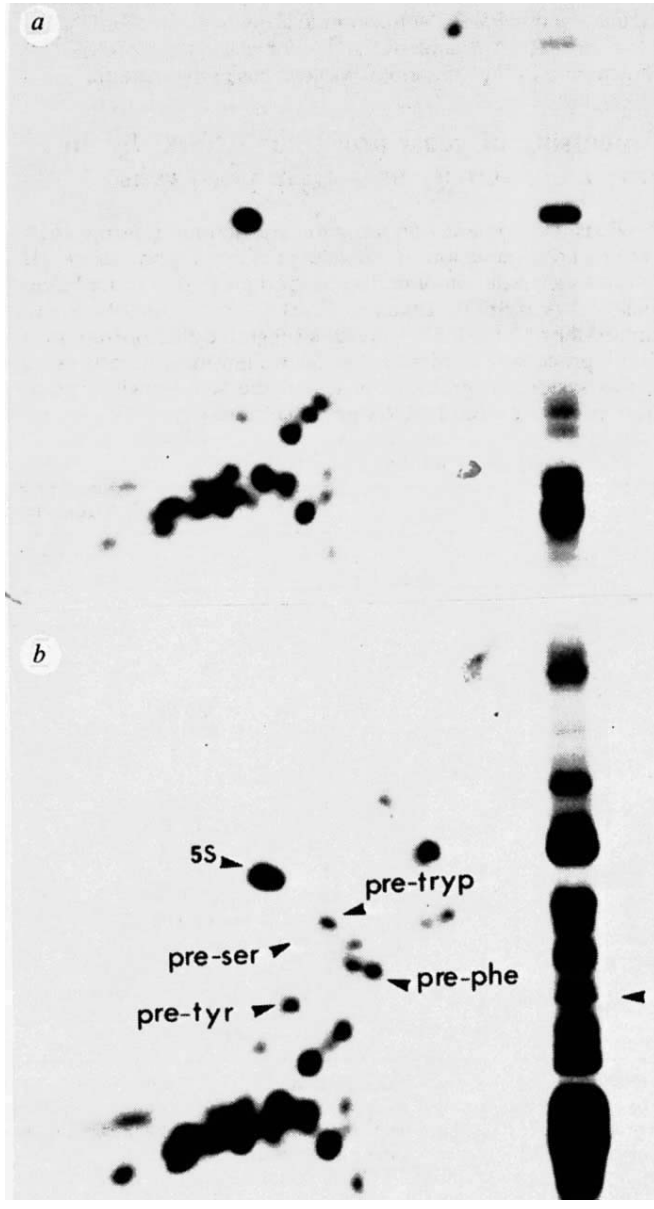


Fig. 1 Two-dimensional electrophoresis of low molecular weight ^{32}P-labelled RNA from wild-type and *ts*136 yeast. Either wild-type A364A or *ts*136 yeast cells[16] were grown at 25 °C to an optical density of 2.0 at 650 nm in YEPD medium[35], collected by centrifugation, washed in 10 mM Tris-HCl, *p*H 7.4, 1 mM EDTA, and resuspended in phosphate depleted YEPD medium[36]. The cells were grown at 25 °C for an additional 2 h, collected and resuspended in 10 mM Tris-HCl, *p*H 7.4, 1 mM EDTA, 2% glucose. Carrier-free ^{32}P-phosphate was added (at room temperature) to a concentration of 1 mCi ml^{-1} and the cells were immediately shifted to 37 °C. After a 15-min labelling period, the cells were chilled to 5 °C and centrifuged. The radioactive cell pellet was resuspended in one volume 100 mM NaCl, 10 mM Tris-HCl, *p*H 7.4, 1 mM EDTA, 0.5% sodium dodecyl sulphate (10 ml for a pellet derived from 50 ml of cell culture), one volume phenol saturated with 10 mM Tris-HCl, *p*H 7.4, and one volume chloroform. The mixture was placed at 68 °C with shaking for 15 min. The suspension was centrifuged, the aqueous phase removed, NaCl added to 0.3 M and the RNA precipitated with ethanol at −20 °C. The nucleic acid pellet was resuspended in 10 mM Tris-HCl, *p*H 7.4, 10 mM $MgCl_2$ and DNase (Worthington, and further purified as in ref. 37) was added to 20 μg ml^{-1} for 30 min at 37°C. After phenol extraction and precipitation by ethanol, the RNA pellet was resuspended in sample buffer for electrophoresis containing 4 M urea, 50 mM NaCl, 5 mM Tris-HCl, *p*H 7.4, 0.5 mM EDTA, 15% sucrose, 0.1% bromphenol blue and 0.1% xylene cyanol. Two-dimensional electrophoresis was carried out by a modification of the procedure of Fradin *et al.*[27]. The first dimension was a 10% acrylamide (19:1, acrylamide:bisacrylamide) separating gel 30 cm high containing 4 M urea in Tris-borate buffer, *p*H 8.3 (ref. 38) and a 5-cm high spacer gel containing 5% acrylamide, 4 M urea in Tris-borate buffer titrated with HCl to *p*H 6.8. Electrophoresis was for 19 h at 250 V. For the second dimension a strip of gel containing RNAs from 4S to 5.8S was embedded in a 20% acrylamide gel containing 4 M urea + Tris-borate buffer, *p*H 8.3, and electrophoresed for 3–4 d at 300 V with several changes of running buffer (Tris-borate, *p*H 8.3). The separation of ^{32}P-labelled low molecular weight RNA is shown for wild-type (*a*) and *ts*136 (*b*) cells. The separation achieved in the first dimension (10% gel) is shown on the right. Pre-tRNA^{Tyr} is indicated by an arrow in *b*. The second dimension is from right to left. Transfer RNA precursors of tRNA^{Tyr} and tRNA^{Phe} were identified by hybridisation of radioactively labelled RNAs to specific DNA probes for either tRNA^{Tyr} (pYT-G, ref. 10) or tRNA^{Phe} (ref. 11). DNA was immobilised onto membrane filters by the procedure of Landy *et al.*[39] (2 μg DNA per 5-mm diameter filter). Hybridisation was carried out in a volume of 100 μl containing 3×SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 10 mM HEPES, *p*H 7.0, and 1X Denhardt solution[40] at 37 °C for 24 h. The filters were washed three times at 37 °C with 2×SSC containing 0.5% SDS and the amount of ^{32}P-RNA hybridised was determined by Cerenkov counting. Pre-tRNA^{Ser} and pre-tRNA^{Tryp} were identified by direct sequence analysis carried out by Etcheverry, Colby and Guthrie (unpublished results and ref. 14).

Their sequence and position in the molecule proves that the intervening sequence in the gene is transcribed and is contained in the precursor. Sequence analysis of the tyrosine genes shows that the intervening sequences differ by a single base change (ref. 10, and see Fig. 3). This heterogeneity is consistent with the sequence of the precursor $tRNA^{Tyr}$; T_1 products 16 and 17 reflect the sequence change from a C to a U, respectively. The quantitation of these two oligonucleotides (Table 1) suggests that there are three genes with a 'C' in the intervening sequence and five genes with a 'T' (U in the RNA) if all eight tRNA genes have intervening sequences and all are transcribed with equal efficiency.

Although analysis of the minor bases occurring in pre-$tRNA^{Tyr}$ is incomplete (Table 1), evidence has been obtained for the following: dihydrouridylic acid (D) in T_1 oligonucleotides nos 9, 11 and 13; ribothymidylic acid (T) in no. 12; pseudouridylic acid (Ψ) in nos 12, 15, 16 and 17; and m^1A in 4. No 2′-*O*-methyl guanylic acid seems to occur, and hence oligonucleotide no. 11 is isolated as DDGp rather than DDGmGp as predicted from the sequence of $tRNA^{Tyr}$ (refs 23, 24). In general pre-$tRNA^{Tyr}$ is apparently undermethylated.

Although pre-$tRNA^{Tyr}$ is undermodified, both the 5′ and 3′ ends are already processed: the 5′ end does not contain additional nucleotides and the 3′ terminal CCA_{OH} has been added. Figure 3 summarises the nucleotide sequences for the $tRNA^{Tyr}$ gene, pre-$tRNA^{Tyr}$ and $tRNA^{Tyr}$. For clarity, pre-$tRNA^{Tyr}$ is shown (and in Fig. 8*b* below) without base modification.

Processing of yeast precursor tRNAs by an enzymatic activity present in yeast cells

Previous experiments on *in vitro* transcription using yeast nuclei and endogenous or exogenous RNA polymerase III showed accumulation of molecules of the size of but not larger than tRNA (4S) (P. Tekamp, G. Bell, P. V. and W. J. R., unpublished results). These results suggested that presumptive tRNA precursors of high molecular weight must be processed in this system. Therefore, we tested the same nuclear preparations as a source of tRNA processing activity.

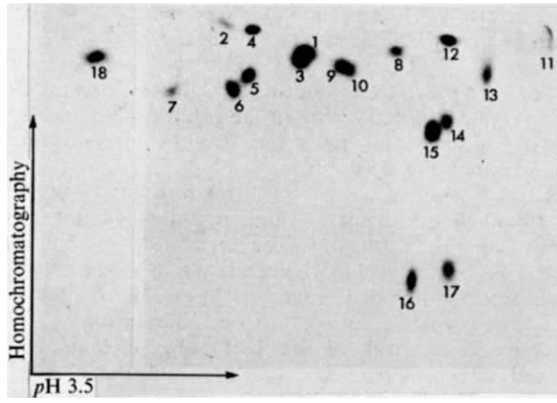


Fig. 2 T_1 RNase oligonucleotides from pre-$tRNA^{Tyr}$. The ^{32}P-labelled band corresponding to pre-$tRNA^{Tyr}$ was cut from a 10% gel and eluted by first grinding the dry gel into small pieces followed by addition of 10 mM Tris-HCl, *p*H 7.4, 1 mM EDTA, 0.3 M NaCl. The gel pieces were eluted overnight at 0 °C with shaking. The eluate was filtered through a nitrocellulose Millex filter (0.45 μm) and precipitated with ethanol. The RNA was hydrolysed by RNase T_1 (Calbiochem) and the resulting oligonucleotides were separated in two dimensions by electrophoresis on cellulose acetate at *p*H 3.5 (ref. 20), and homochromatography on DEAE cellulose at 60 °C using homomix B (ref. 21). The oligonucleotides are numbered 1–18 and are described in Table 1. As oligonucleotide 18 moved much more slowly than the other products in the first dimension, it was chromatographed on a separate homochromatography plate. Pre-$tRNA^{Tyr}$ used for the analysis was prepared as in Fig. 1 except that the cells were labelled to low specific activity as described in Table 2.

Eight different pre-tRNAs were processed *in vitro* to 4S molecules using extracts of nuclei prepared from wild-type yeast. Figure 4 shows the results using five different pre-tRNAs. The preparations of nuclei are reasonably free of nonspecific nucleases, as no degradation of 5S RNA occurs (Fig. 4, lanes 2 and 3).

Table 1 T_1 oligonucleotides of precursor $tRNA^{Tyr}$

Oligo-nucleotide no.	Molar yield Observed	Molar yield Theoretical	Pancreatic RNase products	Minor bases	S_1 nuclease fragment	Sequence
1	6.6	7	G		5′:3′, 3:4	Gp
2	2.1	2	C, G		5′:3′, 1:1	CGp
3	3.2	2	AG		3′	AGp
4	0.54	1	m^1AC, C, U, G	m^1A	3′	m^1ACUCGp
5	1.5	1	C, AAG		5′	CAAGp
6	1.3	1	C, AAG		5′	CCAAGp
7	0.47	1	C, G		3′	CCCCCGp
8	1.4	1	AG, U		5′	UAGp
9	0.96	1	AD, G, C	D	3′	ADCGp
10	1.1	1	AC, G, U		5′	ACUGp
11	1.5	1	DD, G	D	5′	DDGp
12	1.5	1	G, C, T, Ψ	T, Ψ	3′	TΨCGp
13	0.95	1	AAG, DD, D	D	5′	DDDAAGp
14	0.71	1	pCp, G, C, U		5′	pCUCUCGp
15	1.1	1	AAAΨ, G, C, U	Ψ	5′	AAAΨCUUGp
16	0.37		AAU, AC, C, G, U, Ψ	Ψ	—	ΨAAUUUACCACUACGp
17	0.57	1	AAU, AU, AC, C, G, U, Ψ	Ψ	—	ΨAAUUUAUCACUACGp
18	0.95	1	AC, C		3′	$ACCA_{OH}$

The individual oligonucleotides visualised in Fig. 2 were recovered from the homochromatography plate and counted in scrapers by Cerenkov radiation. The molar yields were calculated from the counts incorporated into each oligonucleotide relative to the average number of counts incorporated per phosphate. The composition of each oligonucleotide was determined by hydrolysis with RNase A and subsequent electrophoresis at *p*H 3.5 on DE81 paper[22]. Analysis of minor bases was carried out by T_2 ribonuclease digestion of the RNase A products and two-dimensional thin layer chromatography[33,34]. Oligonucleotides were ascribed to either the 3′ or 5′ side of pre-$tRNA^{Tyr}$ following treatment of the RNA with S_1 nuclease which cleaves tRNA into 'halves' (see Fig. 7). T_1 products 1 and 2 are found in both S_1 'halves' and their molar yields in each half are indicated. Although these data provide only partial sequence analysis, the actual sequence of pre-$tRNA^{Tyr}$ has been deduced by comparison with the sequence of $tRNA^{Tyr}$(refs 23, 24) and the sequence of the $tRNA^{Tyr}$ gene[10].

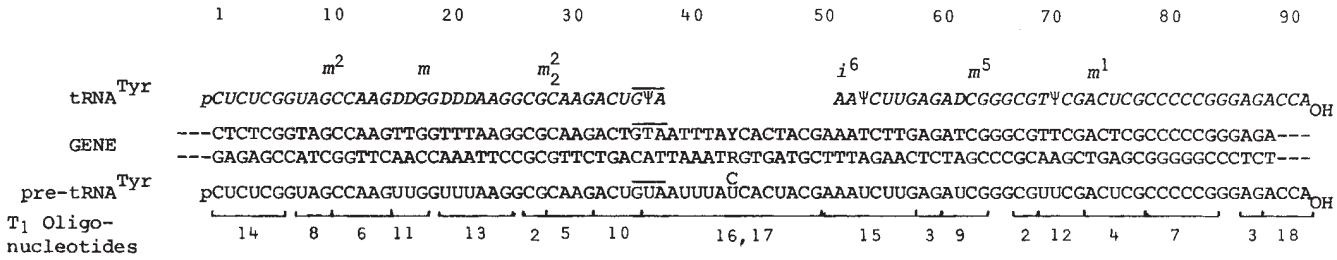
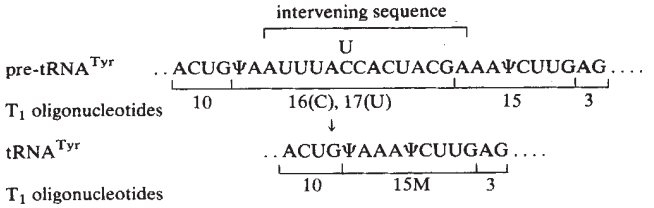


Fig. 3 The nucleotide sequence of yeast tRNA^{Tyr}, the tRNA^{Tyr} gene, and pre-tRNA^{Tyr}. Only the actual coding part of the gene is shown, although the nucleotide sequence on either side has been determined[10]. In addition, there is no nucleotide sequence in the gene for the 3′ terminal CCA_{OH} (ref. 10). The anticodon and its coding triplet are overlined in both RNA and DNA sequences. For simplicity, no minor bases are indicated for pre-tRNA^{Tyr} (see Table 1 for an estimate of their composition). The T_1 oligonucleotides 2–18, shown in Fig. 2 and described in Table 1, are indicated below the linear sequence of pre-tRNA^{Tyr}; T_1 product no. 1 (nucleotide G) is not shown for simplicity. The variable base pairs within the intervening sequence of the gene are indicated by Y (pyrimidine) and R (purine). The position of the intervening sequence displayed is the most reasonable considering the analysis of the conformation of pre-tRNA^{Tyr} in Fig. 7 below.

The processing reaction was optimised for processing activity with respect to NaCl, $MgCl_2$ and ATP concentrations (Fig. 5). The optimal conditions are 50 mM Tris-HCl (*p*H 8), 10 mM 2-mercaptoethanol, 8 mM $MgCl_2$, 160 mM NaCl and 0.8 mM ATP. The reaction is highly dependent on ATP although GTP seems to be able to replace ATP in this crude system. At equivalent concentrations, NaCl may be replaced by KCl, LiCl or NH_4Cl with identical activity. The reaction is inhibited by yeast tRNA.

The processing activity, tRNA excision-ligase, is found in extracts from broken yeast cells. All the enzyme can be recovered in soluble form following centrifugation of extracts at 100,000*g* in 0.5 M KCl (data not shown).

The mobility of the processed precursor tRNA on a denaturing polyacrylamide gel indicates proper excision of the intervening sequence and rejoining of the ends to form mature tRNA (Fig. 4). The fidelity of the *in vitro* reaction was further characterised using pre-tRNA^{Tyr} as a defined substrate. ^{32}P-labelled pre-tRNA^{Tyr} was treated with yeast nuclei and the resulting products analysed by electrophoresis (Fig. 4*b*). T_1 ribonuclease digestion and fingerprint analysis of the 4S RNA shows that it is authentic tRNA^{Tyr} (Fig. 6). All the T_1 oligonucleotides of tRNA^{Tyr} are present. T_1 oligonucleotides 16 and 17 which contain the intervening sequence of the precursor are absent (compare Figs 2 and 6). Oligonucleotide 15 is also absent and is replaced by 15M. The sequence of this T_1 product is ΨAAAΨCUUG (preliminary analysis suggests there is also modified A in this oligonucleotide) and is the critical oligonucleotide proving the precise excision of the intervening sequence and re-ligation to form mature tRNA^{Tyr}. This is schematically shown below.



The predicted intermediates from the *in vitro* processing of pre-tRNA^{Tyr} may also be produced in the *in vitro* reaction. These compounds are visible on the gel as discrete faster migrating bands labelled 'a' and 'b' (Fig. 4*b*). When band 'a' was recovered from the gel and re-electrophoresed in a 20% acrylamide gel it could be resolved into two species 38 and 40 nucleotides long. Subsequent fingerprint analysis of both fragments confirmed that they are the 3′ and 5′ 'halves' of the precursor following removal of the intervening sequence (data not shown). Band 'b' was shown by re-electrophoresis on a 20% gel to contain fragments of RNA similar in size to the intervening sequence excised from pre-tRNA^{Tyr}. The possibility that these compounds are selective degradation products of an S-1 like endonuclease activity in the extracts has not been eliminated.

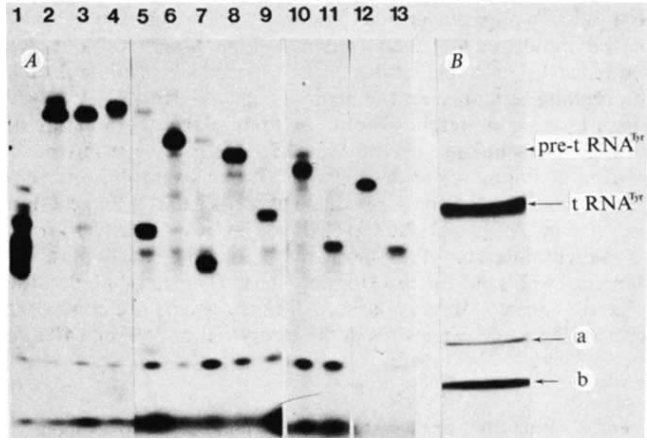


Fig. 4 Processing of yeast tRNA precursors to molecules of 4S size by an enzyme system from yeast nuclei. *A*, Assay of processing activity using several pre-tRNAs and 5S RNA as substrates. Yeast nuclei were isolated from strain B1279 (ref. 41) by the method of May described by Duffus[42] except that spheroplasts were lysed in a Parr bomb[43] at 800 p.s.i. The tRNA precursors used as substrates were isolated from *ts*136 yeast cells following pulse-labelling with ^{32}P and gel electrophoresis in a 10% gel as described in the legend of Fig. 1. Each assay tube contained approximately 5×10^4 c.p.m. of RNA, 50 mM Tris-HCl, *p*H 8, 8 mM $MgCl_2$, 0.8 mM ATP, 10 mM 2-mercaptoethanol, 160 mM NaCl and 5 μl of yeast nuclei (containing the equivalent of 1 μg DNA). After incubation at 27 °C for 25 min, the tubes were centrifuged (3,000*g*) to remove the nuclei and the supernatant was made 0.1% in SDS. After heating at 65 °C for 3 min the tube contents were loaded onto an 11% acrylamide slab gel prepared and run as described by Rubin[44]. 1, Total yeast 4S RNA; 2, 5S RNA; 4, pre-tRNA^{Leu} (J. Abelson, personal communication and ref. 14) 6, unidentified putative pre-tRNA; 8, unidentified putative pre-tRNA; 10, pre-tRNA^{Phe}; 12, pre-tRNA^{Tyr}. Lanes 3, 5, 7, 9, 11 and 13 correspond to the same RNAs as in lanes 2, 4, 6, 8, 10 and 12, respectively, but after incubation with the nuclei system described above. *B*, Preparative processing of pre-tRNA^{Tyr}. ^{32}P-labelled yeast pre-tRNA^{Tyr} (2×10^6 c.p.m.) was incubated in 0.05 M Tris-HCl, *p*H 8, 8 mM $MgCl_2$, 0.8 mM ATP, 10 mM 2-mercaptoethanol, 160 mM NaCl, and 40 μl of yeast nuclei (equivalent to 8 μg of DNA) in a total volume of 0.45 ml. After incubation at 27 °C for 30 min, the tube was centrifuged and the supernatant made 0.1% in SDS. After heating for 3 min at 65 °C, the sample was loaded in a preparative 11% acrylamide slab and electrophoresed as in *A*. RNA bands were located by autoradiography, excised and eluted as described in the legend of Fig. 1.

Conformation of pre-tRNA^{Tyr}

Susceptibility of an RNA molecule to S_1 nuclease digestion has been a successful method for assessing its conformation[25,26]. S_1 nuclease cleaves RNA predominantly in accessible single-stranded regions and in all tRNAs studied hydrolyses primarily at the anticodon loop and the 3′ single-stranded CCA_{OH} terminus producing oligonucleotides with 5′-phosphate termini.[25,26]

^{32}P-labelled pre-tRNA^{Tyr} was digested with S_1 nuclease using limiting conditions such that less than 50% of the molecules were cleaved and purified by gel electrophoresis. The size of the two fragments produced from pre-tRNA^{Tyr} was estimated to be 40–42 nucleotides for the faster migrating fragment and 52–54 nucleotides for the more slowly migrating component (data not shown). The size of the fragments indicates that the site of cleavage of pre-tRNA^{Tyr} is within the intervening sequence.

This conclusion was substantiated by T_1 ribonuclease digestion and fingerprinting of the two fragments obtained after S_1 digestion of pre-tRNA^{Tyr} (Fig. 7). The assignments of oligonucleotides were based on their position in the fingerprint (compare with Fig. 2), RNase A digestion (data not shown) and in certain cases minor base analysis (data not shown).

The fingerprint of the larger fragment (Fig. 7*a*) is unique in that only T_1 oligonucleotides known to arise from the '3′-side' of the molecule are seen (see also Fig. 3 and Table 1 for sequences). Oligonucleotides 16 and 17, which result from the intervening sequence and occur in uncleaved pre-tRNA^{Tyr} (see Fig. 2), are completely absent. In their place are a series of weaker spots in the area of spot 15. These arise from the 3′ portion of oligonucleotides 16 and 17. For example, the relative size and the RNase A products of spots a and b suggest that they are pUACCACUACGp and pUUACCACUACGp from oligonucleotide 16. Other spots in this region of the separation were not analysed but presumably arise from oligonucleotide 17 and contain U in place of C. All these results are consistent with major S_1 cleavage sites in the intervening sequence 40 and 41 nucleotides from the 5′ end of pre-tRNA^{Tyr}. These cleavages then give rise to a 3′ fragment 51 or 52 nucleotides long (Fig. 7*a*) and a 5′ fragment 41 or 40 nucleotides long. (Pre-tRNA^{Tyr} is 92 nucleotides long.)

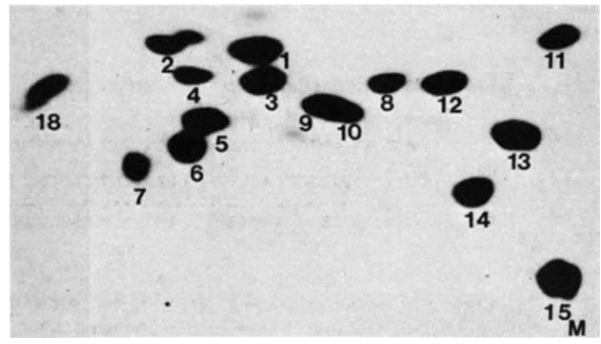
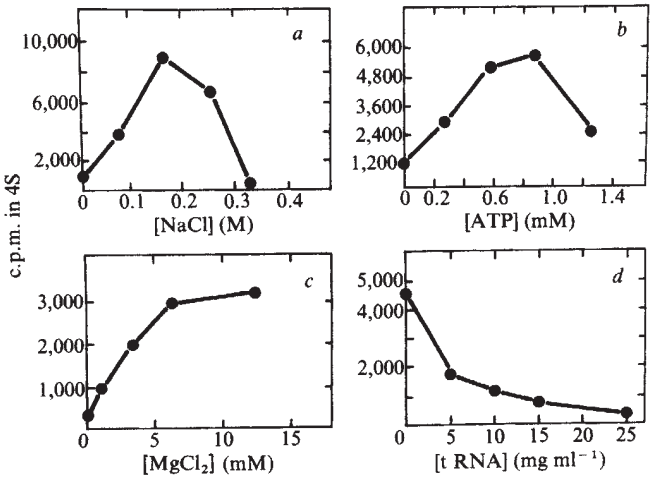
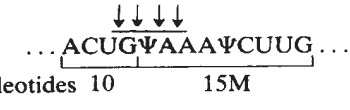


Fig. 6 T_1 RNase oligonucleotides from *in vitro* matured tRNA^{Tyr}. The band migrating in the region of mature tRNA^{tyr} in Fig. 4*b* was eluted, digested with T_1 RNase and the resulting oligonucleotides were resolved as described in Fig. 2. The oligonucleotides which are identical to those in Fig. 2 are labelled 1–14. A new oligonucleotide spot corresponding to the anticodon loop of mature tRNA^{Tyr} is labelled 15 M.

Fig. 5 Properties of a yeast enzyme system that removes intervening sequences from pre-tRNAs (excision-ligase activity). Yeast nuclei (containing the equivalent of 1 μg of DNA) were incubated in 50 mM Tris-HCl, *p*H 8, 10 mM 2-mercaptoethanol, with $2–5 \times 10^4$ c.p.m. of a mix of yeast ^{32}P-tRNA precursors (4.5S RNA) for 30 min at 27 °C. After electrophoresis and autoradiography, the bands migrating in the 4S region were excised and counted. *a*, Effect of [NaCl], tubes contained 0.8 mM ATP and 8 mM $MgCl_2$; *b*, effect of [ATP], tubes contained 160 mM NaCl and 8 mM $MgCl_2$; *c*, effect of [$MgCl_2$], tubes contained 0.8 mM ATP and 160 mM NaCl; *d*, inhibition by total yeast tRNA, tubes contained 0.8 mM ATP, 8 mM $MgCl_2$ and 160 mM NaCl.



Pre-tRNA^{Tyr} was processed into mature tRNA^{Tyr} (for example, Fig. 4*b*). After limited S_1 nuclease cleavage of this RNA the two fragments (35 and 43 nucleotides long) were isolated and digested with T_1 ribonuclease and fingerprinted (Fig. 7*c*, *d*). Analysis of the fragments as described above for pre-tRNA^{Tyr} shows that the larger fragments arise from the 3′ side of the molecule and the smaller fragment from the 5′ portion of the molecule. As the yield of oligonucleotides 10 and 15M are reduced, S_1 cleavage occurs at the anticodon as indicated below:



(The overlined sequence $\overline{G\Psi A}$ is the anticodon. S_1 nuclease cleavage is indicated by the arrows.)

These data are consistent with the secondary structure models of pre-tRNA^{Tyr} and tRNA^{Tyr} shown in Fig. 8. cleavage occurs in accessible single-stranded regions. However, the anticodon is protected in a hydrogen-bonded structure in the precursor (Fig. 8*b*), but is completely single-stranded in the mature tRNA (Fig. 8*a*).

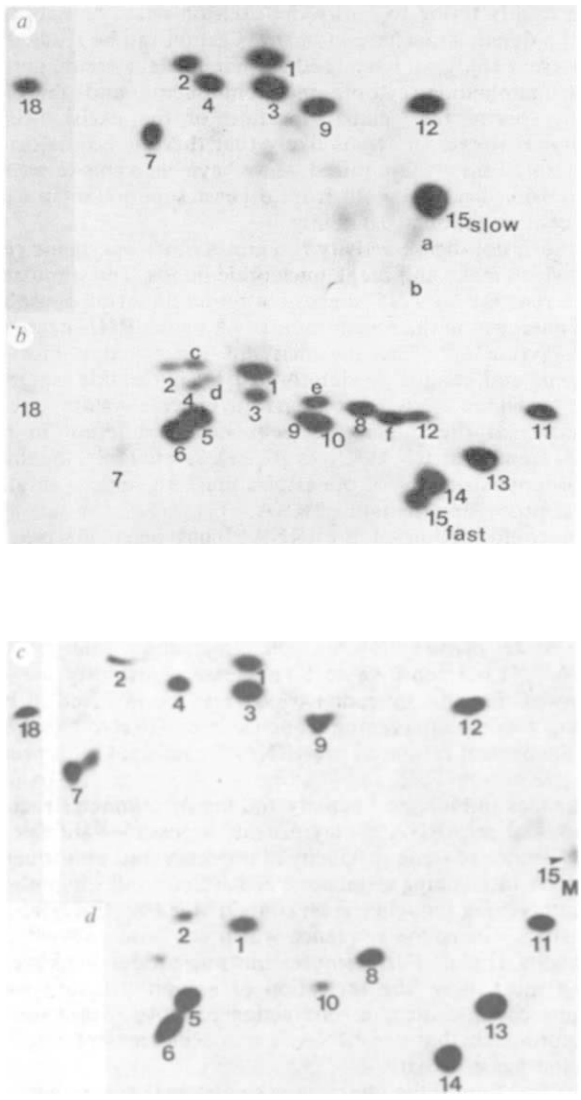


Fig. 7 Analysis of pre-tRNA^{Tyr} and tRNA^{Tyr} treated with S_1 nuclease. S_1 endonuclease was prepared by the method of Vogt[45] and further purified by filtration through Sephadex G75 according to Ando[46]. [^{32}P]pre-tRNA^{Tyr} or [^{32}P]tRNA^{Tyr} was digested with S_1 nuclease at room temperature for 2 h in a reaction mixture containing yeast RNA carrier at 100 μg ml^{-1}, 0.3 M NaCl, 30 mM Na acetate, *p*H 4.5, 3 mM $ZnCl_2$. The reaction was limited to 50% digestion of the RNA. RNA was extracted with phenol, precipitated with ethanol and electrophoresed in a 20% acrylamide gel containing 4 M urea and Tris-borate buffer, *p*H 8.3 (ref. 38). The S_1 cleavage products were located in the gel by autoradiography, excised and eluted as described in legend of Fig. 1. The fragments were hydrolysed with T_1 RNase and the oligonucleotides were separated in two dimensions as in Fig. 2. Individual oligonucleotides were identified by digestion with RNase A and/or RNase T_2 (ref. 22) and are numbered in correspondence with the sequence map presented in Fig. 3 for pre-tRNA^{Tyr} and Fig. 6 for tRNA^{Tyr}. *a*, The T_1 RNase oligonucleotides present in the more slowly migrating or larger S_1 fragment of pre-tRNA^{Tyr}. *b*, The T_1 RNase oligonucleotides present in the faster migrating or smaller S_1 fragment of pre-tRNA^{Tyr}. New T_1 oligonucleotide products in *a* and *b* are labelled a–f. a, b, e and f are discussed in the text. c And d have not been analysed in detail but are likely to be derived from S_1 cleavage at the 3′ terminus in oligonucleotide 18. Oligonucleotide 15 derived from the more frequent cleavage by S_1 is labelled '15 slow' and oligonucleotide 15 derived from the minor cleavage (by S_1) is labelled '15 fast'. *c*, The RNase T_1 oligonucleotides present in the larger S_1 fragment of tRNA^{Tyr}. Oligonucleotide 7 migrated along the edge of the homochromatography plate and appears as two spots. *d*, The RNase T_1 oligonucleotides present in the smaller S_1 fragment of tRNA^{Tyr}.

Pre-tRNA^{Tyr} cannot be aminoacylated *in vitro*

^{32}P-RNA labelled to a low specific activity was prepared and pre-tRNA^{Tyr} isolated in microgram amounts after electrophoresis in a 10% gel. The purity of pre-tRNA^{Tyr} was assessed by fingerprinting (Fig. 2).

Yeast aminoacyl-tRNA synthetases were prepared from wild-type yeast and the enzymes further purified by chromatography on DEAE Sephadex. The preparation was free of nuclease and processing activities, as degradation of precursor tRNA could not be detected after treatment with excess synthetase (data not shown).

The reaction conditions for aminoacylation of tRNA were optimised using highly purified tRNA^{Tyr} as substrate. In these conditions aminoacylation was complete. One mol of ^{3}H-tyrosine was added per mol of mature tRNA^{Tyr}. However, using identical conditions pre-tRNA^{Tyr} could not be aminoacylated (Table 2). The inability of pre-tRNA^{Tyr} to be aminoacylated was not due to contamination; unpurified mature tRNA^{Tyr} isolated from the same gel as the precursor was a good substrate (Table 2). Mixing experiments also indicated that the pre-tRNA^{Tyr} preparation was not an inhibitor of the reaction (Table 2).

Table 2 Inability of pre-tRNA^{Tyr} to be aminoacylated

	^{3}H-Tyrosine c.p.m. incorporated§	
	Expt 1	Expt 2
No RNA	2,300	1,714
tRNA^{Tyr}*	58,000	77,203
tRNA^{Tyr}†	11,299	10,349
pre-tRNA^{Tyr}	1,426	971
pre-tRNA^{Phe}‡	1,601	1,083
tRNA^{Tyr}* + tRNA^{Tyr}†	68,768	50,487
tRNA^{Tyr}* + pre-tRNA^{Tyr}	71,952	52,409
tRNA^{Tyr}* + pre-tRNA^{Phe}‡	64,167	—
tRNA^{Tyr}† + pre-tRNA^{Tyr}	8,695	8,963

Aminoacyl-tRNA synthetases were prepared from 10 g of yeast cells collected in mid-log growth and lysed by grinding with a mortar and pestle in the presence of alumina. The extract was digested for 5 min with DNase (1 μg ml^{-1}) at room temperature in 50 mM Tris-HCl, *p*H 8.0, 5 mM $MgCl_2$ and 50 mM KCl. After low speed centrifugation, the crude extract was adjusted to 1 mM dithiothritol (DTT), and centrifuged at 48,000 r.p.m. for 90 min in a Ti50 rotor. The supernatant was brought to 50 mM Tris-HCl, *p*H 7.9, 50 mM ammonium sulphate, 0.5 mM EDTA, 1 mM DTT, 25% glycerol and loaded onto a DEAE-Sephadex column (1.2 cm × 10 cm) equilibrated with the same buffer without glycerol. The column flow through was collected and precipitated overnight at 0 °C in 70% ammonium sulphate. The precipitate was resuspended in 2 ml, 50 mM Tris-HCl, *p*H 7.9, 0.5 mM EDTA, 1 mM DTT and 50% glycerol. Aminoacylation of tRNAs was carried out in 10 μl reactions containing 0.04 M Tris-HCl, *p*H 7.9, 0.02 M $MgCl_2$, 0.04 M KCl, 5 mM ATP, 10 mM ^{3}H-tyrosine (specific activity of 42 Ci mmol^{-1}) and either 100 ng (or 200 ng) tRNA for reactions containing one (or two) species of tRNA (or pre-tRNA). Using these conditions, aminoacylation is not limited by substrate. After incubation at 37 °C for 10 min, aliquots were removed, spotted onto Whatman 3MM paper and precipitated with cold 10% trichloroacetic acid (TCA). Following several washes with 5% cold TCA, the paper was dried and counted by liquid scintillation. Pre-tRNA^{Tyr}, pre-tRNA^{Phe} and unpurified tRNA^{Tyr} were purified from cells labelled at 0.1 mCi ^{32}P-phosphate per ml.

* Pure tRNA^{Tyr}

† Unpurified tRNA^{Tyr} isolated from same 10% acrylamide gel as pre-tRNA^{Tyr}.

‡ Pre-tRNA^{Phe} isolated from same 10% acrylamide gel as pre-tRNA^{Tyr} and tRNA^{Tyr}.

§ Counting efficiency of ^{3}H-tyrosine on Whatman 3MM paper was determined to be 3%.

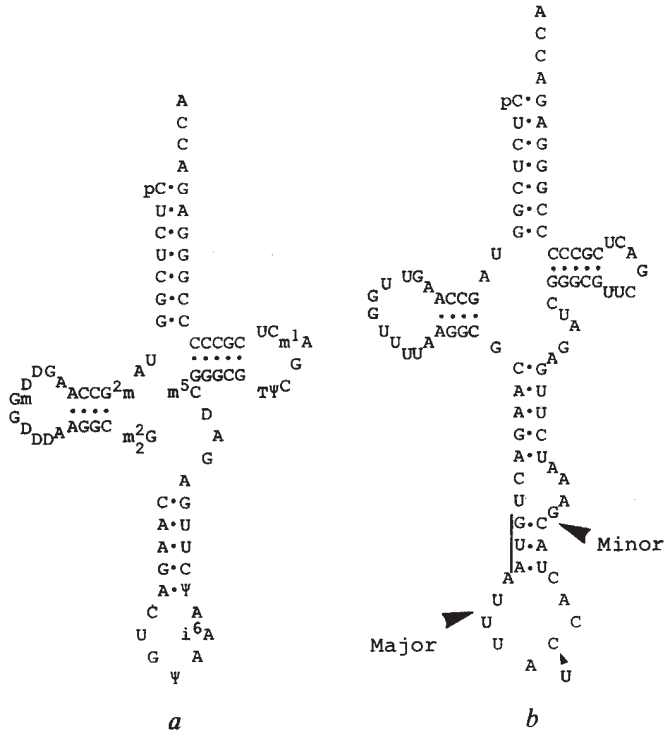


Fig. 8 Secondary structure of $tRNA^{Tyr}$ and pre-$tRNA^{Tyr}$. $tRNA^{Tyr}$ (*a*) is shown as previously described[30,31], and pre-$tRNA^{Tyr}$ (*b*) is shown as predicted from the data presented. The area of the major and minor sites of S_1 cleavage are indicated by arrows. The single base change in the intervening sequence corresponding to different $tRNA^{Tyr}$ genes is also indicated. None of the minor bases in pre-$tRNA^{Tyr}$ are indicated. The anticodon triplet is overlined in *b*.

Discussion

The presence of tRNA precursors in yeast has been reported earlier[27]. It was previously tacitly assumed that pre-tRNAs contain additional sequences at the 5′ and 3′ ends of the molecules as had been determined for *Escherichia coli*[28] and T4 bacteriophage[29] tRNA precursors. We have analysed in detail a particular precursor, pre-$tRNA^{Tyr}$, and have shown that it contains an internal sequence identical to the intervening sequence found in the gene for $tRNA^{Tyr}$, but no additional nucleotides at the termini. Presumably, the end of the primary gene transcript are processed rapidly during synthesis of pre-$tRNA^{Tyr}$ to remove any additional 5′ sequence and to add the 3′ terminal CCA. Thus, the removal of the intervening sequence is apparently a late step in the processing pathway for tRNA at least in the mutant *ts*136. Perhaps the excision-ligase activity which removes intervening sequences is defective in *ts*136 cells.

It is not known what fraction of tRNA genes contains intervening sequences. We can detect about 10 species of RNA which may be precursor tRNAs (see Fig. 1). The *in vitro* processing of all of these pre-tRNAs suggests that they all contain intervening sequences. Although some precursors may not be resolved from each other by the electrophoretic separation, it nevertheless seems that not all tRNAs can be synthesised from precursors which accumulate in the *ts*136 cells used in this study. The physiological reason that some pre-tRNAs contain intervening sequences and others do not is not yet known. There may even be different routes for the synthesis of isoaccepting tRNAs, as it has been shown that $tRNA^{Arg}$, $tRNA^{Asp}$ and some genes for $tRNA^{Ser}$ do not contain intervening sequences (ref. 14 and G. Page, personal communication).

The ability to process pre-tRNAs to mature 4S RNAs *in vitro* provides a model system to study processing of pre-RNAs. We are currently trying to purify the excision-ligase activity such that the detailed mechanism of the reaction can be studied. In the present study we have used a crude nuclei system containing contaminating cytoplasmic components and therefore cannot specify the cellular location of the excision-ligase activity. However, it seems likely that there is no major cell structural component required, as we have been able to recover the excision-ligase activity from a yeast supernatant fraction after centrifugation at 100,000*g*.

The excision-ligase activity resembles other enzymatic reactions which make and break nucleotide bonds. The stimulation of the reaction by ATP suggests a requirement of phosphate bond energy as in the reactions of DNA ligase, RNA ligase and DNA gyrase[30–32]. The specificity of the reaction must be stringent and cannot be determined by nucleotide sequence alone (provided there is not a separate enzyme system for each sequence) as the intervening sequence is different in pre-$tRNA^{Tyr}$ and pre-$tRNA^{Phe}$ (refs 10, 11). Presumably, the three-dimensional structure of pre-tRNA must be such as to allow precise processing to mature tRNA.

The conformation of pre-$tRNA^{Tyr}$ has been analysed by determining the products of cleavage by S_1 endonuclease and its structure is in general similar to that of mature $tRNA^{Tyr}$, as the extended anticodon loop is sensitive to S_1. However, in contrast to mature $tRNA^{Tyr}$ the anticodon triplet of pre-$tRNA^{Tyr}$ is not sensitive to S_1 nuclease. Previously, we had proposed that the anticodon triplet may be involved in base pairing with the intervening sequence in pre-$tRNA^{Phe}$ (ref. 11), and the present results on pre-$tRNA^{Tyr}$ conform to this prediction (see also ref. 14).

The lack of biological activity and the determined structural features of pre-$tRNA^{Tyr}$ may provide a possible rationale for the existence of some specificity of sequence and conformation in tRNA intervening sequences. According to this hypothesis, the intervening sequence must contain at least a three-nucleotide stretch or codon sequence which can base pair with the anticodon triplet. Furthermore, the nucleotides flanking the codon must allow the formation of a loop structure which permits codon–anticodon interaction and provides a tertiary structure such that pre-tRNA is a specific substrate for the excision-ligase activity.

The function of the intervening sequence in the pathway for the production of biologically active tRNA is not fully understood. However two features must be accounted for in any hypothesis; pre-$tRNA^{Tyr}$ cannot be aminoacylated until processed to $tRNA^{Tyr}$, and the anticodon is not accessible for hydrogen bonding. A possible function of the intervening sequence is that other processing and maturation of tRNA may be completed before removal of the intervening sequence and the assumption of biological activity. Of course, there remains the possibility that pre-tRNAs containing intervening sequences have other specific biological functions in the regulation of cell metabolism.

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A specific glycoprotein as the target site of adhesion blocking Fab in aggregating *Dictyostelium* cells

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During the acquisition of aggregation competence a new antigen appears on the surface of Dictyostelium *cells. Univalent antibody fragments (Fab) against this antigen render the cells unable to form the specific type of cell adhesion which is characteristic of aggregating cells. This membrane constituent has been purified and identified as a concanavalin A-binding glycoprotein present at about* 2×10^5 *copies per cell.*

THE molecular basis of cell-to-cell adhesion is being investigated in a variety of systems including sponge cells[1], embryos of sea urchins and mammals[3], sexually differentiated cells of algae[4] and yeasts[5], and in fibroblasts[6,7]. Since the studies of Moscona[8] much work has been concentrated on the reaggregation of trypsin-dissociated cells of the chicken neuroretina[9–11]. Investigation of the molecular basis of cell recognition and adhesion in these different organisms and cell types may reveal some general principles underlying cell interactions[12]. Compared to animal tissues *Dictyostelium discoideum* has the advantage for aggregation studies that single cells are accessible without trypsin treatment. *D. discoideum* can be grown in fermenters, where the cells acquire the ability to aggregate within several hours after the end of nutrient supply. Large quantities of homogeneous, aggregating cells are thus available.

Two types of cell assembly during aggregation

During aggregation the cells of *D. discoideum* elongate and become strongly adhesive at their ends, but they also assemble side-by-side. Both end-to-end and side-by-side adhesion can be completely blocked by univalent antibody fragments (Fab) directed against membrane antigens of aggregation competent cells[13]. The two types of cell assembly are blocked by Fab molecules of different specificities[14]. The corresponding target antigens have been referred to as contact sites A and B (Fig. 1). End-to-end adhesion can also be uncoupled from side-by-side adhesion by EDTA which selectively inhibits the latter[15].

Fab molecules of specificities other than those directed against 'contact sites' do not impair cell adhesion, even when they are bound at up to 2×10^6 molecules per cell to all parts of the cell surface[16]. Of particular importance is the finding that non-blocking Fab remains present at the actual areas of contact between the ends of adjacent cells, as shown by fluorescent-labelled Fab[16]. This observation suggests that Fab molecules of $35 \times 35 \times 60$ Å size can fit within the space between contiguous surfaces without impairing cell adhesion.

Purified glycoprotein reverses adhesion-blocking effect of Fab

Contact sites A (cs-A) are defined as the developmentally regulated target sites of Fab molecules that block the end-to-end attachment of aggregating cells. Cs-A can be specifically assayed by absorption of adhesion blocking Fab. The Fab is titrated with living, aggregation competent cells in the presence of 10 mM EDTA which suppresses cs-B activity almost completely. Absorption of Fab with cs-A containing fractions decreases the adhesion blocking activity, and this decrease can be quantitated by retitration of the Fab with living cells[14]. This immunoassay does not depend on the availability of monospecific antisera, since it measures blockage of a specific function of the cells by certain Fab species, rather than the total number of Fab molecules bound to the cell surface. Neither does the assay depend on the preservation of contact sites in a biologically active state since only their antigenic specificity is crucial. On these grounds the assay proved to be suitable for the purification of cs-A from cell membranes of *D. discoideum*[17] and of a comparable antigen in neuroretina cells[18].

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