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Identification of a protein factor binding to the 5'-flanking region of a tRNA gene and being involved in modulation of tRNA gene transcription *in vivo* in *Saccharomyces cerevisiae*

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ABSTRACT

Control mechanisms of tRNA gene transcription were studied in vivo in Saccharomyces cerevisiae. In order to be able to monitor in vivo transcription products of an individual tRNA gene, a 'tester gene' was used which is readily transcribed in vivo in yeast but does not cross-hybridize with any cellular yeast tRNA. A series of insertion mutants were constructed, modifying thereby the immediate and further distant 5'-flanking region of the 'tester tRNA gene'. Small linker molecules of different length and different sequence were inserted at positions -3 and -56 on the non-coding strand. Resulting tRNA gene variants were transformed into yeast cells and in vivo synthesized products were monitored by primer extension analysis. From the experimental data we suggest that a few essential nucleotides within the flanking region are able to determine the in vivo transcription activity of the 'tester tRNA gene'. Our results are rationalized on a biochemical level by protein binding assays: At least one protein binds to the 5'-flanking region of the 'tester tRNA gene' and different protein complexes are sequestered on active or less active tRNA gene variants.

INTRODUCTION

Although mechanisms of eukaryotic tRNA gene transcription have been studied in great detail (for review see 1), little is known about modulatory or regulatory mechanisms involved in transcription of these genes. Eukaryotic tRNA genes belong to multicopy gene families, up to 20 identical copies for each individual tRNA gene existing in many genomes. Because members of a particular tRNA gene family contain identical promoters (2, 3) modulation or regulation of individual tRNA gene transcription via promoter strength cannot be realized.

A number of in vitro studies (4 - 8) and recent in vivo studies (9 - 12), document modulation of transcription within tRNA gene families. The differences in transcription efficiencies among individual members could be attributed in most cases to differences within the 5'-flanking regions suggesting the existence of positively (5, 7) or negatively (6, 13 - 14) regulating elements located upstream from the mature tRNA coding region. Since these effects lack a firm molecular basis this study was initiated where the 5'-flanking region of a defined 'tester tRNA gene' was systematically altered. The 'tester gene' we used codes for a $tRNA^{Val}$(GUA). It is readily transcribed in vivo in yeast but does not cross-hybridize with any yeast tRNA. Small linker fragments of increasing nucleotide number and characteristic base composition were inserted close to the 5'-end of the mature tRNA coding region as well as relatively distant from the gene, before analysis for in vivo function in S. cerevisiae.

Our results underscore the importance of gene proximal 5'-flanking regions for modulation of gene activity and demonstrate for the first time that proteins can be involved in this modulation.

MATERIALS AND METHODS

Nucleic acids

The 'tester gene' used in this study is a $tRNA^{Val}$(GUA) gene, cloned into the yeast shuttle vector yRMP1 (yValGUA 1.4, see 12). Although isolated from Dictyostelium discoideum this tRNA gene is approximately 72% homology to the corresponding yeast tRNA gene with no alterations in gene internal promoter regions. In order to eliminate any BamH1 sites a dispensable 385bp BamH1 fragment was removed and the plasmid was religated after filling in protruding ends. The resulting plasmid (referred to as PC or 'tester tRNA gene') contained unique restriction recognition sites for EcoR1 (-56), Nde1 (-3), Xho1 (+49) and Cla1 (+137) which were used to construct all variants or to prepare specific DNA fragments for gel retardation experiments (see Fig. 1 and 2).

Linker fragments used in this study were obtained from New England Biolabs. tRNA genes with modified 5'-flanking regions were sequenced as double strand DNAs according to the dideoxy chain termination method (15). Bulk tRNA from transformed yeast cells was prepared as described (12).

Nomenclature

Insertion clones were given logical names starting with NF or EF depending on whether nucleotides were inserted into the filled in Nde1 restriction site or into the filled in EcoR1 restriction site, respectively. The number and the letter following the colon indicate the length and the sequence of the linker used for insertion, being B=BamH1 linker, E=EcoR1 linker, H=Hind3 linker, N=Nde1 linker and X=Xho1 linker. F indicates that protruding ends of the new restriction site were filled in and religated.

Transformation in S. cerevisiae

As a recipient strain for transformation served S. cerevisiae strain YH-D5α (his4-166, leu2-2, trp1, can^r), kindly provided by Dr. Herb Hottinger. The transformation procedure was according to Struhl et al. (16). Transformants were selected for a trp^+ phenotype. Cells were grown in SD-medium (containing per liter 6.7g yeast nitrogen-base w/o amino acids, 20g glucose), supplemented with histidine and leucine. Successful transformation was verified by reisolation of plasmid DNA which was detected after transformation into E. coli strain HB101. These control experiments were carried out with much care under quantitative conditions, in order to correlate the copy number of a given plasmid with the in vivo transcription efficiency of the corresponding tRNA gene variant.

Primer extension analysis

Gene products were detected through primer extension analysis (12). A synthetic DNA fragment complementary to nucleotides 23 - 40 of the 'tester tRNA gene' served as a specific primer for cDNA synthesis. 0.5 A_{260} units of bulk tRNA prepared from yeast cells were coprecipitated with 15pmol ^{32}P-labelled primer fragment ($5x10^5$ - $2x10^6$ Cerenkov cpm). The dry pellets were dissolved in 10µl buffer containing 50mM KCl, 50mM Tris/HCl pH 8.0, and incubated for 2 min at 90°C. After 5 min on ice and 10 min at room temperature 5µl of a buffer was added containing 50mM KCl, 50mM Tris/HCl pH 8.0, 20mM $MgCl_2$, 6mM DTT, 2.5mM dNTPs and 3 units AMV reverse transcriptase. Assays were incubated at 37°C for 1 h and reactions were stopped by ethanol precipitation. The pellets were dissolved in 2.5µl distilled water and 1.5µl loading buffer (95% formamide, 10mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). Products were separated on 8% polyacrylamide gels containing 50% urea. Gels were exposed to X-ray film in the presence of enhancing screens for 1 h at -80°C.

Quantification of cDNA products

The in vivo steady state levels of transcription products of all templates were determined by measuring Cerenkov radiation in gel slices (12). cDNAs corresponding to premature stops, to tRNAs and precursor molecules were measured. Since mature products from all tRNA gene variants are identical with respect to structure, different degradation rates cannot account for differences in the detected amounts of tRNA. Therefore different steady state levels of in vivo synthesized tRNA products represent in a first approximation differences in template activity.

Preparation of nuclear cell-free extract and chromatographic procedures

Nuclear extract was prepared from S. cerevisiae strain 20B-12 (α-trp, pep 4-3). Cells were harvested by centrifugation at room temperature. The cell pellet was resuspended in buffer A (33ml/l culture) containing 1M sorbitol, 50mM Tris/HCl pH 7.8, 10mM $MgCl_2$, 30mM DTT. After centrifugation cells were resuspended in buffer B (33ml/l culture) containing 1M sorbitol, 50mM Tris/HCl pH 7.8, 10mM $MgCl_2$, 3mM DTT. 3.2mg Zymolyase was added and cells were incubated for at least 60min (OD_{600} = 1.0). Spheroblasts were collected by centrifugation and washed twice in buffer B. Thereafter, the spheroblast pellet was carefully resuspended in buffer C (21ml/l culture) containing 10mM Hepes pH 7.8, 15mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 1.5mM DTT. The cell suspension was dounced once using the tight fitting A-pestle and after 20 min on ice 5 times using the B-pestle. After centrifugation for 10min at 12.000rpm in a Sorvall centrifuge the pellet was resuspended gently in buffer C (10ml/l culture) and dounced carefully again (A-pestle). 0.2 volumes of 4M $(NH_4)_2SO_4$ was added and the solution centrifuged for 40min at 100.000 x g. Soluble proteins were precipitated by adding 0.25g/ml solid $(NH_4)_2SO_4$ and collected by centrifugation (20min at 100.000 x g). The pellet was suspended in buffer D (3ml/l culture) and dialyzed 3 times against buffer D containing 100mM KCl, 10% glycerol, 20mM Hepes pH 7.8, 0.1mM EDTA, 30mM DTT.

Nuclear extract was applied to a DEAE-Sephadex A25 (Pharmacia) column equilibrated with buffer E containing 20mM Hepes pH 7.8, 20% glycerol, 10mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and 0.1M KCl. The proteins were sequentially step-eluted with buffer E containing 0.2M, 0,3M, 0.4M, 0.5M and 0.6M KCl. Fractions were pooled and protein was precipitated with 0,35g/ml solid $(NH_4)_2SO_4$. After centrifugation for 20 min at 100 000 x g protein pellets were dissolved and dialyzed 3 times against buffer E containing 0.1M KCl for total 1 h and stored in aliquots at -80°C. The 0.3M KCl step (5mg protein/ml) from the DEAE Sephadex A25 column was used to perform DNase I protection experiments.

Gel retardation assays

To prepare fragments for gel retardation assays, plasmid DNA was digested first with EcoR1 and after filling in the protruding ends with dNTPs and [α-^{32}P]dATP, a second suitable restriction endonuclease was used to release a DNA fragment. Labelled restriction fragments were purified on polyacrylamide gels.

In gel retention assays (17) unlabelled E. coli DNA or poly(dA-dT) was used for unspecific competition. 0.5ng [α-^{32}P] labelled DNA, unspecific competitor DNA (0.037µg - 10.0µg) and nuclear extract were mixed in 24µl buffer containing 0.1% Triton X-100, 4% glycerol, 1mM Na-EDTA, 10mM Tris/HCl pH 7.5, 80mM NaCl. Nuclear extract was added last. Reactions were incubated for 30 min at room temperature and thereafter loaded on a low-ionic-strength 4% polyacrylamide gel (acrylamide/bisacrylamide weight ratio of 30:1) containing 1mM Na-EDTA, 3.3mM Na-acetate, 6.7mM Tris/HCl pH 7.5. Electrophoresis was carried out at 25mA for 2 - 3 h at 4°C.

DNase I protection (footprinting) assay

Protection assays were performed as follows: 0.5ng [α-^{32}P] labelled DNA, 100ng unspecific competitor DNA (poly(dA-dT)$_n$) were mixed in 24µl buffer containing 0.1% Triton X-100, 4% glycerol, 1mM Na-EDTA, 10mM Tris/HCl pH 7.5, 80mM NaCl. Then 10µl buffer E (20mM Hepes pH 7.8, 20% glycerol, 10mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and 0.1M KCl) and nuclear extract were added. Assays were incubated for 20 min at 20°C following a DNase I digestion for 5 min at 20°C. Reactions were stopped by precipitating the DNA with ethanol. DNA fragments were separated on sequencing gels (16% acrylamide).

RESULTS

Construction of variant tRNA^{Val}(GUA) genes

The DNA fragment coding for the 'tester tRNA^{Val}(GUA) gene' contains single restriction enzyme recognition sites within the 5'-flanking region for EcoR1 and Nde1 at positions -56 and -3, respectively (see Fig. 1). These sites were used to insert systematically small linker fragments of increasing size and different nucleotide sequence. Clones were obtained with insertions of 4, 12, 14, 16, 18 and 20bp into the EcoR1 restriction site and with insertions of 2, 10, 11, 12, 14, 16, 18 and 26bp into the Nde1 recognition site. The character of inserted DNA differed through the use of commercially available Nde1 linker, BamH1 linker of different size, EcoR1 linker, Hind3 linker and Xho1 linker (see Fig. 1 and 2). Two derivatives of clones NF:8B and NF:12BF were fortuitously obtained comprising partial deletions within the former Nde1 restriction site. These clones (ΔN:8B and ΔN:12BF) lack nucleotides -1 and -2 in addition to another nucleotide further upstream. Similarly clone NF:9NF was obtained fortuitously. All changes (summarized in Fig. 2) were confirmed by sequence analysis.

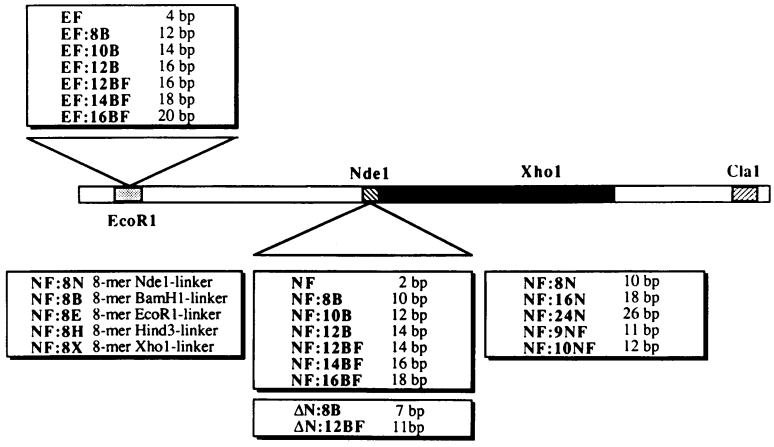

Figure 1. Schematic representation of the 'tRNA tester gene' (PC) and of insertion mutant constructs. The mature tRNA coding region is indicated as a black box. Unique restriction recognition sites are located at position -56 (EcoR1), -3 (Nde1), +49 (Xho1) and +137 (Cla1). The names of the resulting derivatives and the number of inserted nucleotides are depicted.

Expression of 'tester tRNA gene' variants in vivo in S. cerevisiae

Variant plasmids were introduced into yeast by transformation. To analyze in vivo expression properties of tRNA genes with modified 5'-flanking regions, bulk tRNA was isolated from transformants and assayed by primer extension analysis (12). The primer used (see METHODS) recognizes exclusively products of the 'tester tRNA gene' and does not anneal to any other yeast RNA. From the sequences of the primer and of the 'tester tRNA gene' it can be deduced that a 40 nucleotide long cDNA corresponds to mature tRNA (see Fig. 3). Detectable cDNAs larger than 40 nucleotides are synthesized from transcripts with unprocessed 5' ends, while cDNAs smaller than 40 nucleotides represent intermediates that occur due to pausing of reverse transcriptase at the transition from the D-stem to the amino acceptor stem of the tRNA or at positions in the tRNA which are modified (18).

The 40 nucleotide long cDNA, corresponding to mature tRNA products, is observed in most reactions (see Fig. 3). There are, however, notable differences in 'steady state' levels of synthesized RNA depending on length and structure of inserted DNA. Since mature products of all tested gene variants are structurally identical, the possibility of differential degradation of the different gene products is rather unlikely. Although different primary transcripts may be processed with different rates, we have no indication that less efficiently processed primary

::GGTCGGATGGTGTAGTCGGTTATCACGGTTGCTTTACACGCAACAGGTCTCGAGTTCGATCCTCGGTCGGATCA::TATCTTTTT		Relative Activity
CTCCTTCACTATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	PC	1.00
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACCATATGGTAT::	NF:8N	1.30
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACGGATCCGTAT::	NF:8B	0.02
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACCTCGAGGTAT::	NF:8X	1.00
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACAAGCTTGTAT::	NF:8H	0.90
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAGGAATTCCTAT::	NF:8E	0.70
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACGGATCCGTAT::	NF:8B	0.02
TTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACGGGATCCCGTAT::	NF:10B	0.08
TAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACGCGGATCCGCGTAT::	NF:12BF	0.03
ACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACGGGATCGATCCCGTAT::	NF:14BF	0.05
ATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACGCGGATCGATCCGCGTAT::	NF:16BF	0.10
ATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACCATATGGTAT::	NF:8N	1.30
TTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACCATTATGGTAT::	NF:9NF	1.00
TTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACCATATATGGTAT::	NF:10NF	1.25
ATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATACCATATGGCCATATGGTAT::	NF:16N	1.00
TTCACTATTTTAACATGTGGAATTAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF	0.80
TTTAACATGTGGAATTCGGATCCGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF:8B	0.65
TAACATGTGGAATTCGGGATCCCGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF:10B	0.80
ACATGTGGAATTCGCGGATCCGCGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF:12B	0.65
ACATGTGGAATTCGGATCGATCCGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF:12BF	0.80
ATGTGGAATTCGGGATCGATCCCGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF:14BF	0.80
GTGGAATTCGCGGATCGATCCGCGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATAT::	EF:16BF	0.80
ACTATTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATCGGATCCGT::	ΔN:8B	0.20
TTTTAACATGTGGAATTCCAGCTTGGCTGCTGCAGGTATTCTCTCAAACCATCTAAATATATTGTTTCATCGGATCGATCCGT::	ΔN:12BF	0.30

Figure 2. Nucleotide sequences of analyzed tRNA templates. In line 1 the mature tRNA coding region (defined between colon pairs) and part of the 3'-flanking region including the transcription termination signal is shown. This sequence is present in all tRNA gene variants analyzed. Below, the 5'-flanking regions of the 'tRNA tester gene' (PC) and of all derivatives are listed. Inserted nucleotide pairs in 'tRNA tester gene' derivatives are underlined. Rough estimates of relative apparent in vivo transcription activities (activity of PC arbitrarily defined as 1.00) are shown on the right.

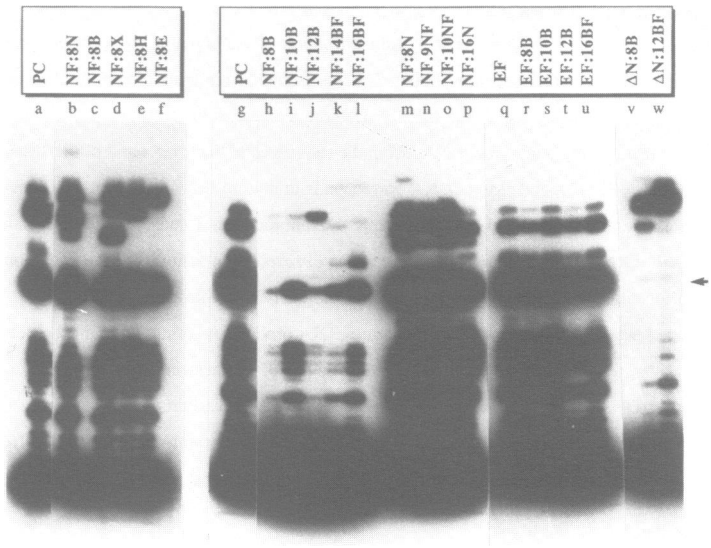

Figure 3. Primer extension analysis of in vivo synthesized tRNAs from the 'tRNA tester gene' and from derivatives of the 'tester gene'. Bulk tRNA was isolated from yeast strains which were transformed with plasmids carrying tRNA gene variants. A ^{32}P-labelled oligonucleotide recognizing exclusively products of the 'tester gene' variants and not annealing to any endogenous yeast tRNA was hybridized with 0.5 A_{260} units of the respective tRNAs. cDNAs were synthesized and size-fractionated on sequencing gels. An extended primer fragment of 40 nucleotides corresponds to mature tRNA (indicated by the arrow head). Larger cDNAs were synthesized on primary transcripts while fragments smaller than 40 nucleotides represent premature cDNAs. These result from pausing of the Reverse Transcriptase at positions of tight secondary structure or at modified nucleotides (12). Analyses of the unmodified 'tester tRNA gene' (PC) are shown in lanes a and g. Derivatives generated by DNA insertions into the NdeI restriction site are depicted in lanes b - f and h - p, while derivatives generated by DNA insertions into the EcoR1 restriction site are depicted in lanes r - u. Clones ΔN:8B and ΔN:12BF contain deletions of nucleotides -1 and -2. Primary transcripts of these templates are incapable of undergoing 5'-end maturation (lanes v - w).

transcripts are unspecifically degraded (see also below). Furthermore - as observed earlier (12) - differences in the detectable amount of tRNA gene products could never be correlated with the plasmid copy number per cell. Thus the observed signal intensities can to a first approximation account for differences in template activity rather than differences in gene doses and be taken as rough quantitative estimates of apparent transcription efficiencies.

Gene proximal parts of the 5'-flanking region can drastically influence the transcription efficiency of the 'tester tRNA gene'

Five different linker molecules of identical length were inserted into the blunt-ended NdeI restriction site at position -3. The derivatives thereby obtained contained different DNA insertions each of 10 additional nucleotides compared to the original 'tRNA tester gene'. Primer extension analyses of tRNAs synthesized from these genes in vivo in yeast demonstrate similar apparent activities for most of the derivatives (Fig. 3, lanes b - f) compared to the parent clone (Fig. 3, lane a) with one drastic exception of the clone where an 8mer BamH1 linker was inserted

(Fig. 3, lane c). This particular derivative, in contrast to all other derivatives including the original 'tester tRNA gene' produces only very small amounts of tRNA.

The length of the inserted DNA fragment may influence the transcription efficiency of 'tester tRNA gene'

To analyze whether the length of inserted DNA in front of the 'tester tRNA gene' could possibly influence in vivo expression of the tRNA gene, further variant genes were constructed (see Fig. 1 and 2) by insertion of different BamH1 or Nde1 linker molecules. In Fig. 3 lanes h - l and lanes m - p the respective primer extension analyses are shown. Two major conclusions can be drawn from these analyses: a) All tRNA gene variants with insertions of BamH1 linker derivatives near the mature tRNA 5'-end are transcribed with significantly lower efficiency in vivo in yeast than variants with Nde1 linker derivatives and b) especially within the BamH1 linker series - but also to a certain degree within the Nde1 linker series - there seem to be some oscillation with respect to the apparent transcription activities depending on the length of the inserted oligonucleotide. Within the BamH1 series two maxima are apparent at insertion lengths of 12bp (NF:10B) and 18bp (NF:16BF) and two clones with insertions of 10bp (NF:8B) and 14bp (NF:12B) seem to contain particularly inefficient templates. This latter result gives an indication that some other regulatory element may be located at a site more distant from the mature tRNA 5'-end, which - by inserting oligonucleotides of different length - changes its position on the helical backbone of the DNA relative to the gene internal promoter elements. Alternatively the observed results may simply be explained by the different structures of inserted DNA fragments (see Fig. 2).

Transcription efficiency of the tRNA gene may be influenced by regions distal to the mature tRNA 5'-end

In order to test whether BamH1 linker derivatives exert their drastic negative effects on in vivo transcription not only if inserted at position -3 relative to the 'tester tRNA gene' but also at a more distal position, another set of derivatives were constructed where the respective DNA fragments were inserted at nucleotide -56 into the filled-in EcoR1 restriction site (see Fig. 1). As shown in Fig. 3, lanes q - u, transcription of these EF:B clones is significantly higher than transcription of clones of the NF:B series (Fig. 3, lanes h - l). In repeated experiments all EF:B clones gave less transcription products than the parent clone, and the slight oscillation pattern visible for the primary transcripts in Fig. 3 (lanes q - u) and for mature products in short exposed autoradiographs was highly reproducible. The nature of this oscillation in apparent transcription activities is similar in both series. The 8B derivatives and the 12B derivatives of the NF and EF series represent more poorly transcribed templates (Fig. 3, lanes h, j, r and t); derivatives 10B as well as 16BF constitute more active templates in the respective NF and EF series (Fig. 3, lanes i, l, s and u). This may indicate that the sequence of the inserted BamH1 linker DNA is responsible for the oscillation in template acticity while the point of insertion relative to the mature tRNA coding region seems to determine active or less active templates. The same linker derivatives inserted either at position -3 or -56 exert effects on template activity differing almost by one order of magnitude.

An extended aminoacyl acceptor stem is necessary for 5'-end maturation

In lanes v and w of Fig. 3 two tRNA gene variants (ΔN:8B and ΔN:12BF) were analyzed which had acquired a fortuitous deletion of nucleotides -1 and -2 during the cloning process (see METHODS). In both cases, primary transcripts accumulate to significant amounts but almost no maturated products can be detected. This result is rationalized by the fact that the 'tester tRNA gene' has a G5 - G69 mismatch within the aminoacyl stem. Therefore a stable aminoacyl stem - necessary for the recognition of 5' and 3' maturation activities - does not

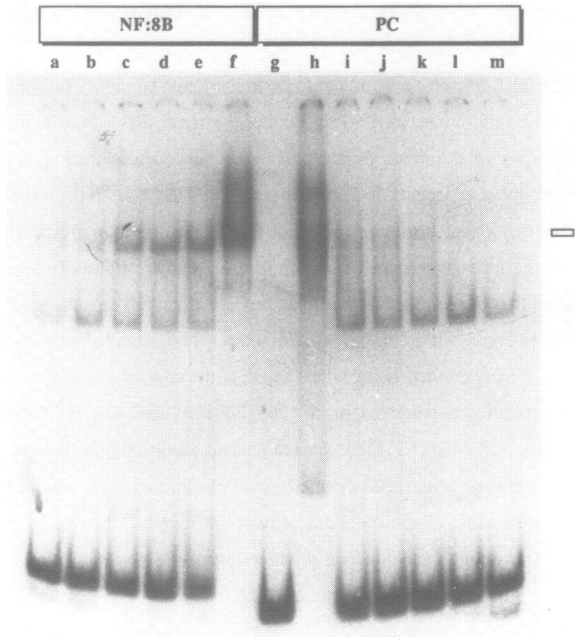

Figure 4. Gel retardation assay with 5'-flanking regions of the 'tRNA tester gene' (PC) and of the NF:8B derivative. One nanogram of a labelled EcoR1/Nde1 fragment isolated from PC and of a labelled EcoR1/ BamH1 fragment isolated from NF:8B was incubated with nuclear extract and with increasing amounts of unspecific competitor DNA. Assays were then subjected to low ionic strength polyacrylamide gel electrophoresis and fragments were visualized by autoradiography. Lane g shows the analysis of the PC fragment incubated in the absence of any proteins. In all other experiments fragments were incubated with nuclear extract plus 0µg, 0.25µg, 0.50µg, 1.0µg, 2.0µg and 4.0µg of poly(dA-dT)$_n$ as unspecific competitor DNA. The respective results are shown in lanes f - a for the NF:8B fragment and in lanes h - m for the PC fragment. At least two retarded DNA/protein complexes can be detected. The slowest migrating complex formed on the NF:8B fragment (indicated by the box) resists significantly higher competitor DNA concentrations than the corresponding complex formed on the PC fragment.

form, unless basepairing of the immediate 5'- and 3'-flanking nucleotides leads to the formation of an extended and thereby more stable aminoacyl-stem. This seems to be realized in primary transcripts of the 'tester gene' and of all other constructed variants of that gene. Primary transcripts of these clones contain an additional 4 nucleotides of 5'- and 3'-flanking sequence complementarity (see Fig. 2). Due to the 2 base pair deletions in clones ΔN:8B and ΔN:12BF the formation of such an extended aminoacyl stem cannot occur in primary transcripts derived from these clones. Free energies of aminoacyl-stems of different tRNAs range from –10kJ/mol up to –15kJ/mol (calculated according to the rules of Salser (19)). Such stability seems crucial, since correct secondary and tertiary structure of a tRNA molecule is a prerequisite for maturation of primary transcripts. Both variants, ΔN:8B and ΔN:12BF, have calculated free energies in their aminoacyl stems of only –4.4kJ/mol.

Although relatively great amounts of primary transcripts from clones ΔN:8B and ΔN:12BF can be detected (Fig.

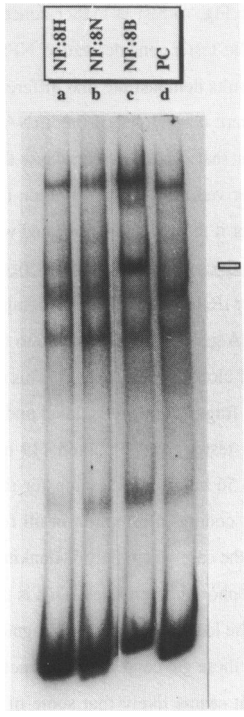

Figure 5. Comparison of DNA/protein complex formation of different 'tRNA tester gene' derivatives. EcoR1/Xho1 fragments of the tRNA templates NF:8H (a), NF:8N (b), NF:8B (c) and PC (d) were isolated and incubated with nuclear extract in the presence of 1000 fold excess of unspecific competitor DNA (1µg poly(dA-dT)$_n$). These fragments contain in addition to 53 nucleotides coding information 56 or 66 nucleotides of the 5'-flanking region. On the NF:8B fragment (lane c) a uniquely strong complex is formed (indicated by a box). Additionally the slowest migrating complex - probably containing components of the RNA polymerase III transcription complex - is significantly stronger for the NF:8B fragment as compared to the corresponding complexes formed on the other tested fragments.

3, lanes v and w), the total amount of cDNA synthesized from these clones range only slightly higher than those of NF:B derivatives. This also demonstrates that primary tRNA gene transcripts seem to be fairly insensitive to unspecific degradation in vivo in yeast.

The 5'-flanking regions of different tRNA gene variants contain specific binding sites for different proteins

In an attempt to explain the observed effects of different 5'-flanking regions on transcription efficiency on a biochemical level nuclear proteins which might specifically bind to this region were analyzed. 1ng of a 53 bp EcoR1/Nde1 fragment isolated from the parent 'tester gene' and 1 ng of a 56 bp EcoR1/BamH1 fragment of the derivative NF:8B were labelled on their coding strands and challenged with yeast nuclear extract in the presence of up to 1µg poly(dA-dT) as unspecific competitor (Fig. 4). In low ionic strength gels two retarded fragments can be detected even in the presence of a 1000 fold excess of unspecific competitor DNA. However, the more slowly

migrating complex (indicated by a box in Fig. 4) resists higher concentrations of unspecific competitor DNA when formed on the 5'-flanking region of the 'tester gene derivative' NF:8B rather when formed on the 5'-flanking region on the parent clone (PC). These results demonstrate that different proteins can be sequestered to specific sequences of 5'-flanking regions of different derivatives of the 'tRNA tester gene' even in the absence of any mature tRNA coding region. The conclusion that indeed proteins cause the retardation in electrophoretic mobilities of labelled 5'-flanking regions could be derived from the observation that complexes do not form if the nuclear extract is preincubated at 60°C for 10 min or if the extract is pretreated with proteinase K (data not shown).

Poorly transcribed 'tester tRNA gene' derivatives form a unique transcription complex

Complex formation by the original 'tester tRNA gene' (PC) was compared with derivatives NF:8B, NF:8N and NF:8H to question, whether any other tRNA gene derivative is able to form a unique complex similar to that detected on the labelled 5'-flanking region of clone NF:8B (Fig. 5). This experiment differed from the experiment shown in Fig. 4 in that now EcoR1/Xho1 fragments were isolated and labelled. Corresponding fragments were 109 nucleotides in length for the original 'tester gene' (PC) and 119 nucleotides long for all other derivatives. These fragments contained in addition to 56 bp flanking region for the 'tester tRNA gene and 66 bp flanking region for the derivatives, 53 nucleotides coding information in all four cases. As shown in Fig. 5 the set of retarded fragments is much greater than in the case where only 5'-flanking nucleotides were tested and some of the fragments show significantly lower electrophoretic mobilities. This is due to the fact that promoter elements for RNA polymerase III directed transcription are located on the tested fragments. Although the retarded electrophoretic mobility of a protein/DNA complex under these gel conditions does not necessarily correlate with the size of the sequestered protein or protein complex, it seems likely that some of the retarded fragments are loaded with components of the RNA polymerase III transcription complex. The pattern of retarded fragments is very similar for DNA fragments isolated from the 'tester gene' and from derivatives NF:8N and NF:8H (Fig. 5, lanes d, a and b), whereas the analysis of the NF:8B fragment does reveal a unique fragment pattern. Another retarded DNA fragment lights up in this analysis (Fig. 5, lane c), most likely corresponding to the additional retarded fragment observed in Fig. 4, where only the 5'-flanking region of NF:8B was analyzed. Additionally, the signal corresponding to the largest retarded fragment, which is present in every analysis shown in Fig. 5, contains significantly more DNA in case of the NF:8B fragment compared to all other tested fragments. Consequently the signal corresponding to unloaded DNA is much less intense for NF:8B DNA than for the other tested DNAs.

High affinity of the transcription complex results in poor template activity

If it is correct that DNA fragments with the lowest electrophoretic mobilities observed in Fig. 5 are associated with parts of the RNA polymerase III transcription complex, one had to conclude that the affinity of this complex to the NF:8B template is greater than to the other tested templates although the gene internal recognition elements are identical for all tested derivatives. To test this hypothesis, DNA/protein complexes formed on different 'tester tRNA gene' derivatives were challenged in addition to constant amounts of 1µg unspecific competitor DNA with a second tRNA gene containing identical gene internal promoter elements but totally unrelated flanking regions. This tRNA gene was added unlabelled in increasing amounts to the complexes formed on the EcoR1/Xho1 fragments isolated from 'tester tRNA gene' (PC) (Fig. 6, lanes a - f), from the NF:8N derivative (Fig. 6, lanes g - l) and from the NF:8B derivative (Fig. 6, lanes m - r). In the presence of excess amounts of competitor tRNA

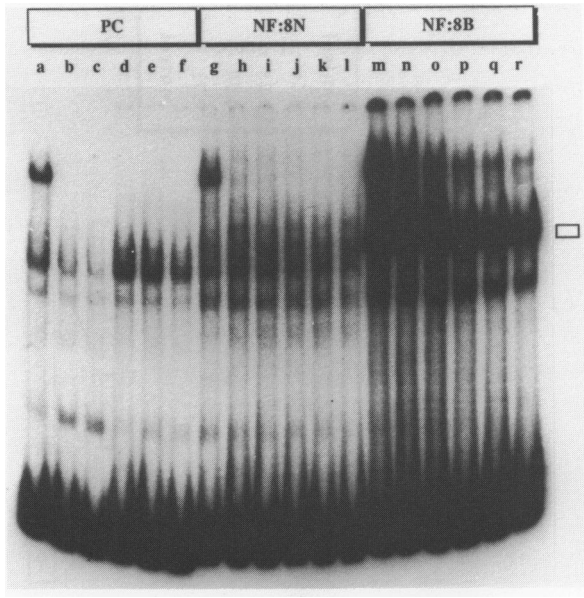

Figure 6. Specific competition of DNA/protein complex formation. Labelled EcoR1/Xho1 fragments of PC (lanes a - f), NF:8N (lanes g - l) and NF:8B (lanes m - r) were incubated with equal amount of nuclear extract, 1000 fold excess of unspecific competitor DNA (1μg poly(dA-dT)$_n$) and with increasing amounts of a second tRNA gene (0ng, 15ng, 30ng, 60ng, 120ng and 240ng). This specific competitor gene contains the identical mature tRNA coding region as the 'tester tRNA gene' variants but an unrelated 5'-flanking region. The slowest migrating DNA/protein complex is readily competed away in PC and NF:8N analyses, suggesting that this complex consists of components of the RNA polymerase III transcription complex. The corresponding complex formed on the NF:8B fragment, however, resists much higher concentrations of specific competitor DNA. Nearly unaffected by the addition of a second tRNA gene is the additional NF:8B specific complex marked by the box.

gene, proteins causing most extensive retardation of DNA fragments are no longer stably bound to the 'tRNA tester gene' and to the NF:8N derivative. However, even at high competitor concentrations only parts of these proteins are competed away from the NF:8B derivative.

Three conclusions can be drawn from these results: i) Proteins causing the most extended degree of fragment retardation are most likely to represent constituents of the RNA polymerase III transcription complex. They can be competed away by a second tRNA gene but competition varies depending on the tRNA gene derivative where the complex is bound to. ii) Since all three tested tRNA gene derivatives contain identical gene internal recognition sites for the RNA polymerase III transcription complex, the 5'-flanking region has to be responsible for different stabilities of complex binding. iii) The fact that the unique retarded fragment of NH:8B DNA is not competed away by increasing amounts of the competitor DNA confirms the location of a protein binding site within the 5'-flanking region of that tRNA gene variant. Similar results were obtained with the NF:12B derivative (data not shown).

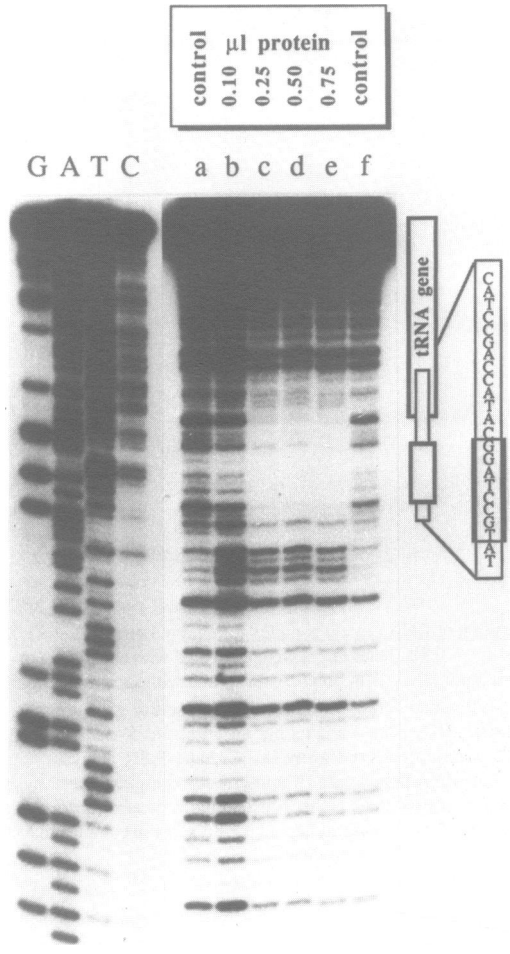

Figure 7. DNase I footprinting of the NF:8B insertion mutant (coding strand). The DNA was ^{32}P-labelled by a fill-in reaction at the EcoR1 site followed by Xho1 digestion. Labelled DNA was incubated with either no protein (lanes a and f) or with increasing amounts of fractionated nuclear extract; 0.5µg, 1.25µg, 2.5µg and 3.75µg protein were added prior to DNase I digestion for 5 min (lanes b - e). Additionally the fragment was subjected to sequence analysis (36). The protected sequence including a strongly protected core (heavily boxed) is indicated. This core resides within the 5'-flanking region. Additionally, some protection within the mature tRNA coding region is observed which covers the 5'end of the tRNA gene up to the 5'ICR.

An (ACGGATCC)-binding protein renders NF:B derivatives less active templates

In order to identify a protein binding site within the 5'-flanking region of the NF:8B derivative, DNase I protection assays were performed. Either an EcoR1/Xho1 fragment labelled at the EcoR1 site (coding strand) or a Cla1/EcoR1 fragment labelled at the Cla1 site (non-coding strand) were incubated with partially purified nuclear extract from yeast cells and limited amounts of DNase I (Fig. 7 and 8, respectively). In both cases a region within

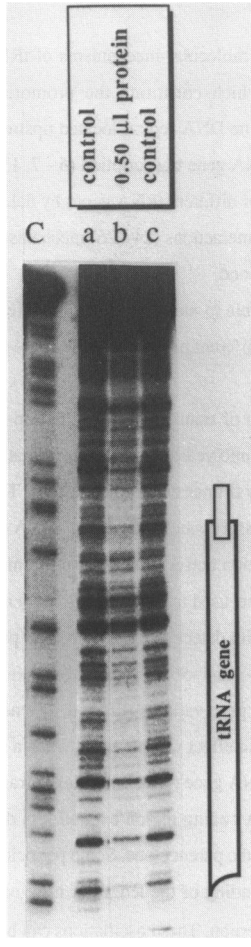

Figure 8. DNase I footprinting of the NF:8B insertion mutant (non-coding strand). The DNA was ^{32}P-labelled by a fill-in reaction at the ClaI site followed by EcoR1 digestion. Labelled DNA was incubated with either no protein (lanes a and b) or with fractionated nuclear extract (2.5µg) prior to DNase I digestion for 5 min (lane b). Additionally the fragment was subjected to sequence analysis (36). Only the C-reaction is shown which corresponds to the G-reaction depicted in Fig. 7. The protected sequence on the non-coding strand corresponds to the protected sequence on the coding strand.

the 5'-flank of the tRNA gene derivative is protected from DNase I digestion. Observed protection is strongest around the core sequence 5'-ACGGATCC-3' but extends up to the 5' internal control region (5'ICR). The same motif is also protected within the 5'-flanking region of derivative NF:12B (data not shown). It can be concluded that this strong protein binding immediately in front of the tRNA gene probably causes the observed low in vivo transcription efficiency of both templates, probably by direct interaction with the RNA polymerase III transcription complex.

DISCUSSION

Previous studies trying to unravel the molecular mechanisms of tRNA gene transcription have revealed two functional regions: a) internal parts which constitute the promoter regions for the RNA polymerase III transcription complex (2, 3, 20); and b) the DNA region located upstream of the mature tRNA coding sequence apparently involved in modulation of tRNA gene transcription (5 - 7, 13 - 14, 21 - 22). Whereas the gene internal promoter regions are well characterized for different tRNA genes by deletion analyses (2 - 3), by mutation analyses (22 - 23) and by analyzing protein/DNA interactions (24 - 26) mechanisms of transcription modulation directed by 5'-flanking regions are still poorly understood.

Using gel retardation assays we were able to identify protein binding sites within the 5'-flanking regions of the different tRNA gene variants and show, different protein complexes are formed on the 5'-flanking regions of active versus inactive tRNA templates.

Insight into the molecular mechanisms of transcription modulation was gained through in vivo analyses of a 'tester tRNA gene' which was introduced into yeast cells by transformation. Transcription products from this gene and from variants of it were monitored by primer extension analyses. The use of a 'tester tRNA' gene instead of a homologous yeast tRNA gene was necessary since eukaryotic tRNA genes belong to multicopy gene families which make in vivo tracing of transcription activity of a particular member of such a gene family impossible. Useful advantages of the 'tester tRNA gene' used in this study were: a) it is efficiently transcribed in vivo in yeast, b) endogenous tRNA does not interfere with detection of transcription products, c) unique cloning sites are located within the 5'-flanking region at positions -3 and -56 for insertion of different linker molecules.

The in vivo activities of different 'tester gene variants' differed, depending on the slight changes made within the 5'-flanking sequence. The most pronounced effect was observed when a set of BamH1 linker derivatives was inserted immediately in front of the 'tester tRNA gene'. The same linker fragments inserted at a more distant position relative to the 5'-end of the mature tRNA coding region exerted less drastic effects although template activities were reproducibly decreased compared to the parent clone. Some periocidicity of template activity was observed for corresponding clones of both series depending of the length of inserted DNA although with drastic quantitative differences depending on the point of insertion. These oscillations can be interpreted if the template activity varies due to the slight difference in nucleotide composition of the inserted DNA fragments. Alternatively, the observed periocidicity in template activity could indicate a stereo-specific alignment of two *trans*-acting factors. Examples for such a mode of action have been demonstrated for the E. coli araBAD operon (27) and for SV40 early genes (28). By inserting nucleotides in small increments in front of the 'tester tRNA gene' the relative position of an upstream located element on the helical backbone of the DNA might be altered, thereby causing the observed differences in apparent template activities. The similar qualitative behavior of corresponding clones of insertions at position -3 or -56, respectively may, however, indicate that parts of the inserted BamH1 linker derivatives are directly involved in the exerted effects. This conclusion is also favored by the fact, that neither insertions of a NdeI linker, nor EcoR1, XhoI or Hind3 linker show similar effects on template activity. The drastic quantitative difference between the two series where BamH1 linker molecules were inserted at position -3 and -56, however, underscore the importance of the position where alterations are made relative to the mature tRNA coding region.

Using gel retardation assays and DNase I protection assays we were able to identify protein binding sites in

variants with BamH1 linker insertions at position -3. These results demonstrate for the first time that proteins can be sequestered to 5'-flanking regions of tRNA genes and that modulation of tRNA gene transcription can be caused by trans-acting factors binding to the 5'-flanking region of a tRNA gene. The exact mode of action, however, is still uncertain. It can be excluded from our data that the factor binding to BamH1 insertion clones act similar to factors which bind to classic RNA polymerase II enhancer or silencer elements. The latter are able to exert their influence on a particular gene rather independently of their orientation and distance from the transcription start site. The protein binding site identified on BamH1 insertion clones, however, must be located relatively close to the 5'-end of the mature tRNA coding region in order to influence transcription efficiency most severely.

There seem to be some interactions between the 5'-flanking protein detected on BamH1 insertion clones and parts of the RNA polymerase III transcription complex: Components of this complex bind much stronger to BamH1 insertion clones compared to the other, more active derivatives (see Fig. 6, lanes m - r). From experiments in progress we have some indications that an angular displacement on the DNA helix of this protein binding site relative to the gene internal promoter elements of the tRNA gene may interfere with these protein/protein interactions. Which components of the RNA polymerase III transcription complex are responsible for the observed effects are not known. The fragments we used in our gel retardation experiments contain the entire 5'ICR but no parts of the 3'ICRs. 5'ICR and 3'ICRs (24, 29 - 30), and even regions extending past the transcription termination site (31 - 32) are involved in stable complex formation. Two transcription factors, TFIIIB and TFIIIC, are sequestered onto those regions, which act stoichoimetrically and are not recycled anymore once the complex has been formed (25). Although TFIIIB has not been shown to interact directly with DNA, its presence is essential for stabilization of TFIIIC binding on a tRNA gene (25, 33). Formation and stability of the transcription complex can be monitored in template exclusion assays (24, 32). Whether our results from gel retardation experiments in the presence of a second tRNA gene (see Fig. 6) visualize the situation in template exclusion assays is questionable, since only parts of the target sequences for stable complex formation are present and it is quite possible that only parts of the complex are competed away.

Recently published reports discuss other models of how transcription efficiencies of tRNA genes are effected by 5'-flanking regions. Loftquist and Sharp (8) suggest RNA polymerase III itself being a limiting component which may be sequestered to different extents onto different $tRNA^{Asn}$ genes but they explicitly leave the possibility open that other as yet unknown factors might be involved. Raymond and Johnson (34) concluded from template exclusion assays that 5'-flanking sequences upstream of a conserved region present in some yeast tRNA genes affect formation of stable preinitiation complexes. Also Arnold and Gross (35) suggest sequences upstream of a human $tRNA^{Val}$ gene affect the extent of complex formation but not its stabilization. Although all these models might be correct we favor the involvement of *trans*-acting factors in modulation of tRNA gene transcription. Studies are in progress to uncover the mode of action of these factors and to test other tRNA genes in other biological systems in order to establish whether this regulatory system is a more general one.

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