The role of the 5'-flanking sequence of a human tRNA^{Glu} gene in modulation of its transcriptional activity *in vitro*

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The role of a tRNA-like structure within the 5'-flanking sequence of a human tRNA^{Glu} gene in the modulation of its transcription *in vitro* by HeLa cell extracts has been investigated using several deletion mutants of a recombinant of the gene which lacked part or all of the tRNA-like structure. The transcriptional efficiency of four mutants was the same as that of the wild-type recombinant, two mutants had decreased transcriptional efficiency, one was more efficient, and one, lacking part of the 5' intragenic control region, was inactive. Correlation of the transcriptional efficiencies with the position and the size of the 5'-flanking sequence that was deleted indicated that the tRNA-like structure may be deleted without loss of transcriptional efficiency. Current models for the modulation of tRNA gene transcription by the 5'-flanking sequence are assessed in the light of the results obtained, and a potential model is presented.

INTRODUCTION

Although it is well established that the transcription of eukaryotic tRNA genes is directed by intragenic control regions (De Franco *et al.*, 1980; Galli *et al.*, 1981; Sharp *et al.*, 1985; Geiduschek & Tocchini-Valentini, 1988), flanking sequences also appear to modulate transcription. In several instances tRNA genes with an identical coding sequence but having different 5'-flanking sequences differ in their promoter strength (Lofquist & Sharp, 1986; Gouilloud & Clarkson, 1986; Arnold *et al.*, 1986, 1988; Doran *et al.*, 1987, 1988; McKay *et al.*, 1988).

With only few exceptions (e.g. Shaw & Olson, 1984; Schaack & Soll, 1985; Raymond *et al.*, 1985; Marschalek & Dingermann, 1988), the role of flanking sequences has only been investigated *in vitro*. The predominant observation of these *in vitro* studies is that deletions into 5'-flanking regions diminish, and in some instances (Larson *et al.*, 1983) almost abolish, transcription. However, in a few cases increased transcription has been found, suggesting that positive as well as negative modulator sequences are present in the 5'-flanking sequences of some tRNA genes. Several studies have demonstrated transcription factor binding to these 5'-flanking sequences (Raymond & Johnson, 1987; Marschalek & Dingermann, 1988; Rooney & Harding, 1988; Kassavetis *et al.*, 1989). In addition, 3'-flanking sequences are implicated in modulation of some tRNA genes, e.g. *Bombyx mori* tRNA^{Ala} (Wilson *et al.*, 1985) and *Drosophila* tRNA^{Arg} (Sharp *et al.*, 1983).

Positive modulators have been identified in the 5'-flanking sequences of several tRNA genes (Sprague *et al.*, 1980; Larson *et al.*, 1983; Johnson & Raymond, 1984; Raymond *et al.*, 1985; Morry & Harding, 1986; Arnold & Gross, 1987; Sajjadi & Spiegelman, 1987; Rooney & Harding, 1988; Lofquist *et al.*, 1988; Horvath & Spiegelman, 1988). These sequences, of 5–35 nucleotides, are located in the first 53 nucleotides upstream of various tRNA genes. Comparison of the sequences of these positive modulators has revealed no common features. Potential Z-DNA sequences, present in the 5'-flanking regions of several tRNA genes, appear to inhibit their transcription (Hipskind & Clarkson, 1983; Nordheim & Rich, 1983; Santoro *et al.*, 1984;

Glew *et al.*, 1986), as does an undecanucleotide sequence in the 5'-flanking region of a *Drosophila* tRNA^{Lys} gene, the position of which relative to some element downstream influences the extent of transcription repression (De Franco *et al.*, 1981). Both inhibitory and stimulatory sequences have been identified in the 5'-flanking regions of some *Drosophila melanogaster* tRNA genes (Schaack *et al.*, 1984; Sajjadi *et al.*, 1987).

Finally, several authors have demonstrated that 5'-flanking sequences modulate transcription of tRNA genes differently in different transcription systems (Raymond & Johnson, 1983; Johnson *et al.*, 1984; Schaack & Soll, 1985; Drabkin & RajBhandary, 1985). Such observations are consistent with reports that 5'-flanking sequences elements are responsible for tissue-specific expression of tRNA genes in *B. mori* (Young *et al.*, 1986) and *Caenorhabditis elegans* (Hodgkin *et al.*, 1987; Kondo *et al.*, 1988). In conclusion, the studies so far have not revealed any common feature of flanking sequences by which the transcription of tRNA genes is modulated. The requirements for faithful transcription might differ from species to species or even from tissue to tissue in the same eukaryote.

We have previously described a human tRNA^{Glu} gene which has high transcriptional activity when micro-injected into *Xenopus* oocyte nuclei, producing more tRNA than a cluster of seven active *Xenopus* tRNA genes. The 5'-flanking sequence preceding this gene has a potential for forming a tRNA-like structure at residues 292–347 (Goddard *et al.*, 1983). These properties have led us to investigate whether or not this tRNA-like structure within the 5'-flanking sequence affects the transcriptional efficiency of the gene. The same gene, together with 355 bp of 5'-flank and 54 bp of 3'-flank, was subcloned as a 481 bp fragment into M13mp9 to generate the recombinant MtGlu6, and several deletion mutants, lacking part or all of the tRNA-like structure, were constructed. Preliminary experiments using a heterologous transcription system, in which two deletion mutants were tested in parallel with MtGlu6, showed that part of the 5'-flanking sequence is required for maximum tRNA^{Glu} gene transcription (Goddard *et al.*, 1985). In this paper the transcriptional efficiency of a wider range of these deletion mutants has been compared with that of the wild-type

Abbreviations used: RF, replicative form; TF, transcription factor.

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recombinant MtGlu6 in a homologous transcription system using a HeLa cell nuclear extract as the source of RNA polymerase III and transcription factors.

MATERIALS AND METHODS

Materials

Radiochemicals were purchased from Amersham International. Restriction enzymes were from Gibco-BRL, Pharmacia or Northumbria Biologicals Ltd. Klenow fragment and proteinase K were from Boehringer, crude *Escherichia coli* tRNA from M.R.E., Porton Down, Salisbury, Wilts., U.K. and exonuclease *Bal31* from New England Biolabs (supplied by CP Labs., Bishops Stortford, Herts., U.K.).

Plasmid Recombinants

The recombinant MtGlu6 contains residues 1–481 of the tRNA^{Glu} gene and the flanking sequence previously reported (Goddard *et al.*, 1983). These were removed, as a 481 bp *EcoRI*–*NaeI* fragment, from a recombinant containing residues 1–937 of that sequence as a *SmaI*–*XhoI* fragment in M13mp9 and inserted into *SmaI*+*EcoRI*-cut M13mp9. Recombinant M13T-(su⁺) (Laski *et al.*, 1982a,b) was a gift from Dr. A. Fire. General recombinant DNA techniques were performed essentially as described by Maniatis *et al.* (1982) except when otherwise stated. Recombinant M13 replicative form (RF) DNAs were prepared from 1 litre exponential phase cultures of infected *E. coli* JM105 or *E. coli* JM109 by the method of Birnboim & Doly (1979) and purified through two CsCl gradients. The DNA consisted of $\geq 95\%$ supercoiled covalently closed circles, as judged by gel electrophoresis.

Generation and characterization of deletion mutants of MtGlu6

Recombinant MtGlu6 RF DNA, which contains unique sites for *SstI* and *StuI* at residues 148–153 and 297–302 respectively of the insert sequence, was linearized with *SstI* and partially digested with exonuclease *Bal31* for 1, 5, 10 or 20 min as described by Maniatis *et al.* (1982). The extent of digestion was monitored by the sizes of fragments released by digestion with *EcoRI* or *HindIII*. Appropriate fractions were either ligated or digested further with *StuI* before purification on agarose gels and ligation. The ligated samples were used to transform *E. coli* JM101 using the method of Hanahan (1983). The transformants were initially screened by restriction digest analysis and further characterized by determination of the sequence of the insert DNA (Sanger *et al.*, 1980).

An extract was prepared by the method of Weil *et al.* (1979) from 3-litre cultures of HeLa S3 cells (Gibco) grown in suspension in supplemented Eagle's medium (Gibco) to approx. 5×10^5 cells/ml. The final S100 extract, containing 30 mM-Hepes, 120 mM-KCl, 3 mM-MgCl₂ and 20% glycerol and stored in 400 μ l portions at -70°C , was used in all of the experiments described below.

Transcription assay

The following assay, initially based on a consensus of earlier methods (Segall *et al.*, 1980; Laski *et al.*, 1983; Makowski *et al.*, 1983; Murphy & Baralle, 1983; Schaack *et al.*, 1984), was refined by series of optimization experiments. A typical transcription reaction mixture (20 μ l) contained 14 mM-Hepes/KOH (pH 7.9), 80 mM-KCl, 20 mM-NaCl, 3.5 mM-MgCl₂, 10% (v/v) glycerol, 0.3 mM-dithiothreitol (as final concentrations derived from components added in the extract as well as exogenously), 600 μ M unlabelled ATP, CTP and GTP, 2.5 μ Ci of [α -³²P]UTP (Amersham; 400 Ci/mmol), 25 μ M unlabelled UTP, 10 mM-phosphocreatine, 10 μ l of HeLa S100 extract, the desired amount of

supercoiled recombinant DNA and M13mp12 DNA 'carrier' to give 150 ng of total DNA in the assay. The mixture was incubated at 30°C for 60 min and the reaction was stopped by the addition of 20 μ l of a solution containing 1% SDS, 2 mg of proteinase K/ml and 0.5 mg of *E. coli* crude tRNA/ml. The reaction mixture was maintained at 37°C for 20 min and then an equal volume of 1 M-ammonium acetate was added. The mixture was extracted once with phenol/chloroform (1:1, v/v) and the phenol/chloroform layer was re-extracted with an equal volume of 0.5 M-ammonium acetate. The RNA was precipitated with ethanol, resuspended in 10 μ l of TE (0.01 M-Tris/HCl/1 mM-EDTA, pH 7.5), mixed with 10 μ l of formamide dye mix (0.03% Bromophenol Blue/20 mM-EDTA in deionized formamide) and electrophoresed at 250 V for 2.5 h on a 10% (w/v) polyacrylamide/4 M-urea vertical gel (20 cm $\times$ 20 cm $\times$ 0.1 cm). Soon after autoradiography, the gel and the autoradiographs were aligned and regions corresponding to transcripts were excised, and the RNA content was calculated from the measured Cerenkov c.p.m. All assays were performed in duplicate and the results were corrected for gel background and decay.

RESULTS

Construction and characterization of deletion mutants

The mutants used in the experiments described below were constructed from MtGlu6 as described (see the Materials and methods section) to generate two classes of deletions (Fig. 1). The first class, constructed by simple deletion around the *SstI* site 54 bp upstream from the start of the coding region, is represented by MtGluD33, MtGluD44, MtGluD92 and MtGluD104. In the second class, represented by MtGluD5, MtGluD7, MtGluD20 and MtGluD47, all residues between the *StuI* site (204 bp upstream of the gene) and the *SstI* site are removed in addition to residues closer to the gene. Although the high transformation efficiency (approx. $10^8/\mu\text{g}$) afforded by the method of Hanahan (1983) yielded 114 mutants which were approximately equally distributed between these two classes, the range of the regions deleted was not uniform, presumably due to the sequence-dependent efficiency of the *Bal31* exonuclease used. The above mutants, representing deletions within the first 204 bp of the 5'-flank, were chosen for quantitative transcriptional studies.

Characterization of the transcription system

Initial experiments were carried out using MtGlu6 RF DNA as template in order to establish the optimal conditions for tRNA^{Glu} gene transcription *in vitro*. Preliminary experiments using 10 μ l of extract in a 20 μ l reaction volume showed that, after a short lag, transcript production increased linearly with time at 30°C for at least 2 h and was maximal at 100–120 ng of template DNA. In order to establish that MtGlu6 transcripts were RNA polymerase III products, a standard transcription assay using 150 ng of MtGlu6 was performed in the presence of 0.5 μ g of α -amanitin/ml, sufficient to inhibit the activity of RNA polymerase II. The nature and amount of the transcripts was the same in the presence or the absence of α -amanitin (results not shown), so subsequent assays were performed without α -amanitin. The optimal ionic conditions were established by successive variation of the final concentrations of MgCl₂, NaCl and KCl. The reaction was found to have a relatively sharp optimal [MgCl₂]_{final} of 3.5 mM, to be inhibited at [NaCl]_{final} > 40 mM and to be strongly inhibited at [KCl]_{final} > 100 mM.

The dependence of the system upon exogenous tRNA^{Glu} template was then investigated. Transcription was detectable of 5 ng of MtGlu6/20 μ l of assay, and increased approximately sigmoidally with increasing template concentration to reach

Fig. 1. Schematic representation of the tRNA^{Glu} gene and flanking sequences in the M13mp9 recombinant MtGlu6 and its deletion mutants

The sequence of the gene (boxed) and the tRNA-like structure of its immediate 5'-flanking sequence is shown (top). Residue numbers are according to Fig. 4 of Goddard *et al.* (1983) and indicate residues present in the deletions. Deleted regions (---) and restriction sites in the multiple cloning site or used in mutant construction are indicated (E, *Eco*RI; H, *Hind*III; N, *Nae*I; P, *Pst*I; S, *Sma*I).

Fig. 2. Transcription products of MtGlu6 and its deletions (40 ng plus 110 ng of M13 carrier DNA) separated by PAGE and identified by autoradiography

The different templates used in otherwise identical assays are shown. The blank control (BI) contained 150 ng of M13 carrier DNA but no template. The size marker (M) is periodate-treated human placental tRNA[³²P]pCp.

saturation at approx. 120 ng/assay. The low levels of transcription observed at low concentrations of MtGlu6 were increased up to 5-fold by addition of non-template DNA carrier in the form of vector M13mp12 RF DNA (see Fig. 3a).

The transcriptional efficiency of MtGlu6 was found to be significantly dependent on its conformation. When the supercoiled covalently closed circular MtGlu6 was linearized by

*Bgl*II (at residue 6935 of the M13 vector, approx. 700 bp from the multiple cloning region), the template efficiency was reduced 3-fold. Similar results were also found for all of the deletion mutants tested.

Transcriptional efficiency of MtGlu6 and its deletions

When the extents of transcription from MtGlu6 and its deletion mutants at non-saturating concentrations (40 ng of template DNA plus 110 ng of carrier DNA per 20 μ l of assay) were compared, it was observed that several of the deletions (MtGluD20, MtGluD33, MtGluD44 and MtGluD104) had a similar efficiency to that of MtGlu6, but that MtGluD47 was more efficient, MtGluD5 and MtGluD7 were less efficient and MtGluD92 was inactive (Fig. 2). Since the amount of transcript is dependent upon the amount of template DNA in the assay, it was decided to compare the amount of transcript produced using a wide range of concentrations of the tRNA^{Glu} gene or of its deletion mutants as templates, although previous studies on the transcriptional efficiency of genes have usually reported the extent of transcription at only one concentration of template DNA.

Samples of 0, 10, 20, 40, 60, 80, 100, 125 and 150 ng of transcript from each mutant, in the presence of M13mp12 DNA carrier to give a final concentration of 150 ng/20 μ l, were assayed as described in the Materials and methods section. Concurrent with each mutant assayed, MtGlu6 was also assayed at two concentrations (40 ng plus 110 ng of carrier DNA and 150 ng/20 μ l of assay) to allow the direct comparison of the efficiency of the mutants with that of MtGlu6. The extent of transcription from MtGlu6 in these assays showed a day-to-day

Fig. 3. Extent of transcription from various amounts of MtGlu6 and its deletions

All assays were performed under identical conditions (60 min, 30 °C) with carrier M13 DNA added to give 150 ng of total DNA, except where indicated. (a) Transcripts from MtGlu6 (○) and MtGluD5 (●); the extent of transcription from MtGlu6 in the absence of M13 DNA carrier is also shown (□). (b) Transcripts from MtGluD7 (□), MtGluD20 (●) and MtGluD47 (○) compared with MtGlu6 (---, *a* above). (c) Transcripts from MtGluD33 (□) and MtGluD44 (●) compared with MtGlu6 (---). (d) Transcripts from MtGluD104 (□) and MtGluD92 (●) compared with MtGlu6 (---). Error bars indicate S.D. for four samples.

variation of $\leq 10\%$. The results of such analyses are presented in Figs. 3(a)–3(d). The results obtained for mutants MtGluD20 (Fig. 3b), MtGluD104 (Fig. 3d), MtGluD33 and MtGluD44 (Fig. 3c) show no significant difference from those for MtGlu6. Mutant MtGluD47, although its transcriptional efficiency was essentially the same as that of MtGlu6 (105%), was more efficient than MtGlu6 at lower, non-saturating, amounts of template DNA (Fig. 3b). However, mutants MtGluD5 and MtGluD7 produced considerably fewer transcripts than MtGlu6 at 150 ng of template (60% and 74% of MtGlu6 respectively) and at all limiting template DNA concentrations (Figs. 3a and 3b). Finally, mutant MtGluD92 was not transcribed at all, presumably due to its lack of residues 1–12 of the tRNA^{Glu} gene, which include the first four nucleotides of the 5' ICR (Fig. 3d).

Competition experiments

These apparent differences in promoter strength were investigated further in assays in which MtGlu6 or its deletion mutants were used to compete with another tRNA gene for RNA polymerase III and/or transcription factors. The recombinant used for this purpose, M13tT-(su⁺), contains an amber suppressor tRNA gene, derived from a *X. laevis* tRNA^{Tyr}_{GTA} gene, (Laski *et al.*, 1982a,b). Since this recombinant has a 13-nucleotide intervening sequence, the size of the transcript (86 nucleotides) readily distinguishes it from the shorter transcription products of MtGlu6 or its deletion mutants. The template

efficiency of this gene was found to be lower than that of MtGlu6. When 80 ng of M13tT-(su⁺) was transcribed in the presence of 40 ng of MtGlu6 or its deletions (plus carrier DNA to 150 ng), the extent of transcription was decreased (Fig. 4). Transcript A (Fig. 4), of size 200–250 nucleotides, is produced by RNA polymerase III. The region of M13tT-(su⁺) transcribed is not established. The competitive strengths of MtGlu6 and its mutants, indicated both by the decreased transcription from M13tT-(su⁺) and the amount of tRNA^{Glu} produced, were MtGluD47 > MtGlu6 $\approx$ MtGluD20 $\approx$ MtGluD33 $\approx$ MtGluD44 $\approx$ MtGluD104 > MtGluD7 $\approx$ MtGluD5.

The relatively poor competition by MtGluD7 and MtGluD5 was confirmed by competitive transcription assays containing 0, 20, 40, 60 or 80 ng of MtGlu6, MtGluD5, MtGluD7, MtGluD33 or MtGluD104 (plus carrier DNA to give a total of 80 ng) and 120 ng of M13tT-(su⁺). MtGlu6 (150 ng) transcribed in the absence of M13tT-(su⁺) served as a control and reference. The results are shown in Figs. 5(a) and 5(b). The number of transcription products from 120 ng of M13tT-(su⁺), in the absence of MtGlu6, represented 58% of that from 150 ng of MtGlu6, and this was lowered in the presence of increasing amounts of MtGlu6 or its deletions. The extent of this decrease varied. MtGluD5 only marginally lowered M13tT-(su⁺) transcription, and MtGluD7 was less effective than MtGlu6 (Fig. 5a). MtGluD33 and MtGluD104 were indistinguishable from MtGlu6, with 80 ng decreasing the relative transcription efficiency

of M13tT-(su⁺) from 58 %, in the absence of competitor, to 38 %, 41 % and 39 % respectively (results not shown). These differences were also reflected in the relative amounts of tRNA^{Glu} produced (Fig. 5b). The high promoter strength of MtGluD47 was confirmed in a similar experiment using 0, 20, 40, 80 or 120 ng of MtGlu6, MtGluD20, MtGluD44 or MtGluD47 (plus carrier DNA to give a total of 120 ng) with 80 ng of M13tT-(su⁺). Similar results (not shown) were obtained with MtGlu6, MtGluD20 and MtGluD44, but low amounts of MtGluD47 decreased M13tT-(su⁺) transcription more effectively than did MtGlu6 (Fig. 5c). The number of transcription products derived from 80 ng of M13tT-(su⁺) in the absence of MtGlu6 was 33 %

of the number of transcription products derived from 150 ng of MtGlu6. However, when 80 ng of M13tT-(su⁺) was assayed together with 120 ng of MtGlu6, M13tT-(su⁺) efficiency was lowered 2-fold (16 %); the ratio of 120 ng of MtGlu6/80 ng of M13tT-(su⁺) transcription products was 5.7:1.0. When the same amount of M13tT-(su⁺) was assayed together with 120 ng of MtGluD20, MtGluD44, MtGluD47 or MtGluD92, its efficiency was decreased from 33 % to 15 %, 14 %, 14 % and 20 % respectively.

DISCUSSION

The transcription system

The properties of the transcription assay system described above are essentially similar to those described by other workers. HeLa cell nuclear extracts were prepared by three different methods (Weil *et al.*, 1979; Dignam *et al.*, 1983; Shapiro *et al.*, 1988) and tested in an assay using 150 ng of MtGlu6 template DNA. In our hands, the extract prepared by the method of Weil *et al.* (1979) was most active, and one such extract was used in

Fig. 4. Transcription products from competing templates of M13tT-(su⁺) (80 ng/assay) and MtGlu6 or its deletions (40 ng plus 30 ng of M13 carrier DNA) separated by PAGE and identified by autoradiography

The different MtGlu templates used in otherwise identical assays are shown. M13tT-(su⁺) and MtGlu transcripts and size marker M (periodate-treated tRNA^{[32P]pCp}) are identified. Transcript A is discussed in the text.

Fig. 6. Transcriptional efficiencies of MtGlu6 deletion mutants

The mutants were present at 150 ng/assay (●) or 40 ng plus 110 ng of carrier DNA/assay (□). Efficiencies relative to MtGlu6 (....., 150 ng; - - - - - , 40 ng + 110 ng of carrier) are shown as a function of intact immediate 5'-flanking sequence. The location of the tRNA-like structure within the 5'-flanking sequence is shown.

Fig. 5. Competitive strength of MtGlu6 and its deletions as templates in the presence of M13tT-(su⁺)

The extent of transcription (fmol of transcript) is expressed relative to that from 150 ng of MtGlu6 in the absence of carrier or competing M13tT-(su⁺) template. (a) Relative efficiency of 80 ng of M13tT-(su⁺) in the presence of various amounts of MtGlu6 (○), MtGluD5 (●) or MtGluD7 (□). (b) Relative efficiency of various amounts of MtGlu6 (○), MtGluD5 (●) or MtGluD7 (□) in the presence of 120 ng of M13tT-(su⁺). (c) Relative efficiency of 120 ng of M13tT-(su⁺) in the presence of various amounts of MtGlu6 (○) or MtGluD47 (□).

all experiments. With this extract (10 μ l/20 μ l of assay), after a lag which presumably reflects the time taken in the formation of active initiation complexes, the number of full-length transcripts increased linearly with time for at least 2 h and several transcripts per gene were produced, indicating that polymerase is released to permit multiple rounds of transcription of the same gene. Stimulation of transcription by a DNA carrier most probably arises by removal of sequence-independent DNA-binding proteins that can inhibit transcription by randomly binding to DNA, masking the promoter to prevent factor binding (Shapiro *et al.*, 1988). Since each assay in this study contained the same amount of the same extract, and therefore the same amount of sequence-independent DNA-binding proteins, carrier DNA was added to give constant total DNA (150 ng/20 μ l) in each assay. The observed dependency of template efficiency upon conformation is consistent with the finding that transcription of an *E. coli* tRNA^{Tyr} gene was dependent on the extent of negative supercoiling of its DNA (Lamond, 1985). A similar 2–3-fold decrease in the transcription efficiency was observed on linearization of covalently closed circular DNA containing the adenovirus major late promoter (Shapiro *et al.*, 1988).

Recognition sequences of the 5'-flank

For four of the deletion mutants tested over a wide range of template concentrations (10–150 ng/assay), the extent of transcription was the same, within experimental error, as that found for MtGlu6, which has an intact 5'-flank. However, two mutants showed decreased transcriptional efficiency, one (MtGluD92) was inactive and one had enhanced template activity (Figs. 2 and 3). These relative promoter strengths (MtGluD47 > MtGlu6 $\approx$ MtGluD20 $\approx$ MtGluD33 $\approx$ MtGluD44 $\approx$ MtGluD104 > MtGluD7 > MtGluD5) were the same as the relative competitor strengths, measured by their ability to compete with another tRNA gene [M13t(su⁺)] for RNA polymerase III and its factors.

The diverse effects of the 5'-flanking sequence on tRNA gene transcriptional activity observed for a wide variety of genes and systems has led to several hypotheses on the features of the 5'-flanking sequence which are involved in transcriptional modulation (reviewed in Geiduschek & Tocchini-Valentini, 1988). Correlation of the transcriptional activity of the MtGlu6 mutants with the size and position of the upstream sequences which are deleted (see Fig. 1) is not easily reconciled with these hypotheses.

Sequences which have been reported to modulate (positively or negatively) transcription of eukaryotic genes are, with one exception, absent from the 5'-flanking sequence of the tRNA^{Glu} gene. A sequence of the general form TNNCT (and TCGCT especially), proposed as a positive transcription modulator for a class of *Drosophila* tRNA genes (Sajjadi *et al.*, 1987), is present seven times in the 250 nucleotides upstream of the tRNA^{Glu} gene. Deletion mutants MtGluD47 and MtGluD104 both lack three of these sequences (TNNCT at 175–179, TCGCT at 198–202 and 312–316), but the transcriptional activity of MtGluD104 is similar to that of MtGlu6, and that of MtGluD47 is enhanced.

In some cases, modulation of transcriptional activity by flanking sequences has been shown to be sensitive to precise spacing. The use of two different classes of deletion mutants allowed us to examine the effect on transcriptional activity of the distance of a possible modulator sequence, present 5' to the *StuI* site used in the construction of class II mutants. Our results provide no evidence for such a sequence. MtGlu6 deletion mutants D20, D33, D44 and D104, which lack from 8 to 192 nucleotides between the *StuI* site and the gene, have the same transcriptional activity as MtGlu6, whereas MtGluD47, which has a deletion of essentially the same size as that in MtGluD104, shows enhanced transcriptional activity. Any such modulator

sequence(s) must therefore be located between the *StuI* site and the gene.

Both positive and negative transcription modulators are present in the 5'-flanking sequences of eukaryotic tRNA genes (Schaack *et al.*, 1984) and could explain the results reported here. MtGluD20, MtGluD104 and MtGluD44, which contain immediate 5'-flanking sequences of 12, 13 and 25 nucleotides respectively, have the same transcriptional efficiency as MtGlu6 (Fig. 6). The decreased activity of MtGluD5, containing 51 nucleotides of immediate 5'-flanking sequence (i.e. lacking residues 152–304, Fig. 4), suggests that a positive modulator may reside within this deleted region. Such a hypothesis requires that residues 305–318 contain a negative modulator, since MtGluD7 and MtGluD47 (lacking residues 152–311 and 152–318 respectively) have higher transcription efficiencies than MtGluD5. This hypothesis is supported by the fact that, as more of the sequence 305–318 is removed, the maximum transcriptional efficiency of the gene is increased, and this is consistent with the results reported here for other deletions. Further studies of different mutants will test this hypothesis and help to identify more precisely those sequences which modulate the transcriptional efficiency of the tRNA^{Glu} gene.

Relevance to transcription *in vivo*

Some insight into tRNA gene transcription *in vivo* may be gained from comparison of the findings reported here with those of our earlier experiments in which the mutants MtGluD5 and MtGluD7 were tested in parallel with MtGlu6 by micro-injection into *Xenopus* oocyte nuclei (Goddard *et al.*, 1985). These showed that removal of increasingly large portions of the tRNA-like structure led to increasingly large decreases in transcription, suggesting that the tRNA-like structure in the immediate 5'-flanking sequence considerably stimulates the transcription of the gene. The more extensive and detailed study reported here is inconsistent with such a simple hypothesis for transcription *in vitro* in a homologous system. Whereas MtGluD5 and MtGluD7 (which lack part of the tRNA-like structure) have decreased transcriptional activity in both systems, other deletion mutants in which the 5'-flanking sequence is removed have the same transcriptional activity as MtGlu6. The similar transcriptional activities of MtGluD20 and MtGlu6 show that all of the tRNA-like structure of the 5'-flanking sequence to within 12 nucleotides from the tRNA^{Glu} gene can be deleted without affecting the transcriptional efficiency of the gene.

These differences in transcriptional efficiency of the tRNA^{Glu} gene deletion mutants in different transcription systems strongly suggest that the precise role of the 5'-flank is dependent upon the nature of the tissue-specific protein factors present to enhance or inhibit the formation of the initiation complex. In formation of that complex, transcription factor IIIB (TFIIIB) acts as the sole initiation factor by directing the binding of RNA polymerase III. TFIIC, binding at the intragenic promoter, acts as an assembly factor in order to position TFIIB upstream of the gene, but is not required for transcription (Kassavetis *et al.*, 1990). Modulation of transcription, e.g. in development, may thus be effected via changes in the activity of TFIIB (White *et al.*, 1989) or by the presence of tissue-specific factors which recognize different upstream sequences that positively or negatively regulate TFIIB binding. In order to establish how closely assays *in vitro* mimic the activity of tRNA genes *in vivo*, the transcripts of different members of a given tRNA gene family in different tissues must be identified. Schmutzler & Gross (1990) have recently done this for the human tRNA^{Val} gene family, showing differential expression of family members in HeLa cells and placenta. It thus now becomes feasible to develop cell-free systems in which tissue-specific tRNA gene expression, reflecting that found *in vivo*,

occurs, and so to isolate those factors which interact with the 5'-flanking sequence.

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