A human tRNA\textsubscript{Glu} gene of high transcriptional activity

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**SUMMARY**

A mixture of low molecular weight RNAs, in which only tRNAs were radio-labelled, was used as a hybridisation probe to select for tRNA-like sequences within a bank of human genomic DNA in λ Charon 4A.

A restriction enzyme digest of one of the selected λ Charon 4A recombinants contained two fragments (2.4 Kb & 1.8 Kb) which hybridised tRNA and which, when subcloned into pAT153, were transcribed in Xenopus oocyte nuclei. Analysis of the subcloned 2.4 Kb fragment, which was of remarkably high transcriptional activity, revealed the presence of a single gene for tRNA\textsubscript{Glu} in the middle of the fragment. The sequence immediately preceding the gene has the potential for forming a tRNA-like structure.

**INTRODUCTION**

During the past few years, the application of gene cloning and DNA sequencing has led to a great increase in our knowledge of the structure and function of tRNA genes of higher eukaryotes (1, 2). Studies so far on tRNA gene organisation show that many types of gene arrangement occur. There are examples of clusters of different tRNA genes in Xenopus and Drosophila (3, 4). In Xenopus the studied cluster occurs as a 300-fold tandem repeat, whereas in most other organisms tRNA genes are widely scattered throughout the genome. Relatively few studies have been made of mammalian tRNA genes. In rat, different tRNA genes appear to occur in small interspersed clusters (5). One of these clusters is repeated about ten times on the haploid DNA and shows sequence variation between the repeats including the presence of incomplete genes (pseudogenes) (6). In man, tRNA\textsuperscript{Met} genes are widely scattered (7), but clusters of different tRNA genes have also been found (8). Clearly further studies are required to relate this apparent anarchy of gene organisation to, for example, differences in the expression of tRNA genes in different tissues or at different stages of development of an organism.

Expression studies, on the other hand, have produced clearer, if unexpected, results. In Xenopus, Drosophila and C. elegans the promoter for transcription of tRNA genes comprises noncontiguous regions within the coding
sequence (for review see (1)). Modulation of the transcription is less well understood but the sequence preceding the gene has been implicated in some cases (9-11). No comparable studies have been made on mammalian tRNA genes.

In this paper we report the isolation of several tDNA-containing clones from a human genomic library and the structure and expression in vivo of a tRNA gene from one of these.

MATERIALS & METHODS

MATERIALS

The human genome library of 15-20 Kb fragments of foetal liver DNA cloned in λ Charon 4A (12) was a gift from T. Maniatis. pBR322 clones of X. laevis tDNAs (13), used as control during library screening and clone characterization were supplied by S.G. Clarkson. tRNA nucleotidyl transferase was prepared from Baker's Yeast by the method of Sternbach et al (14). DNA ligase was a gift from D. Bentley. RNA ligase was from P.L. Biochemicals, snake venom phosphodiesterase from Worthington and restriction enzymes Sst I and Sst II from BRL (UK). All other enzymes were from Biolabs. All radiochemicals were from Amersham International.

Radioactive tRNA for Hybridisation Probes

Crude tRNA was prepared from human placenta by the method of Roe (15). Three different probes were prepared from this. (a) "Crude tRNA [32P]PcP" (2 to 5 x 10^7 cpm µg^-1) was obtained by ligation of cytidine 3', 5' [5'-32P] bisphosphate (> 3,000 Ci/mmol) to the tRNA for 2-12 hours using the method of England et al (16); (b) Crude tRNA was aminoacylated using a freshly desalted mixture of aminoacyl tRNA synthetases from human placenta by the method of Roe (15) except that twenty amino acids were used, ten of which (Phe, Pro, Tyr, Ser, Arg, Ala, Asp, Glu, Asn, Leu) were 14C labelled at 0.1 Ci:mmol. The mixed aminoacyl tRNAs (average specific activity 48 pmol each aminoacid/A 260 unit) were treated with periodate by the method of Traboni et al (17) and excess periodate removed by addition of rhamnose to 0.1 M. The protected aminoacyl tRNAs were then deacylated by incubation with 0.5 M lysine pH 8.8 which also caused $\beta$-elimination of the periodate oxidised uncharged RNA. This material, chargeable to the same extent as untreated tRNA, was radiolabelled as described in (a) above to yield "periodate-treated tRNA [32P]PcP" (approx. 2 x 10^7 cpm/µg); (c) Crude tRNA (1 µg) was treated with snake venom phosphodiesterase and repaired with yeast tRNA-nucleotidyl transferase, CTP and [α-32P] ATP by the method of Silberklang et al (18), modified to reduce the reaction volume from 10 µl to 4 µl. This gave
Isolation of λ clones

This and subsequent manipulations were performed in compliance with GMAG requirements.

Grids of 400 mixed plaques were prepared from nearly confluent plates of the Maniatis library grown on E. coli K803. Filters (Millipore HA or Schleicher and Schuell BA85) were prepared from these grided plates and hybridised with crude tRNA \(^{32}\)PpCp or periodate-treated tRNA \(^{32}\)PpCp for 16-24 hr in 50% formamide, 5 x SSC at 42°C. Filters were washed (x 3) at room temperature in hybridisation buffer and (x2) in 2 x SSC, dried and autoradiographed. Plaques which hybridised either form of tRNA were purified and rescreened. Clones showing hybridisation to periodate-treated tRNA \(^{32}\)PpCp were selected.

Production, separation, hybridization subcloning and sequencing of restriction fragments

These were performed using standard methods essentially the same as those compiled by Maniatis et al (19). DNA fragment size markers were λ DNA digested with Hind III and Ml3mp2RF DNA digested with Hae III. The conditions of RNA-DNA hybridisation were the same as for plaque filters. The vectors used were pAT153 (20, 21), Ml3mp8(RF) and Ml3mp9(RF) (22). Nucleotide sequences of fragments subcloned in M13 were determined by the method of Sanger et al (23).

Oocyte injection and RNA analysis

Plasmid DNA (5-10 ng/oocyte) was co-injected with \([\alpha^{32}\)P]GTP (410 Ci/mmol; 0.5 μCi/oocyte) into oocyte nuclei as described by Kressman et al (24) and the oocytes were incubated overnight. Total RNA was then extracted (25) and separated on 10% polyacrylamide gels containing 4 M urea (26).

RESULTS AND DISCUSSION

Isolation and physical mapping of a tDNA fragment

A mixture of radiolabelled tRNAs was chosen to probe the Maniatis human genomic library in order to increase the likelihood of selecting a wider variety of tRNA genes than might be expected using either a purified single species tRNA or eukaryotic tDNA as a probe. Furthermore it was hoped that the use of mixed tRNAs would favour selection of those genes the products of which predominate in crude tRNA and which, therefore, are present in high copy number or of high transcriptional activity. In the initial screening of 4,000 mixed plaques (estimated to contain up to ten or more different recom-
binants per plaque), 268 putative positives were selected of which approx. 50 showed strong hybridization, approx. 50 hybridised weakly and the rest showed intermediate hybridisation to crude tRNA[^32P]pCp. Screening with periodate-treated tRNA[^32P]pCp, plaque purification and rescreening, permitted selection of eight recombinants. Only one of these originated from a mixed plaque which strongly hybridised to crude tRNA[^32P]pCp, but three were from weakly hybridising plaques. The clone reported here, λ htl37, originally showed weak to intermediate hybridisation.

In view of the high incidence of 'false' positives, which we tentatively ascribe to radiolabelled small RNAs (e.g. 5S RNA, degraded rRNA etc.) present in crude tRNA[^32P]pCp but not radiolabelled in periodate-treated tRNA[^32P]pCp, the selected clones were further screened using another independent and unambiguous probe. The post-transcriptional addition and repair of the 3' terminus by tRNA nucleotidyltransferase permitted selective labelling of RNAs with a tRNA-like structure to less than 10^6 cpm μg^-1, irrespective of the time (zero to 20 min) of the prior treatment of the tRNA with venom phosphodiesterase. This precluded the probe's use in plaque hybridisation but was just sufficient for hybridisation of Southern blots of the restriction fragments of the recombinant DNAs.

The result for the recombinant λ htl37 is shown in Figure 1. Digestion
Figure 2
Restriction map of the 2.4 Kb Hind III fragment (box) showing hybridization of tRNA to restriction fragments.

with Hind III released, inter alia, two fragments of 10 Kb and approx. 2.4 Kb which hybridised to both periodate-treated \(^{32}P\)pCp and tRNA CpC\(^{32}P\)pA.

Further digestion with EcoRI appeared not to cut the 2.4 Kb Hind III fragment but the larger fragment was cut to release a 1.8 Kb fragment which hybridised to both probes. The 2.4 Kb and 1.8 Kb fragments were isolated and subcloned into Hind III-cut pAT153 or (Hind III + EcoRI)-cut pAT153 to yield PLB4 and pTC163 respectively.

In order to establish the location of the tRNA gene(s) within the 2.4 Kb fragment, PLB4 was linearised by digestion with EcoRI which cuts the DNA uniquely at a position 49 bases from the unique Hind III site into which the 2.4 Kb fragment was inserted (20, 21). The linearised plasmid was then digested with one of several restriction endonucleases which recognise sequences of 6 nucleotides absent from pAT153 or present in a few precisely known positions. The size of the fragments measured directly, and in most cases unambiguously, the position of restriction sites, if any, to the right of the unique EcoRI site. These are shown in Figure 2. Ambiguity with respect to the position of one of the Sma I sites was removed by digestion of the circular plasmid recombinant with Sma I. This released a fragment of 400 bp consistent with Sma I sites at approx. 900 bp and 1300 bp from the EcoRI site. The fragments which hybridised crude tRNA \(^{32}P\)pCp, shown in Figure 2, indicated tRNA-like sequences were confined to the central portion of the 2.4 Kb fragment.

Transcriptional studies

The RNA produced by Xenopus oocytes following microinjection of the
Figure 3
(a) RNAs synthesised in Xenopus oocytes following nuclear injection with recombinant DNAs and [α-32P]-GTP. M = Marker tRNA [32p]pCp (left, crude tRNA; right, "periodate-treated tRNA"); pTC163 = 1.8 Kb Hind III-EcoRI fragment from λ htl37 in pAT153; pNB21 = A pBR322 subclone of human DNA being characterized; pLB4 = The 2.4 Kb Hind III fragment containing the tRNA(Glu) gene; pt210 = 3.18 Kb X. laevis tDNA cluster in pBR322 (only in the right hand lane were conditions identical to all other experiments). C = Control (only [α-32p]GTP)
(b) Fragments of the 2.4 Kb subcloned into M13 for sequence analysis and transcriptional studies. The direction and approximate extent of sequence data is shown by the arrows. An additional subclone equivalent to F but in M13mp9 was used to determine the sequence 1300 to 1600. The
conclusion from these studies is shown diagrammatically below the parent fragment B.

(c) Autoradiograph of gel containing RNAs produced by subclones A-G (Fig. 3b) following their nuclear co-injection, with \( {\alpha}^{-32P} \)GTP, into *Xenopus* oocytes.

subcloned fragment into their nuclei was resolved by polyacrylamide gel electrophoresis. In this system endogenous transcription of the oocyte tRNA genes is very low and almost all the tRNA transcripts are derived from the large number of injected tRNA genes. Brief (2 hr) autoradiography of the gel (Fig. 3a) indicated that probably only one tRNA species, of relatively high electrophoretic mobility, was transcribed following microinjection of either pLB4 or pTC163. The discontinuous gel system used (26) resolved most of the species transcribed from the pBR322 clone of the *Xenopus laevis* tDNA cluster, pt210 (3, 13). A longer exposure (16 hr - data not shown) revealed minor tRNA 4S bands and endogenous transcripts. The former were larger than the major species and may be ascribed either to tRNAs produced by less active tRNA genes in the recombinant or to precursors to the major tRNA species. The most striking feature of Figure 3a is the amount of tRNA produced by oocytes microinjected with pLB4 compared to that produced on microinjection of the same amounts of either pTC163 or the control recombinant pt210. This control contains within its 3.18 Kb insert a cluster of 8 tRNA genes of which 7 are transcriptionally active in the homologous *Xenopus* oocyte nuclei or germinal vesicle extracts (3). The density of the autoradiograph bands (Fig. 3a) indicate that the major tRNA bands produced by pLB4 is approximately 10 times that from pTC163, 3 times that of the strongest band from pt210 and is 10-20% greater than the sum of all of the tRNA bands produced by pt210.

The 2.4 Kb fragment contained the restriction sites Xma I, Hind III and Xho I (compatible with Sal I). This permitted subcloning of suitable portions of the fragment into Ml3mp8 or Ml3mp9 for both sequence analysis and further transcriptional studies (Fig. 3b). Analysis of the RNA produced in oocytes following nuclear injection of RFDNA from several of these Ml3 recombinants is shown in Figure 3c. No RNA was produced from the Xho I-Hind III fragment in Ml3mp9 (E in Fig. 3b and c) confirming the hybridization studies which suggested no tRNA-like sequence in the region 1800-2400. Subclones of the two fragments from the central region, Xma I (900)-Xma I (1300) (A in Fig. 3b, c) and Xma I (1300)-Xho I (1800) (F in Fig. 3b, c) also produced no RNA although the larger Xma I (900)-Xho I (1800) fragment in either Ml3mp8 or Ml3mp9 (D and G in Fig. 3b, c) produced tRNA of the same size and quantity as the
Figure 4

The nucleotide sequence from the XmaI site at approximately 900 on the restriction map of Figure 2. The tRNA_{Glu} gene is boxed. The restriction sites found experimentally (Figure 2) are shown.

parent clone pLB4 (B in Fig. 3b, c) or the Hind III-Xho fragment (C in Fig. 3b, c). The minor 4S bands (Fig. 3c; lanes C, D and G) we ascribe to precursors of the tRNA which have been fully processed in the pLB4 parent clone transcript (Fig. 3c; lane B). The high molecular weight products, present in all lanes of Fig. 3c and strong in lanes A and D are presumably endogenous transcripts. This suggested that the 2.4 Kb fragment contained only one tRNA gene which was split by Xma I (900) to yield two transcriptionally inactive fragments.

Sequence Studies

Eleven of the M13 recombinants were sequenced as indicated in Figure 3b. The orientation of the Xma I fragment within the 2.4 Kb sequence was determined from the position of the Sst I site (at approx. 1250 bp from EcoRI - Fig. 2) within the Xma I fragment sequence. All other fragments were of known orientation permitting the alignment of sequence from approx. 900 bp to 2150 bp from the EcoRI site of Figure 2. This is shown in Figure 4.

The position of the only tRNA gene in the sequence (356 to 427, corres-
Figure 5

(A) Sequence of the tRNA^Glu^ gene (non-coding strand) arranged in a cloverleaf secondary structure with the yeast tRNA^Phe^ numbering. Conserved and semi-conserved nucleotides are boxed.

(B) A possible secondary structure in the 5' flanking sequence (residues 292-347) with features resembling that of a tRNA. The tRNA^Glu^ gene, drawn in the same manner, is also shown (residues 356-end).

Corresponding to approx. 1250 to approx. 1320 on the map in Fig. 2) confirmed the inference from transcriptional studies that this inadvertently disrupted human tRNA gene, like tRNA genes of other eukaryotes, contains an intragenic split promoter, both parts of which are required for transcription. Xma I cuts within the gene after residue 402, corresponding to the junction of the variable loop and the Tyc stem of the tRNA.

The structure of the tRNA^Glu^ gene is shown in Figure 5A where the non-coding (tRNA-like) strand is folded as a cloverleaf with standard numbering of residues according to those found in yeast tRNA^Phe^ (27). The most unusual structural feature is the presence of an C2-A^71^ pair in the acceptor stem, the first example of its occurrence in a eukaryotic tRNA (28). Another unexpected feature is the presence of T(U) at position 73, instead of a purine previously found in every tRNA^Glu^ sequence (27). The general structural properties of tRNAs, including all the conserved or semi-conserved nucleotides are present. Some features characteristic of tRNA^Glu^, e.g. the absence of residues 17 and 47 and an extra residue (20:1) in the D loop, are found in this gene. The sequence reported here differs from the rat tRNA^Glu^AA (29) tDNA^Glu^GAG of rat (6) and tDNA^Glu^ of Drosophila (30) by 19, 7 and 17 residues respectively.

It is premature to attempt to generalise upon the organisation of tRNA genes within the human genome. Earlier studies (7) on tRNA^Met^ genes show
that about twelve genes are scattered in the genome, whereas more recently a
cluster of three different tRNA genes, each separated by approx. 0.5 Kb, have
been found in a human Λ recombinant which contains several tRNA genes (8).
The single tRNA^Glu^ gene reported here is separated from other tRNA genes by
at least 1 Kb, being centrally located within a 2.4 Kb fragment. However
there is evidence for a least one more tRNA gene within the clone, Λ htl37
from which the 2.4 Kb fragment was derived (see Fig. 1).
We have inspected the sequence surrounding the tRNA^Glu^ gene (Fig. 4).
No other tRNA genes were found using the program of Staden (31). No open
reading frames of significant length were detected. However several repet-
tive sequences and inverted repeats of eight or more nucleotides were found.
The 5' flanking sequence preceding the tRNA^Glu^ gene shares the feature,
noted for other human tRNAs (8), of a block of purines followed by a block of
pyrimidines although their lengths in the present sequence are considerably
larger than those noted previously. In addition we note the presence of a
7 bp alternating purine-pyrimidine tract capable of forming left handed DNA
which has been suggested may be an inhibitory sequence in a variant tRNA^Met^
that of Xenopus laevis (32).

The interesting feature of this region however is that residues 292-347
have the potential to form a secondary structure resembling that of part of a
tRNA. This is shown in Figure 5B. It would be misleading to call this
sequence a tRNA pseudogene. In spite of the presence of TTC in the region
corresponding to the anticodon loop it shows low homology with the adjacent
tRNA^Glu^ gene, nor does it contain a convincingly large proportion of the
conserved or semi-conserved nucleotides noted for tRNA - the most generous
interpretation permits only about half of these residues to be present. The
strongest resemblance of this sequence to tRNA lies in its secondary structure
and the relative spacing of the arms to each other and to these in the tRNA
gene. Although this is not perfect (with 6 and 8 residues in the anticodon
and T=C loops instead of the normal 7 residue), we have been unable to gener-
ate a structure resembling this from other reported 5' flanking sequences.
When two eukaryotic tRNA genes are in tandem they are usually separated by a
greater distance than the structures shown in Figure 5B and, if of the same
polarity, are separated by a T-rich "transcription termination" sequence
(33). Although the RNA polymerase promoter is contained within the
transcribed gene, there is evidence that the transcriptional control region
for tRNA genes (9-11), are in the 5' flanking sequence immediately preceding
the gene (for review see 32). For example Drosophila tRNA^Lys^ gene is re-
pressed by an oligo T-containing undecanucleotide 13 nucleotides upstream from the tRNA (9, 11), whereas the normal 5' flanking sequence of Bombyx mori tRNA^{Ala} is required for transcription by a homologous cell-free extract (10). It has been proposed that the intragenic promoter of tRNA genes is recognised as a tertiary structure that partially resembles tRNA itself (32). The present gene is remarkably active in transcription. We are therefore tempted to speculate that this peculiar structural feature of the 5' flanking may serve to enhance transcription of the gene.

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REFERENCES

1. Kubli, E., Experientia 37, 1-9 (1981).
2. Soll, D., Abelson, J.N. and Schimmel, P.R. (1980) Transfer RNA: Biological Aspects, Cold Spring Harbor Laboratory.
3. Yen, P.H. and Davidson, N. (1980) Cell 22, 137-148.
4. Müller, P. and Clarkson, S.G. (1980) Cell 19, 345-353.
5. Lasser-Weiss, M., Bawnik, N., Rosen, A., Sarid, S. and Daniel, V. (1981) Nucl. Acids. Res. 9, 5965-5978.
6. Shibuya, K., Noguchi, S., Nishimura, S. and Sekiya, T. (1982) Nucl. Acids. Res. 10, 4441-4448.
7. Santos, T. and Zasloff, M. (1981) Cell 23, 699-709.
8. Roy, K.L., Cooke, H. and Buckland, R. (1982) Nucl. Acids. Res. 10, 7313-7322.
9. DeFranco, D., Schmidt, O. and Soll, D. (1980) Proc. Natl. Acad. Sci. USA 77, 3365-3368.
10. Sprague, K.U., Larson, D. and Morton, D. (1980) Cell 22, 171-178.
11. DeFranco, D., Sharp, S. and Soll, D. (1981) J. Biol. Chem. 256, 12424-12429.
12. Lawn, R.M., Fritsch, E.F., Parker, R.C., Blake, G. and Maniatis, T. (1978) Cell 15, 1157-1174.
13. Clarkson, S.G., Kurer, V. and Smith, H.O. (1978) Cell 14, 713-724.
14. Sternbach, H., von der Haar, F., Schlimme, E., Gaertner, E. and Cramer, F. (1971) Eur. J. Biochem. 22, 166-172.
15. Roe, B.A. (1975) Nucl. Acids. Res. 2, 21-42.
16. England, T.B., Bruce, A.G. and Uhlenbeck, O.C. (1980) in Methods in Enzymology Vol. 65 pp 65-74, Academic Press, New York.
Nucleic Acids Research

17. Traboni, C., Cortese R. and Salvatore F. (1980), Nucl. Acids. Res. 8, 5223-5232.
18. Silberklang, M., Gillum, A.M. and RajBhandary, U.L. (1979) in Methods in Enzymology Vol. LIX pp 58 -109, Academic Press, New York.
19. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory.
20. Twigg, A.J. and Sherratt, D. (1980) Nature 283, 216-218.
21. Sutcliffe, J.G. (1978) Cold Spring Harbor symp. Quant. Biol. 43, 77-90.
22. Messing, J. and Vieira, J. (1982) Gene 19, 269-276.
23. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.H. (1980) J. Mol. Biol. 143, 161-178.
24. Kressmann, A., Clarkson, S.G., Pirrotta, V. and Birnstiel, M.L. (1978) Proc. Natl. Acad. Sci. USA 75, 1176-1180.
25. Bienz, M. and Gurdon, J.B. (1982) Cell 29, 811-819.
26. Fradin, A., Gruhl, H. and Feldmann, H. (1975) FEBS Letters 50, 185-189.
27. Sprinzl, M. and Gauss, D.H. (1983) Nucl. Acids Res. 11, r1-r55.
28. Grosjean, H. Cedergren, R.J. and McKay, W. (1982) Biochemie 64, 387-397.
29. Chan, J.C., Yang, J.A., Dunn, M.J., Agris, P.F. and Wong, T-W. (1982) Nucl. Acids Res. 10, 4605-4608.
30. Indik, Z.K. and Tartof, K.D. (1982) Nucl. Acids Res. 10, 4159-4172.
31. Staden, R. (1980) Nucl. Acids Res. 8, 817-825.
32. Hall, B.D., Clarkson, S.G. and Tocchini-Valentini, G. (1982) Cell 29, 3-5.
33. Korn, L.J. and Brown, D.D. (1978) Cell 15, 1145-1156.