The tRNA\textsuperscript{Tyr}-isoacceptors and their genes in the ciliate \textit{Tetrahymena thermophila}: cytoplasmic tRNA\textsuperscript{Tyr} has a Q\textsuperscript{Ψ}A anticodon and is coded by multiple intron-containing genes

Volker Junker, Thomas Teichmann*, Armin Hekele§, Christiane Fingerhut and Hildburg Beier*

Institut für Biochemie, Bayerische Julius-Maximilians-Universität, Biozentrum, Am Hubland, D-97074 Würzburg, Germany

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ABSTRACT

In the ciliated protozoa \textit{Tetrahymena thermophila} introns have been detected in rRNA and mRNAs until now. We have isolated and sequenced seven tRNA\textsuperscript{Tyr} genes from the \textit{T.thermophila} nuclear genome. All of these genes contain introns of identical length and sequence. The 11 bp long intervening sequences are located 1 nt 3’ to the anticodon as found in other eukaryotic nuclear tRNA genes. \textit{Tetrahymena} tRNA\textsuperscript{Tyr} genes are efficiently transcribed in HeLa cell nuclear extract. Moreover, processing and splicing occurred in HeLa as well as in wheat germ extracts, supporting the notion that \textit{Tetrahymena} tRNA\textsuperscript{Tyr} introns can be classified as authentic tRNA introns. We have also isolated cytoplasmic tRNA\textsuperscript{Tyr} from \textit{Tetrahymena} cells. This tRNA\textsuperscript{Tyr} isoacceptor has a Q\textsuperscript{Ψ}A anticodon and is not a UAG suppressor as shown in \textit{in vitro} translation studies. Since UAG and UAA codons are used as glutamine codons in \textit{Tetrahymena} macro-nuclear DNA, the presence of a strong natural UAG suppressor such as tRNA\textsuperscript{Tyr} with Q\textsuperscript{Ψ}A anticodon should cause misreading of the glutamine as tyrosine codons and the absence of the latter had thus been predicted. Furthermore we have studied the organization of tRNA\textsuperscript{Tyr} genes in the genome of \textit{T.thermophila} and have found two types of tRNA\textsuperscript{Tyr} gene arrangement. A minimum of 12 tRNA\textsuperscript{Tyr} genes are present as single copies in genomic DNA HindIII restriction fragments ranging in size from 0.6 to 7 kb. Additionally one cluster of tRNA\textsuperscript{Tyr} genes consisting of six members has been detected in a 2.3 kb HindIII fragment.

INTRODUCTION

Ciliated protozoa emerged as an evolutionary group more than 10\textsuperscript{9} years ago before the appearance of fungi, plants and animals (1). Despite their great genetic diversity, ciliates have in common the presence of nuclear dimorphism: a germ-line micronucleus, which is transcriptionally inactive and a vegetative macronucleus which is responsible for the transcription. Some ciliates, such as \textit{Tetrahymena}, \textit{Stylonichia} and \textit{Paramecium} use an altered genetic code. It was reported by several workers independently that a number of macronuclear genes in these ciliates contain internal TAA and TAG codons. A comparison of the derived amino acid sequences with other known homologous protein sequences revealed that these two codons which are stop codons in the universal genetic code appear to be used as glutamine codons (2,3). This assumption was confirmed when two unusual glutamine tRNA isoacceptors with CUA and UmUA anticodon, in addition to the normal tRNA\textsuperscript{Gln}UmUG, were purified from \textit{Tetrahymena thermophila} cells (4,5). Moreover it was demonstrated that tRNA\textsuperscript{Gln}UmUA reads the UAA and UAG codons \textit{in vitro}, whereas tRNA\textsuperscript{Gln}CUA recognizes only UAG (5), indicating that \textit{Tetrahymena} uses UAA and UAG as glutamine codons and that UGA may be the only functional termination codon employed by this organism in the nuclear genome.

We have previously shown that tobacco and wheat cytoplasmic tRNA\textsuperscript{Tyr} with GΨA anticodon is a powerful natural UAG and to a lesser extent a UAA suppressor, whereas tRNA\textsuperscript{Tyr} with QΨA or GU anticodon is unable to interact with these two stop codons (6–8). Since UAG and UAA are sense codons in \textit{Tetrahymena} mRNAs, we postulated that a tRNA\textsuperscript{Tyr} isoacceptor with GΨA anticodon should cause misreading of the glutamine as tyrosine codons and that UGA may be the only functional termination codon employed by this organism in the nuclear genome.

The synthesis of pseudouridine in the centre of the tRNA\textsuperscript{Tyr} anticodon, i.e., Ψ\textsubscript{35} has been shown to be dependent on the presence of an intron in the corresponding tRNA precursors in yeast (9), animals (10,11) and plants (8). Until now, intron-containing tRNA genes have not been found in the nuclear genome of ciliated protozoa. For that reason it seemed to be of general interest to learn if tRNA\textsuperscript{Tyr} genes from \textit{Tetrahymena} accommodate intervening sequences. We first used tRNA\textsuperscript{Tyr}-specific sequences in order to amplify tRNA\textsuperscript{Tyr} genes present in the \textit{Tetrahymena} nuclear genome and subsequently isolated seven full-length tRNA\textsuperscript{Tyr} genes and a single truncated tRNA\textsuperscript{Tyr} gene

*To whom correspondence should be addressed. Tel: +49 931 888 40 31; Fax: +49 931 888 40 28

Present addresses: * MPI für Züchtungsforschung, Carl-von-Linné Weg 10, D-50829 Köln, Germany and §Boehringer-Manheim GmbH, Nonnerwald 2, D-82377 Penzberg, Germany
from a genomic library. All of the seven tRNA\textsuperscript{Tyr} genes contain identical 11 bp long intervening sequences. Furthermore, we show here that Tetrahymena tRNA\textsuperscript{5Sr} genes are transcribed in HeLa cell nuclear extract and that intron-containing tRNA\textsuperscript{5yr} precursors are processed and spliced in HeLa as well as in wheat germ extracts.

**MATERIALS AND METHODS**

**Enzymes and reagents**

Restriction endonucleases, nuclease P1, calf intestinal alkaline phosphatase, T4 RNA ligase, T4 DNA ligase and T4 polynucleotide kinase were from Boehringer, Mannheim. [\(^3\)H]Tyrosine with a specific activity of 1 Tbq/mmol was obtained from Amersham Buchler (Braunschweig) and all other radiochemicals were from Hartmann Analytic, Braunschweig. The Sequenase kit (7-deaza-dGTP version) from USB was used for sequencing reactions. Untreated wheat germ was a gift from SynPharma (Frankfurt) and all other radiochemicals were obtained from Amersham Buchler (Braunschweig) and all other radiochemicals were obtained from Amersham Buchler (Braunschweig).

**Bacterial strains and plasmids**

*Escherichia coli* JM109 was used as a host for propagation of plasmid DNA. The recombinant plasmid pAy3II carries an *Arabidopsis thaliana* tRNA\textsuperscript{Ile} gene on a 1.4 kb RsoI fragment cloned into pUC19 DNA (12). *Escherichia coli* DH5\textsuperscript{α} was purchased from Biozym.

**Tetrahymena tRNA and aminocyl-tRNA synthetase**

Unfractionated tRNA and aminocyl-tRNA synthetase from *Tetrahymena* were isolated from the cells of *Thermophila*, mating type IV essentially as described by Kuchino et al. (4). Fractionation of tRNAs by BD-Cellulose column chromatography, purification of tRNAs by successive polyacrylamide gel electrophoresis and sequencing of tRNA by post-labelling techniques was carried out according to Beier et al. (6) and Zerfass and Beier (8).

**Isolation of DNA from Tetrahymena**

About 10\textsuperscript{8} cells from a fast growing culture of *Thermophila*, mating type IV were harvested by centrifugation, washed with cold 10 mM Tris–HCl, pH 7.4 and suspended in 1 ml of the same buffer. The cell suspension was mixed with 10 ml lysis buffer (10 mM Tris–HCl, pH 7.4, 0.1 M Na\textsubscript{2}EDTA, 1% SDS) and 6 ml phenol and incubated for 10 min at 60°C, then twice extracted with chloroform/isoamylalcohol (24:1). The aqueous phase was recovered, dialyzed against SSC and subsequently treated with 100 µg RNase A/ml and 100 µg proteinase K/ml.

**Synthesis and labelling of oligodeoxyribonucleotides**

Oligonucleotides were synthesized with the Gene Assembler Plus from Pharmacia LKB. They were end-labelled with [\(^32\)P]ATP using T4 polynucleotide kinase and purified by using Nucleobond AX 5 columns (Macherey-Nagel, Düren). Labelled DNA fragments of high specific activity were prepared by using the random primed DNA labelling kit from Boehringer, Mannheim.

**DNA amplification by polymerase chain reaction (PCR)**

The amplification reaction was carried out in a total volume of 100 µl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.1% gelatin, 200 µM each of dGTP, dATP, dTTP and dCTP, 1 µM of each primer: (Tyr 1: 5′-CCGAACTTAGCT-CAGTTGG-3′, identical to nucleotides 1–19 and Tyr 2: 5′-TCCGTTCTCCGGGTTTG-3′, complementary to nucleotides 56–73 of Tetrahymena tRNA\textsuperscript{5yr} shown in Fig. 1B), 100 ng genomic DNA from *Thermophila* and 2.5 U AmpliTaq\textsuperscript{TM} DNA polymerase (Perkin Elmer Cetus). The mixture was subjected first to a 3 min denaturation at 95°C, followed by 30 cycles of 60 s at 95°C, 60 s at 55°C and 60 s at 72°C in a Perkin Elmer Cetus Thermocycler.

**Isolation of genomic clones**

Approximately 100 µg of genomic DNA from *Thermophila* was digested with HindIII and the fragments were separated on a preparative 0.8% agarose gel employing Seakem GTG agarose from Biozym. HindIII fragments of 2.0–2.3 kb were isolated by electroelution and ligated into pUC19 DNA which had been cleaved with HindIII and treated with alkaline phosphatase. The DNA mixture was used to transform the *E.coli* strain DH5α. Colony screening was carried out with [\(^32\)P]-labelled oligonucleotides as probes. Prehybridization of the nitrocellulose filter was in 6× SSC, 10× Denhardt's, 0.01 M phosphate buffer, pH 6.8, 1 mM ATP, 0.05% SDS, 10 mM Na\textsubscript{2}EDTA, 0.1 mg/ml herring sperm DNA at 42°C for 4 h. Hybridization was at 42°C for 18 h in the same buffer and 3 × 10\textsuperscript{6} c.p.m. [\(^32\)P]-labelled probe/ml. After hybridization, the filter was washed two times with 6× SSC, 0.05% SDS at room temperature, once at 42°C for 30 min and once at the stringent temperature (50–53°C) for 7 min.

**DNA sequencing**

Direct sequencing of plasmid DNAs was performed according to Hattori and Sakaki (13), employing the method of ‘primer walking’. The clone pTetY3 was partially sequenced by MediGene AG DNA-Analytik, München.

**In vitro transcription in HeLa cell nuclear extract**

Nuclear extract was prepared from HeLa S3 cells essentially as described by Dignam et al. (14). Transcription assays and the
elution of precursors from preparative gels were performed as described by Stange and Beier (15).

**In vitro** processing and splicing of pre-tRNAs in wheat germ extract

Cell-free wheat germ S23 extract was prepared from wheat embryos according to Stange and Beier (15). In **in vitro** processing and splicing of tRNA precursors was performed in a total volume of 30 µl, containing 6 µl S23 extract (25 mg/ml), 20 mM Tris–HCl, pH 7.4, 100 mM potassium acetate, 6 mM magnesium acetate, 80 µM spermine, 10 mM creatine phosphate, 0.8% Triton X-100 (Surfact-Amps X-100 from Pierce), 1 mM ATP, 0.1 mM CTP and 10⁶ c.p.m. precursor tRNA.

**RESULTS**

Purification and characterization of Tetrahymena cytoplasmic and mitochondrial tRNA<sup> Tyr </sup> isoacceptors

Total tRNA from *T. thermophila* was fractionated by BD-cellulose chromatography. A linear gradient of 0.35–1 M NaCl (0.01 M MgCl₂, 0.02 M NaOAc, pH 4.5) was first applied and the hydrophobic tRNAs were then eluted with 2 M NaCl/15% ethanol. Aliquots of appropriate fractions were selected for aminoclayation assays. The presence of tRNA<sup> Tyr </sup> was determined by using [³H]tyrosine and a crude synthetase preparation from *T. thermophila* cells. All tRNA<sup> Tyr </sup> isoacceptors eluted late from the BD-cellulose column (i.e., fraction IV) and were found also in the ethanol eluate (i.e., fraction V). The tRNAs<sup> Tyr </sup> in each fraction were further purified by successive gel electrophoresis in a native 10% polyacrylamide gel, pH 8.3, containing 10% glycerol, a 10% denaturing polyacrylamide gel, pH 3.5, and then in a 12.5% denaturing polyacrylamide gel, pH 8.3. The nucleotide sequences of the purified tRNAs<sup> Tyr </sup> were determined according to Stanley and Vassilenko (16).

Two mitochondrial tRNA<sup> Tyr </sup> isoacceptors with GUA and QUA anticodon (Fig. 1A) were present in fraction V in about equal amounts whereas a single cytoplasmic tRNA<sup> Tyr </sup> isoacceptor with QUA anticodon was observed in fractions IV and V (Fig. 1B). Since we had used a crude synthetase preparation for the detection of tRNAs<sup> Tyr </sup> it was no surprise that we had identified the mitochondrial isoacceptors in addition to the desired cytoplasmic tRNA<sup> Tyr </sup>. The presence of tRNA<sup> Tyr </sup> was determined by using [³H]tyrosine and a crude synthetase preparation from *T. thermophila* cells. All tRNA<sup> Tyr </sup> isoacceptors eluted late from the BD-cellulose column (i.e., fraction IV) and were found also in the ethanol eluate (i.e., fraction V). The tRNAs<sup> Tyr </sup> in each fraction were further purified by successive gel electrophoresis in a native 10% polyacrylamide gel, pH 8.3, containing 10% glycerol, a 10% denaturing polyacrylamide gel, pH 3.5, and then in a 12.5% denaturing polyacrylamide gel, pH 8.3. The nucleotide sequences of the purified tRNAs<sup> Tyr </sup> were determined according to Stanley and Vassilenko (16).

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The sequence of cytoplasmic tRNA<sup> Tyr </sup> is the first one for this isoacceptor characterized in any ciliate organism until now and shows the typical features of eukaryotic as compared to prokaryotic tRNA<sup> Tyr </sup>, i.e., a short extra arm of 5 nt and two cytidines at positions 1 and 2 (18). The modified nucleoside in the first anticodon position of mitochondrial and cytoplasmic tRNAs<sup> Tyr </sup> is the known queuosine found also in bacterial and plant tRNAs<sup> Tyr </sup> as deduced from its chromatographic migration pattern in two different solvents (19).

Suppressor activity of *Tetrahymena* mitochondrial and cytoplasmic tRNAs<sup> Tyr </sup> was examined in a messenger-dependent reticulocyte lysate to which tobacco mosaic virus (TMV) RNA and the corresponding RNA were added. Tobacco tRNA<sup> Tyr </sup> with GUA anticodon stimulates readthrough over the leaky UAG termination codon at the end of the 126K cistron of TMV RNA to a great extent (Fig. 2, lane d) as has been previously demonstrated (6), whereas the two *Tetrahymena* tRNA<sup> Tyr </sup> isoacceptors exhibit no UAG suppressor activity in this assay (Fig. 2, lanes b and c).

Amplification of *Tetrahymena* nuclear tRNA<sup> Tyr </sup> genes by polymerase chain reaction

We used the nucleotide sequence established for *Tetrahymena* cytoplasmic tRNA<sup> Tyr </sup> (Fig. 1B) to design primers hybridizing to the 5' and 3' ends of the putative gene(s) for the amplification reaction. The PCR products were separated on a 2% agarose gel. A number of products of variable size were detected in ethidium bromide stained gels. The products were blotted onto a nylon membrane and identification of tDNA<sup> Tyr </sup>-containing fragments was achieved by hybridization with a tRNA<sup> Tyr </sup>-specific probe (i.e., Tyr 3) identical to nucleotides 18–37 of cytoplasmic tRNA<sup> Tyr </sup> (Fig. 1B). A strong hybridizing fragment of ∼345 bp and two weakly hybridizing fragments of ∼600 and ∼780 bp were detected. The purified 346 bp PCR fragment was subsequently cloned into SmaI-cleaved pUC19 DNA. The nucleotide sequence of this DNA fragment is shown in Figure 3. The amplified genomic DNA fragment contains two tRNA<sup> Tyr </sup> genes separated by a spacer region of 179 bp. Both tRNA<sup> Tyr </sup> genes have intervening sequences of identical length (i.e., 11 bp) and sequence. The first tRNA<sup> Tyr </sup> gene codes for the sequenced tRNA<sup> Tyr </sup> shown in Figure 1B, whereas the second gene differs in eight positions from the latter. The presence of amplified products of even higher size than 346 bp indicated that tRNA<sup> Tyr </sup> genes might be clustered at a single site in *Tetrahymena* macronuclear DNA.

Organization of tRNA<sup> Tyr </sup> genes in the *Tetrahymena* nuclear genome

In order to obtain more precise information about the tRNA<sup> Tyr </sup> gene organization in the *Tetrahymena* genome we used first the
Figure 3. Nucleotide sequence of a 346 bp DNA fragment deduced from PCR-amplified genomic DNA of *Tetrahymena*. The fragment includes two tRNA^Tyr^ genes (identified by bold letters) and an intergenic region of 179 bp. The fragment shown in Figure 3 includes two tRNA^Tyr^ genes located on the amplified PCR fragment as expected (Fig. 5). We have elucidated the complete nucleotide sequences of the inserts contained in pTetY2 and pTetY3 and part of the 2.3 kb insert of pTetY3. The sequenced region of pTetY1 and the complete nucleotide sequences of pTetY2 and pTetY3 are available from the EMBL database under accession numbers Y14523, Y14524 and Y14525.

Figure 4. Southern hybridization analysis of genomic *Tetrahymena* DNA. *Tetrahymena* genomic DNA (10 μg) was digested with 5 U EcoRl/mg DNA (lane a), 5 U HindIII/mg DNA (lane b) and 5 U XhoI/mg DNA (lane c) for 6 h at 37°C followed by electrophoresis on a 0.8% agarose gel. Hybridization to Southern blots was carried out with the PCR-amplified randomly primed 346 bp fragment shown in Figure 3. EcoRl-digested SPP1 DNA samples were run as markers in adjacent lanes. Prehybridization and hybridization was performed at 65°C in 6×SSC, 10× Denhardt’s solution, 0.01 M phosphate buffer, pH 6.8, 0.05% SDS, 1 mM ATP, 10 mM EDTA and 100 μg/ml denatured salmon testes DNA. The nylon membrane was washed successively in 3× SSC for 1 h, 1.5× SSC for 1 h and 0.75× SSC for 30 min at 65°C. The numbers indicate the length of hybridizing HindIII fragments (left) and XhoI fragments (right), respectively, in kb.

random-primed 346 bp long PCR fragment as a probe for hybridization to *EcoRl*, *HindIII* and *XhoI*-digested genomic DNA from *Tetrahymena*. Southern blot analysis revealed the existence of at least 11 hybridizing fragments in each digest, ranging in size from 0.6 to >10 kb (Fig. 4). If we utilized 5′-labelled oligonucleotide Tyr 3 as a probe, essentially the same hybridization pattern was obtained (not shown). A remarkably strong hybridization fragment was seen in each digest which had the approximate length of 10, 2.3 or 1.6 kb upon cleavage with *EcoRl*, *HindIII* and *XhoI*, respectively (Fig. 4). We subsequently isolated HindIII fragments in the range from 2.0 to 2.3 kb from a preparative agarose gel for generating a mini genomic library assuming that the DNA section corresponding to the 346 bp fragment amplified by PCR (Fig. 3) was contained in one of them.

**Isolation and nucleotide sequences of eight tRNA^Tyr^ genes from the *Tetrahymena* nuclear genome**

About 1.5 × 10^4 white colonies containing HindIII fragments of 2.0–2.3 kb from *Tetrahymena* genomic DNA ligated into the HindIII site of pUC19 were screened with 32P-labelled tRNA^Tyr^-specific probe Tyr 3 (Fig. 1B). About 10 hybridizing colonies were thus identified and repeated rescreening with the same probe yielded eventually two positive clones which were called pTetY1 and pTetY2. Unexpectedly, preliminary sequence data revealed that neither clone accommodated the genomic equivalent to the 346 bp PCR fragment shown in Figure 3. The remaining positive clones were therefore screened with a labelled oligonucleotide complementary to a sequence of the spacer region between the two tRNA^Tyr^ genes located on the amplified PCR fragment as indicated in Figure 3. This probe, i.e., IR-346, enabled us to identify unambiguously the desired clone, which was called pTetY3. All three clones contain HindIII fragments of 2.2–2.3 kb as expected (Fig. 5). We have elucidated the complete nucleotide sequences of the inserts contained in pTetY2 and pTetY3 and part of the 2.3 kb insert of pTetY1. Cleavage of the latter clone with a variety of other restriction enzymes did not reveal the presence of more than one tRNA^Tyr^ gene on this individual fragment. Likewise the clone pTetY2 contains only a single tRNA^Tyr^ gene. However, the 2.3 kb HindIII fragment contained in pTetY3 harbours five tRNA^Tyr^ genes and a pseudogene, consisting of a truncated 5′ half of the coding region. Moreover, this fragment contains the 346 bp region amplified earlier by PCR as indicated in Figure 5. The six tRNA^Tyr^ genes of pTetY3 have the same orientation and are separated by spacer regions ranging from 166 to 179 bp. Five of the eight tRNA^Tyr^ genes in clones pTetY1, pTetY2 and pTetY3 correspond exactly to the sequenced cytoplasmic tRNA^Tyr^ (Figs 1B and 6). The tRNA^Tyr^ genes TetY4 and TetY7 vary in a number of positions from the major species as indicated in the secondary structure models shown in Figure 6. Some of these mutations result in mismatches in the anticodon and/or T stem. Disruption of base pairs in either stem has been shown to generate processing-defective pre-tRNA molecules in HeLa and plant extracts (20), indicating that TetY4 and TetY7 may be pseudogenes. All seven full-length tRNA^Tyr^ genes have 11 bp long introns of identical sequence (Figs 3 and 9).
Figure 6. Nucleotide sequences of the coding region of eight nuclear tRNA$^{\text{Tyr}}$ genes from *T.thermophila* shown as cloverleaf models. The genes are grouped according to their sequence similarity. TetY5 consists only of a truncated 5$'$ half of a tRNA$^{\text{Tyr}}$ gene. The seven full-length tRNA$^{\text{Tyr}}$ genes contain identical 11 bp long intervening sequences at the signified position. The nucleotides in which TetY4 and TetY7 differ from the other five tRNA$^{\text{Tyr}}$ genes are indicated by white letters on a black background.

Comparison of the nucleotide sequences flanking the eight tRNA$^{\text{Tyr}}$ genes reveals few significant similarities beyond the regions of 20 bp upstream and downstream of the coding regions with the exception of the spacer regions connecting TetY3/TetY4 and TetY4/TetY5 (Fig. 5). These sections of ∼150 bp comprise an overall sequence homology of >90%.

**In vitro transcription of *Tetrahymena* tRNA$^{\text{Tyr}}$ genes in HeLa cell nuclear extract**

The HeLa nuclear extract has been shown to support RNA polymerase III-dependent transcription of numerous heterologous tRNA genes originating from yeast, plants and animals (21–24). Transcription is initiated generally upstream of the tRNA gene at a purine neighbouring a pyrimidine and is terminated at a stretch of at least five consecutive thymidine residues either immediately following the end of the tRNA gene or within ∼20 bp downstream (25). The seven full-length *Tetrahymena* tRNA$^{\text{Tyr}}$ genes all contain putative initiation signals at positions −3 and/or −6 and a stretch of T residues immediately flanking the tRNA genes consisting of six (TetY3, TetY4, TetY7), eight (TetY6), nine (TetY8), 14 (TetY2) and 15 (TetY1) residues. Consequently they should be substrates for the HeLa RNA polymerase III.

We selected TetY2* for our studies, located on a 236 bp DraI fragment derived from the original 2162 bp HindIII insert of pTetY2 as indicated in Figure 5. **In vitro transcription** of pTetY2* in HeLa cell nuclear extract in the presence of 4 mM MgCl$_2$ yielded two major primary transcripts of 91 and 94 nt after 20 min and upon further incubation a processed intermediate product of 87 as well as the mature tRNA of 76 nt (Fig. 7). The two high molecular weight polynucleotides which were synthesized also at 1 mM MgCl$_2$, i.e., in the absence of processing events (12), are probably transcripts which have originated by initiation of transcription at two different sites, since a very strong termination signal of 14 T residues is located immediately downstream of the tRNA$^{\text{Tyr}}$ gene. A similar pattern of products is generated upon **in vitro** transcription of a Tetrahymena tRNA$^{\text{Tyr}}$ gene in HeLa cell extract. The DNA of pTetY2* was incubated at 30°C in HeLa nuclear extract in the presence of [α$^32$P]GTP and 4 mM MgCl$_2$ for the times indicated above. The synthesized products were analysed on a 12.5% polyacrylamide/8 M urea gel. P 94 and P 91 indicate primary transcripts, whereas I 87 refers to the intron-containing intermediate product with processed 5$'$ and 3$'$ ends and M 76 indicates mature tRNA.

**DISCUSSION**

Natural suppressor tRNAs encoded by nuclear DNA have been characterized in a number of eukaryotic organisms (27,28). With the exception of selenocysteine tRNA(U$^\text{CA}$) all of them are normal cytoplasmic tRNAs. Accordingly, misreading of either stop codon involves unconventional base pairing. And in return unorthodox codon reading depends on the nucleotides surrounding splicing in a cell-free S23 extract from wheat germ. We had previously demonstrated that homologous and heterologous tRNA precursors are faithfully processed, that is removal of 5$'$ and 3$'$ flanking sequences was equally efficient with plant, animal and yeast pre-tRNAs. However, intron excision by the wheat germ splicing endonuclease appeared to request exclusively homologous substrates for its action (26). Figure 8 shows the processing pattern of Tetrahymena intron-containing pre-tRNA$^{\text{Tyr}}$ (i.e., P 94) and for comparison, the maturation of an Arabidopsis pre-tRNA$^{\text{Tyr}}$ (i.e., P 113) derived from transcription of pAtY3II (12). The latter is first converted to intron-containing pre-tRNA with processed ends (i.e., I 88) followed by intron excision and ligation of the resulting tRNA halves to produce mature tRNA (M 76). Maturation of either of the two Tetrahymena primary transcripts (i.e., P 91 and P 94) proceeds essentially by the same pattern albeit with a lower efficiency.
that the presence of a tRNA Tyr isoacceptor with G instead of Q at these sites is abolished. This implies that the potential UAG suppressor activity of tRNA Tyr G in this organism (5,30). It is conceivable that the release factor in the cytoplasm of Tetrahymena cells should be more deleterious than in other organisms and, as shown here, is in fact avoided. 

A single tRNA Tyr isoacceptor with Q anticodon was identified in the cytoplasm of T. thermophila (Fig. 1B). In vitro translation experiments clearly demonstrated that this tRNA is not a UAG suppressor in vitro (Fig. 2, lane b), confirming earlier results with tRNA Tyr Q isolated from Drosophila (29) and wheat (7). Due to an altered genetic code employed by Tetrahymena, UAA and UAG are glutamine codons in the nuclear genome of this organism (5,30). It is conceivable that the release factor in Tetrahymena has become specific for UGA and thus should be unable to recognize UAA and UAG codons any longer. Consequently, competition between release factor and any putative suppressor tRNA at these sites is abolished. This implies that the presence of a tRNA Tyr isoacceptor with Q anticodon in the cytoplasm of Tetrahymena cells should be more deleterious than in other organisms and, as shown here, is in fact avoided. 

Bacterial tRNAs normally are completely modified with respect to Q (31). However, in eukaryotes cytoplasmic tRNAs exhibit a variable Q content depending on the developmental state. Sheep reticulocytes and fetal liver contain significant amounts of tRNA Tyr Q-Glu whereas in adult sheep and bovine liver only tRNA Tyr Q can be detected (32,33). Significant changes in the amount of Q-containing tRNAs isolated from different ontogenetic stages during the development of Drosophila have been observed (34). Interestingly we have found that tRNA Tyr Q-Glu is exclusively present in mature tobacco and wheat leaves, whereas tRNA Tyr Q is abundant in wheat germ (6,7). In the latter case it could be argued that in fully differentiated leaves the potential UAG suppressor activity of tRNA Tyr Q-Glu causes less harm than in embryonic tissue where fidelity of codon reading is more important. 

A strict correlation between the presence of a pseudouridine in the middle of the anticodon of cytoplasmic tRNA Tyr (i.e., Ψ) and the presence of introns in the corresponding genes has been demonstrated in yeast (9), plants (8) and animals (10,11). As shown in this study, tRNA Tyr genes in the nuclear genome of the ciliate protozoa T. thermophila also contain introns (Fig. 3). Together with the presence of Ψ in the anticodon of Tetrahymena tRNA Tyr this implies that the acquisition of introns into tRNA Tyr genes and the adaptation of a putative Ψ synthase to utilize intron-containing pre-tRNAs as substrates has evolved before the separation of ciliates from the major eukaryotic line. 

Until now, two distinct classes of introns have been identified in Tetrahymena: the group I self-splicing intron in 26 S rRNA (35) and a number of nuclear pre-mRNA introns (36). The introns of nuclear-encoded tRNA Tyr genes of Tetrahymena described here are located 1 nt 3’ to the anticodon (Figs 6 and 9), i.e., at the conserved position found in all other eukaryotes, indicating that they represent a third class of introns that are excised by a pre-tRNA endonuclease (37).

The seven Tetrahymena tRNA genes isolated and sequenced by us (Fig. 5) all comprise introns of identical length and sequence. This is reminiscent of the situation in yeast: the eight nuclear-encoded tRNA Tyr genes of Saccharomyces cerevisiae have almost identical 14 bp long introns differing only in one position (38). However, in animal and plant tRNA Tyr genes the length and sequence of introns can vary considerably within one species (10,22,23,39,40). One of the Tetrahymena tRNA Tyr genes was selected for in vitro transcription and processing studies. It was shown that this gene is efficiently transcribed in a HeLa cell nuclear extract and that the primary transcript is converted to mature tRNA (Fig. 7). Given the eukaryotic nature of the Tetrahymena tRNA Tyr gene and the almost universal capability of the HeLa extract to transcribe and process homologous and heterologous substrates (21–23) this result had been anticipated. In contrast to the HeLa splicing tRNA endonuclease it was recently demonstrated by us that the wheat germ tRNA endonuclease displays a pronounced preference for plant intron-containing pre-tRNAs (26,41). Further studies revealed that two elements are essential for pre-tRNA splicing in plants: a
defined intron secondary structure with the 5′ and 3′ splice sites separated by 4–5 bp in the extended anticodon stem and a number of specific nucleotides or base pairs in the mature domain (41). Although the nucleotide sequence of *Tetrahymena* tRNA^Tyr^ exhibits only ~80% homology to that of plant tRNA^Tyr^, the nucleotides critical for recognition by plant splicing endonuclease are present in the former. Moreover, the intron of *Tetrahymena* pre-tRNA^Tyr^ can adopt a structure similar to that of the plant substrate (Fig. 9). In vitro splicing studies with *Tetrahymena* pre-tRNA^Tyr^ revealed that this heterologous pre-tRNA is indeed processed and spliced in wheat germ extract (Fig. 8) confirming that the two features mentioned above are sufficient for substrate recognition by the plant pre-tRNA endonuclease.

Little is known about the organization of tRNA genes in the *Tetrahymena* genome. One EcoRI fragment of 2.5 kb has been previously isolated from a genomic phage library of *T.thermophila* and shown to contain four tRNA^Gln(TTA)^ genes, the fourth one being truncated due to an EcoRI site acquired by a single point mutation in the T stem (4). A second tRNA gene cluster has been identified on a genomic 4 kb HinIII fragment from the related *Tetrahymena pyriformis*. The sequenced region spans 1.4 kb and encodes three tRNA^Ser(AGA)^ and a single tRNA^Asp(GTT)^ gene (42). We have detected at least 11 restriction fragments in Southern blots of digested genomic *Tetrahymena* DNA hybridizing to a tRNA^Tyr^-specific probe (Fig. 4). Three HinIII fragments of similar size (i.e., 2.2–2.3 kb) were cloned and shown to contain altogether seven full-length tRNA^Tyr^ genes as well as a truncated tRNA^Tyr^ gene (Fig. 5) so that the total number of individual tRNA^Tyr^ genes in the *Tetrahymena* genome amounts to a minimum of 18 copies. Considering the relatively small size of 2.2 × 10^6 bp per haploid genome estimated for the micronuclear DNA of *Tetrahymena* (1), the large number of tRNA^Tyr^ genes appears to be unusual. In higher eukaryotes, the calculated number of tRNA^Tyr^ genes ranges between 8 in the *Drosophila* (43) and ~12–15 copies in the human and tobacco genome, respectively (11,23). From the overall hybridization pattern seen in Figure 4 it can be concluded that tRNA^Tyr^ genes in the nuclear genome of *T.thermophila* are mainly organized as a dispersed multigene family and that clustering of tRNA genes as found in pTetY3 (Fig. 5) is rather an exception than a general feature. Micro- and macronuclei of *T.thermophila* each contain ~30 clusters of 55 RNA genes per haploid genome, the average number of genes per cluster being five. The 55 RNA gene clusters are widely separated in the genome (44). Similarly an equal number of ~800 tRNA genes per haploid genome has been determined in the micro- and macronucleus of *Tpyriformis* (45). Assuming that a maximum of 40 different tRNA isoacceptors are encoded by the *Tetrahymena* genome, this would amount to 20 copies per isoacceptor in agreement with our results.

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