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All human $tRNA^{Tyr}$ genes contain introns as a prerequisite for pseudouridine biosynthesis in the anticodon

Hans van Tol and Hildburg Beier

Institut für Biochemie, Bayerische Julius-Maximilians-Universität, Röntgenring 11, D-8700 Würzburg, FRG

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ABSTRACT

Two synthetic oligonucleotides, one specific for the 5' exon, the other spanning the splice junction, were used to show that (a) the human haploid genome contains at least 12 independent gene loci for $tRNA^{Tyr}$, and (b) that all of them carry an intron. From one of the cloned human $tRNA^{Tyr}$ genes (pHtT1) the 20 bp intron was deleted to generate pHtT1Δ. Homologous *in vitro* transcription, fingerprint analyses of the products and elucidation of their nucleoside composition revealed that the pseudouridine (Ψ_{35}) in the center of the anticodon of $tRNA^{Tyr}$ was synthesized in the intron-containing precursor whereas this U to Ψ modification did not take place in precursors or mature $tRNA^{Tyr}$ derived from pHtT1Δ. On the basis of these results and of studies from other laboratories we suggest that the evolutionary pressure for maintaining introns in eukaryotic $tRNAs^{Tyr}$ is this strict intron-requirement for Ψ_{35} synthesis. Taking into account that all eukaryotic cytoplasmic $tRNAs^{Tyr}$ contain a Ψ_{35} we discuss here a special need for this modified nucleoside in stabilizing codon-anticodon interactions involving (a) classical base pairing upon translation of tyrosine codons and (b) unconventional interactions during UAG amber codon suppression by $tRNA^{Tyr}_{G\Psi A}$ in eukaryotic cells.

INTRODUCTION

Intervening sequences are found in a wide variety of nuclear tRNA genes of lower eukaryotes like yeasts, fungi and certain protists, e.g., in those specific for Ser, Leu, Ile, Lys, Phe, Trp, Pro and Tyr (1). It is noticeable that within the same tRNA gene family, even in the same species, some of these genes occur with and without intron, respectively (2-5).

In contrast to lower eukaryotes, the majority of nuclear tRNA genes of higher eukaryotes does not appear to contain introns. An inspection of the respective gene sequences (6) reveals that introns have only been found in genes coding for tRNAs of the Leu and Tyr families and there is the striking observation that - without any exception - all known $tRNA^{Tyr}$ genes, from as different organisms as yeast, *Nicotiana*, *Drosophila*, *Xenopus* and human, contain introns (7-13). Beyond that, all eukaryotic cytoplasmic $tRNAs^{Tyr}$ are exceptional in that they contain a pseudouridine (Ψ) in the center of their GΨA or QΨA anticodons. GUA and QUA anticodons, as present in procaryotic,

chloroplast and mitochondrial tRNAs^{Tyr}, have never been found in their eukaryotic counterparts (6). Hence, there may be a relation between the obviously obligatory presence of introns in tRNA^{Tyr} genes and the general presence of Ψ in the center of the tRNA^{Tyr} anticodons, i.e., there may be a strong pressure in favour of maintaining both, introns in the genes and Ψ in the anticodons of eukaryotic tRNAs^{Tyr} throughout evolution. In fact, Johnson and Abelson (14) have found that deletion of the intron from a yeast tRNA^{Tyr} ochre suppressor gene abolishes pseudouridine biosynthesis in the corresponding suppressor tRNA *in vivo*.

Here we show that an intron-less tRNA^{Tyr} gene constructed by intron deletion from a tRNA^{Tyr} gene recently isolated from human DNA (13) produces mature tRNA^{Tyr} with a GUA anticodon in a HeLa cell nuclear extract. In contrast, the wild-type gene generates a tRNA^{Tyr} with GΨA anticodon. Moreover, we demonstrate that virtually all human tRNA^{Tyr} genes contain introns.

MATERIALS AND METHODS

Enzymes and reagents

RNase T1 and T2 were purchased from Calbiochem and Sankyo, respectively. T4 polynucleotide kinase was from NEN. [α-^{32}P]GTP and [α-^{32}P]ATP with specific activities of 111 TBq/mmol were from Amersham. All other enzymes and chemicals were purchased from Boehringer, Mannheim.

Bacterial strains and plasmids

The vector plasmid pUC19 was obtained from Dr. J. Messing, Minnesota, USA. *E. coli* JM109 was used as a host for propagation of plasmid DNAs. The plasmid pSVtTsu$^-$ was provided by Dr. U.L. RajBhandary, Cambridge, USA. It contains a Xenopus tRNA^{Tyr} gene (15). The pHtT1 clone consists of a HaeIII fragment of 334 bp derived from a human genomic library (16) which was subcloned into the SmaI site of a pUC19 vector. This restriction fragment harbours an intron-containing tRNA^{Tyr} gene (13).

Construction of an intron-less tRNA^{Tyr} gene

The construction was performed by oligonucleotide directed deletion of the intron in the tRNA^{Tyr} gene of pHtT1 using a combination of methods described by Wallace *et al.* (17), Chen and Seeburg (18) and Eghtedarzadeh and Henikoff (19). Briefly, 10 µg supercoiled pHtT1 DNA was denatured in 40 µl 0.2 M NaOH, 0.2 mM EDTA. After 5 min at 37°C the solution was neutralized by the addition of 4 µl 2 M NH_4OAc, pH 5.0, DNA then precipitated with 2 volumes of ethanol. The remaining double-stranded DNA was digested with HaeIII and HinfI in the presence of calf intestinal alkaline phosphatase. A synthetic

20-mer oligonucleotide complementary to nucleotides 29-48 (= probe 2, Figure 1) of mature human $tRNA^{Tyr}$ was used as a primer on single-stranded pHtT1 DNA. Annealing and synthesis of circular double-stranded heteroduplex DNA was performed according to Eghtedarzadeh and Henikoff (19). The resulting heteroduplex DNA was used to transform E. coli JM109. The transformants were identified by hybridization to [5'-^{32}P]-labeled probe 2. One colony that hybridized was recloned and sequenced. This clone contained no intron in the $tRNA^{Tyr}$ gene but in additon the 3' exon was deleted downstream from nucleotide 46 of mature $tRNA^{Tyr}$. The only two SauI restriction sites in the 5'-flanking sequence (bp -30 to -24) and near the splice junction (bp 60 to 66) of the $tRNA^{Tyr}$ gene (13) were now used to exchange the intron-containing SauI-fragment of pHtT1 against the intron-less SauI-fragment of the crippled clone, thus generating pHtT1Δ.

DNA Sequencing

Direct sequencing of plasmid DNAs was performed according to Chen and Seeburg (18) using two different primers for rapid sequencing of DNA inserts from both ends. DNA sequence analysis was done by the dideoxy chain termination method (20).

Synthesis of 20-mer oligodeoxyribonucleotides

The 20-mer oligodeoxyribonucleotides complementary to nucleotides 18-37 (probe 1) and nucleotides 29-48 (probe 2), respectively, of $tRNA^{Tyr}$ from human placenta (13) were synthesized by the phosphoramidite method (21). They were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified on a 20% polyacrylamide/4M urea gel.

Isolation of high molecular weight placenta DNA

Fresh human placenta was chilled on ice and cut in small pieces which were immediately frozen in liquid nitrogen and stored at -80°C. The frozen tissue was homogenized in a mortar under liquid nitrogen and DNA was extracted from the powder essentially as described by Blin and Stafford (22). Only high molecular weight DNA preparations of more than 100 kbp were used for restriction enzyme digestions.

Hybridization conditions

Transfer of DNA to nitrocellulose was by the method of Southern (23). Prehybridization of the filter and hybridization was carried out at 40°C essentially as described (13). Wash temperatures have been varied and are mentioned in the legends to the corresponding figures.

In vitro transcription in HeLa cell nuclear extracts

Nuclear extracts were prepared from HeLa cells according to Dignam et

al. (24). Transcription assays were performed as described previously (13). For quantitation of the various RNA products, bands were excised from the gels and quantitated by Cerenkov counting.

Analysis of in vitro transcripts.

Digestion of RNAs with RNase T1 and fingerprint analyses were performed according to Silberklang et al. (25). The oligonucleotides were eluted from DEAE-cellulose plates and digested with RNase T2 as described by Stange and Beier (26). Identification of the labeled nucleotides was by one-dimensional chromatography on cellulose thin-layer plates (27).

RESULTS

1. Construction of a human intron-less $tRNA^{Tyr}$ gene

A human intron-containing $tRNA^{Tyr}$ gene was recently isolated from a genomic library of human fetal liver DNA. A HaeIII fragment of 334 bp of the human DNA was subcloned into a pUC19 vector. This recombinant plasmid is referred to as pHtT1 (13). The pHtT1 plasmid was utilized for the deletion of the 20 bp intron by oligonucleotide directed mutagenesis. An oligonucleotide, complementary to residues 29 to 48 of mature $tRNA^{Tyr}$, spanning 9 bp upstream and 11 bp downstream of the splice site (Figure 1, probe 2) was used to create the desired deletion (see Material and Methods).

To test which of the transformed E. coli JM109 cells contained the altered tyrosine tRNA gene, we used probe 2 as a hybridization probe for colony screening. Under appropriate conditions this oligonucleotide will hybridize to the deleted gene, but not to the parent containing the intron (see Figure 6). The pure deletion plasmid, referred to as pHtT1Δ, was isolated by retransformation and rescreening. As a final control the exact nucleotide sequence of the deleted gene was confirmed by DNA sequencing (18, 20). The sequence of pHtT1Δ is identical with that of pHtT1 minus intron.

2. In vitro transcription of intron-containing (pHtT1) and intron-less (pHtT1Δ) $tRNA^{Tyr}$ genes and analysis of their transcripts by RNase T1 fingerprints

A HeLa cell nuclear extract was used to transcribe pHtT1 and pHtT1Δ DNA in vitro. Transcription reactions were carried out essentially as described by van Tol et al. (13) with the variation that S-adenosyl-L-methionine was added to the HeLa extract at a concentration of 0.02 mM (28). Figure 2 shows the transcription and processing products of the two $tRNA^{Tyr}$ genes. Incubation of pHtT1 DNA results in seven discrete RNAs as revealed by polyacrylamide gel electrophoresis (Figure 2a, b). Characterization of these RNA species has been described elsewhere (13). Briefly, the RNA species H2 is the major

Figure 1. Nucleotide sequence of the noncoding strand of the structural gene for human $tRNA^{Tyr}$ cloned in pUC19. The sequence shown is that of the mutated intron-less $tRNA^{Tyr}$ gene (i.e., pHtT1Δ). The arrow indicates the position of the 20 bp intervening sequence found in the wild type $tRNA^{Tyr}$ gene (i.e., pHtT1; 13). The 5'-terminal nucleotide of the structural gene has been designated number 1. The anticodon sequence is boxed. The bars represent the two synthetic 20-mer deoxyoligonucleotides used in this work: probe 1 is complementary to nucleotides 18-37 and probe 2 is complementary to nucleotides 29-48, respectively, of mature $tRNA^{Tyr}$.

transcript of the $tRNA^{Tyr}$ gene, with a 5' leader, intervening sequence, and a 3' trailer. Excision of the intron (not visible) yields tRNA halves with unprocessed ends (i.e., H6 and H7). Ligation of these halves leads to H4 which is processed to mature $tRNA^{Tyr}$ H5, by removal of 5' leader and 3' trailer. The minor RNA species H1 is an independent transcript with a longer 5' leader but with the same 3' trailer as shown for H2.

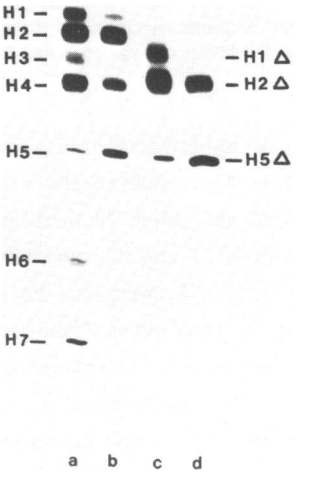

Figure 2. Gel electrophoretic analysis of in vitro transcripts of pHtT1 and pHtT1Δ DNAs synthsized in a HeLa cell nuclear extract. Transcription assays were performed in 10 µl reaction mixtures containing pHtT1 (a, b) and pHtT1Δ DNA (c, d), respectively. Incubation was in the presence of [α-^{32}P]GTP for 60 min at 30°C (a, c). 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 mM, and incubation was then continued for another 90 min (b, d). Samples were analyzed on a 12.5% polyacrylamide/7 M urea gel followed by autoradiography. The RNA species are designated H1-H7 according to their decreasing size (13).

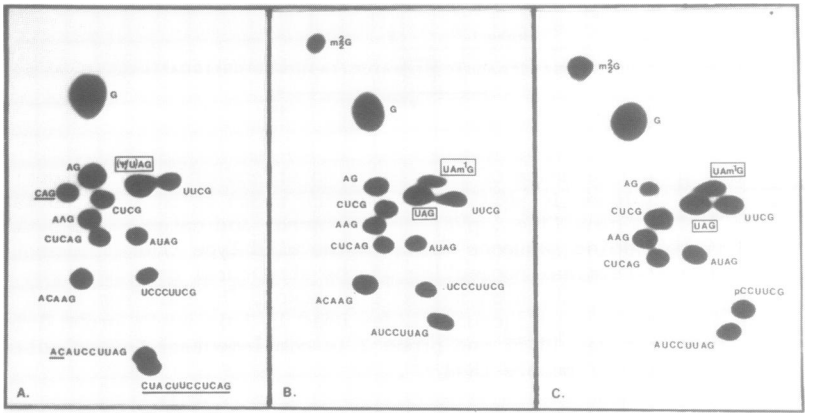

Figure 3. RNase T1 fingerprint analysis of ^{32}P-labeled pre-tRNAs^{Tyr} and mature tRNA^{Tyr} derived from pHtT1 and pHtT1Δ. [α-^{32}P]ATP-labeled RNA transcripts H2 (A), H2Δ (B), and H5Δ (C) were recovered from a preparative gel (numbering of the RNA species as in Figure 2) and digested with RNase 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. (43) and by comparison with the DNA sequence (13). Oligonucleotide sequences derived from the intron are underlined. Oligonucleotides which were further characterized with respect to modified nucleosides are boxed.

In vitro transcription of pHtT1Δ DNA displays the expected pattern of only three RNA species (Figure 2 c). Due to the missing intron, the primary transcripts (i.e. H1Δ and H2Δ) are about 20 nucleotides shorter as compared to the corresponding RNA species H1 and H2, and tRNA halves are absent from this pattern. The third product, H5Δ, migrates to the same position as mature tRNA^{Tyr}, H5, indicating that it originates from H1Δ and H2Δ by processing of 5'- and 3'-flanking sequences. This relationship between the three RNAs was further shown in a pulse-chase experiment (Figure 2 d).

Quantitation of all transcription and processing products (average values from 4 different assays) revealed that pHtT1Δ DNA is transcribed slightly better than pHtT1 (130% versus 100% transcription efficiency).

The exact composition of RNA species H1Δ, H2Δ and H5Δ was established by RNase T1 fingerprints (Figure 3 B, C). For comparison the fingerprint of RNA species H2 is also shown (Figure 3 A). As mentioned above, this species contains a 20 bp intron in addition to 5'- and 3'-flanking sequences. The oligonucleotides originating from the intron of H2 (i.e., CAG, CUACUUCCUCAG and the first two nucleotides of ACAUCCUUAG) are underlined in Figure 3 A.

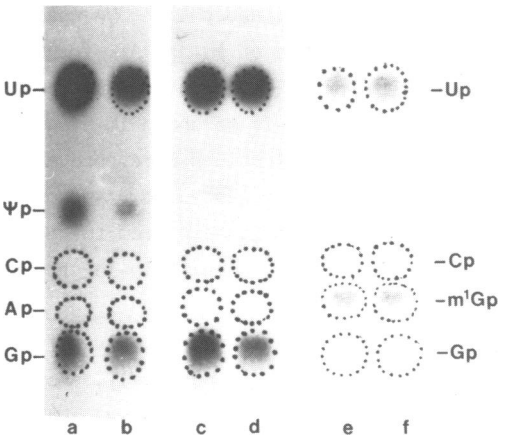

Figure 4. Analysis of modified nucleosides present in the anticodon region of precursor tRNAs H2, H2Δ and mature tRNAs^{Tyr}. The oligonucleotides derived from [α-^{32}P]ATP-labeled RNAs were recovered from fingerprints by scraping the corresponding spots from the DEAE-cellulose plates. Digestion of the eluted oligonucleotides was carried out with RNase T2. Identification of the labeled nucleotides was by one-dimensional chromatography on cellulose thin-layer plates in isopropanol/concentrated HCl/water (27). Unlabeled 3' nucleotide markers were run in parallel and were visualized under u.v. light. a. (Ψ/U)AG from H2 fingerprint (Figure 3A); b. (Ψ/U)AG from H5 fingerprint (not shown); c. UAG from H2Δ fingerprint (Figure 3B); d. UAG from H5Δ fingerprint (Figure 3C); e. UAm^{I}G from H2Δ fingerprint (Figure 3B); f. UAm^{I}G from H5Δ fingerprint (Figure 3C).

An inspection of the fingerprint of H2Δ (Figure 3 B) reveals the absence of these oligonucleotides, thus again proving the absence of the intron in the tRNA^{Tyr} gene of pHtT1Δ. The minor species H1Δ, as compared with H2Δ, contains only one additional oligonucleotide, UACACG, which is derived from the 5' flank (13), thus identifying it as an independent transcript (not shown). Processing of H2Δ leads to H5Δ. The fingerprint of H5Δ (Figure 3 C) contains the oligonucleotide pCCUUCG typical for the mature 5' end, whereas UCCUUCG (derived from part of the 5'-flanking sequence) and ACAAG (derived from the 3'-flanking sequence) are missing (compare Figure 5). The fingerprint patterns of H5 (not shown) and H5Δ (Figure 3 C) are identical.

3. Comparison of base modifications made in pre-tRNAs and mature tRNAs^{Tyr} derived from pHtT1 and pHtT1Δ

One of the reasons why we constructed an intron-less tRNA^{Tyr} gene was to evaluate in a homologous *in vitro* system whether the presence of the intron is necessary for the modification of uridine to pseudouridine (i.e., Ψ_{35}), in the anticodon of human tRNA^{Tyr}.

In vitro transcription of pHtT1 and pHtT1Δ DNA was performed in a HeLa cell nuclear extract in the presence of [α-^{32}P]ATP for 90 min at 30°C. The RNA species H2, H4, H5, H2Δ and H5Δ were eluted from preparative gels, followed by RNase T1 fingerprint analysis as shown in Figure 3 for RNA species H2, H2Δ and H5Δ. The boxed oligonucleotides were recovered from the corresponding fingerprints and completely digested to 3'-mononucleotides with RNase T2, whereby the labeled phosphate originates from the nearest 3'-neighbour. The digests were then analysed by cellulose thin-layer chromatography (Figure 4).

Analysis of the UAG oligonucleotide spot derived from fingerprints of H2 (Figure 3A), H4 and H5 (not shown) respectively, shows a Ψp in addition to labeled Gp and Up mononucleotides (Figure 4a, b). UAG is present twice in pre-tRNAs and tRNA^{Tyr}. The first one is located at positions 20-22, whereas the second one derives from the anticodon loop of mature tRNA^{Tyr} (pos. 35-37). A pseudouridine does not occur in human tRNA^{Tyr} at pos. 20 (13), thus the observed Ψp originates from the uridine in position 35. The estimation of molar yields reveals that pseudouridine modification of all three RNA species (i.e. H2, H4 and H5) synthesized in the HeLa cell extract had occurred only partially (∿ 50%). A pseudouridine mononucleotide could not be detected in the corresponding analysis of UAG oligonucleotides derived from H2Δ and H5Δ, respectively (Figure 4c, d).

Inspection of the oligonucleotide which migrates slightly faster in both dimensions than UAG (derived from H2Δ and H5Δ, respectively, Figure 3B, C) reveals the presence of m^{1}Gp and again not of Ψp (Figure 4e, f). A slightly different migration behaviour of oligonucleotides containing methylated guanosines in fingerprints has been described (25). Since RNase T2 cannot generate a [^{32}P]Ap from [α-^{32}P]ATP-labeled UAm^{1}G, the identification of [^{32}P]m^{1}Gp is unambigous although it migrates like a Ap in the solvent used for thinlayer chromatography (27). In the pre-tRNA H2 fingerprint this specific oligonucleotide is not present (Figure 3A), indicating the absence of m^{1}G modification, whereas it is visible in H5 fingerprints (not shown) due to the m^{1}G-specific mobility shift.

A summary of the base modifications found in the anticodon region of intron-containing and intron-less pre-tRNAs^{Tyr} and their corresponding mature tRNAs is presented in Figure 5. The conversion of the uridine at position 35 to pseudouridine takes place only in the intron-containing pre-tRNA H2 and not at all in pre-tRNA H2Δ or in mature tRNA^{Tyr}. On the other hand, a methylated guanosine at position 37 has been identified only in intron-less

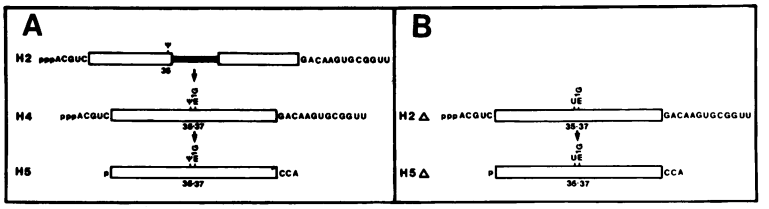

Figure 5. Base modifications in the anticodon region of intron-containing (A) and intron-less (B) pre-tRNAs^{Tyr} and their corresponding mature tRNAs synthesized in a HeLa cell extract. A. The major transcript H2 of the human tRNA^{Tyr} gene (i.e., pHtT1) is a precursor of 111 nucleotides with a 5' leader, the 5' exon (open box), a 20-base intron (black bar), the 3' exon (open box), and a 3' trailer. Removal of the intron and ligation of tRNA halves yield the pre-tRNA H4 and finally mature tRNA^{Tyr} H5 by removal of 5' leader and 3' trailer. B. The major transcript of pHtT1Δ is a precursor of 91 nucleotides (i.e., H2Δ) with a 5' leader, the tRNA sequence without an intron, and a 3' trailer which is processed to mature tRNA^{Tyr} (i.e., H5Δ) by removal of the flanking sequences. Numbers refer to positions in mature tRNA.

precursors (i.e. H4 and H2Δ) and mature tRNAs^{Tyr} (i.e., H5 and H5Δ).

Queosine (Q) which is present in the first position of the anticodon of human tRNA^{Tyr} (13) was not found in mature tRNAs^{Tyr} produced in the HeLa cell extract.

4. Identification of tRNA^{Tyr} genes in the human genome

In the previous chapter we have demonstrated that in vitro transcription of an intron-less tRNA^{Tyr} gene results in the absence of pseudouridine modification in the anticodon of mature tRNA^{Tyr}. Assuming a special need for the presence of this particular pseudouridine in the anticodon of eukaryotic cytoplasmic tRNAs^{Tyr} and with respect to the results mentioned above it appeared possible that all eukaryotic nuclear tRNA^{Tyr} genes contain introns.

To corroborate this assumption we chose the human tRNA^{Tyr} gene family for more detailed studies. We had recently cloned and sequenced two human tRNA^{Tyr} genes, both containing introns (13) and the sequences of two more intron-containing human tRNA^{Tyr} genes have been described by MacPherson and Roy (12). Instead of cloning and sequencing all of the remaining human tRNA^{Tyr} genes we used an indirect approach to demonstrate the absence of intron-less tRNA^{Tyr} genes in the human genome.

Figure 6 demonstrates the selective hybridization of the oligonucleotide probe 2 (see Figure 1) to the intron-less tRNA^{Tyr} gene pHtT1Δ as compared to pHtT1. The theoretic dissociation temperature (T_d) of probe 2 is 60 °C for hybridization to a tRNA gene without intron, and 28-32 °C for a tRNA gene with intron (29). In order to estimate the actual T_d, hybridization of probe 2

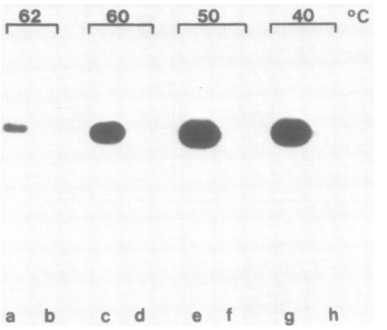

Figure 6. Hybridization of ^{32}P-labeled 20-mer probe 2 to pHtT1 and pHtT1Δ. 0.2 µg of pHtT1 DNA (b, d, f, h) and pHtT1Δ DNA (a, c, e, g), respectively, were subjected to electrophoresis on a 0.8% agarose gel. The DNA was then transferred to nitrocellulose (23) and hybridization was carried out with ^{32}P-labeled oligonucleotide probe 2 (see Figure 1) as described in Material and Methods. After hybridization, the filter were washed five times with 6 x SSC at room temperature, once for 30 min at 40°C and once for 10 min at the temperatures indicated above the Southern blot, followed by autoradiography.

to pHtT1 and pHtT1Δ, respectively, was performed at different wash temperatures. The hybridization signals are of about the same strength between 40° and 60° C for pHtT1Δ. At a wash temperature of 62 °C the signal appears much weaker, indicating that the actual T_d is around 60 °C (Figure 6a, c, e, g). Hybridization of probe 2 to pHtT1 DNA yields only a very weak signal at 40 °C (Figure 6 h). The estimated T_d difference of more than 21 °C should allow the unambigous discrimination between tRNA genes with and without introns in hybridization studies using oligonucleotide probe 2. Consequently, this approach was used to evaluate whether the human genome contains intron-less $tRNA^{Tyr}$ genes.

First we determined the number of individual $tRNA^{tyr}$ gene loci in the human haploid genome by hybridization of [5-^{32}P]-labeled oligonucleotide probe 1 (see Figure 1) to Southern blots of restriction digests of high molecular weight DNA from human placenta. We had previously established stringent hybridization conditions with two Xenopus laevis $tRNA^{Tyr}$ clones, i.e., $pSVtTsu^-$ and $pSVtTsu^+$, which differ in a single base pair in the anticodon sequence of the $tRNA^{Tyr}$ gene (13). EcoRI digestion of placenta DNA yields 10 fragments to which probe I has hybridized (Figure 7d). An additional fragment of 0.6 kbp was identified if the digests had been separated on a 1 % rather than on a

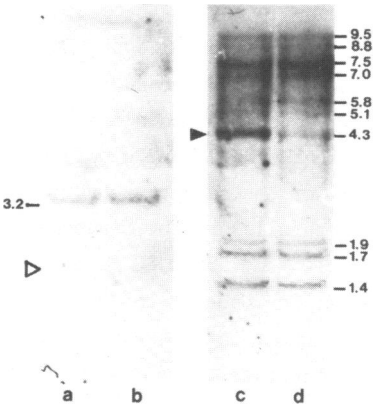

Figure 7. Hybridization of ^{32}P-labeled 20-mer oligonucleotide probes 1 and 2 to EcoRI-digested genomic human placenta DNA. 20 µg placenta DNA were digested with EcoRI and subjected to electrophoresis on a 0.6% agarose gel together with 3 gene equivalents of pHtT1 DNA (a) and 3 gene equivalents of BamHI-digested pSVtTsu$^-$ DNA (c). Markers of HpaI- and EcoRI-digested T7 and λ Charon 4A DNA were run in adjacent lanes (not shown). Hybridization to Southern blots containing the restriction fragments of placenta DNA was carried out with ^{32}P-labeled oligonucleotide probe 1 (c, d) and probe 2 (a, b), respectively (see Figure 1). After hybridization, the filter were washed five times with 6 x SSC at room temperature, two times for 20 min at 40°C and once for 10 min at 55°C (a, b) and 62°C (c, d), respectively. The numbers refer to the length of the fragments (kbp) to which the 20-mer probes 1 and 2 hybridized as visualized by autoradiography. The triangles (▲, Δ) indicate the position of the tRNA^{Tyr} gene-containing BamHI fragment of pSVtTsu$^-$ (c) and of the supercoiled form of pHtT1 plasmid DNA (a), respectively, which were added to the placenta DNA digests.

0.6 % agarose gel (not shown). The stronger hybridization signal of the 7.5 kbp fragment may indicate that (i) either different tDNA-carrying restriction fragments were not resolved on the gel or that (ii) this fragment may contain more than one tRNA^{Tyr} gene. Thus, a minimal number of 12 individual tRNA^{Tyr} gene loci appears to be present in the haploid human genome.

A single mismatch in the duplex between the tDNA^{Tyr} and probe 1 reduces the T_d by about 10 °C as established with the two Xenopus clones mentioned above. This correlates well with the values of 6-12 °C published by others (30, 31). Using wash temperatures as low as 54 °C did not increase the strength of the hybridization signal of any of the genomic fragments which hybridized to probe 1, indicating that all of the 12 individual tRNA^{Tyr} genes identified in the human genome do not carry mutations in the region 18-37 of mature tRNA. Since probe 1 hybridizes exclusively to the 5' half of tRNA^{Tyr} genes, independently whether they contain introns or not, nothing can be said about the

presence or absence of introns in any of these genes. On the other hand, as pointed out earlier, probe 2 will hybridize at the selected wash temperature only to $tRNA^{Tyr}$ genes containing no introns.

Hybridization of probe 2 to Southern blots of EcoRI digested placenta DNA revealed a single fragment of 3.2 kbp to which probe 2 has hybridized (Figure 7 b). As a control for the selective hybridization, 3 gene equivalents of pHtT1 DNA were added in parallel to the placenta digests (Figure 7 a). Reducing the wash temperature from 55 to 50 °C does not lead to the occurrence of additional signals, nor does it change the intensity of the 3.2 kbp signal. Thus, the latter fragment very likely contains a DNA sequence without intron, complementary to nucleotides 29-48 of mature $tRNA^{Tyr}$. However, the same DNA fragment should carry at least one mutation in the region between nucleotides 18-28, since hybridization of probe 1 to genomic blots has not revealed a 3.2 kbp fragment, not even when a wash temperature of 54 °C was used. Whatever the nature of this putative $tRNA^{Tyr}$ gene is, it can clearly be said that all of the 12 $tRNA^{Tyr}$ genes identified in a genomic blot of human placenta DNA (Figure 7 d) contain introns.

DISCUSSION

Since the discovery of intervening sequences in yeast tRNA genes (7) the question about the purpose they might serve has been of major interest. Here we show that the intron is necessary for the biosynthesis of an important modified nucleoside, namely pseudouridine (Ψ_{35}) which is present in the center of the anticodons of all eukaryotic cytoplasmic $tRNAs^{Tyr}$. We have compared the expression of a human nuclear intron-containing $tRNA^{Tyr}$ gene (pHtT1) with that of its intron-less derivative (pHtT1Δ) in a HeLa cell extract. Interestingly, both $tRNA^{Tyr}$ genes which differ only in the 20 bp intervening sequence produce the same ratio of minor and major transcription products due to initiation at bp -11 and bp -5, respectively (Figure 2). This indicates that the distance difference of 20 bp between both internal promoter elements does not at all influence the selection of the transcription initiation sites. However, the transcription efficiencies of 100% versus 130% for pHtT1 and pHtT1Δ, respectively, should be noted.

The fingerprint and nucleotide analyses of the intron-containing pre-$tRNA^{Tyr}$ (H2), of the intron-less precursors (H4 and H2Δ) and of the mature $tRNAs^{Tyr}$ (H5 and H5Δ) revealed different modification patterns in the anticodon loop (Figures 3, 4, 5). Both mature $tRNAs^{Tyr}$ contain 1-methylguanosine at the 3' side of the anticodon (m^1G_{37}), whereas pseudouridine (Ψ_{35}) is to-

tally absent in H5Δ. Exactly the same result is found for the intron-less precursors H4 and H2Δ. In the intron-containing precursor H2, however, m^1G_{37} does not yet occur, but Ψ_{35} is already present. This indicates that the intron interferes with G_{37} methylation, whereas it is absolutely required for the biosynthesis of Ψ_{35}.

Johnson and Abelson (14) have shown an intron-dependence for the Ψ_{35} synthesis in a yeast ochre suppressor $tRNA^{Tyr}$ in vivo, however, not in a wild type yeast strain but in a rnal mutant strain which, for unknown reasons, accumulates intron-containing pre-tRNAs (32). A yeast cell-free extract was only able to synthesize Ψ_{39} in the anticodon stem but not Ψ_{35} in the anticodon, suggesting that at least two pseudouridine synthases exist in yeast and that the Ψ_{35}-synthase was inactive (14). Here we present evidence that in an in vitro system, i.e., in a HeLa cell nuclear extract (in which the Ψ_{35} synthase is active) the U_{35} to Ψ_{35} modification in a human $tRNA^{Tyr}$ gene is in fact intron-dependent and furthermore, that the Ψ_{35} synthases appear to display the same specificity for intron-containing precursors in lower as well as in higher eukaryotes.

Another case of intron-dependent modification in the anticodon has been reported by Strobel and Abelson (33). The yeast amber suppressor tRNA gene SUP53 contains a 32 bp intron and encodes a $tRNA^{Leu}$ with a 5-methylcytidine (m^5C_{34}) in the wobble position of the anticodon. The intron-less derivative of this gene transcribed in a S. cerevisiae nuclear extract displays an unmodified C_{34}. In this context we would like to predict that the two intron-containing Drosophila tRNA genes coding for $tRNA^{Leu}_{CAA}$ (3) also produce a m^5C_{34} wobble base in the mature tRNAs.

In summary, it has now been demonstrated that only the intron-containing pre-tRNAs are the substrates for the enzymes which generate the Ψ_{35} modification in virtually all $tRNAs^{Tyr}$ and the m^5C_{34} modification at least in yeast $tRNA^{Leu}$. However, introns do not appear to be neccessary for proper base modifications in the anticodon loop of other tRNAs (34).

The fact that all cytoplasmic $tRNAs^{Tyr}$ have a Ψ_{35} in the anticodon, together with the absolute requirement of the Ψ_{35} synthase for an intron, implies that all eukaryotic nuclear tRNA genes should contain introns. Using two different synthetic oligonucleotides as hybridization probes we could show that at least 12 independent $tRNA^{Tyr}$ gene loci exist in the human genome and that all of them contain an intron (Figure 7). Noticeably, the oligonucleotide probe spanning the splice junction of $tRNA^{Tyr}$ genes (i.e. probe 2) hybridized to a single EcoRI fragment which was not among the fragments to which

our exon-specific probe (i.e. probe 1) hybridized. This putative intron-less $tRNA^{Tyr}$ gene does certainly not derive from mitochondrial DNA, since the corresponding $tRNA^{Tyr}$ differs considerably from the cytoplasmic species (13, 35). We rather believe that this is a $tRNA^{Tyr}$ pseudogene. This notion is supported by the fact that a $tRNA^{Tyr}$ with a GUA anticodon was not detected by us in human placenta (not shown) or by others in any eukaryotic $tRNA^{Tyr}$ (6).

Remarkably, the human $tRNA^{Tyr}$ gene family appears to be the only one which contains introns (6). There are only two members of another tRNA gene family known in higher eukaryotes to contain introns, namely the $tRNA^{Leu}_{CAA}$ genes of Drosophila (3). Compared with the eight known intron-containing tRNA gene families in Saccharomyces cerevisiae (1), this indicates a dramatic loss of introns during evolution. Although we cannot interpret the obvious loss of introns in higher eukaryotes, we can at least offer an explanation for the selective evolutionary maintenance of $tRNA^{Tyr}$ and $tRNA^{Leu}$ gene introns: their function for providing appropriate substrates for essential nucleoside modifications in the anticodon.

What is then the need, for instance, for $tRNAs^{Tyr}$ to retain the pseudouridine in their anticodon? Several studies have shown that generally undermodification of the wobble base results in an alteration of the codon-anticodon interaction leading both to an increase or decrease of base pairing capacity (36-38).

Pseudouridine differs in so far from all other nucleosides as it is the only modified nucleoside found in the middle position of the anticodon. Furthermore, Ψ_{35} displays several features which might explain its importance in stabilizing codon-anticodon interactions (39, 40). In this context it should be remembered that a chemically induced yeast ochre suppressor $tRNA^{Tyr}$ with a U*UA anticodon shows reduced suppressor activity as compared to the $tRNA^{Tyr}$ having a U*ΨA anticodon (14).

Ψ also plays a role in "natural" suppression: major cytoplasmic $tRNAs^{Tyr}$ with a GΨA anticodon recognize the amber codon UAG. The best studied example is the UAG codon at the end of the 126 K cistron of tobacco mosaic virus (TMV), which is suppressed by $tRNAs^{Tyr}_{GΨA}$ *in vivo* and *in vitro* (41, 42). For UAG suppression the Ψ in the anticodon is believed to be an absolute necessity. We postulate that natural UAG suppression by $tRNAs^{Tyr}_{GΨA}$ is not alone needed for the replication of eukaryotic viruses, but also for the generation of cellular readthrough proteins with essential functions in certain stages of development and/or differentiation. Consequently, the need for such proteins may have been the evolutionary force for having Ψ in $tRNA^{Tyr}$ anticodons, and this re-

quirement for Ψ may in turn have been the motive for maintaining introns in the cytoplasmic $tRNAs^{Tyr}$ of eukaryotes.

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