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A human and a plant intron-containing tRNA^{Tyr} gene are both transcribed in a HeLa cell extract but spliced along different pathways

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tRNA splicing enzymes had been identified in mammalian and plant cells long before homologous intron-containing tRNA genes were detected. The tRNA^{Tyr} gene presented here is the first intron-containing, human tRNA gene for which transcription and pre-tRNA maturation has been studied in a homologous system. This gene is disrupted by a 20-bp long intron and encodes one of the two major human tRNAs^{Tyr} which have been purified and sequenced. A tRNA^{Tyr} gene recently isolated from *Nicotiana* also contains an intron and codes for a functional, major cytoplasmic tRNA^{Tyr}. Both tRNA genes are efficiently transcribed in a HeLa cell nuclear extract. Each of them produces two independent primary transcripts because of two initiation and termination sites, respectively. The maturation of the tRNA^{Tyr} precursors proceeds along different pathways. The intervening sequence of the human pre-tRNA^{Tyr} is excised first, followed by ligation of the tRNA halves and maturation of the flanks, as has been shown for all intron-containing tRNA genes transcribed in HeLa extract. The order of maturation steps is reversed for the plant pre-tRNA^{Tyr}: processing of the flanking sequences precedes intron excision. This maturation pathway corresponds to that observed *in vivo* for tRNA biosynthesis in *Xenopus* oocytes and yeast.

Key words: introns/*in vitro* transcription/plant and human tRNA^{Tyr} genes/pre-tRNA^{Tyr} splicing

Introduction

HeLa cell extracts have become the universal transcription system for polymerase III genes *in vitro* and thus have achieved a general importance comparable with that of the reticulocyte lysate for *in vitro* translation. Even genes of unrelated organisms like tRNA genes of *Drosophila*, yeast, prokaryotes and plant chloroplasts are transcribed and processed in this extract (Schaack and Söll, 1985; Standring *et al.*, 1981; Gruissem *et al.*, 1982). Transcription and splicing of intron-containing yeast and *Xenopus* tRNA genes has been studied in HeLa cell extracts and revealed that the 2′,3′-cyclophosphate of the 5′ exon forms the phosphodiester bond between the halves of the spliced, mature tRNA gene (Filipowicz and Shatkin, 1983). However, transcription and splicing of a homologous, intron-containing human tRNA gene has not been demonstrated until now. Since all known yeast and *Xenopus* tRNA^{Tyr} genes contain introns (Sprinzl *et al.*, 1985a), we concluded that this should also be the case for corresponding human genes. We therefore isolated a human tRNA^{Tyr} gene from a genomic library, determined its nucleotide sequence and described its transcription and splicing in the homologous extract. We show here that this gene encodes one of the two major cytoplasmic tRNAs^{Tyr} of human placenta, both of which we isolated and sequenced.

In parallel we cloned and characterized a nuclear plant tRNA^{Tyr} gene which also contains an intron. Since a homologous *in vitro* transcription system for polymerase III genes of plant origin is not available, we used the HeLa cell extract, and show here that this plant tRNA^{Tyr} gene is actively transcribed and faithfully processed. Interestingly, the primary transcripts of the human and the plant gene are spliced along different pathways.

Results

Isolation and nucleotide sequences of human and tobacco tRNA^{Tyr} genes

A human genomic library in a λ Charon 4A vector was screened for tyrosine tRNA genes as described in Materials and methods using a synthetic 20-mer oligodeoxyribonucleotide as a probe. Two recombinant λ clones (λHtT1 and λHtT2), each carrying a tRNA^{Tyr} gene were obtained. Restriction enzyme digests indicated that these tRNA^{Tyr} genes are derived from different regions of the human genome.

To establish conditions under which the formation of mis-

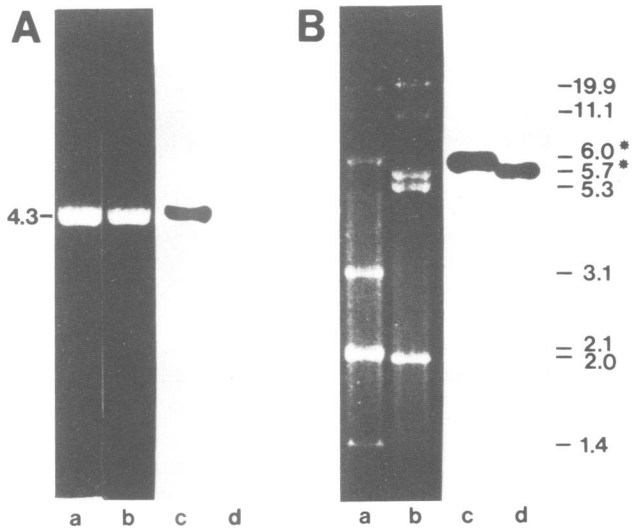

Fig. 1. Restriction endonuclease digests and hybridization of the 20-mer probe to λHtT1 and λHtT2 DNA. Equimolar amounts of **(A)** plasmid DNA (pSVtTsu$^-$, **lanes a** and **c**; pSVtTsu$^+$, **lanes b** and **d**) and **(B)** λDNA (λHtT1, **lanes a** and **c**; λHtT2, **lanes b** and **d**) were digested with *Bam*HI and *Eco*RI, respectively, and subjected to electrophoresis on a 0.8% agarose gel. Markers of *Hpa*I- and *Eco*RI-digested T7 and λ Charon 4A DNA, respectively, were run in adjacent lanes (not shown). Hybridization to Southern blots containing the restriction fragments of plasmid and λDNA was carried out with a ^{32}P-labeled 20-mer oligonucleotide probe as described in Materials and methods. At the left-hand side of panels A and B ethidium bromide stained gels **(lanes a** and **b)** are shown, whereas at the right side the corresponding autoradiograms of Southern blots are displayed **(lanes c** and **d)**. The numbers indicate the length of the fragments (kb) as deduced from the sizes of DNA fragments of digested marker DNAs. The fragments to which the 20-mer oligonucleotide hybridized are indicated by an asterisk (*).

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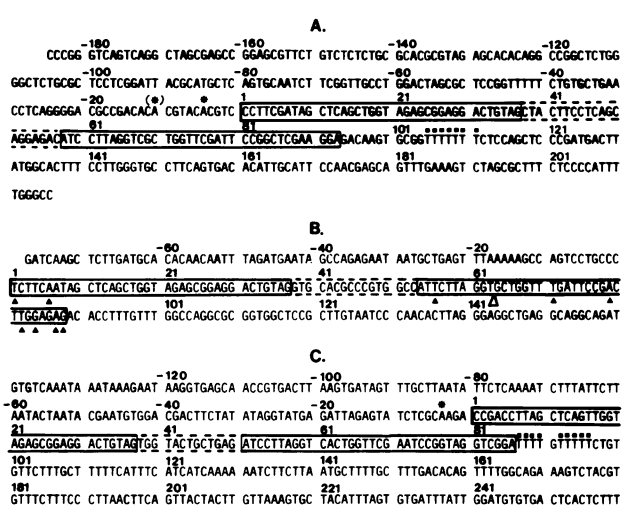

Fig. 2. Nucleotide sequences of the noncoding strands of the tRNA^{Tyr} genes containing regions of pHtT1 **(A)**, pHtT2 **(B)** and pNtT1 **(C)**. The two exons of each gene are shown in boxes, the intervening sequences within dashed lines. The 5′-terminal nucleotide of the structural gene has been designated number 1, upstream nucleotides have negative numbers. Asterisks (*) mark the transcription initiation sites, the stretches of squares (■) above the sequence locate the transcription termination sites. The positions in which the structural tRNA^{Tyr} gene of pHtT2 differs from pHtT1 are indicated by black triangles (▲). The open triangle (△) in **(B)** points to a nucleotide deletion.

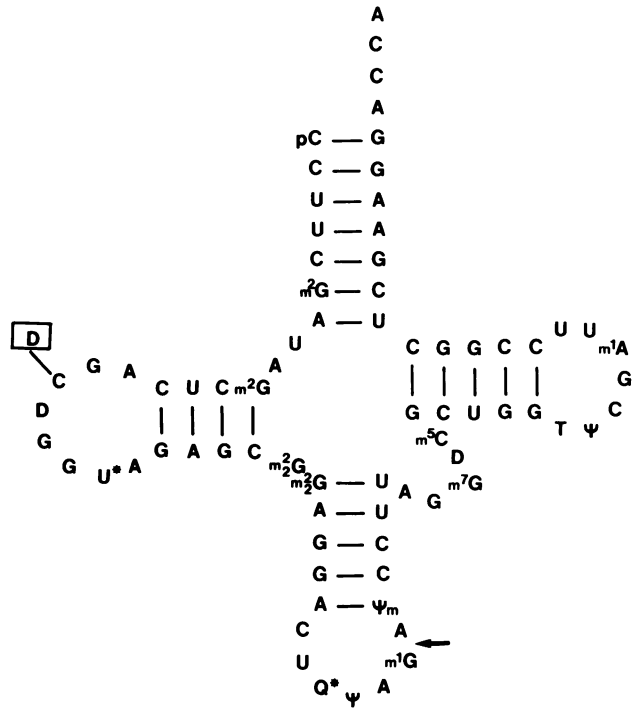

Fig. 3. Nucleotide sequences of placenta tRNA$_1^{Tyr}$ and tRNA$_2^{Tyr}$. The clover leaf model shows the nucleotide sequence of tRNA$_1^{Tyr}$. Dihydrouridine (D) is found in tRNA$_2^{Tyr}$ at position 16 (boxed). The nucleotide sequence of tRNA$_2^{Tyr}$ is identical with that determined for bovine liver tRNA^{Tyr} (Johnson *et al.*, 1985). Human tRNA$_1^{Tyr}$ and *X. laevis* tRNA^{Tyr} are identical in sequence, but differ in various modifications (Laski *et al.*, 1982). U* in position 20 is 3-(3-amino-3-carboxypropyl)uridine, acp^{3}U, and Q* is β-D-galactosyl-queuosine. The arrow indicates the position of the intron in the pre-tRNA^{Tyr}.

matched duplexes of the DNA/oligomer hybrid would be eliminated (Wallace *et al.*, 1979; Szostak *et al.*, 1979) we used in parallel two *Xenopus laevis* tRNA^{Tyr} clones, i.e. pSVtTsu$^-$ and pSVtTsu$^+$ which differ in a single base pair in the anticodon sequence of the structural genes. A wash temperature of 60°C resulted in almost complete dissociation of the pSVtTsu$^+$ DNA/20-mer duplex (Figure 1A,d) while the pSVtTsu$^-$ DNA/20-mer duplex remained stable (Figure 1A,c). Thus, we performed all Southern hybridization analyses at wash temperatures of 60°C to identify tRNA^{Tyr} genes under stringent hybridization conditions.

Among the four *Eco*RI fragments of the 12.6-kb human fragment of λHtT1, the 6.0-kb fragment hybridized to the 20-mer probe (Figure 1B,c). Further restriction enzyme cleavages yielded a *Sma*I/*Hae*III fragment of 401 bp which was subcloned into the *Sma*I site of pUC19 (= pHtT1). The sequence of the 401-bp fragment is shown in Figure 2A. The λHtT2 phage DNA contains a 13-kb insert which can be cleaved into three fragments by *Eco*RI. The tRNA^{Tyr} gene is located on the 5.7-kb fragment (Figure 1B,d). Further restriction enzyme digestions of a corresponding subclone yielded a *Sau*3A fragment of 416 bp (= pHtT2). The sequence of 238 bp of this clone is shown in Figure 2B.

The tRNA^{Tyr} gene of pHtT1 (nucleotides 1−93) differs from the known tRNA^{Tyr} species of bovine liver (Johnson *et al.*, 1985) at nucleotide 16 where a dihydrouridine (D) has been identified, whereas the human tDNA sequence of pHtT1 contains cytidine in this location. To clarify this point we isolated the major tRNAs^{Tyr} from human placenta and determined their sequences by post-labeling procedures. These two tRNAs^{Tyr} differ in nucleotide 16 only, which is C in tRNA$_1^{Tyr}$ and D in tRNA$_2^{Tyr}$ (Figure 3). Thus the structural gene of pHtT1 (Figure 2A) encodes tRNA$_1^{Tyr}$. The ratio of tRNA$_1^{Tyr}$ to tRNA$_2^{Tyr}$ is about 7:3 in unfractionated placenta tRNA. The only other vertebrate tRNA^{Tyr} besides that of bovine liver (Johnson *et al.*, 1985) which has a C in position 16 and is thus identical in nucleotide sequence to tRNA$_1^{Tyr}$ from human placenta is tRNA^{Tyr} from *X. laevis* (Laski *et al.*, 1982).

Interesting features of the gene for human tRNA$_1^{Tyr}$ are a 20-nucleotide-long intron and a transcription termination signal of seven consecutive thymidines located 11 bp from the 3′ end of the structural gene (Figure 2A). The second tRNA^{Tyr} gene also contains an intron which is 16 nucleotides long. There is no sequence homology between the introns and the flanking sequences of the two tRNA^{Tyr} genes. The structural gene of pHtT2 (without intron) differs from pHtT1 in nine positions and by a deletion (Figure 2B). These mutations are located outside the region which is complementary to our 20-mer hybridization probe. Most of the mutations are compensatory in that basepairs on the tRNA level are maintained. However, A64 disrupts the secondary structure of the T-arm and the absence of C48 prevents the tertiary structure interaction with G15 (Quigley and Rich, 1976) and U in position 56 is abnormal (Sprinzl *et al.*, 1985b). This gene has all the following properties of a tRNA pseudogene. (i) It is inefficiently transcribed (Figure 4a) presumably due to transition of the invariant C56 within the B-box of the internal promoter to T, a mutation which also impairs transcription drastically in a human tRNA^{Gly} pseudogene (Pirtle *et al.*, 1986). (ii) There is no effective transcription signal. The $(T)_3G(T)_3$ sequence in the 3′-proximal region of this gene is inefficient in this respect, since even a $(T)_3G(T)_4$ tract is a rather weak terminator (Gouilloud and Clarkson, 1986). (iii) As mentioned above, at least two of the mutations may disturb the secondary and tertiary structure of the pre-tRNA and thus impair processing. (iv) A corresponding functional tRNA^{Tyr} is not known.

The minimal number of individual tRNA^{Tyr} gene loci appears

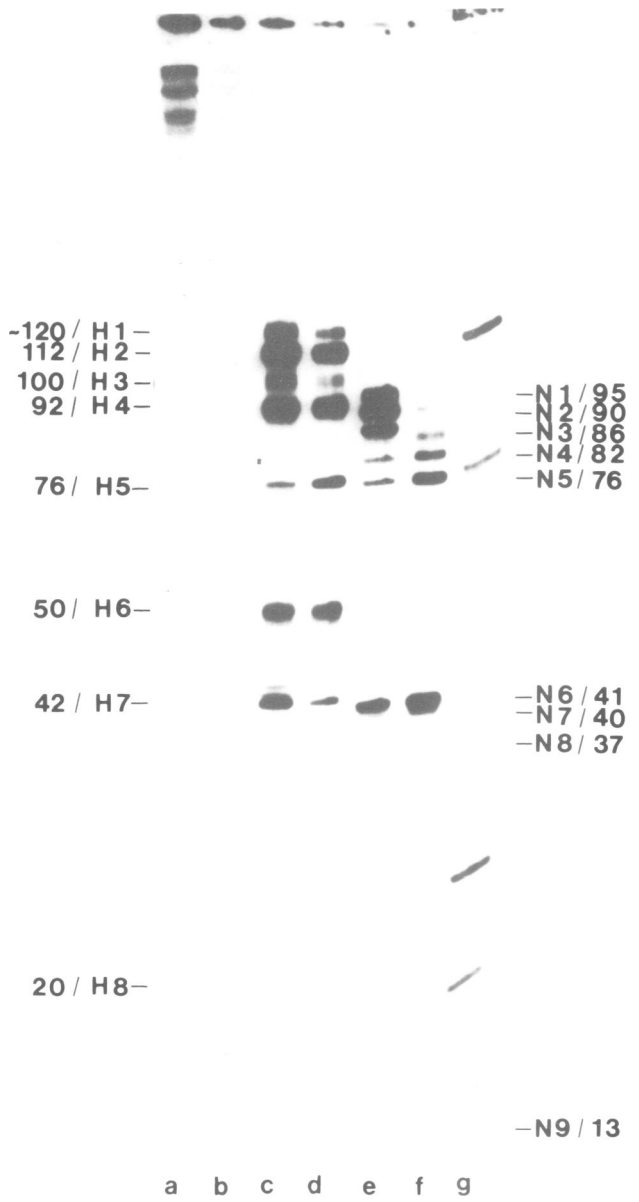

Fig. 4. Gel electrophoretic analysis of *in vitro* transcripts of pHtT1, pHtT2 and pNtT1 DNAs synthesized in a HeLa cell nuclear extract. Transcription assays were performed essentially as described in Materials and methods in 10 μl reaction mixtures containing 3.5 nM template DNA of pHtT2 **(lanes a** and **b)**, pHtT1 **(lanes c** and **d)** and pNtT1 **(lanes e** and **f)**. Incubation was in the presence of [α-^{32}P]GTP for 60 min at 30°C **(lanes a, c** and **e)**. In pulse-chase experiments, incubation was first at 30°C for 60 min after which unlabeled GTP and ATP were added at final concentrations of 1.4 and 1.0 mM, respectively, and incubation was continued for 90 min **(lanes b, d** and **f)**. Samples were analysed on a 12.5% polyacrylamide/8 M urea gel (0.4 mm thick, 40 cm long). Autoradiography was for 10 h at −70°C. The major transcripts are identified by their genomic origin (H = human, N = *Nicotiana*), by a number according to their decreasing size (1−8), and by their length in nucleotides. The size of the transcripts was calculated from 5′-^{32}P-labeled standards, i.e. rabbit liver 5S RNA (~118 nucleotides), placenta tRNA^{Tyr} (76 nucleotides), 26-mer and 20-mer synthetic oligonucleotides **(lane g)**.

to be 12 in the haploid human genome as evidenced by stringent hybridization of ^{32}P-labeled 20-mer to Southern blots of *Eco*RI digested genomic placenta DNA (not shown). The hybridization pattern indicates that human tRNA^{Tyr} genes are mainly organized as a dispersed multigene family. This corroborates well with the minimum number of 12 and 13 dispersed tRNA$_i^{Met}$ (Santos and Zasloff, 1981) and tRNA^{Val} genes (Arnold *et al.*, 1986), respectively, in the human genome.

The sequence of the tRNA^{Tyr} gene from tobacco nuclear DNA is shown in Figure 2C. Like the two human tRNA^{Tyr} genes it contains an intron. This gene encodes tRNA$_1^{Tyr}$, one of the two major tyrosine tRNAs from tobacco leaves (Beier *et al.*, 1984). A special feature of this gene is a $(T)_4G(T)_5$ termination signal immediately adjacent to the 3′ end of the structural gene which resembles the situation in yeast tRNA^{Tyr} genes (Goodman *et al.*, 1977).

In vitro transcription of tRNA^{Tyr} genes and pre-tRNA processing in a HeLa cell nuclear extract

Only very few tRNA genes containing introns have been transcribed *in vitro* in HeLa cell extracts until now, i.e. yeast tRNA^{Leu}, yeast tRNA^{Ser} and *X. laevis* tRNA^{Tyr} (Standring *et al.*, 1981; Filipowicz and Shatkin, 1983; Laski *et al.*, 1983). The question then arose whether a homologous intron-containing human tRNA gene would be transcribed and spliced in the same manner as the heterologous tRNA genes mentioned above. In the previous section we have characterized two tRNA^{Tyr} genes from human DNA (Figure 2A,B). Incubation of pHtT1 DNA in a HeLa extract results in eight discrete RNAs as revealed by polyacrylamide gel electrophoresis (Figure 4c). These products are designated H1−H8 according to their decreasing size. The second human tRNA^{Tyr} gene (i.e. pHtT2) was poorly transcribed and yielded no products of similar size. Only two high mol. wt RNAs of about 400−500 nucleotides were visible (Figure 4a).

Surprisingly, the tRNA^{Tyr} gene of plant origin was transcribed very efficiently in the HeLa extract. Nine discrete RNA species were produced and designated N1−N9 (Figure 4e). The predominant products of pHtT1 and pNtT1 after 60 min are two RNAs of 112 (H2) and 92 nucleotides (H4) and three RNAs of 95 (N1), 90 (N2) and 86 nucleotides (N3), respectively. The RNA species H5 and N5 represent the mature-sized tRNAs as evidenced by their fingerprints and their electrophoretic mobility which is indistinguishable from authentic tRNA^{Tyr} (Figure 4g).

To investigate whether the products of tRNA size (i.e. H5 and N5) were derived from larger precursors, pulse-chase experiments were performed. The tRNA genes were first incubated with [α-^{32}P]GTP for 1 h and then excess unlabeled GTP was added and incubation was continued for 90 min. As can be seen in Figure 4 (lanes d and f), products of tRNA^{Tyr} size accumulate at the expense of precursors H2 and H4, and N1, N2 and N3, respectively. *In vitro* transcription of all three tRNA^{Tyr} genes was sensitive to 200 μg/ml, but not to 20 μg/ml α-amanitin, indicating that the observed transcripts were synthesized by RNA polymerase III.

Transcription of a 900-bp *Eco*RI/*Pst*I fragment from λHtT1 yields no further transcripts, suggesting that there are no functional tRNA genes in the near neighbourhood of the tRNA^{Tyr} gene (not shown).

Quantitation of all transcription and processing products H1−H8 and N1−N9 revealed that the transcription efficiency of the human (pHtT1) and the plant tRNA^{Tyr} genes (pNtT1) in the HeLa cell nuclear extract are about the same.

Fingerprint analysis of in vitro transcripts and processing products

The relationship between RNA species H1−H8 and N1−N9, respectively, was further established by RNase T1 fingerprints. All major *in vitro* transcripts derived from pHtT1 and pNtT1 were recovered from preparative gels and characterized in this way. Figure 5 shows three selected fingerprint patterns of RNA

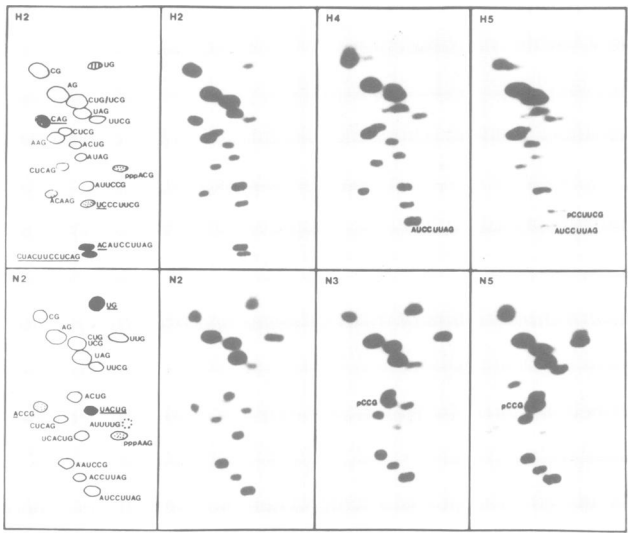

Fig. 5. RNase T1 fingerprints of pre-tRNAs and mature tRNAs^{Tyr} derived from pHtT1 and pNtT1. [α-^{32}P]GTP-labeled RNA transcripts were recovered from a preparative gel and digested with RNase T1. Oligonucleotide fractionation was by electrophoresis at pH 3.5 on cellulose acetate in the first dimension (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). The oligonucleotides were identified by their position according to Domdey *et al.* (1978), by comparison with the DNA sequence and by the estimation of molar yields. The designation of the RNA transcripts is as in Figure 4. At the left is a schematic presentation of the oligonucleotides derived from H2 and N2, respectively. Open circles, from mature tRNAs^{Tyr}; stippled spots, from the 5′-flanking sequences; hatched spots, from the 3′-flanking sequences; solid spots, from the intron. Oligonucleotides unique to H4, H5, N3 and N5 are indicated by their sequence in the autoradiographs. The dotted circle in the N2 scheme locates an oligonucleotide AUUUUG characteristic for N1.

species from both genes. The sequences and the molar yields of the oligonucleotides match those predicted by the DNA sequences (Figure 2). As mentioned above, H2 and N2 are the major products synthesized *in vitro* after 60 min of incubation (Figure 4). These RNAs contain introns and flanking sequences as follows. (i) There are oligonucleotides which exclusively or partially originate from the introns (solid spots, intron-derived nucleotides underlined in the schematic fingerprints). (ii) The precursor H2 contains 5′ flanking sequences (stippled spots) as demonstrated by the unique spots UCCCUUCG (flanking nucleotides underlined) and pppACG. This latter spot disappears from the respective fingerprints upon phosphatase treatment of the pre-tRNAs H2 and H4 and appears instead as ACG. The 5′-terminal oligonucleotide pppAAG of N2 was identified accordingly. The minor species H1, as compared with H2, contains only one additional oligonucleotide, UACACG, which is derived from the 5′ flank, thus identifying H1 as an independent transcript with a longer 5′ leader (data not shown). (iii) Species H2 contains a relatively long 3′ trailer as evidenced by the specific oligonucleotides UG and ACAAG (hatched spots). The other trailer-specific oligonucleotides are masked by fragments from other regions of the pre-tRNA sequence. N2 does not show a trailer-derived oligonucleotide, indicating that transcription termination occurs in the oligo(T) tract immediately adjacent to the structural gene. The minor precursor N1, however, contains an extra oligonucleotide AUUUUG (dotted circle) which is obviously generated by transcription over the first oligo(T) stretch and termination within the succeeding oligo(T) sequence (Figure 2C).

An inspection of the fingerprint of H4 reveals that this RNA contains the flanking sequences, but not the intron. Correspondingly, H3 has derived from H1 by removal of the intron (not shown). The fingerprint of the mature tRNA^{Tyr} (H5) indicates the presence of oligonucleotide pCCUUCG typical for the mature 5′ end, whose 5′ phosphate was again identified by its shift in the fingerprint after phosphatase treatment of the tRNA. The RNAs H6 and H7 comprise oligonucleotides typical for the unprocessed 5′ and 3′ tRNA halves, respectively. The fingerprint of H8 displays the three oligonucleotides deriving from the intron (not shown).

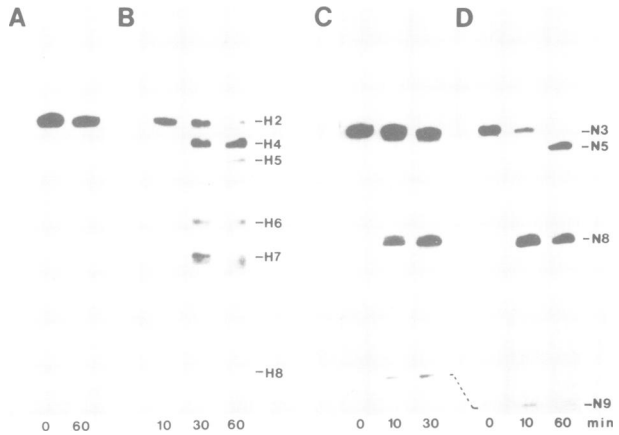

Fig. 6. *In vitro* splicing in a HeLa cell nuclear extract of pre-tRNAs^{Tyr} derived from pHtT1 and pNtT1. The [α-^{32}P]GTP-labeled RNA precursors H2 and N3 were eluted from preparative gels. H2 (**A** and **B**) and N3 (**C** and **D**) pre-tRNAs were incubated for the times indicated either in the presence of 2 mM $MgCl_2$/0.2 mM ATP (**A** and **C**) or in the presence of 7 mM $MgCl_2$/1 mM ATP (**B** and **D**). The RNAs were separated on a 10% (**A** and **B**) and a 12.5% (**C** and **D**) polyacrylamide/8 M urea gel (0.4 mm thick, 20 cm long), respectively. The products derived from splicing of the pre-tRNAs are identified as in Figure 4 at the right side of the gels.

Species N3, unlike the spliced precursor H4, still contains the intron, but not the flanking sequences. The minor RNA N4 contains no intron, but the flanking sequences, thus resembling H4. The fingerprint of the mature tRNA (N5) contains pCCG, the oligonucleotide from the mature 5′ end. RNA N6 is the 5′ half of the 5′ leader, whereas N8 is a mixture of the 5′ half with the mature 5′ end and, to some extent, the 3′ half. N7 also contains the 3′ half, because of its size presumably with unprocessed trailer (data not shown). RNA N9 is easily identified as the correctly excised intron due to its characteristic fingerprint. It seems noteworthy that the intron as well as the tRNA 3′ halves H6 and N7 were partially 5′ phosphorylated (20−50%) if transcription was performed at high ATP concentrations of 1 mM (not shown). According to Filipowicz and Shatkin (1983), such 5′ phosphorylated 3′ halves are not substrates for the HeLa RNA ligase which joins the tRNA halves. Some indirect evidence for post-transcriptional modification in the HeLa extract is provided by the fingerprints of N2, N3 and N5: the oligonucleotide UCACUG becomes weaker and new faint spots arise in its neighbourhood during tRNA maturation from N2 via N3 to N5, presumably due to partial modifications of G46 and/or U47.

High Mg^{2+} concentrations are required for intron excision from the human but not from the plant tRNA^{Tyr}

To elucidate the sequence of processing and splicing events by which the mature human and plant tRNAs^{Tyr} are generated from their precursors H2 and N3, respectively, we isolated these pre-tRNAs and incubated them in the HeLa extract under two different conditions. In most polymerase III transcription and splicing systems, Mg^{2+} concentrations around 5 mM are used (Koski *et al.*, 1980; Laski *et al.*, 1983; Filipowicz and Shatkin, 1983;

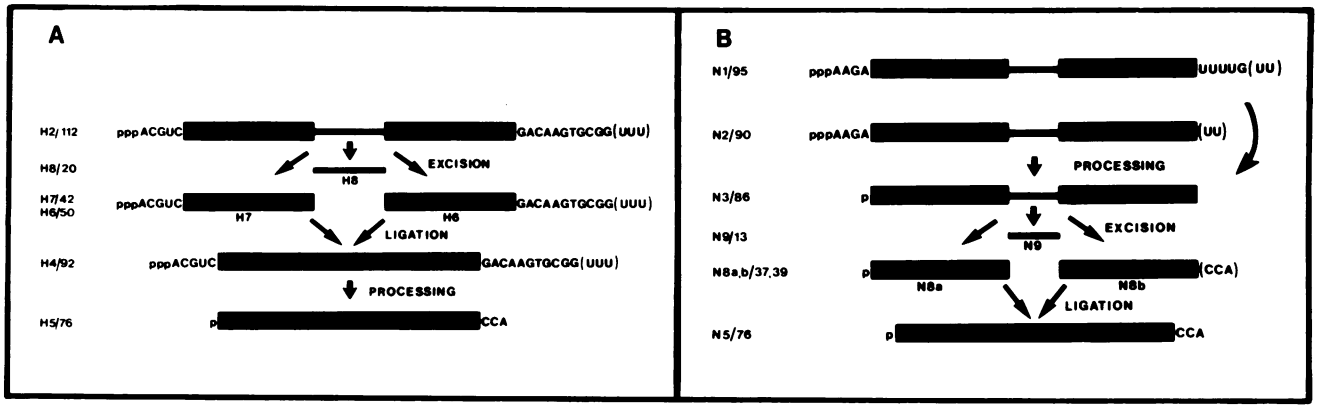

Fig. 7. Processing pathways of intron-containing pre-tRNAs^{Tyr} of human and plant origin. **(A)** The major transcript (H2) of the human tRNA^{Tyr} gene is a precursor of 112 ± 2 nucleotides with a 5′ leader, intervening sequence, and a 3′ trailer. This pre-tRNA^{Tyr} is first cleaved to yield tRNA halves with unprocessed ends (H6 and H7) and the intron (H8). These tRNA halves are then ligated to H4 which is processed to mature tRNA^{Tyr} (H5) by removal of 5′ leader and 3′ trailer. **(B)** A minor transcript of this gene, N1, is a precursor of 95 ± 2 nucleotides which differs from N2 only by its longer 3′ trailer. N1 and N2 appear to be independent transcription products resulting from terminatation at the two oligo(T) stretches adjacent to the tRNA gene (Figure 2C). N1 and N2 are both first processed to N3, the intron-containing precursor with mature ends. N3 is then cleaved to yield the intron (N9) and the tRNA halves (N8a,b) which are subsequently ligated to mature tRNA^{Tyr} (N5).

Drabkin and RajBhandary, 1985). Another special requirement for the splicing reaction, in addition to Mg^{2+}, is a high ATP concentration of about 1 mM (Peebles *et al.*, 1979). Therefore we studied *in vitro* splicing in the presence of 7 mM $MgCl_2$ and 1 mM ATP. Incubation of the human pre-tRNA^{Tyr} H2 under these conditions yields halves (H6 and H7) and the intron (H8) after 10 min. The ligated RNA with unprocessed flanks (H4) appears after 30 min and the mature tRNA (H5) after 60 min (Figures 6B and 7A). The processing of purified H4 to H5 occurs very slowly in the HeLa extract as observed in parallel assays. Even after 60 min of incubation, only 10% of H4 was processed to mature tRNA H5. The minor transcript H1 which contains a longer 5′ leader than H2 was spliced to H3, the ligated RNA with unprocessed flanks (not shown).

Incubation of the plant tRNA^{Tyr} precursor N3, which contains the intron but no flanks, yields the comigrating half molecules N8 and intron N9 after 10 min incubation under standard conditions, and the mature tRNA^{Tyr} N5 is efficiently formed after 60 min (Figure 6D). In parallel experiments the purified precursors N1 and N2 were both processed to N3 (not shown) as presented in Figure 7B.

As expected, the precursor H2 remains unspliced upon incubation in the HeLa extract at low Mg^{2+} concentrations (Figure 6A). Surprisingly, the precursor N3 is efficiently cleaved into halves (N8) and the intron (N9) under these conditions (Figure 6C). Ligation is not observed in this case because of the low ATP concentration (0.2 mM).

Splicing of a human and a plant pre-tRNA^{Tyr} occurs along different pathways

The steps involved in the maturation of a human and a plant tRNA^{Tyr} as observed in HeLa cell nuclear extracts are schematically presented in Figure 7. The major primary transcript of the human tRNA^{Tyr} gene (pHtT1) is about 112 nucleotides long and contains a 5′ leader of five, an intron of 20 and a 3′ trailer of about 14 nucleotides (H2, Figures 4, 5 and 7A). The data from Figure 6B and the corresponding fingerprints (Figure 5) provide unequivocal proof for the following steps: intron excision and ligation of the resulting halves to pre-tRNA H4 with unprocessed flanks. This precursor is then slowly processed to mature tRNA^{Tyr} H5 (Figure 6B). The minor primary transcript H1 of pHtT1 differs from the major one (H2) only by its longer 5′ leader and is about 120 nucleotides long (Figure 4). The maturation of H1 to mature tRNA^{Tyr} via H3 follows exactly the same steps as established for H2 (not shown).

The two primary transcripts of the human tRNA^{Tyr} gene, H1 and H2, result from transcription initiation at two independent sites, whereas the two primary transcripts N1 and N2 derived from the plant tRNA^{Tyr} pNtT1 are generated by transcription termination at two sites. The major precursor N2 is about 90 nucleotides long and contains a 5′ leader of four, an intron of 13 and a 3′ trailer of one or two uridines (Figures 4, 5 and 7B). The minor precursor N1 is about 95 nucleotides long and differs from N2 only in the 3′ trailer as evidenced by the corresponding fingerprints (Figure 5). The maturation of these two pre-tRNAs is summarized in Figure 7B: the first step for both, N1 and N2, is the processing of the flanking sequences, yielding the precursor N3. This has been documented by incubation of the isolated precursors in a HeLa extract according to Figure 6D. The intermediate RNA N3 yet contains the intron, but not the flanks (Figures 4 and 5). The last maturation step of the plant pre-tRNA is intron excision from N3 and ligation of the resulting halves to the mature tRNA^{Tyr} (Figure 6D).

A minor maturation pathway from N1 and N2 starts with intron excision, leading to the 5′ and 3′ halves N6 and N7 with unprocessed flanks, followed by ligation to the intermediate N4 (Figure 4). This maturation process resembles that found for the human pre-tRNA^{Tyr} (Figure 7A) and becomes more prominent at elevated Mg^{2+} concentrations (>5 mM).

Discussion

We present here the first intron-containing human tRNA gene for which homologous *in vitro* transcription and maturation is shown. Among the reports on mammalian tRNA genes isolated and sequenced so far (Sprinzl *et al.*, 1985a), there is only one concerning intron-containing tRNA genes (MacPherson and Roy, 1986). About 10–20% of all yeast tRNA genes appear to have introns (Guthrie and Abelson, 1982), whereas tRNA introns seem to be rare in higher eukaryotes (Clarkson, 1983) and especially in mammalia. Only the tRNA^{Tyr} genes of all eukaryotes sequenced until now including those from yeast, *Xenopus* and man contain introns (Goodman *et al.*, 1977; Müller and Clarkson, 1980; MacPherson and Roy, 1986). Consequently we expected that this is

also the case for nuclear plant tRNA^{Tyr} genes. In fact we showed that the tRNA^{Tyr} gene, the first nuclear plant tRNA gene for which a functional tRNA is known (Beier *et al.*, 1984), contains an intron (Stange and Beier, 1986). Hence there seems to be an evolutionary pressure on the maintenance of introns in tRNA^{Tyr} genes in eukaryotes, which is perhaps related to the finding that the intron of tRNA^{Tyr} genes is involved in the correct modification of its tRNA product (Johnson and Abelson, 1983). A comparison of the intron sequences from Figure 2 and those from tRNA^{Tyr} genes of other higher eukaryotes (Müller and Clarkson, 1980; Gouilloud and Clarkson, 1986; MacPherson and Roy, 1986) show that they differ in length and share no sequence homologies. It therefore seems to be an exception that three tRNA^{Tyr} genes of yeast contain almost identical intron sequences (Goodman *et al.*, 1977). One of the common features of all intron-containing pre-tRNAs seems to be a secondary structure involving an 'extended anticodon stem' due to base pairing between one (Müller and Clarkson, 1980), two (Colby *et al.*, 1981) or three nucleotides (Ogden *et al.*, 1981) of the anticodon triplet and the intervening sequence. A similar secondary structure is possible for the transcripts of the human and the plant tRNA^{Tyr} genes described here (Figure 2A and C). Only a less stable secondary structure can be drawn for a putative pre-tRNA of the second human tRNA^{Tyr} gene (Figure 2B) which is a pseudogene as discussed in Results.

The fact that we identified intron-containing tRNA^{Tyr} genes is not so unexpected since human (Standring *et al.*, 1981; Filipowicz and Shatkin, 1983; Laski *et al.*, 1983) and plant cells (Tyc *et al.*, 1983) have all the enzymes for pre-tRNA splicing. It was therefore most important to establish how a homologous (pHtT1) and in parallel a non-homologous (pNtT1), intron-containing tRNA gene would be transcribed and processed in the HeLa extract. In fact, these two genes are transcribed with equal efficiency, and more efficiently than the *Xenopus* tRNA^{Tyr} gene in pSVtTsu$^-$ (not shown). For each of the tRNA^{Tyr} genes we find two independent primary transcripts which differ in their 5′ leader and 3′ trailer, respectively. The transcription of the human tRNA^{Tyr} gene initiates at two positions. The major site, accounting for about 70% of the initiation events, is the A residue at position −5; the minor site is the A at position −11. Transcription terminatation occurs for both transcripts within the 3′ flanking $(T)_7$ cluster (Figures 2A and 5). Transcription initiation of the plant tRNA^{Tyr} gene starts at the A residue at −4, whereas termination occurs at two different sites. The major site is the $(T)_4$ sequence immediately following the 3′ end of the structural gene and accounts for about 80% of the primary transcripts. The minor termination signal is the $(T)_5$ stretch which is separated by a G from the major termination site (Figures 2C and 5). Accordingly, analyses of the ratios of the primary transcripts H1/H2 and N1/N2, respectively, during the time course of incubation up to 180 min did not reveal precursor/product relationships.

As shown in Figure 7A, the maturation of the primary transcript of the human tRNA^{Tyr} gene is initiated by intron excision, followed by ligation of the resulting halves to pre-tRNA with unprocessed 5′ leader and 3′ trailer, from which mature tRNA^{Tyr} is generated by slow processing. This splicing and processing pathway is in complete agreement with that for all intron-containing tRNA genes transcribed in HeLa extracts (Standring *et al.*, 1981; Filipowicz and Shatkin, 1983; Laski *et al.*, 1983). All these observations are in contrast to the results obtained *in vivo*, i.e. in yeast and in *Xenopus* oocytes, where the order of maturation events is just reversed (Etcheverry *et al.*, 1979; Melton *et al.*, 1980; Standring *et al.*, 1981; Colby *et al.*, 1981; Hopper and Kurjan, 1981). An explanation for this difference between tRNA maturation pathways *in vitro* and *in vivo* has been proposed by Standring *et al.* (1981): a deficiency of processing nucleases in transcription extracts. The sequence of maturation steps of the plant pre-tRNA^{Tyr}, however, shows that this argument does not apply here. The first step of the two primary transcripts N1 and N2 (Figure 7B) is the efficient processing of the 5′ leader and 3′ trailer sequences (Figure 4, lanes c and f). Only then follows intron excision and ligation of the tRNA halves to mature tRNA^{Tyr}. Surprisingly, this is exactly the order of maturation steps as observed *in vivo*. Furthermore, this result indicates that intron excision and ligation of half molecules proceeds with pre-tRNAs with and without flanking sequences as well.

The primary transcripts of the human and the plant tRNA^{Tyr} gene not only differ in their maturation pathways, but also in the Mg^{2+} dependence of intron excision (Figure 6). Standard conditions for pre-tRNA splicing require high (5−8 mM) Mg^{2+} concentrations (O'Farrell *et al.*, 1978; Laski *et al.*, 1983). This is also true for the human pre-tRNA^{Tyr} (Figure 6B) which is stable in HeLa extract at 2 mM Mg^{2+} (Figure 6A). In contrast, intron excision from the plant pre-tRNA^{Tyr} occurs already at the low Mg^{2+} concentration of 2 mM (Figure 6C). The reason for this difference may be that a pre-tRNA with flanks may need more Mg^{2+} for establishing the tertiary structure which is the optimal substrate for the splicing endonuclease. Our observation that intron excision of the plant pre-tRNA occurs readily at low Mg^{2+} concentrations would imply that this reaction precedes processing as in the case of the human pre-tRNA^{Tyr}. This does not happen, most likely because the plant tRNA^{Tyr} is a better substrate for the HeLa 5′ and 3′ processing enzymes, and because, for sterical reasons, enzymes for processing of flanking sequences and for the excision of introns cannot operate simultaneously on the same pre-tRNA substrate. Consequently, the different maturation pathway for the plant pre-tRNA^{Tyr} in the HeLa extract, which resembles the sequence of events observed *in vivo*, is certainly related to its favourable structure and not at all to its origin from the plant genome.

Materials and methods

Enzymes and reagents

Restriction endonucleases, T4 DNA ligase, DNA polymerase I (Klenow fragment), calf intestinal alkaline phosphatase and deoxynucleoside triphosphates and sequencing primers were purchased from Boehringer, Mannheim. Dideoxynucleoside triphosphates were obtained from P-L Biochemicals. T4 polynucleotide kinase was from NEN, T1 RNase was from Calbiochem. 5S rRNA from rabbit liver was prepared in our laboratory. Unfractionated tRNA from human placenta was a gift of Dr B.Roe, Oklahoma, USA.

Bacterial strains and bacteriophages

A genomic library of human fetal liver DNA in bacteriophage λ Charon 4A was obtained from Dr T.Maniatis, Cambridge, USA. *Escherichia coli* LE 392 supE supF served as a host for propagation of λ clones. The vector plasmid pUC19 was provided by Dr J.Messing, Minnesota, USA. *E. coli* JM109 was used as a host for propagation of plasmid DNAs. The plasmids, pSVtTsu$^-$ and pSVtTsu$^+$, were provided by Dr U.L.RajBhandary, Cambridge, USA. pSVtTsu$^-$ is a recombinant of pBR322, SV40 and a 263-bp fragment of *X. laevis* DNA which contains the tRNA^{Tyr} gene; pSVtTsu$^+$ contains an amber suppressor tRNA^{Tyr} gene (Laski *et al.*, 1983).

Isolation and characterization of genomic clones

The human genomic library was screened according to the method of Woo *et al.* (1978), using a ^{32}P-labeled synthetic 20-mer oligonucleotide as a probe (specific activity: 4×10^8 c.p.m./μg DNA). DNA preparations from phages containing tRNA^{Tyr} genes, subcloning into pUC19 vector and propagation of plasmids was performed according to standard procedures (Maniatis *et al.*, 1982). Tobacco leaf nuclear DNA was isolated from pure nuclei via protoplasts. A λ library was prepared by *Eco*RI digestion of total nuclear DNA. The screening procedure and

the characterization of the recombinant plasmid pNtT1, carrying a tobacco tRNA^{Tyr} gene, has been reported elsewhere (Stange and Beier, 1986).

DNA sequencing

Direct sequencing of plasmid DNAs was performed according to Heinrich (1986) using two different primers for rapid sequencing of DNA inserts from both ends. DNA sequence analysis was done by the dideoxy chain termination method (Sanger *et al.*, 1977).

Synthesis and labeling of a 20-mer oligodeoxyribonucleotide

The 20-mer oligodeoxyribonucleotide complementary to nucleotides 18–37 of tRNA^{Tyr} from human placenta (Figure 3) and tRNA^{Tyr} from tobacco (Beier *et al.*, 1984) was synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981). It was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified on a 20% polyacrylamide/8 M urea gel.

Hybridization conditions

Transfer of DNA to nitrocellulose was by the method of Southern (1975). Prehybridization of the filter was in 5 × SSC, 5 × Denhardt's 0.01 M phosphate buffer pH 6.8, 0.2% SDS, 1 mM ATP, 1 mM EDTA, 150 μg/ml denatured salmon testes DNA at 42°C for 5 h. Hybridization was at 42°C for 16 h in the same buffer and ~1 × 10^7 c.p.m. ^{32}P-labeled 20-mer probe. After hybridization, the filter was washed five times with 6 × SSC at room temperature, twice at 42°C for 30 min and once at 60°C for 10 min.

In vitro transcription in HeLa cell nuclear extracts

Nuclear extracts were prepared from HeLa cells according to the method of Roeder (Dignam *et al.*, 1983). Transcription assays were performed under template-saturating conditions in 10 μl reaction mixtures containing 6 μl extract, 3.5 nM template DNA, 30 μg/ml total DNA (adjusted with pUC19), 12 mM Hepes buffer pH 7.9, 12% (v/v) glycerol, 85 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.6 mM each ATP, CTP, UTP, 0.014 mM GTP, 74 KBq [α-^{32}P]GTP, 10 mM creatine phosphate, 0.02 mg/ml α-amanitin, 0.3 mM DTT and 0.3 mM phenylmethylsulfonyl fluoride. Incubation was for 60 min at 30°C. For quantitation of the various RNA products, bands were excised from the gels and quantitated by Cerenkov counting.

Analysis of in vitro transcripts

T1 RNase digestion of RNA and fingerprint analyses were performed essentially as described by Silberklang *et al.* (1979). In order to identify the 5'-phosphorylated termini of transcription products, the eluted RNAs were treated with alkaline phosphatase for 2 h at 37°C, phenol extracted to remove the phosphatase and fingerprinted as above.

Fractionation and isolation of tRNAs^{Tyr} from human placenta

Fractionation of tRNAs^{Tyr} was by BD-cellulose and DEAE Sephadex A50 column chromatography; further purification was by gel electrophoresis. Sequence analysis of tRNA by post-labeling techniques was as described (Beier *et al.*, 1984).

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