The EMBO Journal vol.7 no.12 pp.3823–3828, 1988

Wheat germ splicing endonuclease is highly specific for plant pre-tRNAs

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Communicated by H.J.Gross

Intron-containing pre-tRNAs from organisms as different as yeast, *Nicotiana*, *Xenopus* and man are efficiently spliced and processed in a HeLa cell extract. They are also correctly processed in a wheat germ extract; however, the intron is removed only from the tobacco pre-tRNA. To determine whether plant pre-tRNA introns have any specific structural and/or sequence feature we have cloned two intron-containing $tRNA^{Tyr}$ genes from the plant *Arabidopsis*. Comparison of these genes, of the *Nicotiana* $tRNA^{Tyr}$ gene and of a *Glycine max* $tRNA^{Met}$ gene reveals that plant introns from three different species have no sequence homology and are only 11 to 13 nucleotides long. Thus, short length may be one important feature of plant introns. Furthermore, the 5′ and 3′ splice sites are separated by 4 bp in the extended anticodon stems of these pre-tRNA structures. In contrast, yeast and vertebrate introns are rather variable in length and the splice sites are separated by 5 or 6 bp. These differences in distance and relative helical orientation of the splice sites in plant pre-tRNAs versus pre-tRNAs from other organisms are obviously tolerated by the vertebrate splicing endonuclease, but not at all by the plant enzyme.

Key words: Arabidopsis $tRNA^{Tyr}$ genes/plant pre-tRNA introns/pre-tRNA splicing/wheat germ splicing endonuclease

Introduction

Since the discovery of intron-containing tRNA genes (Goodman *et al.*, 1977), the process of 'splicing', i.e. intron excision and exon ligation, has attracted much interest. Several *in vitro* systems have been established for dissecting and studying this tRNA maturation step in detail. It is known from two commonly used extracts that they are capable of excising introns from heterologous pre-tRNAs. Thus, HeLa cell nuclear extracts efficiently and correctly splice pre-tRNAs from species as diverse as *Nicotiana* (van Tol *et al.*, 1987), yeast (Standring *et al.*, 1981; Filipowicz and Shatkin, 1983) and *Xenopus* (Filipowicz and Shatkin, 1983; Laski *et al.*, 1983; Gouilloud and Clarkson, 1986). Similarly, pre-tRNAs from *Saccharomyces cerevisiae* and *Drosophila* are spliced in *Xenopus* oocytes or corresponding extracts (Mattoccia *et al.*, 1979; Ogden *et al.*, 1979; Melton *et al.*, 1980; Suter, 1987). However, nothing is known about the specificity of splicing activity in plant extracts. We have recently described the first cell-free plant system for faithful pre-tRNA processing, splicing and modification using an intron-containing tobacco tRNA precursor (Stange and Beier, 1987). Here we show that this extract from wheat germ efficiently removes the 5′ and 3′ flanking sequences from human, *Xenopus* and yeast pre-tRNAs. Surprisingly, however, introns are not excised. These findings raise interesting questions about the molecular basis for the pronounced specificity of plant splicing endonuclease for homologous substrates.

Only a few plant nuclear tRNA genes containing intervening sequences have been cloned and sequenced thus far. Besides a tobacco $tRNA^{Tyr}$ gene (Stange and Beier, 1986) there is only one more report about an intron-containing *Glycine max* $tRNA^{Met}$ gene (Waldron *et al.*, 1985). To determine whether plant tRNA introns have any specific and common structural feature, we felt the urgent need for more information about their primary sequences and secondary structures. Therefore, we isolated two more intron-containing $tRNA^{Tyr}$ genes from the plant *Arabidopsis*. Here we compare secondary and tertiary structure models of the four plant pre-tRNAs and show that the introns have a consensus structure which differs from that in yeast and in vertebrates.

Results

Characterization of in vitro synthesized yeast and vertebrate tRNA precursors

For studying *in vitro* splicing of heterologous pre-tRNAs in a wheat germ extract we selected tRNA genes for which efficient transcription in HeLa cell extracts was known, a prerequisite for our studies since an *in vitro* polymerase III transcription system from plant cells does not yet exist. Transcription in HeLa cell extracts has been established for the two yeast $tRNA^{Ser}_{CGA}$ and $tRNA^{Leu}_{3}$ genes (Filipowicz and Shatkin, 1983; Ganguly *et al.*, 1988), for the *Xenopus* (Laski *et al.*, 1983; Gouilloud and Clarkson, 1986) and the human $tRNA^{Tyr}$ gene (van Tol *et al.*, 1987). We had planned to include several yeast $tRNA^{Tyr}$ genes in our studies since $tRNA^{Tyr}$ genes were also available from *Nicotiana*, *Xenopus* and man. Surprisingly, three $tRNA^{Tyr}$ genes (Goodman *et al.*, 1977) from different loci of the *S.cerevisiae* genome (i.e. pPM57, containing the SUP2 gene, pYSUP6 and pPM35, containing the SUP8 gene) were not transcribed in HeLa extracts. A possible explanation for this could be the nonconsensus nucleotides at positions 52 and 62 of the yeast $tRNA^{Tyr}$ B-block (Hall *et al.*, 1982). A G_{62} to C_{62} transversion is in fact a strong up mutation in competition experiments with a reference gene in yeast extracts (Allison *et al.*, 1983).

For the preparative isolation of the pre-tRNAs, *in vitro* transcription was performed at low Mg^{2+} concentrations as described earlier (Stange and Beier, 1987). Under these conditions transcription proceeds efficiently, but splicing of the pre-tRNAs does not occur, leading to the accumulation of the primary transcripts. The pre-tRNAs were eluted from the gel and characterized in detail by fingerprint analysis.

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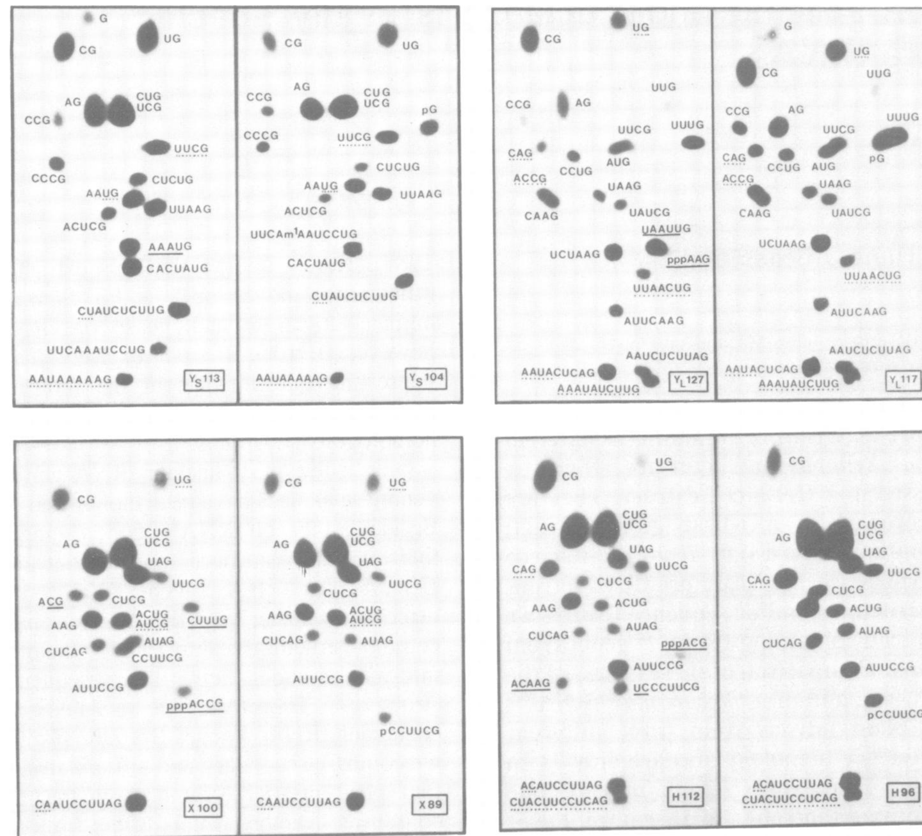

Fig. 1. RNase T1 fingerprint analysis of ^{32}P-labelled pre-tRNAs produced in a wheat germ S100 extract. Each of the four panels shows on the left the RNase T1 fingerprint pattern of unprocessed pre-tRNA synthesized in a HeLa cell nuclear extract and on the right the fingerprint of the major product originating from processing of the corresponding pre-tRNA in the wheat germ extract. The four tRNA genes described in the legend to Figure 2 were transcribed in a HeLa cell nuclear extract in the presence of [α-^{32}P]GTP. The primary transcripts Y_S113, Y_L127, X100 and H112 were recovered from a preparative gel and digested with RNase T1. Oligonucleotide fractionation was by electrophoresis at pH 3.5 on cellulose acetate in the first dimension (from left to right) and by homochromatography in a 30 mM KOH 'homomix' on DEAE-cellulose thin-layer plates at 65°C in the second dimension (from bottom to top) according to Silberklang *et al.* (1979). Aliquots of the ^{32}P-labelled pre-tRNAs were incubated for 120 min in the wheat germ S100 extract and the resulting RNA species Y_S104, Y_L117, X89, and H96 (seen in Figure 2) were also analysed by fingerprinting as described above. The oligonucleotides were identified by their position on the fingerprint (Domdey *et al.*, 1978) and by comparison with the corresponding DNA sequences (Olson *et al.*, 1981; Laski *et al.*, 1983; van Tol *et al.*, 1987; Ganguly *et al.*, 1988). Oligonucleotides specific for the 3'- and 5'-flanking sequences are underlined, those deriving from the intron are dotted.

The patterns of all four pre-tRNAs comprise the expected oligonucleotides including those derived from the introns (dotted) and 5'- and 3'-flanking sequences (underlined), respectively (Figure 1). In detail, the yeast pre-tRNA^{Ser} (Y_S113) contains 4 intron-related oligonucleotides and AAAUG from the 5' leader, the terminal G being the first nucleotide of the 5' exon (Olson *et al.*, 1981). Transcription initiates at G in position −5, since neither A at position −4 nor oligonucleotides from farther upstream were found to be 5' phosphorylated. Yeast pre-tRNA^{Leu} (Y_L127) comprises the intron-specific sequences and two oligonucleotides originating from the 5'-flank, i.e. UAAUUG and pppAAG, both migrating closely together. Hence, transcription starts at the A residue at position −8 (Ganguly *et al.*, 1988). [α-^{32}P]UTP labelling of the pre-tRNAs followed by fingerprinting (not shown) revealed that transcription termination in both cases occurs in stretches of 7 and 8 consecutive T residues, respectively, which almost immediately follow the end of the 3' exon (Filipowicz and Shatkin, 1983; Ganguly *et al.*, 1988).

The fingerprint of *Xenopus* pre-tRNA^{Tyr} (X100) synthesized in the HeLa cell extract contains pppACCG, indicating that transcription initiates at position −5 (Gouilloud and Clarkson, 1986). The oligonucleotides ACG and CUUUG define the 3' trailer (Laski *et al.*, 1983). The fingerprint pattern of the major transcript H112 of the human tRNA^{Tyr} gene is identical with that of the previously designated H2 by van Tol *et al.* (1987). It should be pointed out that transcription initiation of all tRNA genes studied here, including the plant tRNA^{Tyr} gene (van Tol *et al.*, 1987), always starts at a purine which is preceded by a C residue, in accordance with previous results (Clarkson, 1983).

Heterologous tRNA precursors are not spliced in wheat germ extract

The four heterologous pre-tRNAs were incubated in a wheat germ S100 extract in which processing of flanking sequences, excision of the intron and ligation of the resulting halves proceeds very efficiently with a *Nicotiana* pre-tRNA^{Tyr} (Stange and Beier, 1987). Up to 90% of the *Xenopus* and of the two yeast pre-tRNAs are converted to intron-containing pre-tRNAs with mature ends, whereas only 65% of the pre-tRNA H112 had reacted (Figure 2). The identity of these products was established by fingerprint analysis (Figure 1). The oligonucleotide patterns of the [α-^{32}P]GTP- and [α-^{32}P]UTP-labelled processed pre-tRNAs

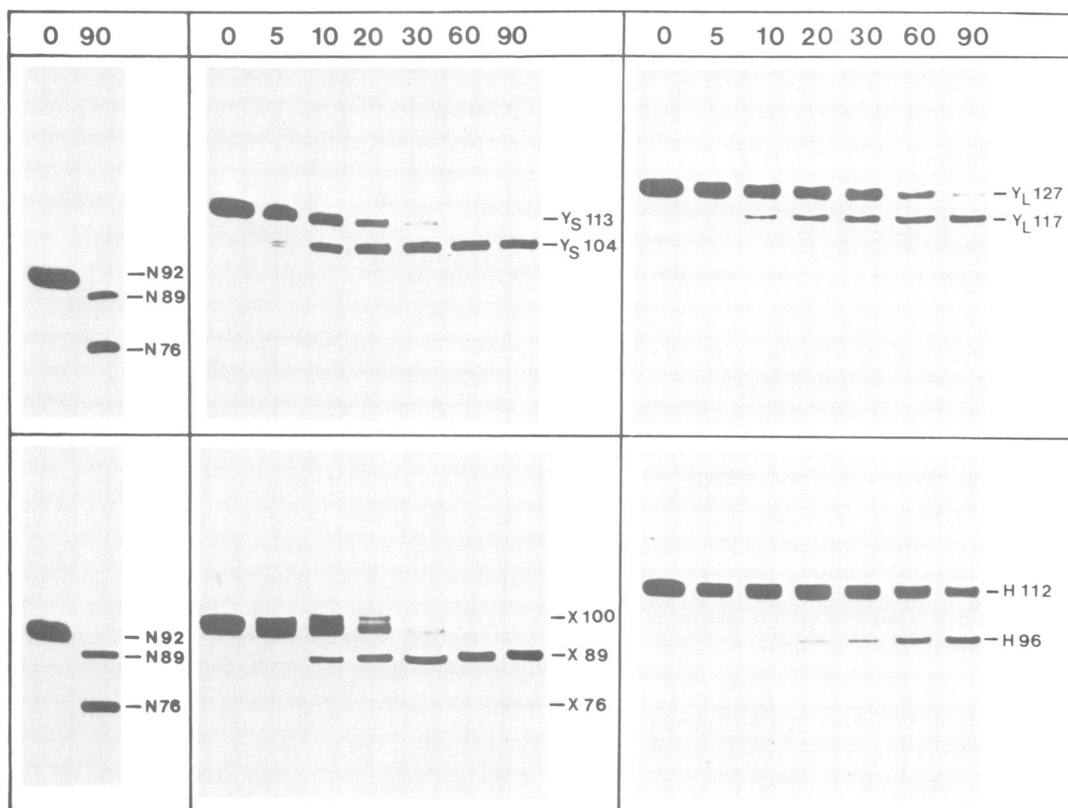

Fig. 2. Time course of *in vitro* processing of heterologous intron-containing tRNA precursors in a wheat germ S100 extract. Two yeast tRNA genes coding for $tRNA^{Ser}$ and $tRNA^{Leu}$, respectively, a $tRNA^{Tyr}$ gene from *X.laevis* and a human $tRNA^{Tyr}$ gene were transcribed *in vitro* in a HeLa cell nuclear extract. The major primary transcripts were recovered from a preparative gel and were used for studying their processing in a wheat germ S100 extract. Aliquots were removed after the times (min) indicated at the top and analysed on a 12.5% polyacrylamide/8M urea gel. The corresponding pre-tRNAs are identified according to their genomic origin (Y_S = $tRNA^{Ser}$ and Y_L = $tRNA^{Leu}$ from *Saccharomyces cerevisiae*; X = *Xenopus laevis*, H = human) and to their length in nucleotides as deduced by fingerprint analyses (Figure 1). For comparison the processing products of the plant $pre\text{-}tRNA^{Tyr}$ (N = *Nicotiana*) after 90 min of incubation in the wheat germ extract are shown in the panels on the left. N92 RNA contains a 5′ leader, a 3′ trailer and a 13-base intron; N89 contains the intron and no flanking sequences and N76 is the mature $tRNA^{Tyr}$ (Stange and Beier, 1987).

Y_S104, Y_L117, X89 and H96 show that they have mature ends but yet contain their introns. This is demonstrated by the absence of oligonucleotides derived from flanking sequences and the presence of all nucleotides which exclusively or partially originate from the introns (Figure 1). As shown previously, the 3′-terminal CCA sequence is rapidly added to all processed tRNAs in wheat germ extract (Stange and Beier, 1987).

Mature tRNA, corresponding in size to N76 (Figure 2) was not produced from any of the heterologous pre-tRNAs with the exception of X100 which yielded up to 10% of mature tRNA. These results were not influenced by variation of the Mg^{2+} and Triton X-100 concentrations over a broad range (not shown). The mature *Xenopus* $tRNA^{Tyr}$ was first detected after 60 min, at which time almost all of the X100 pre-tRNA had been converted to X89 (Figure 2). The RNA X76 was also characterized by fingerprint analysis (not shown). The intron-specific oligonucleotides were not detected, instead, the long oligonucleotide $\text{UAG}{<}^{\text{P}}_{\text{P}}{>}\text{AUCCUUAG}$ containing the RNase T1-resistant, plant-specific ligation site with the 2′-phosphate (Filipowicz and Gross, 1984) appears.

Two Arabidopsis tRNA^Tyr genes contain intervening sequences

We have cloned and sequenced two intron-containing $tRNA^{Tyr}$ genes from *Arabidopsis thaliana* genomic DNA, designated pAtT1 and pAtT3. Both tRNA genes contain an intervening sequence (Figure 3). The 5′ and 3′ exons are

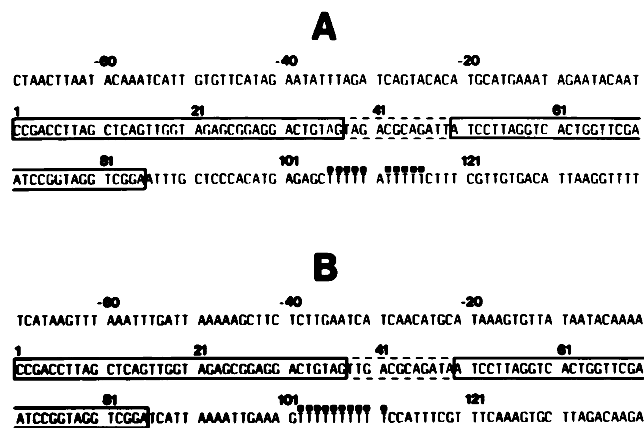

Fig. 3. Nucleotide sequences of two $tRNA^{Tyr}$ genes from *Arabidopsis thaliana*. The noncoding strands of the structural genes and part of their flanking regions contained in pAtT1 (**A**) and pAtT3 (**B**), respectively, are shown. The 5′-terminal nucleotide of the structural genes has been designated number 1. The two exons of each gene are shown in boxes, the intervening sequences within dashed lines. The stretches of squares (■) above the sequence locate the transcription termination sites.

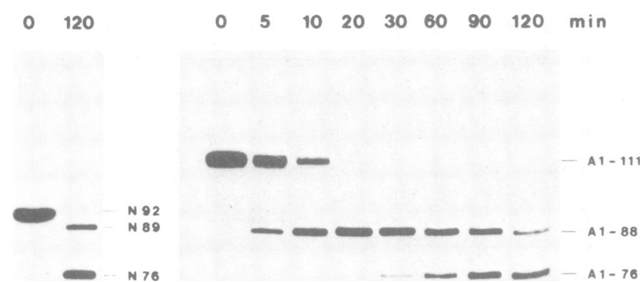

Fig. 4. Time course of *in vitro* processing and splicing of *Arabidopsis* $tRNA^{Tyr}$ precursor in a wheat germ S100 extract. pAtT1 DNA, containing a $tRNA^{Tyr}$ gene from *Arabidopsis* (Figure 3) was transcribed in a HeLa cell extract. The major primary transcript A1-111 was recovered from a preparative gel and incubated in a wheat germ S100 extract. Aliquots were removed after the times (min) indicated at the top and analysed on a 12.5% polyacrylamide/8M urea gel. The products are identified by their length in nucleotides. A1-111 is first processed to A1-88 by removal of 5'- and 3'-flanking sequences. Splicing then yields mature tRNA (i.e. A1-76) as deduced by RNase T1 fingerprint analysis (not shown). The same pattern was observed after incubation of the major transcript from pAtT3 DNA. For comparison the processing products of the *Nicotiana* pre-$tRNA^{Tyr}$ (N92) are shown on the left.

identical with those of the *Nicotiana* $tRNA^{Tyr}$ gene (Stange and Beier, 1986). The 12-bp long introns of the two *Arabidopsis* $tRNA^{Tyr}$ genes differ only in two positions from each other (Figure 3) and show no homology to the 13-bp long intervening sequence of the *Nicotiana* $tRNA^{Tyr}$ gene. These *Arabidopsis* genes are efficiently transcribed in a HeLa cell extract and the major transcripts are processed and accurately spliced in the wheat germ S100 extract. The time course of the *in vitro* processing and splicing of the two *Arabidopsis* pre-tRNAs resembles that of the tobacco pre-$tRNA^{Tyr}$ in S100 extract (Stange and Beier, 1987). The pre-tRNAs are first processed to intron-containing precursors with mature ends, followed by removal of the introns and ligation of the halves. Incubation up to 120 min yields 60–80% of mature $tRNA^{Tyr}$ (Figure 4). Hence, all three intron-containing plant pre-$tRNAs^{Tyr}$ are correctly spliced by the wheat germ endonuclease, whereas yeast or vertebrate pre-tRNAs are only processed at their 5' and 3' ends (Figure 2). This result explains why earlier attempts to detect a splicing endonuclease in wheat germ extracts using intron-containing yeast pre-tRNAs as substrates were not successful (Gegenheimer *et al.*, 1983).

Discussion

It is becoming increasingly apparent that tRNA secondary and tertiary structures may define the selection of cleavage sites by the splicing endonuclease (Lee and Knapp, 1985). Our data (Stange and Beier, 1986; Figure 3) and a $tRNA^{Met}$ gene sequence from *Glycine max* (Waldron *et al.*, 1985) show that there is no homology among the primary sequences of plant tRNA introns. In order to define a consensus structure for these introns and its difference versus that of yeast and vertebrates we compared secondary and tertiary structure models. The use of structure-specific chemical and enzymatic probes has provided evidence that pre-tRNAs have a tRNA-like structure in the mature domain of the molecule and that the intron and a portion of the anticodon loop form a helical, extended anticodon stem (Swerdlow and Guthrie, 1984; Lee and Knapp, 1985). The length of 11 (Waldron *et al.*, 1985), 12 and 13 nucleotides, respectively, of the four plant tRNA introns results in a rather short extended anticodon stem (Figure 5). This does not allow the formation of branched structures as found in most yeast pre-tRNAs (Ogden *et al.*, 1984). Thus, short length may be one important feature of plant introns.

Due to the specific pairing between intron bases and those in and next to the anticodon, only four base pairs separate the splice sites in the four plant pre-tRNAs (Figure 5), including pre-$tRNA^{Met}$ from *Glycine max* (Waldron *et al.*, 1985), whereas they are separated by five or six base pairs in all yeast (Ogden *et al.*, 1984) and human pre-tRNAs (MacPherson and Roy, 1986; van Tol *et al.*, 1987). The tertiary structure models show that both splice sites are located on the same side of the helical structure, probably facing the 'inside' of the 'L'-shaped pre-tRNA. The presence of four base pairs between the splice sites in plant pre-tRNAs and of five or six base pairs in yeast and vertebrate pre-tRNAs causes a difference in distance and relative helical orientation of the splice sites (Figure 5) which is obviously tolerated by the HeLa splicing endonuclease, but not by the plant enzyme. The *Xenopus laevis* pre-$tRNA^{Tyr}$ has a short intron of 13 nucleotides and yet it is spliced poorly by the wheat germ enzyme (Figure 2), indicating that the structural features discussed above may indeed play an important role. Since this pre-tRNA cannot form an extended anticodon stem, it is not clear what its conformation is in the complex with the splicing endonuclease.

There are several features of the splice sites in plant pre-tRNAs common to all pre-tRNAs: the 3' splice site is in most cases a loop of three to five nucleotides in a fixed position relative to the mature domain, and the 5' splice site is also single-stranded or located in a labile double-stranded region (Ogden *et al.*, 1984; Strobel and Abelson, 1986; Greer *et al.*, 1987).

Pre-tRNA 5'- and 3'-processing enzymes are generally sensitive indicators for an appropriate mature tRNA domain structure. The corresponding plant enzymes are obviously not species-specific since they recognize a wide variety of heterologous substrates, quite in contrast to the splicing endonuclease (Figure 2). This indicates that plant versus yeast and vertebrate pre-tRNAs do not differ in their mature domain structure in the wheat germ extract.

Our results raise the question of whether the ability to excise introns from heterologous pre-tRNAs is a common or an exceptional property of splicing endonucleases. The HeLa and *Xenopus* enzymes may not be typical in this respect. For instance, the *S.cerevisiae* splicing endonuclease may possibly be as specific for homologous substrates as the plant enzyme, since three *Schizosaccharomyces pombe* pre-tRNAs (i.e. $tRNA^{Lys}$, $tRNA^{Ser}$ SUP12 and $tRNA^{Leu}$ SUP8) are very inefficiently spliced in *S.cerevisiae* extracts (Gamulin *et al.*, 1983; Sumner-Smith *et al.*, 1984).

Another interesting feature of plant versus yeast pre-tRNA splicing is that the ligase involved is specific for tRNA halves in yeast, whereas it is highly unspecific in wheat germ (Filipowicz and Gross, 1984). Thus, the specificity of pre-tRNA splicing is maintained in plants by the endonuclease alone, and in yeast perhaps by both components of the pre-tRNA splicing apparatus.

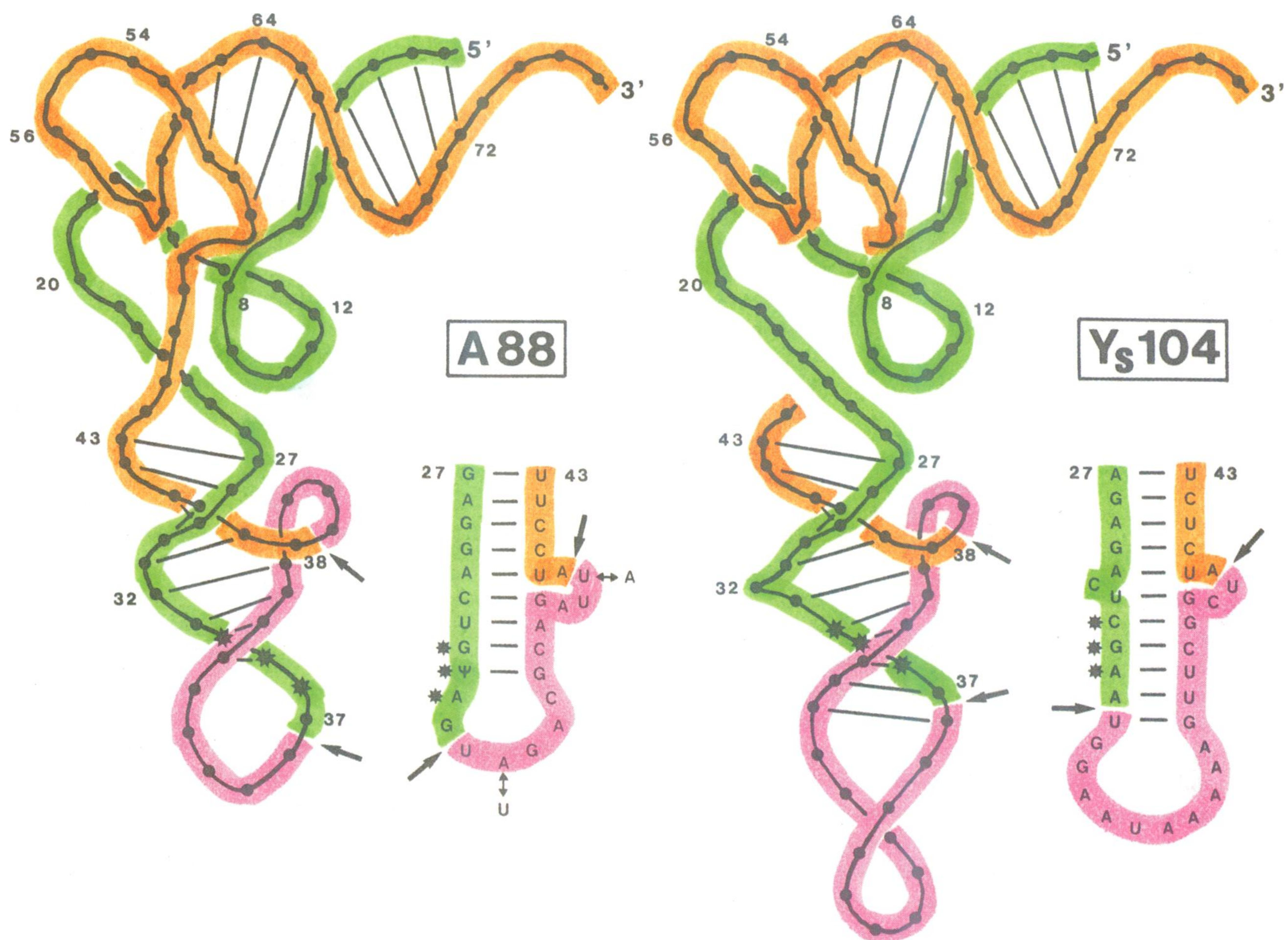

Fig. 5. Secondary and tertiary structure models of intron-containing plant and yeast pre-tRNAs. Three-dimensional structures with mature 5' and 3' ends were drawn without computer assistance by extrapolation of the known tRNA model (Rich and Kim, 1978). The solid line follows the polynucleotide backbone, the bases are represented by dots. Base interactions are shown only for the acceptor stem and T stem, and for the extended anticodon stem. Corresponding secondary structures of the extended anticodon stem are also shown. Arrows next to nucleotide 37 point to the 5' splice site, those next to nucleotide 38 to the 3' splice site; stars identify the anticodons. Numbers locate bases in the standard tRNA cloverleaf. The extended anticodon stems were folded into secondary structures of minimal free energy, using the Zuker-Nussinov algorithm (Steger *et al.*, 1984). ΔGs are -16.8 kJ/mol or -20.0 if A_{31} and U_{39} are unpaired (A88) and -36.1 kJ/mol (Y_S104). G:ψ pairs were treated as G:U pairs. Since X-ray structures of bulge loops are not available, our models were drawn in such a way that a continuous helical extended anticodon stem is maintained. A88: two pre-tRNAs^{Tyr} from *Arabidopsis thaliana* with sequence variations in the introns as indicated; Y_S104: pre-tRNA^{Ser} from *S.cerevisiae*, from which the long 'extra arm' has been omitted since its orientation is not known. The extended anticodon stems of pre-tRNA^{Tyr} from *Nicotiana* (Stange and Beier, 1986) and pre-tRNA^{Met} from *Glycine max* (Waldron *et al.*, 1985) form secondary structures as shown above for A88, with the 5' and 3' splice sites separated by four base pairs. Pseudouridine (ψ_{35}) is included in the anticodon of pre-tRNA^{Tyr} because it is synthesized only in the intron-containing pre-tRNAs (Stange and Beier, 1987; van Tol and Beier, 1988). Green: 5' exon; pink: intron; orange: 3' exon.

Materials and methods

Enzymes and reagents

T4 polynucleotide kinase and RNase T1 were purchased from NEN and Calbiochem, respectively. [α-^{32}P]GTP with a sp. act. of 111 TBq/mmol was from Amersham. All other enzymes and chemicals were obtained from Boehringer, Mannheim. Untreated wheat germs were a gift from Keimdiät GmbH, Augsburg.

Plasmids

The vector plasmid pUC19 was obtained from Dr J.Messing, Minnesota, USA. *Escherichia coli* JM109 was used as a host for propagation of plasmid DNAs. The recombinant plasmids used in this study were: pPM5 and pAA101, which contain the tRNA$^{Ser}_{CGA}$ gene on a 2.5 kb *Bam*HI$-$*Hin*dIII-fragment and the tRNA$^{Leu}_{3}$ gene on a 2.2 kb *Xho*I$-$*Sal*I-fragment, respectively, from *S.cerevisiae* DNA subcloned into pBR322 (Olson *et al.*, 1981; Ganguly *et al.*, 1988); pSVtTsu^{-}, a recombinant of pBR322, SV40 and a 263-bp *Hae*II$-$*Hha*I-fragment of *X.laevis* DNA, which encodes a tRNA^{Tyr} gene (Laski *et al.*, 1983); pHtT1, which consists of a *Hae*III-fragment of 334 bp derived from human DNA, harbouring a tRNA^{Tyr} gene (van Tol *et al.*, 1987) and pNtT1, carrying a tRNA^{Tyr} gene on a 3 kb *Eco*RI-fragment from *Nicotiana rustica* DNA (Stange and Beier, 1986), both subcloned into a pUC19 vector.

Isolation of genomic clones

Total leaf DNA was isolated from young leaves of *Arabidopsis thaliana*. A λ library was prepared by *Eco*RI-digestion of total DNA. Ligation of the resulting fragments into lambda gt 11 vector DNA and *in vitro* packaging was performed according to standard procedures. A synthetic oligonucleotide comprising nts. 18$-$37 of cytoplasmic tobacco tRNA^{Tyr} (Beier *et al.*, 1984) was used for screening of the λ library. Two hybridizing *Eco*RI-fragments of $\sim$3700 and 1500 bp, respectively, were identified and subcloned into pUC19, yielding the plasmids pAtT1 and pAtT3.

DNA sequencing

Direct sequencing of plasmid DNAs was performed according to a modified dideoxy chain termination method (Hattori and Sakaki, 1986).

In vitro transcription in HeLa cell nuclear extracts

Nuclear extracts were prepared from HeLa cells according to Dignam et al. (1983). Transcription assays and the elution of tRNA precursors from preparative gels were performed as described by Stange and Beier (1987).

In vitro processing and splicing of tRNA precursors in wheat germ extracts

Cell-free wheat germ S100 extracts were prepared from wheat embryos as recently described (Stange and Beier, 1987). *In vitro* processing of tRNA precursors was performed in a total volume of 100 μl, containing 15 μl S100 extract, 20 mM Tris HCl, pH 7.5, 100 mM KOAc, 80 μM spermine, 6 mM $Mg(OAc)_2$, 0.15 mM DTT, 0.1 mM CTP, 1 mM ATP, 0.8% Triton X-100 and $\sim 2.5 \times 10^4$ c.p.m. of pre-tRNA.

RNase T1 fingerprint analysis

Digestion of RNAs with RNase T1 and fingerprint analyses were performed according to Silberklang et al. (1979).

Acknowledgements

We wish to thank Professor U.L.RajBhandary for critical reading of the manuscript, Prof. A.R.Kranz (Frankfurt) for *Arabidopsis thaliana* seeds, Dr R.Masterson (Köln) for plant material and U.Thomann from our laboratory for HeLa cell nuclear extract. We thank our colleagues for recombinant plasmids: H.Domdey, München (pYSUP6), H.Feldmann, München (pPM5), C.Greer, Irvine (pPM57), M.V.Olson, St Louis (pPM35), U.L. RajBhandary, Cambridge, USA (pSVtTsu$^-$ and pAA101) and H.van Tol, Würzburg (pHtT1). We are grateful to Dr G.Steger (Düsseldorf) for the calculation of minimal energies using the Zuker-Nussinov algorithm. This work was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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Received on July 6, 1988; revised on August 25, 1988