Characterization of nuclear $tRNA^{Tyr}$ introns: their evolution from red algae to higher plants

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Abstract **We have previously isolated numerous intron-containing nuclear $tRNA^{Tyr}$ genes derived from either monocotyledonous (*Triticum*) or dicotyledonous (*Arabidopsis*, *Nicotiana*) plants by screening the corresponding genomic phage libraries with a synthetic $tRNA^{Tyr}$-specific oligonucleotide. Here we have characterized additional $tRNA^{Tyr}$ genes from phylogenetically divergent plant species representing red algae (*Champia*), brown algae (*Cystophyllum*), green algae (*Ulva*), stonewort (*Chara*), liverwort (*Marchantia*), moss (*Polytrichum*), fern (*Rumohra*) and gymnosperms (*Ginkgo*) using amplification of the coding sequences from the corresponding genomic DNAs by polymerase chain reaction (PCR). All novel $tRNA^{Tyr}$ genes contain intervening sequences of variable sequence and length ranging in size from 11 to 21 bp. However, two features are conserved in all plant pre-$tRNA^{Tyr}$ introns: they possess a uridine and less frequently an adenosine at the 5′ boundary and can adopt similar intron secondary structures in which an extended anticodon helix of 4–5 bp is formed by base-pairing between nucleotides of the intron and the anticodon loop. In order to elucidate the potential role of the highly conserved uridine at the first intron position, we have replaced it by all other nucleosides in an *Arabidopsis* pre-$tRNA^{Tyr}$ and have studied in wheat germ extract its effect on splicing and on conversion of U to Ψ in the GΨA anticodon. Furthermore, we discuss the putative acquisition of $tRNA^{Tyr}$ introns at an early step of evolution after the separation of *Archaea* and *Eucarya*.**

Key words: Nuclear $tRNA^{Tyr}$ intron; Evolution

1. Introduction

All eukaryotic cytoplasmic tyrosine tRNAs are exceptional in that they contain a pseudouridine in the middle of their GΨA or QΨA anticodons at position 35 (Ψ_{35}). It has been shown that the Ψ_{35} synthase from *Saccharomyces cerevisiae* [1], *Xenopus laevis* [2], *Homo sapiens* [3] and higher plants [4,5] requires intron-containing pre-tRNAs as substrates. Thus, there exists a strict correlation between the presence of Ψ_{35} in cytoplasmic $tRNA^{Tyr}$ and the occurrence of introns in the corresponding nuclear genes from higher eukaryotes.

In *Bacteria* and *Archaea*, $tRNA^{Tyr}$ genes do not contain introns and the $tRNAs^{Tyr}$ have a GUA or QUA anticodon [6]. Consequently, the acquisition of introns and the adaptability of a pseudouridine synthase to utilize intron-containing substrates must have evolved after the divergence of *Archaea* and *Eucarya* and before the appearance of higher plants and animals.

We have previously isolated numerous nuclear $tRNA^{Tyr}$ genes from genomic libraries of *Triticum* [7], *Arabidopsis* [8] and *Nicotiana* [9]. Here we have characterized additional $tRNA^{Tyr}$ genes from one higher plant (*Pisum*) and eight phylogenetically divergent lower plant species from red algae to gymnosperms employing polymerase chain reaction (PCR). We have found that all novel $tRNA^{Tyr}$ genes also contain introns at the same position, one nucleotide 3′ of the anticodon. Remarkably, these introns vary considerably in sequence even within one species, quite in contrast to the nucleotide sequence of the mature domain which has remained almost unaltered in the sequenced region between the D and T stems of the secondary structure in the whole plant kingdom.

The only obvious common feature of plant introns at the sequence level is the prevalent occurrence of a uridine at the 5′ end (iU1). In order to reveal a possible function for its conservation we have substituted the iU1 by A, C and G and examined its effect on Ψ_{35} synthesis and/or splicing. As we will demonstrate in this report, the exchange of iU1 for any other nucleoside (with the exception of C) has no influence on splicing efficiency, whereas substitution of iU1 by A, C or G reduces Ψ_{35} synthesis by more than 50%.

2. Materials and methods

2.1. Enzymes and reagents

Restriction endonucleases, calf intestinal alkaline phosphatase, T4 DNA ligase and T4 polynucleotide kinase were from Boehringer (Mannheim). RNase T2 was obtained from Calbiochem. [α-^{32}P]ATP and [α-^{32}P]GTP with specific activities of 15 Tbq/mmol were from Hartmann Analytic (Braunschweig). T7 RNA polymerase was prepared according to Zawadzki and Gross [10]. Untreated wheat germs were a gift from SynPharma GmbH (Augsburg).

2.2. Plant material

Six different land plant species were used in this study: *Arabidopsis thaliana* (ecotype Landsberg *erecta*), *Pisum sativum* (pea), *Ginkgo biloba*, *Rumohra standishii*, Ching (fern), *Polytrichum juniperinum* (moss) and *Marchantia paleacea*, var. diptera (liverwort). *Chara braunii* (stonewort) plants were from a pond at Matsue (Japan) and three multicellular algae, *Ulva pertusa* (green alga), *Cystophyllum sisymbrioides* (brown alga) and *Champia parvula* (red alga), were collected from the seashore of the Sea of Japan.

2.3. Preparation of plant DNAs

High molecular weight DNA was isolated from whole plants of *Arabidopsis*, *Ruhmora*, *Polytrichum*, *Chara* and three different marine algae, from leaves of *Ginkgo* and from embryonic axes of *Pisum* essentially as described [11]. *Marchantia* total DNA was provided by Dr. S. Takio (Hiroshima, Japan).

2.4. Synthesis of oligodeoxyribonucleotides

The four oligodeoxyribonucleotides Tyr 1 to Tyr 4 utilized as primers for PCR analysis were synthesized with a Cyclone Plus DNA Synthesizer (Millipore). Tyr 1 (5′-CCGACCTTAGCTCAGTTGG-3′) corresponds to the 5′ distal end and Tyr 2 (5′-TCCGACCTACCGGATTC-3′) is complementary to the 3′ distal region of the *Arabidopsis* $tRNA^{Tyr}$ gene AtY3II [8,12], whereas primers Tyr 3 and Tyr 4 are identical to nts 1–19 (5′-CCCCTTGTAGCTCAGTTGG-3′) and com-

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plementary to nts 56–73 (5′-TCCCCTCGACCGGATTCG-3′) of *Scenedesmus obliquus* cytoplasmic $tRNA^{Tyr}$ [13].

2.5. PCR amplification of nuclear $tRNA^{Tyr}$ genes from plants

The amplification reactions were carried out in a total volume of 100 μl containing 0.1–1 μg plant DNA, 0.2 μM of each relevant primer, 250 μM dNTP and 1 unit of Taq DNA polymerase (Toyobo). The mixture was subjected to 30 cycles in a Perkin-Elmer Thermocycler (GeneAmp PCR system 9600). Each cycle consisted of 95°C, 55°C and 72°C for 30 s, 60 s and 30 s, respectively. PCR products were applied onto a 12% polyacrylamide gel. For cloning, appropriate DNA fragments were eluted from the gel, kinased at the 5′ end with 100 μM ATP and 10 units of T4 polynucleotide kinase and ligated into the *Eco*RV site of the pBluescript II SK^+ vector (Stratagene). The resulting DNA samples were used to transform competent cells of *E. coli* strain XL1-blue. Colonies of transformed bacteria were transferred to Hybond N^+ membranes (Amersham) and screened with a $tDNA^{Tyr}$-specific probe as described [14].

2.6. Construction of tDNA clones

A *Bam*HI/*Sal*I fragment of 202 bp, harboring an intron-containing $tRNA^{Tyr}$ gene from *Arabidopsis*, was excised from the original pAtY3II plasmid DNA [12] and religated into pUC19 DNA cleaved with *Bam*HI and *Sal*I. This clone has been designated pAtY3II*. Subsequent oligonucleotide-directed mutagenesis [15] yielded a construct in which the $tDNA^{Tyr}$ gene was flanked by a T7 RNA polymerase promoter directly at the 5′ side, by a *Bst*NI restriction site at the 3′ side and in which the first base pair in the aminoacyl stem of the tRNA had been exchanged from C1:G72 to G1:C72. The mutated DNA fragment of 224 bp was recloned into the *Bam*HI/*Sal*I sites of pUC19. This clone was named pAtY3II*-T7. The derivatives of pAtY3II* and of pAtY3II*-T7, carrying mutations at the splice sites, were obtained by megaprimer PCR mutagenesis [16] using appropriate synthetic oligonucleotides as primers and Pfu DNA polymerase (Stratagene).

2.7. In vitro transcription of tRNA genes with T7 RNA polymerase

Transcription of pAtY3II*-T7 and its derivatives by T7 RNA polymerase was carried out in 20 μl volumes using 1 μg *Bst*NI-linearized plasmid DNA and 0.6 μg T7 RNA polymerase (400 U/μg). Incubation was for 1 h at 37°C in 40 mM Tris-HCl, pH 8.1, 12 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 20 μCi of [α-^{32}P]ATP, 0.2 mM of unlabelled ATP and 1 mM of GTP, UTP and CTP. The transcripts were purified by repeated phenol/$CHCl_3$ extractions and electrophoresis in a 12.5% polyacrylamide/8 M urea gel.

2.8. In vitro splicing of pre-tRNAs in wheat germ extract

Cell free wheat germ S23 extract (30 mg protein/ml) was prepared from wheat embryos as described [4]. In vitro splicing of tRNA precursors was performed in a volume of 10 μl (analytic) or 30 μl (preparative), containing 2 and 6 μl, respectively, of S23 extract, 20 mM Tris-HCl, pH 7.4, 110 mM potassium acetate, 6.6 mM magnesium acetate, 80 μM spermine, 0,2 mM DTT, 1 mM ATP, 0.1 mM CTP, 0.8% (v/v) Triton X-100 and appropriate amounts of labelled precursor tRNA.

2.9. Analysis of pseudouridine (Ψ_{35}) modification

Pre-tRNAs labelled with [α-^{32}P]ATP were digested with 1 unit of RNase T2 for 5 h at 37°C in 20 μl 5 mM NH_4OAc, pH 4.6 in the presence of 5 μg calf liver tRNA. Identification of the labelled nucleoside 3′-phosphates was performed by one-dimensional chromatography on cellulose thin layer plates as described [17]. The amount of radioactivity in the AMP and ΨMP spots was determined using a PhosphorImager (Molecular Dynamics GmBH, Krefeld).

3. Results and discussion

3.1. Detection of novel plant $tRNA^{Tyr}$ genes by PCR

Cytoplasmic $tRNAs^{Tyr}$ in higher plants as diverse as *Arabidopsis* [8], *Lupinus* [18], *Nicotiana* [9,19] and *Triticum* [7,20] exhibit identical nucleotide sequences with the exception of one base pair at the basis of the TΨC stem: $tRNA_1^{Tyr}$ has an A:U pair and $tRNA_2^{Tyr}$ has a G:C pair at the corresponding position. In order to identify $tRNA^{Tyr}$ genes by PCR analysis in green plants representing Gymnospermae (*Ginkgo*), Pteridophyta (*Rumohra*), Bryophyta (*Polytrichum*, *Marchantia*) and Charophyta (*Chara*) we employed primers Tyr 1 and Tyr 2, based on 5′ and 3′ distal sequences of $tRNA_1^{Tyr}$. For the characterization of $tRNA^{Tyr}$ genes in multicellular algae we designed primers, i.e. Tyr 3 and Tyr 4, corresponding to the 5′ and 3′ ends of cytoplasmic $tRNA^{Tyr}$ from the unicellular green alga *Scenedesmus obliquus* [13], the only known $tRNA^{Tyr}$ sequence in algae. Amplified products were resolved by polyacrylamide gel electrophoresis, giving rise to one major and some minor products in the different PCR reactions. The sizes of the major amplified fragments ranged between 85 and 92 bp. Major and minor products were recovered from the polyacrylamide gel and subcloned into the pBluescript II SK^+ vector for subsequent sequence analysis. A large number of clones were characterized and revealed the presence of intron-containing $tRNA^{Tyr}$ genes in all of them (Fig. 1). PCR amplification of potential $tRNA^{Tyr}$ genes in marine alga DNAs (*Ulva*, *Cystophyllum* and *Champia*) yielded two different major products of about 75 and 85 bp. Sequencing of the shorter fragments showed a high homology to either chloroplast $tRNA^{His}$ or $tRNA^{Thr}$, whereas the longer ones encoded intron-containing nuclear $tRNA^{Tyr}$ genes.

As in higher plants, the coding region – between the two PCR primers – of $tRNA^{Tyr}$ genes from *Champia* to *Ginkgo* was found to be highly conserved, representing either $tRNA_1^{Tyr}$ or $tRNA_2^{Tyr}$. In *Champia* we have detected two $tRNA^{Tyr}$ genes in which the A28:U42 base pair of the anti-

Plant genus	Ribonucleotide sequence	Length (nts)
Champia	**A**UGGUAACAGCU	12
Rhodophyta	**A**UGGUAACAGUA	12
	UGGUAUAAACGGCU	14
Cystophyllum	**A**UGGUAACAGUA	12
Phaeophyta	**U**AGGUUGUGCAGAG	14
Ulva	**A**UGGUAAUAGUA	12
Chlorophyta	**A**UGGUAACAGUA	12
Chara	**U**GUUGCAGAUCA	12
Charophyta	**U**UGUUGCAGAUAA	13
	UUGACACUGAUUGCC	15
	UUGAUAUUGAUUGCC	15
	UUGAUACUGAUUGCC	15
Marchantia	**U**GAGUAACCAGAC	13
Bryophyta	**U**ACAUGAACAGAC	13
Hepaticae	**U**GGACCCUCAGGC	13
Polytrichum	**U**UGGUGCAGAU	11
Bryophyta	**A**GUCCCCAGGU	11
Musci	**A**GUGCGCAGGU	11
	AGUGUGCAGGU	11
	ACGUGUACAGAU	12
Rumohra	**U**GCAAACUGGU	11
Pteridophyta	**U**GUGCAACUGAA	12
	UCGGAAGUCUGGAA	14
	UUGGCUAAACUGGAU	15
	UUUGCUGAACUGGAU	15
Ginkgo	**U**GGGUUGCAGGUA	13
Gymnospermae	**U**GGUUUGGCAGGUAA	15
	UGGACAAUUUCUCUGUGGAU	20
	UUGGACAAAUCCUGACAGAUA	21
	UGGGCGCAGCCUUGUCAGAUA	21
	UGGGCAUUGCUUUGCAGUAAA	21
Pisum	**U**UGUCAAACAGAC	13
Angiospermae	**U**UGUCAAACGGAC	13
Dicotyledoneae	**U**GGUUAAGCAGAU	13
	UUUGGGUAUCAUGCUGUC	18

Fig. 1. Survey of introns present in nuclear $tRNA^{Tyr}$ genes from phylogenetically divergent plant species. All intron sequences are derived from PCR-amplified $tRNA^{Tyr}$ genes as described in this work.

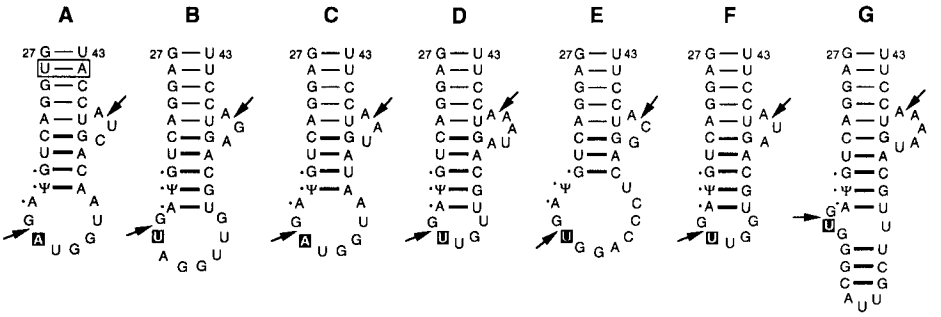

Fig. 2. Putative secondary structures of the extended anticodon stem regions of selected plant intron-containing pre-tRNAs^{Tyr}. A = *Champia*, B = *Cystophyllum*, C = *Ulva*, D = *Chara*, E = *Marchantia*, F = *Polytrichum* and G = *Ginkgo*. Base pairs which are formed by interacting bases from the intron and the anticodon loop are indicated by bold lines, dots identify the anticodon. Arrows point to the 5′ and 3′ splice sites. The conserved nucleoside at the 5′ end of the intron is emphasized by a white letter on a black background. The base pair in which two *Champia* tRNA^{Tyr} genes differ from the conserved sequence is boxed.

codon stem was replaced by U28:A42 (Fig. 2A) and in *Marchantia* we found a single tRNA^{Tyr} gene with two different base pairs in the anticodon stem: G28:C42 and A29:U41 instead of A28:U42 and G29:C41. Of course we cannot rule out that sequence variations exist in other not sequenced regions of the mature domain, like the acceptor stem.

A survey of all plant tRNA^{Tyr} intron sequences characterized in this work is presented in Fig. 1. It reveals some interesting features. Among them, diversity of length and composition of the intron sequence should be noted. For example, in algae as diverse as *Rhodophyta*, *Phaeophyta* and *Chlorophyta*, the introns are short, ranging in size from 12 to 14 bp and starting with a uridine or adenosine at the 5′ end. Remarkably the intron sequence 5′-AUGGUAACAGUA-3′ is found in each group. The reason for the conservation beyond the division is not known. Surprisingly, in almost all other plant species including *Triticum* [7], *Arabidopsis* [8] and *Nicotiana* [9] a uridine is present exclusively at the 5′ end of the intron and a tendency for the occurrence of longer introns ranging in size from 20 to 25 bp can be seen in some higher plants (*Ginkgo* and *Nicotiana*).

Besides tRNA^{Tyr} genes, the only other intron-containing tRNA family known in plants are the tRNA^{Met} genes, recently characterized in eight phylogenetically distinct plant species. The length and sequence of introns in this tRNA family appear to be more conserved than in plant tRNA^{Tyr} genes: they are 10–13 bp long and all carry a GC motif at the 5′ and a GAG motif close to the 3′ end. The latter trinucleotide is a prerequisite for the formation of a putative conserved structure of pre-tRNA^{Met} [14].

A remarkable common feature of introns found in nuclear genes of all angiosperms is that they can adopt similar secondary intron structures: an extended anticodon stem is formed by interactions between bases of the anticodon loop and the intron [7,9,12,14]. We have previously established a cell-free plant extract from wheat germ which contains all necessary factors for efficient and correct pre-tRNA processing, splicing and modification [4]. Unexpectedly we discovered that wheat germ splicing endonuclease displays a high specificity for homologous substrates [21]. Further studies disclosed that one prerequisite for efficient intron excision by wheat germ splicing endonuclease is a relatively strict requirement for a defined intron secondary structure. Thus we have demonstrated that after disrupting the intron-anticodon interaction by single nucleotide exchanges, splicing was severely impaired [22]. As seen in Fig. 2 most introns from tRNA^{Tyr} genes of algae and lower land plants can also fold into this type of secondary structure, in which the two splice sites are located in single-stranded regions and are separated by four or five base pairs. In a few cases an extended anticodon stem of only three base pairs can be formed which still appears to be tolerated by the plant splicing endonuclease (Fig. 2E). In *Ginkgo* (Fig. 1) and *Nicotiana* [9] relatively long intervening sequences of 20–25 bp have been identified. Pre-tRNAs with long introns can assume a secondary structure in which the two splice sites reside in small single-stranded loops separated by four base pairs (Fig. 2G). We have previously shown that these long introns are excised from the corresponding pre-tRNAs^{Tyr} in wheat germ extract as efficiently or even better than short ones [9].

3.2. *Effects of nucleotides adjacent to the 5′ and 3′ cleavage sites on intron excision by wheat germ tRNA endonuclease*

As stated above, an obvious feature of plant tRNA^{Tyr} introns is the prevalence of a uridine at their 5′ ends (Fig. 1). Taken into consideration the demand of plant tRNA endonuclease for homologous substrates, quite in contrast to human and yeast splicing endonucleases [21], it seemed reasonable to assume that the nucleotide at the 5′ boundary of plant tRNA introns plays a decisive role in intron excision. Therefore we substituted the uridine by any other nucleoside at this position choosing an *Arabidopsis* tRNA^{Tyr} gene for our studies. Furthermore we also examined the influence of nucleoside exchanges at all other positions neighboring the 5′ or 3′ splice sites (Fig. 3).

In order to study the effect of mutations at the splice sites in wheat germ extract, we first synthesized the primary transcripts in HeLa cell nuclear extract using the corresponding plasmid DNAs, i.e. pAtY3II* and its derivatives, and recovered the processed intermediates (i.e. I 88) from a preparative gel. These products still carry an intron, but have correct mature 5′ and 3′ ends (Fig. 3A). The wild-type pre-tRNA^{Tyr} is efficiently spliced in wheat germ extract. After 120 min of incubation at 30°C, about 85% of the precursor I 88 is converted to mature tRNA (Fig. 3B/C). We have also employed the corresponding transcript synthesized by T7 RNA polymerase (see Fig. 4A) as substrate for splicing in wheat germ extract, but discovered that splicing efficiency was reduced to about 35% as compared to 85% seen with the HeLa transcript (not shown). This phenomenon is not quite unknown and has first been described by Reyes and Abelson [23] who

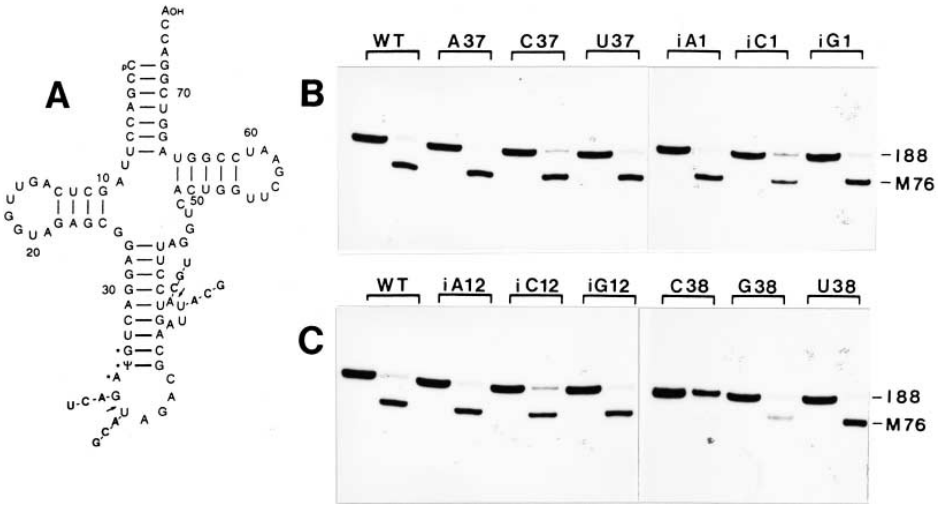

Fig. 3. In vitro splicing of intron-containing wild-type and mutated pre-tRNAs^{Tyr} in wheat germ extract. A: The putative secondary structure of wild-type pre-tRNA^{Tyr} derived from pAtY3II* is shown to the left. The mutated pre-tRNAs^{Tyr} carry base substitutions at the 5′ and 3′ splice sites. G37 has been converted to A, C and U; the uridine at the 5′ end of the intron (i.e. iU1) has been substituted by A, C and G; the uridine at the 3′ end of the intron (i.e. iU12) has been exchanged to A, C and G and A38 has been substituted by C, G and U as indicated in the structure model. B and C: Pre-tRNAs, containing the intron and mature 5′ and 3′ ends (i.e. I 88) were synthesized in HeLa cell nuclear extract in the presence of [α-^{32}P]GTP. They were recovered from a preparative gel and incubated in wheat germ extract at 30°C. The products at 0 and 120 min were analyzed on a 10% polyacrylamide/8 M urea gel. M = mature tRNA.

observed that an intron-containing pre-tRNA^{Phe} synthesized by T7 RNA polymerase was spliced with reduced efficiency in yeast nuclear extract. The synthetic transcripts carry a 5′ triphosphate terminus, are completely unmodified and contain a G1:C72 (instead of a C1:G72) base pair at the beginning of the acceptor stem, in the case of pre-tRNA^{Tyr} (Figs. 3A and 4A). Any of these features could contribute to the fact that a synthetic pre-tRNA is not an optimal substrate for wheat germ tRNA endonuclease.

As shown in Fig. 3B, exchange of nucleosides at the 5′ splice site, i.e. at position 37 in the exon and at the first position of the intron (i1), has no marked effect on splicing. A significant reduction of splicing efficiency from 85 to 60% was observed if the uridine at position i1 had been substituted by C. Thus we conclude that the conservation of a uridine and in parts of an adenosine at this position in introns of plant tRNA^{Tyr} genes cannot be explained by a preference for either nucleoside by plant tRNA endonuclease. Since all introns in plant tRNA^{Met} genes begin with a guanosine [14], we had assumed that at least an exchange of iU1 to G would not interfere with splicing.

Substitution of the uridine at the last position of the intron (i.e. i12) from U to A, C or G had no major influence on splicing efficiency either (Fig. 3C). A lower yield (~70%) of mature tRNA was observed only in the case of pre-tRNA^{Tyr}-iC12 (Fig. 3C). These results are in accordance with the fact that plant tRNA^{Tyr} introns exhibit no preference for any nucleoside at this position (Fig. 1) and that a cytidine is avoided in plant tRNA^{Met} introns [14]. An interesting observation was made when the highly conserved adenosine at the exon posi-

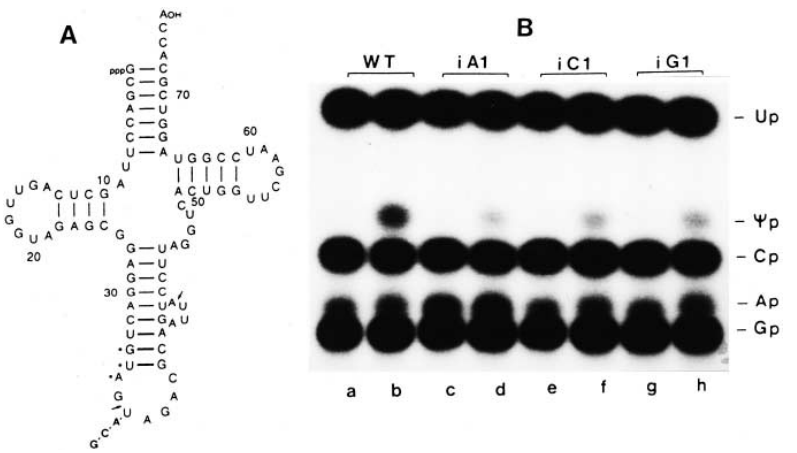

Fig. 4. Pseudouridine 35 synthesis of wild-type and mutated pre-tRNAs^{Tyr} in wheat germ extract. A: The putative secondary structure of wild-type pre-tRNA^{Tyr} derived from pAtY3II*-T7 is shown to the left. The mutated pre-tRNAs^{Tyr} carry base substitutions at the 5′ end of the intron as indicated, i.e. iU1 has been exchanged to A, C and G. B: The corresponding plasmid DNAs were transcribed by T7 RNA polymerase in the presence of [α-^{32}P]ATP. The transcripts were incubated in wheat germ extract at 30°C. The intron-containing pre-tRNAs^{Tyr} were analyzed for the presence of Ψ_{35} at 0 min (a, c, e and g) and 120 min (b, d, f and h) by chromatography on cellulose thin layer plates after RNase T2 digestion as described [17].

tion 38 was replaced by U, G or C. Pre-tRNA^{Tyr}-U38 was spliced as readily as the wild-type, whereas pre-tRNA^{Tyr}-G38 was spliced with reduced efficiency and accuracy and splicing of pre-tRNA^{Tyr}-C38 was severely impaired (Fig. 3C). At the moment we have no explanation for the latter effect, but we can rule out that pre-tRNA^{Tyr}-C38 exhibits conformational alterations due to the exchange of A38 to C. Chemical structure probing in the presence of 2.5 mM Pb^{2+} according to Perret et al. [24] has revealed identical cleavage patterns (not shown). It has been demonstrated that cleavage by yeast or *Xenopus* tRNA endonucleases requires specific recognition elements in the immediate neighborhood of the two splice sites. Thus, the exchange of A38 for C in yeast pre-tRNA^{Phe} leads to inaccurate cleavage at the 3′ splice site by *Xenopus* tRNA endonuclease [25].

3.3. *Influence of nucleotides located at the 5′ end of the intron on Ψ_{35} synthesis*

A characteristic feature of cytoplasmic tRNA^{Tyr} is the presence of a pseudouridine (Ψ_{35}) in the center of the GΨA or QΨA anticodon. The conversion of U_{35} to Ψ_{35} by Ψ_{35} synthase has been shown to require the presence of an intron-containing pre-tRNA^{Tyr} in yeast [1,26], *Xenopus* oocytes [2], HeLa cells [3] and wheat germ extract [5]. Furthermore it was demonstrated that the consensus sequence U_{33} N_{34} U_{35} A_{36} Pu_{37} in the intron-containing precursor is absolutely required for Ψ_{35} synthesis in HeLa cell and wheat germ extracts [17], indicating that Ψ_{35} synthase recognizes the tertiary structure as well as specific sequences surrounding the nucleoside to be modified. In the latter study it was ruled out that nucleosides neighboring the purine at position 37 might play a direct role in the recognition process, because all four nucleosides were identified at the 5′ end of the intervening sequence in pre-tRNAs^{Tyr} originating from *Xenopus* [27], man [28–30] and *Drosophila* [2] and because at that time the conservation of a uridine in plant tRNA^{Tyr} introns was not yet known. Since the possibility existed that plant Ψ_{35} synthase displays a preference for homologous substrates as does the plant tRNA endonuclease, we examined the influence of the nucleoside following the purine at position 37 on Ψ_{35} synthesis in wheat germ extract. For these studies we chose intron-containing pre-tRNAs^{Tyr} synthesized in vitro by T7 RNA polymerase in the presence of [α-^{32}P]ATP (Fig. 4A). The pre-tRNAs were first incubated for 120 min in wheat germ extract and were then recovered from a polyacrylamide gel for further analysis. The Ψ content was analyzed after complete hydrolysis of the pre-tRNA with RNase T2. Since RNase T2 produces nucleoside 3′-monophosphates, the labelled phosphate is exclusively derived from the nearest adenosine. This ensures that only Ψ_{35} will be labelled, since the other two potential pseudouridines in tRNA^{Tyr} (i.e. Ψ_{39} and Ψ_{55}) are followed by a cytidine [17].

The T7 transcripts, which were poorly spliced as mentioned earlier, were excellent substrates for Ψ_{35} synthesis (Fig. 4B, lane b). Between 90 and 100% of U_{35} was reproducibly converted to Ψ_{35} in wild-type pre-tRNA^{Tyr} in wheat germ extract. The kinetics of Ψ formation indicated that the maximum value was reached within 20–30 min of incubation (not shown).

Mutation of the uridine in the first position of the intron to A, C or G resulted repeatedly in a significant reduction of Ψ_{35} synthesis: 25–30% of Ψ_{35} was found in pre-tRNA^{Tyr}-iA1, 45–50% in pre-tRNA^{Tyr}-iC1 and 50–60% in pre-tRNA^{Tyr}-iG1 (Fig. 4B, lanes d, f and h). Although this is not a dramatic effect, it might nevertheless explain the prevalence of uridine at this position in introns of plant tRNA^{Tyr} genes.

3.4. *Origin of introns in nuclear-encoded tRNA^{Tyr} genes and the acquisition of Ψ in the tRNA^{Tyr} anticodon*

Recently it has been proposed by Woese et al. [31] that the three major groups of living organisms commonly known as *Bacteria*, *Archaea* and *Eucarya* should be called domains. The evolution of tRNAs^{Tyr} and their genes is of special interest, because three major alterations have taken place from *Bacteria* to *Eucarya*.

First, a switch occurred from prokaryotic to eukaryotic type of tRNA^{Tyr} in the overall nucleotide sequence of the mature domain. The prokaryotic tRNA^{Tyr} has a minimum of two G:C pairs at the onset of the acceptor stem (G1:C72 and G2:C71), a D stem of only three base pairs and a long extra arm consisting of 13–15 nucleotides. This refers to all known members of *Bacteria*, including *Mycoplasma*, *Bacillus subtilis* and *Escherichia coli* [6]. Principally the same features are found in all tRNAs^{Tyr} encoded by organelles (with the exception of animal mitochondria), reflecting their prokaryotic origin. The cytoplasmic eukaryotic tRNA^{Tyr} has at least two C:G pairs at the beginning of the acceptor stem (C1:G72 and C2:G71), a D stem of four base pairs and a short extra arm of five nucleotides. In the domain *Archaea*, a eukaryotic type of tRNA^{Tyr} has been characterized on the DNA [32] and on the tRNA level [33]. The phylogenetic tree as proposed by Woese et al. [31] predicts that *Bacteria* evolved first from the common universal ancestor, followed by the divergence of *Archaea* from the line that led eventually to *Eucarya*. Consequently, *Archaea* and *Eucarya* share more features with regard to molecular processes than *Bacteria* and *Archaea*. This refers to many properties of the translational apparatus [34] and especially it appears that tRNA isoacceptors from *Archaea* have more similarities with their eukaryotic counterparts than with bacterial tRNAs [6].

Second, an intron was acquired at a conserved position one nucleotide 3′ of the anticodon in *Eucarya*. Introns have not been identified in tRNA^{Tyr} genes from *Bacteria*, organelles and *Archaea*. It should be noted that only a few sequence data are yet available from *Archaea* derived from the genus *Methanococcus* [6] and from *Thermococcus celer* [35], both representing the kingdom *Euryarchaeota*.

Extensive information exists about tRNA^{Tyr} genes and tRNAs^{Tyr} in *Eucarya*. In higher animals, including insects and vertebrates, all tRNA^{Tyr} genes contain introns [6]. In the whole plant kingdom from multicellular algae to higher plants, tRNA^{Tyr} genes encode introns as shown in this work (Fig. 1). In the fungus *Saccharomyces cerevisiae* all eight tRNA^{Tyr} genes comprise almost identical introns 14 bp in length [36]. A single copy of an intron-containing tRNA^{Tyr} gene has been detected in the genome of the slime mold *Dictyostelium discoideum* [37]. We have recently characterized seven tRNA^{Tyr} genes in the macronuclear genome of the ciliate protozoon *Tetrahymena thermophila*. All of these genes contain 11 bp long introns of identical sequence [38]. In the nuclear genome of *Trypanosoma brucei* a single copy of a tRNA^{Tyr} gene exists, which harbors an 11 bp long intervening sequence [39]. *T. brucei* belongs to a group of protozoa which has emerged early in the evolution of eukaryotes [31]. Hence, it is the most ancient eukaryotic representative known so far

that encodes an intron-containing $tRNA^{Tyr}$ gene. Consequently, we postulate that introns have been acquired by $tRNA^{Tyr}$ genes after divergence of *Archaea* and *Eukarya* at a very early time of eukaryotic development. It should be emphasized that two tendencies can be observed during the evolution of nuclear $tRNA^{Tyr}$ introns. They are of identical length and sequence within one species and are generally short (i.e. 11–14 bp) in more ancient organisms representing flagellates (*Trypanosoma*), slime molds (*Dictyostelium*), ciliates (*Tetrahymena*) and fungi (*S. cerevisiae*), whereas in animals and higher plants introns vary in length and sequence. The longest intron of 113 bp has been found in the nuclear genome of *Drosophila melanogaster* [2].

Third, only the anticodons of eukaryotic cytoplasmic tRNAs contain a Ψ_{35}. Since introns are a prerequisite for Ψ_{35} synthesis as mentioned above, Ψ_{35} synthase must have evolved after the acquisition of introns. Moreover, we have shown that a mutant pre-$tRNA^{Tyr}$ in which U_{35} was converted to C_{35} was spliced as efficiently as wild-type pre-$tRNA^{Tyr}$ [17], indicating that splicing was not affected by the absence of Ψ_{35} modification in pre-$tRNA^{Tyr}$. This observation further supports the notion that Ψ_{35} synthesis may have evolved independently from the splicing process. The cytoplasmic $tRNAs^{Tyr}$ have not yet been characterized in the flagellate *Trypanosoma* or in the slime mold *Dictyostelium*, so that we have no knowledge whether these organisms have already a Ψ_{35} synthase. However, we know that the ciliate *Tetrahymena thermophila* has a cytoplasmic $tRNA^{Tyr}$ with QΨA anticodon [38], indicating that Ψ_{35} synthesis must have evolved before ciliates branched off from the common eukaryotic line.

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