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The role of non-coding DNA sequences in transcription and processing of a yeast tRNA

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ABSTRACT

We have tested the hypothesis that conserved sequences in the intervening sequence (IVS) and 5'-flanking region of a yeast $tRNA_3^{Leu}$ gene serve some function. Genes with deletions of 8, 10, 13 and 20 bp in the IVS are all active as templates *in vitro*. Yeast extracts produce mature $tRNA_3^{Leu}$ from Δ 8, Δ10 and Δ13 genes. *Xenopus* extracts do not detectably ligate the 5' and 3' half-molecules resulting from IVS excision. Neither extract is able to excise the IVS from Δ20 precursors. Genes with introns enlarged by 10, 21 or 30 bp of DNA produce mature tRNA. Insertion of 103 bp results in reduced levels of transcription, little if any end maturation, and no detectable mature product. A conserved 15 bp sequence is present at the 5'-end of the tRNA sequence. Replacement of yeast DNA up to position -22 leaves the tRNA gene transcriptionally active. With replacement extended to -2 the gene is active in *Xenopus* extracts but nearly inert in yeast extracts. We conclude that tRNA transcription in yeast is insensitive to IVS structure but can be positively influenced by 5'-flanking sequence.

INTRODUCTION

The presence of intervening sequences in a sub-set of yeast tRNA genes has been thoroughly documented (1-5). The effect of these sequences on the biological activity of tRNA genes remains largely unknown. The position of the IVS in tRNA genes is conserved in all cases identified and within families of genes coding for a single species of tRNA the nucleotide sequence of the IVS is also conserved (1, 2, 5-7) including a sequence of nucleotides capable of base pairing with the anticodon. This degree of conservation may not be carried between species (8, 9). Both the size and sequence of the IVS found in different tRNA gene families show considerable heterogeneity (2, 4). The strong sequence conservation seen in the IVS of yeast tRNA genes supports the conclusion that these sequences are involved in gene function. The central region of some tRNA genes and other RNA polymerase III sensitive genes is known to play either an active or passive role in promoting transcription (10-20). Other studies place control regions in the DNA sequences flanking the 5'-end of polymerase III sensitive genes (19-23). The effects of 5'-flanking

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sequence on the template activity of the adjacent gene can be either positive (21, 23) or negative (19, 22).

To identify a role for conserved sequences in non-coding regions, we have modified a yeast $tRNA_3^{Leu}$ gene by introducing deletions in the 5'-flanking region and by inserting or deleting nucleotides at a Hpa I cleavage site which occurs near the center of the IVS. Insertion of a 21 bp fragment of synthetic DNA corresponding to the sequence of the lac operator region was previously shown to have no deleterious effect on transcription, end-maturation or splicing of this gene in Xenopus germinal vesicle extracts (24). Subsequent modification by insertion of DNA fragments of 10, 30 and 103 bp has not eliminated the template activity of the gene. Xenopus extracts are less sensitive to the insertions than yeast preparations. This suggests that the sequences downstream from the Hpa I restriction site in the IVS are not essential for promoting transcription but may modulate the extent of transcription in homologous systems or they are essential but not sensitive to the presence of the additional nucleotides.

$tRNA_3^{Leu}$ Genes with 8, 10, 13 or 20 bp of DNA deleted from the IVS are also transcribed in vitro. These genes are used with approximately the same efficiency as the unaltered form when yeast extracts are used for transcription, whereas Xenopus extracts show a modest reduction in efficiency of transcription with these templates. End-processing proceeds normally and the excision of the IVS occurs such that ½-tRNA fragments accumulate in reaction mixtures using the Δ8, Δ10, and Δ13 forms as templates in both yeast and oocyte systems. Ligation of these tRNA fragments does not occur to any measurable degree in the Xenopus extracts but does proceed efficiently in the yeast extracts. Transcripts from the Δ20 form of the gene are end-processed efficiently but excision of the IVS does not take place in either type of cell extract.

Other investigators have examined the in vitro transcription of RNA polymerase III sensitive genes including tRNA (14-17, 20) , 5S RNA (10, 11), and adenovirus VA RNA genes (12, 13) in which the normal 5'-flanking sequence had been altered by deletions. In most cases, these deletions have no discernable effect on the transcription of the structural portion of the gene save the precise site of initiation. The unusual conservation of 5'-flanking sequence noted with S. cerevisiae $tRNA_3^{Leu}$ genes suggested there might be a functional constraint therein with this particular gene. To test this possibility, yeast DNA sequences upstream from the structural region of the gene were deleted and replaced by vector DNA to either position -22 or -2 with

respect to the nucleotide which corresponds to the 5'-end of mature $tRNA_3^{Leu}$. The extent of transcription seen when genes deleted to -22 were tested in yeast cell extracts was found to equal that of the unmodified gene but was highly dependent on the reaction conditions. When the -2 form of the gene was tested, it was found to be almost completely inert in the yeast transcription extracts but completely active in the *Xenopus* oocyte preparations. This dramatic difference in template activity may reflect a fundamental difference in the recognition signals used by the RNA polymerase III systems in higher and lower eukaryotes (see also 21-23, 26). Alternatively, it may reflect the specialization of oocytes for extensive RNA synthesis.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Cloning of a 2.5 kb fragment of yeast DNA containing a $tRNA_3^{Leu}$ gene in the vector pBR322 to produce the recombinant plasmid pJD137 has been described (24). Modifications of this gene include insertion of 1 or 3 copies of a synthetic decanucleotide, $\frac{\text{CCAAGCTTGG}}{\text{GGTTCGAACC}}$, (Collaborative Research, Inc.). The fragment was ligated to Hpa I (Bethesda Research Labs) cleaved pJD137 plasmid DNA using T4 DNA-ligase (Bethesda Research Labs). Recombinant DNA molecules were selected by resistance to Hpa I digestion then cloned in *E. coli* C600SF8 and analyzed by restriction mapping and DNA sequencing (27) to verify their identity. A second fragment of DNA, derived from the control region of the *lac* operon by restriction with Alu I, was also inserted at the Hpa I site of the $tRNA_3^{Leu}$ gene. The Alu I fragment extends from position 25, in the CAP binding site, through the RNA polymerase binding site and operator sequence to 2 nucleotides 5' from the AUG codon of the Z-gene (28). The fragment was cloned by ligation into pBR322 which had been digested with EcoRI plus Bam H1 and the resulting staggered ends filled in using the Klenow fragment of *E. coli* DNA polymerase I (Bethesda Research Labs, Inc.) to create blunt ends. The lac DNA was then excised as an EcoRI-Bam H1 fragment, the staggered ends filled-in as described and then blunt-end ligated into Hpa I cleaved pJD137. This results in a 103 bp insertion. Clones representing both orientations of this insertion were isolated and their identity verified by DNA sequence determinations (29). The DNA sequence analyses of both these inserts revealed two discrepancies with the published sequence (28) at positions 112 and 113 in the ALU 2 gene (Fig. 3) where two A residues replace a GT sequence.

Nucleotides were deleted from the central region of the IVS by brief nuclease Bal 31 digestions of Hpa I cleaved pJD137 DNA. The DNA was then

recircularized by ligation, Hpa I digested to remove unreacted molecules and transformed into *E. coli* C600SF8 cells. Individual colonies were screened for deletions by Hae III restriction of DNA isolated using the rapid method of Birnboim and Doty (30). The extent and position of each deletion was determined by DNA sequence analysis (27). A gene with approximately 400 bp of yeast DNA deleted from the 5'-end of the tRNA gene was generated by opening the parent plasmid, pJD137, with Hind III followed by nuclease Bal 31 digestion, then circularizing with T4 DNA ligase and recloning in *E. coli* C600SF8. Amongst the transformants we identified a clone which had lost all but 2 bp of the normal 5'-flanking sequence. DNA sequence analysis of this clone, Δ5'-2L, shows the yeast sequence to be fused to pBR322 DNA at map position 331 (25). The deletion of 5'-flanking sequence upstream from position -22 with respect to the coding region was accomplished by subcloning a 300 bp FnuD II fragment which includes the entire tRNA gene and approximately 170 bp of 3'-flanking DNA. This was cloned into pBR322 which had been digested with EcoRI and Bam H1 then filled in as described previously. The designation of L or R following a clone name indicates transcription of the yeast gene occurs leftward or rightward with respect to the usual pBR322 representation (25).

Transcription Extracts and Reactions

A *Xenopus* oocyte S100 fraction was prepared as described by Ng *et al.* (31). Reactions were done essentially as described except 50mM HEPES buffer adjusted to pH 7.9 with KOH was used in place of Tris·Cl. Alpha ^{32}P-labeled UTP or GTP (ICN Radiochemicals, 400-450 Ci/mM) were used to label transcription products for subsequent polyacrylamide gel electrophoresis and RNA-fingerprint analyses, in some cases. 10-20μCi of ^{32}P- nucleotide triphosphate, at a final nucleotide concentration of 25μM, were used in each 50μl reaction mixture.

Transcriptions performed with yeast cell-free extracts followed closely the methods described by Kleklamp and Weil (32). Each 50 μl reaction mixture contained approximately 400 ng of yeast protein and 300 ng of DNA. The NaCl concentration was 140 mM. Extracts prepared by $(NH_4)_2SO_4$ precipitation of a cleared yeast lysate were used for most transcriptions. A partially purified fraction, the 0.6M NaCl eluate from phosphocellulose chromatography of the crude extract, was used for some experiments and was a generous gift from M. Kleklamp and P. A. Weil. The transcription reactions using yeast cell free extracts were incubated for 30 minutes at 20°C and the reaction products analyzed by electrophoresis on 10% polyacrylamide gels containing 4M urea

(24). Radioactive bands in the gel were located by exposure of the gel to Kodak XAR5 film for approximately 12 hours using a Dupont lightning-plus intensifier screen. For quantitative estimations of the extent of accumulation of transcripts, individual bands were excised and radioactivity determined by Cerenkov counting. Radioactivity in some gel bands was eluted using the method of Maxam and Gilbert (27) for further analyses by either RNA-fingerprinting (33) or the S1 nuclease mapping technique (34) using restriction fragments produced by Hinf I digestion of pJD137 hybridized to the transcription products.

RESULTS

DNA Sequences Conservation of Yeast $tRNA_3^{Leu}$ Genes

The $tRNA_3^{Leu}$ gene is present in multiple copies in *S. cerevisiae* (7, G.Raymond and J. Johnson, unpublished observations) and the DNA sequences of several independent clones which hybridize with radioactive $tRNA_3^{Leu}$ have been determined (5-7, 35). A comparison of these sequences shows the region which specifies the nucleotide sequence of mature $tRNA_3^{Leu}$ to be identical in all copies of the gene. The genes also show extensive sequence homology in the 5'-flanking and intervening sequence regions (Fig. 1). An A-rich sequence of fifteen nucleotides, contiguous with the 5'-end of mature $tRNA_3^{Leu}$, is nearly perfectly conserved in all four DNA fragments even though the sequences just upstream from this region bear only slight similarities. Further, both the position and nucleotide sequence of the IVS show almost complete conservation in all 4 copies of the gene. Two single-base substitutions and one insertion/deletion are the only differences seen. The 3'-flanking nucleotides diverge considerably following a cluster of T residues, the transcription termination signal, 4-5 bp downstream from the 3'-end of the encoded portion of mature tRNA sequence. As the presence of conserved nucleotide sequences in non-coding regions of DNA is considered to be a reflection of functional constraints, we investigated possible involvement of such sequences in the processes involved in the production of a functional tRNA. To this end, we have generated variants of a normal gene, form 4 in Fig. 1, by introducing insertions and deletions in the intervening sequence or substituting plasmid sequences for yeast DNA in the 5'-flanking region. Partial deletion of the 400 nucleotides present at the 5' side of the gene in the parental EcoRI fragment of yeast DNA was done using either a restriction site 22 bp upstream (Fig. 1; Δ5'-22L, Δ5'-22R) or Bal 31 nuclease resection (Fig. 1; Δ5'-2L) as described in Experimental Procedures. The IVS region of the gene was modified

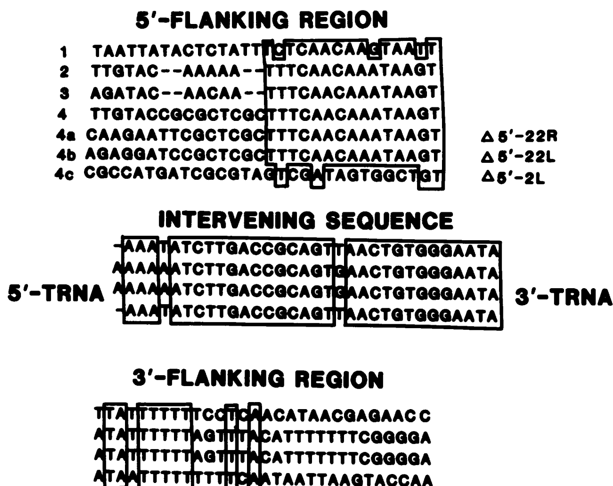

Figure 1. DNA Sequences of Non-coding Regions of some Yeast tRNA Genes. DNA sequences of the non-coding strand are compared for the 5'-flanking, intron and 3'-flanking regions from different yeast $tRNA_3^{Leu}$ genes. The 5' and 3' coding regions of the genes are identical in all cases. Nucleotides present in the non-coding regions of all forms of the gene are boxed. Forms 1-4 represent non-allelic copies of the gene which have been isolated from yeast genomic DNA libraries [1(35), 2(7), 3(6), 4(5)]. Forms 4a, 4b and 4c are derivatives of gene 4, plasmid pJD137 (24), in which the 5'-flanking region has been modified as described in Experimental Procedures. L or R indicates that direction of transcription of the tRNA gene proceeds leftward or rightward with respect to the usual pBR322 representation (25).

by deletion or insertion of nucleotides at the Hpa I cleavage site 19 nucleotides into the form 4 IVS (Fig. 1). Genes bearing partially overlapping deletions of 8, 10, 13 and 20 bp or insertions of 10, 21, 30 and 103 bp have been studied.

In vitro Transcription of Genes Modified in the IVS

The first test of a modified $tRNA_3^{Leu}$ gene involved insertion of a 21 bp synthetic DNA fragment corresponding to the *E. coli* lac operator (24). The ability of this modificant to efficiently produce mature $tRNA_3^{Leu}$ in *Xenopus* germinal vesicle extracts encouraged further investigation of the effects of IVS modification on the transcription of these genes and processing of pre-$tRNA_3^{Leu}$ molecules. As the small insertion was well tolerated by the *Xenopus* transcription apparatus, we tested the effects of small deletions. Further, the recent availability of a cell-free extract from *Saccharomyces* that efficiently transcribes class III genes allowed the use of a homologous test system (32). Figure 2A shows the pattern of transcripts produced when these

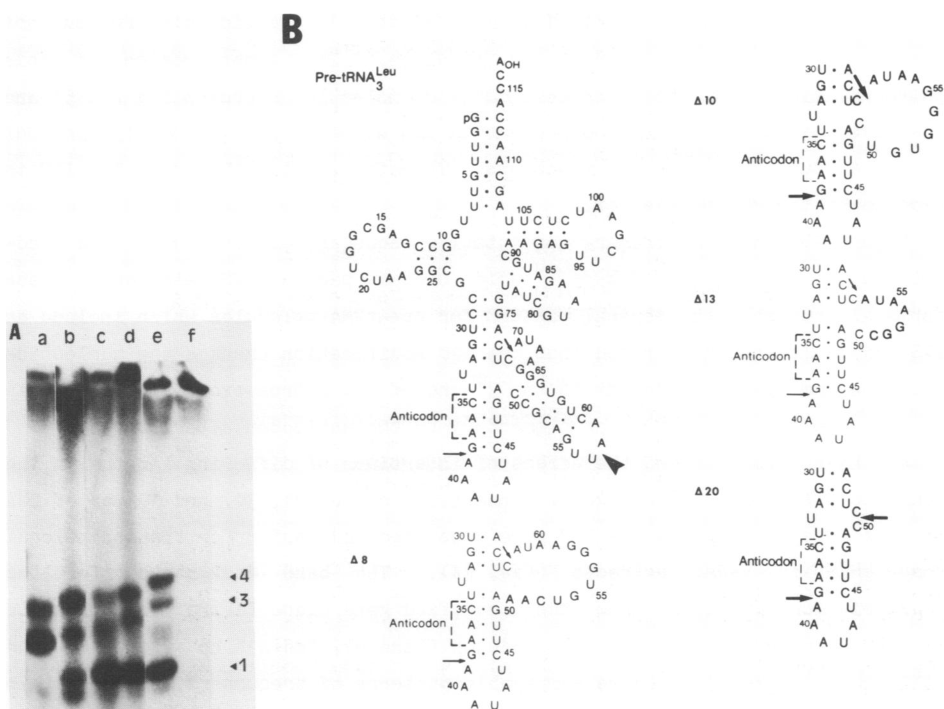

Figure 2. A) Transcription of Genes with Partial Deletions of the Intervening Sequence. Plasmids bearing IVS deletions were transcribed in a yeast cell-free extract as described in Experimental Procedures. Products of the reactions, labeled with α-^{32}P-GTP, were analyzed by electrophoresis in 10% polyacrylamide gels containing 4M urea. The wet gel was used for fluorography to visualize transcription products. Band 4 contains pre-tRNA molecules which are both 5' and 3' end-extended and contain an IVS. Band 3 is a tRNA precursor with mature 5' and 3' ends, including -CCA_{OH}, and an IVS. Band 1 is mature $tRNA_3^{Leu}$. The template plasmids are: a= Δ20, b= Δ13, c= Δ10, d= Δ8, e=unmodified pJD137 and f=pBR322. B) The predicted secondary structure of pre-tRNA molecules from band 3 is shown for the normal gene. The IVS boundaries are marked by small arrows and the Hpa I cleavage site by a heavy arrowhead. The primary structure and a computer generated secondary structure (37) are presented for the anticodon stem-loop and the IVS of each of the deletion mutants.

mutants, cloned in pBR322, are used as templates in yeast cell-free extracts. The accumulation of transcripts from all of these genes proceeds to about the same extent as with the unmodified gene. End-extended and end-matured precursors which still contain the IVS transcript accumulate in the unmodified, Δ 8, Δ 10, Δ 13 and Δ 20 reactions (bands 4 and 3, Fig. 2A). The size of these precursors diminishes in proportion to the size of the deletion indicating accurate transcription of the IVS. Mature tRNA (band 1,

Fig. 2A) is found in the unmodified, Δ 8, Δ 10 and Δ 13 reaction mixtures but not when the Δ 20 gene is used as a template. The lower region of this polyacrylamide gel shows trace amounts of molecules representing the 5' and 3'-halves of mature tRNA to be present in the normal, Δ 8, Δ 10 and Δ 13 product mixtures but not in the Δ 20 reactions. Therefore, the excision of the IVS is blocked by the 20 bp deletion.

Figure 2B shows the primary and putative secondary structures of the end-matured precursor forms of $tRNA_3^{Leu}$ which contain partial IVS deletions. These forms all resemble the general pattern for pre-tRNA molecules which include an IVS (36). It should be noted that the Δ20 modification changes the nucleotide sequence immediately adjacent to the 3'-end of the intron-exon junction and in no case are the nucleotides complementary to the anticodon altered.

We have also tested the effect of insertions of differing lengths on the template efficiency of this gene. Insertions of 10, 21, 30, and 103 bp of DNA at the Hpa I site in the IVS were generated and tested for transcriptional competence in yeast extracts (Fig. 3A). The band designations for the transcription products from the unmodified gene (lane f) are the same as in Figure 2A. The smaller inserts, DI=10 bp (lane e), lac=21 bp (lane d) and DIII= 30 bp (lane c), produce comparable patterns of precursors, which contain complete IVS transcripts. However, the amount of mature $tRNA_3^{Leu}$ formed from these enlarged precursors is diminished. The identity of the band just above mature tRNA in lane c is unknown. Transcription products from genes expanded by 103 bp, the ALU 2 and ALU 1 forms in Figure 3B, are seen in Figure 3A, lane a and b, respectively. RNA-fingerprinting has shown that the large band in each of the two lanes contains complete, end-extended transcripts from the expanded genes. The extent of transcription of the 103 bp insertion forms is reduced from that seen with the unmodified gene. Figure 3B depicts the nucleotide sequence and possible secondary structures for the pre-tRNA molecules generated by transcription of the mutant forms with insertions in their IVS. Computer predictions (37) for the base-pairing patterns in the transcripts from clones bearing the ALU 1 insertion indicate a different base-pairing pattern is also possible. A small but reproducible difference in the electrophoretic mobilities of the ALU 2 and ALU 1 transcription products (Fig. 3A, lanes a and b and Fig. 5, lanes c and d) could be explained by an alternative secondary structure. The transcriptional activity of these largest forms of the gene is in qualitative agreement with the results of Carrara _et al._ (38) who studied transcription of the same $tRNA_3^{Leu}$ gene bearing a 300 bp insertion. However, we see quantitative differences in the

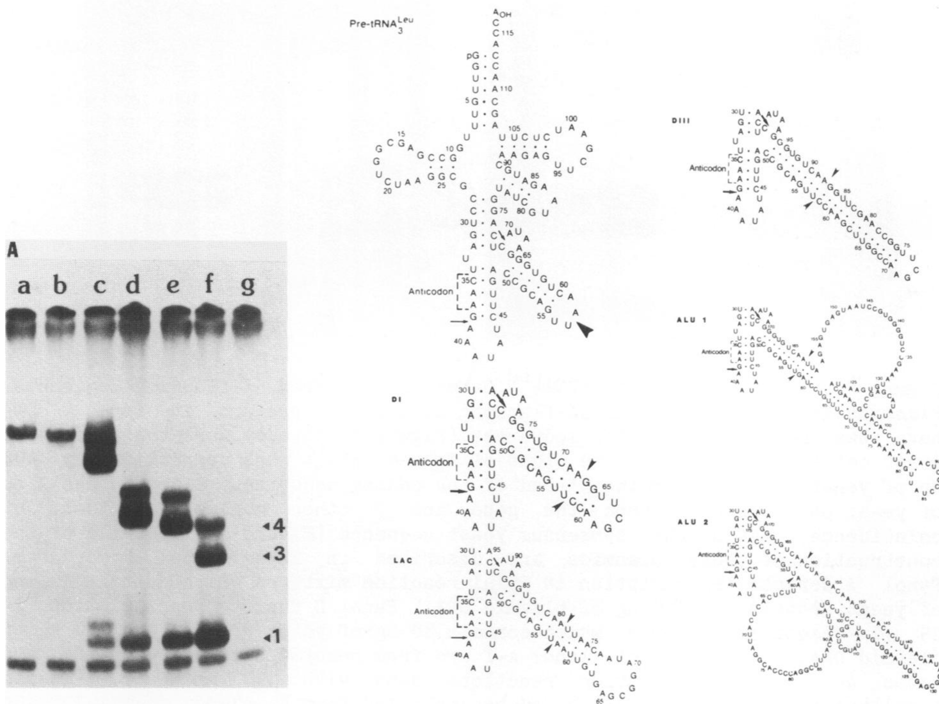

Figure 3. A) Transcription of Genes with Insertions IVS. Plasmids bearing insertions were transcribed in a yeast cell-free extract as described in the legend to Figure 2. The plasmid used as template is: lane a, ALU 2; lane b, ALU 1; lane c, DIII; lane d, LAC; lane e, DI; lane f, unmodified; lane g, pBR322. The identity of products in bands 4, 3 and 1 are described in Figure 2. The band just below the position of mature $tRNA_3^{Leu}$ is not dependent on added template. The identity of the band just above mature tRNA in lane c is not known. B) Pre-$tRNA_3^{Leu}$ shows the primary and predicted secondary structure of the band 3 product from transcription of the form 4 gene. Small arrows mark the boundaries of the IVS and the Hpa I cleavage site is indicated by the heavy arrowhead. The primary structure and a putative secondary structure are presented for the anticodon stem-loop region and IVS of transcripts from each of the insertion mutants.

efficiency with which these genes are recognized by the yeast transcription machinery compared to the unmodified form (Fig. 3A, lanes a, b and f). Differences seen in accumulation of RNA products from the modified genes might be due to a decrease in stability of the unusual pre-tRNA molecules in the transcription reaction mixtures. To test this possibility, a 25-fold excess of unlabeled GTP was added to reaction mixtures at the end of the normal

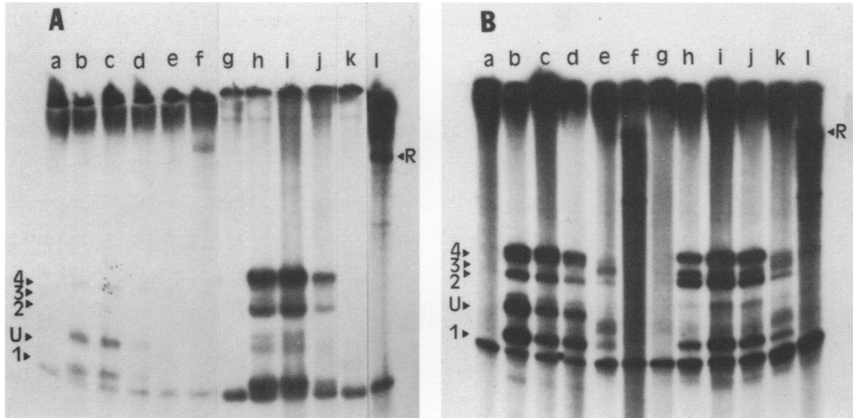

Figure 4. Transcription of $tRNA_3^{Leu}$ Genes with Altered 5'-flanking Sequences. Plasmids in which the normal 5'-flanking nucleotides present in form 4 DNA have been replaced by vector sequences (Figure 1, 4a,b,c) were transcribed in yeast cell-free extracts. The clone of form 4 $tRNA_3^{Leu}$ has approximately 400 bp of yeast DNA flanking the 5'-end of the coding sequence. Clone 4c has 2 bp of yeast DNA upstream from the gene and 2 other nucleotides that, by coincidence, match the consensus yeast sequence (Figure 1). Details of the construction of these plasmids are described in Experimental Procedures. Panel A depicts transcription in 50 µl reaction mixtures which contain 400 ng of yeast protein and 300 ng of plasmid DNA. Panel B depicts transcription in 25 µl reaction mixtures which contain 80 ng of yeast protein and 150 ng of plasmid DNA. In each panel, lanes a-f are from reactions in 90 mM NaCl and lanes g-l are transcription reactions done with 140 mM NaCl. Plasmid templates are a and g=pBR322, b and h=unmodified form 4, c and i= Δ5'-22R, d and j= Δ5'-22L, e and k= Δ5'-2L, f and l=5'-half of form 4 $tDNA_3^{Leu}$ cloned into the Hind III site of pBR322. The identities of RNA molecules in bands 4, 3 and 1 are described in Figure 2A. Band 2 is a end-processing pre-tRNA molecule lacking the $2A'-CCA_{OH}$. Band R is the runoff transcript promoted by the 5'-half of the $tRNA_3^{Leu}$ gene and terminated at the Bam H1 cleavage site of the vector. Band U is an uncharacterized processing intermediate which accumulates in appreciable amounts only under conditions described for panel B.

incubation period and reactions were allowed to proceed for 15 or 30 minutes. The experiments revealed no differential stability of the transcripts from any of the IVS insertion or deletion mutants (data not shown).

Transcription of $tRNA_3^{Leu}$ Genes with Altered 5'-Flanking Sequences

The nearly perfect conservation of 15 nucleotides contiguous with the 5'-end of the coding region in four non-allelic copies of the $tRNA_3^{Leu}$ gene represents a very unusual circumstance for a class III gene. This led us to generate sub-clones in which all but 22 bp of yeast DNA upstream from the structural region of the gene were replaced by plasmid sequences (Fig. 1. forms 4a and 4b). This preserved the conserved region while deleting about

370 bp of yeast DNA present in the parent clone. Further deletion, by nuclease Bal 31 resection, to position -2 removes nearly all of the conserved sequence and leaves the structural portion of the gene intact (Fig. 1, form 4c). Transcription of these clones by cell-free extracts from yeast under our standard assay conditions revealed a dramatic loss of template capacity when the 5'-flanking region of the gene was modified (Fig. 4, panel A, lanes i, j and k) The two clones, Δ5'-22L and Δ5'-22R, are transcribed by the yeast RNA polymerase III but the left orientation is clearly less active than the right (lane i and j) while the Δ5'-2L form is completely inert (lanes e and k). Mixtures of the unmodified gene and Δ5'-2L DNA are transcribed hence the loss of transcription is not due to an inhibitory substance in the Δ5'-2L preparations (data not shown).

Parallel investigations by D. Stillman and P. Geiduschek (unpublished observations) indicated that the Δ5'-2L gene was transcribed under some conditions. To investigate the basis for the observed differences, we compared our transcription conditions with those suggested by D. Stillman and P. Geiduschek (personal communication). Panel B of Figure 4 represents transcriptions done in a 25 ul reaction volume in which the amount of DNA is reduced to 150 ng and extract protein to 80 ng compared to our standard (Panel A) of 300 ng DNA and 400 ng of yeast protein in a 50 ul reaction. Further, we investigated the effect of ionic strength under both sets of conditions by comparing the results seen in 90 and 140 mM NaCl (lanes a-f and g-h, respectively, in both panels of Figure 4). The extent and selectivity of transcription is dramatically influenced by _in vitro_ conditions. The lower NaCl concentration (lanes a-f) inhibits transcription of all templates under conditions used in panel A in agreement with Kleklamp and Weil (1982), whereas the extent of accumulation of RNA products is largely unaffected in panel B. Further transcription of plasmids in which the tRNA gene is oriented in a leftward sense (lanes d, e, j and k) is selectively enhanced under panel B conditions. We observe approximately equal transcription of the unmodified, the Δ5'-22R and Δ5'-22L forms of the gene when panel B conditions are employed with 140 mM NaCl (Fig. 4, panel B, lanes h, i and j). This is in contrast to the clear reduction in template activity seen with Δ 5'-22L under panel A conditions at the same ionic strength (Fig. 4, panel A, lane j). The Δ5'-2L template also shows some weak, ionic strength dependent activity under panel B conditions (lanes e and k). The Δ 5'-22L and Δ 5'-2L yeast fragments are bounded by the pBR322 sequence adjacent to the Bam H1 restriction site of this vector (see Experimental Procedures).

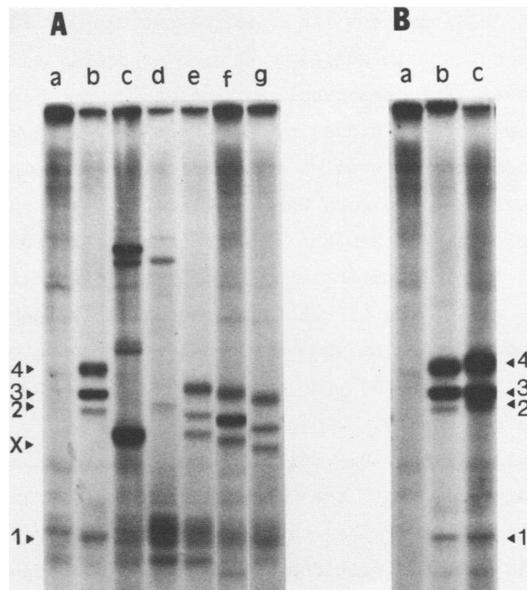

Figure 5. Transcription of Modified Yeast $tRNA_3^{Leu}$ Genes in Xenopus Oocyte Extracts. Plasmids with normal and modified yeast tRNA genes were transcribed in Xenopus oocyte extracts and the reaction products analyzed as described in Figure 2. Panel A presents transcription products from genes with alterations in the IVS. The template DNA for lane a=pBR322, b=pJD137, c=ALU 2, d=ALU 1, e= Δ8, f= Δ10, and g= Δ13. Panel B presents transcription products from deletion of 5'-flanking sequence to the -2 position. The template DNA for lane a=pBR322, b=pJD137, c= Δ5'-2L. Identities for the bands are described in Figures 2A. Band x results from premature termination of transcription of the ALU 2 gene at positions 92-94 (Figure 3B).

The 5'-half of the gene, along with 400 bp of 5'-flanking yeast DNA also functions as a weak template when cloned into the Hind III site of pBR322 (Fig. 4, Panels A and B, lanes f and l). The plasmid used as template was cleaved with Bam H1 to produce run-off transcripts about 415 bp long. Neither pBR322 nor pJD137, the unmodified clone, produce any run-off transcription products. Transcription of the cloned 5' half-gene in the presence of cordycepin triphosphate produced a characteristic set of transcripts not seen with pBR322 or pJD137 (data not shown).

Transcription of Modified Yeast $tRNA_3^{Leu}$ Genes in Xenopus Oocyte Extracts

The yeast $tRNA_3^{Leu}$ gene is actively transcribed in cell free extracts from Xenopus germinal vesicles (24, 38) and Hela cells (6). The template activities of several forms of the gene with modifications in the IVS have also been studied in the Xenopus germinal vesicle extracts (24, 38). Figure 5

depicts the template activity of several forms of the $tRNA_3^{Leu}$ genes in Xenopus oocyte S100 preparations (31), which can be compared with the yeast cell-free system. Insertion of the 103 bp sequences shown in Figure 3B as ALU 2 and ALU 1 give the patterns seen in lanes c and d of panel A (Fig. 5). Both forms direct the synthesis of a transcript which is 5' and 3'-end-extended and contains all the oligonucleotides derived from the gene plus inserted sequences. The ALU 2 orientation is transcribed somewhat more efficiently than ALU 1. Further, a major transcription product seen with the ALU 2 insert, designated band x in panel A, lane c, results from premature transcription termination at positions 92-94 of ALU 2 sequence depicted in Figure 3B. This is a U rich region of the RNA and apparently is a moderately strong termination signal for the Xenopus transcriptional apparatus. A similar band can be seen in the transcription products produced by yeast extracts from this form of the gene but it is present in much smaller amounts relative to the complete transcript (Figure 3A, lane a). RNA Fingerprint analyses of the band just below the largest transcript from the ALU 2 form of the gene (Fig. 5, Panel A, lane c) did not show any spots corresponding to those derived from the mature ends. We were unable to establish the identity of the RNA seen in the position at or near that of mature $tRNA_3^{Leu}$ (band 1) from lanes c-g by RNA-fingerprint analysis. However, it was clear that mature $tRNA_3^{Leu}$ was not present in detectable amounts. Panel B of Figure 5 compares the template activities of the unmodified gene and the 5'-deletion mutant, Δ5'-2L (lane b and c, respectively). The Xenopus transcription machinery utilizes the 5'-deleted form of the gene as well or better than the unmodified version. This result is clearly different than that seen when the yeast extracts are used as the source of transcription components (Figure 4, panels A and B; lanes h and k). The Δ5'-2L transcripts are end-matured and spliced normally.

DISCUSSION

We have attempted to identify functions for two regions of a yeast tRNA gene which do not contain information present in the mature tRNA. Both the 32 bp IVS and the 15 bp adjacent to the 5'-end of the tRNA coding sequence are strongly conserved in non-allelic copies of the gene (Fig. 1) (5-7, 35). The degree and extent of sequence conservation in the $tRNA_3^{Leu}$ 5'-flanking region is, to our knowledge, unprecedented with respect to other RNA polymerase III sensitive genes whose sequence is known. Morton and Sprague (40) have recently found common sequence elements in the 5'-flanking regions

of *Bombyx* Class III genes in a region previously shown to be important for transcription by homologous cell-free extracts (21). We report that for the yeast tRNA$_3^{Leu}$, this region is essential for efficient transcription in homologous, but not heterologous, cell-free extracts (Fig.4 and 5). We do not know whether this inactivity results from a loss of recognition of the gene by some component of the transcription apparatus or from a loss of the initiation site. A similar result has been seen with three other tRNA genes in which the normal 5'-flanking regions have been replaced by vector DNA. A *Bombyx mori* tRNA gene truncated 11 bp upstream from the 5'-end of the coding sequence did not produce detectable amounts of tRNA in extracts from *Bombyx* ovary or silk gland tissue but were approximately as active as the wild-type gene in *Xenopus* germinal vesicle preparations (21). Sharp *et al.* (20) tested the template activity of a number of modified forms of a *Drosophila* tRNA^{Arg} gene in homologous and heterologous extracts. They identify two internal control regions for this gene. However, their data also shows a loss of transcription with a gene deleted to within 8 bp of the 5'-terminus when tested with an extract from *Drosophila* Kc cells. The same gene is active in both *Xenopus* and Hela cell extracts. This result has recently been extended by Dingermann *et al.* (23) who demonstrate differential transcription of 4 identical *Drosophila* tRNA^{Arg} genes by exchanging 5'-flanking sequences. These results are comparable to the effects we observe with the yeast tRNA gene in homologous and heterologous cell extracts. There is, however, some difference in the *Drosophila* tRNA^{Arg} and yeast tRNA$_3^{Leu}$ promoters because other work by Sharp *et al.* (26) indicates that a subclone of the *Drosophila* tRNA^{Arg} containing the normal 5'-flanking sequences and the "A"-block is not transcribed in homologous cell extracts. A comparable subclone of the tRNA$_3^{Leu}$ shows that these two elements are sufficient to promote transcription (Fig. 4, lanes f and l, Stillman and Geiduschek, personal communication; 38). The "B"-block cloned separately does not produce any detectable transcripts from either the *Drosophila* or yeast tRNA with any extract (26; Raymond and Johnson, unpublished observations). Mattoccia *et al.* (42) have shown that the tRNA$_3^{Leu}$ gene with a 3 bp substitution in the "A"-block can be efficiently transcribed in *Xenopus* germinal vesicle extracts.

The yeast *SUP4*-0-tRNA^{Tyr} gene has a 15 bp sequence at the 5'-end of the gene, TTCAACAATTAAATA, very similar to that of the tRNA$_3^{Leu}$ gene. Koski *et al* (43) have assayed the template activity of various mutants of this gene and, surprisingly, see no effect on transcription when this sequence is replaced by

vector DNA. The *in vivo* studies of the same *SUP4* gene (44) support the *in vitro* studies. We are presently testing our mutant tRNA genes *in vivo*. DeFranco *et al*. (19, 22) have identified a sequence of 11 nucleotides present near several *Drosophila* $tRNA^{Lys}$ genes which inhibits transcription of the adjacent gene. Our results are consistent with these and support the conclusion that different organisms, presently only discriminating between vertebrates and invertebrates, do not use identical signal elements to promote polymerase III transcription. Yeast, *Drosophila* and *Bombyx* tRNA genes have all been shown to utilize internal sequences to cue both homologous and heterologous transcription systems (20, 21, 26, 45) as do tRNA genes from higher eukaryotes (14-17, 46). Numerous studies have shown that *Xenopus* and human enzyme systems can efficiently produce tRNA molecules from cloned yeast or *Drosophila* genes (6, 24, 38, 47-51). From the observation that *Xenopus* extracts are able to transcribe at least one bacteriophage T4 tRNA gene (52) and the recent demonstration that both *E. coli* and chloroplast tRNA genes are transcribed by Hela cell extracts (53) it would seem likely that these transcription systems can be completely directed by common features within the tRNA gene while some component(s) of the yeast and insect transcription apparatus are also sensitive, at least in some cases, to DNA sequences outside the coding region. Inhibition of the *Xenopus* transcription apparatus by a 5'-flanking sequence found in *Drosophila* $tRNA^{Lys}$ genes (22) indicates that transcription may be sensitive to some non-coding sequences in higher eukaryotes as well. A caveat to these results may be the largely unexplored effects of vector DNA sequence on transcription of adjacent genes. The different activities of the Δ5'-22L and Δ5'-22R clones seen when the template DNA: protein ratio and ionic strength are varied points out the need for careful controls (Fig. 4, lanes c-d and i-j). A similar effect has recently been noted by Larson et al. studying transcription of a *Bombyx mori* tRNA gene (54).

Transfer RNA gene promoters have been identified as two short sequences which are found in the regions 8-25, A-block, and 50-72, B-block, in 3 different tRNA genes which have been dissected by deletion analysis (14-17, 20). The nucleotide sequence between these two regions does not seem to include any essential recognition elements. However, deletion and substitution experiments have provided evidence suggesting that transcription efficiency is sensitive to the spacing provided by this region and, to a lesser degree, the nature of the sequence itself (14,16,17). The normal spacing for the three genes tested in these studies ranged from 29-38 bp.

The $tRNA_3^{Leu}$ gene we have studied has 65-70 bp between the putative A and B regions including a 32 bp intervening sequence. The tRNA genes studied previously do not contain intervening sequences. Our results demonstrate that deletions in the IVS of up to 20 bp did not impair the use of the gene by cell-free extracts (Fig. 2,5). Expansion of this region to a total length of 170-175 bp also left the gene transcriptionally competent albeit with impaired efficiency (Fig. 3,5). We therefore conclude, as have others (24,44,55), that the integrity of the IVS is not essential for RNA polymerase III transcription. With regard to the spacing function of this region it is somewhat unusual that the gene remains functional when the A-B separation is varied from as little as 45-50 bp to as much as 170 bp. We must, therefore, consider some alternative explanations for the relative insensitivity of this gene to size changes as compared to other tRNA genes. As has been pointed out by Hall et al. (41), size variation from 30-75 nucleotides can be found in this region of normal tRNA genes. The apparent flexibility of the $tRNA_3^{Leu}$ gene, when compared to the others might be due to a lack of sensitivity to either the presence or position of a B-block sequence. Alternatively, the modifications we have generated may not change the spacing of the A and B promoter regions in an active gene. Yeast $tRNA_3^{Leu}$ transcription in Xenopus extracts has been reported to be insensitive to the presence or absence of yeast DNA sequences downstream from position 57, the mid-point of the transcribed region (38). The authors conclude that the 3'-half of the $tRNA_3^{Leu}$ gene is not essential for promoter activity. Our clone of the 5' half of the gene, including about 400 bp of 5'-flanking yeast DNA, also shows some template activity in yeast transcription extracts (Fig. 4) also suggesting that the 3'-proximal promoter region is not always essential for RNA polymerase III recognition. However, Drosophila work (20) and our results in yeast suggest that the 3'-sequence is important for quantitative promoter function in homologous systems. The results of Klemenz et al. (56) support this interpretation by demonstrating that yeast cell extracts contain a protein(s) which specifically binds to the B-block nucleotides of the $tRNA_3^{Leu}$ gene. Further, Kurjan et al. (44) and Koski et al. (45) have elegantly demonstrated that, for a yeast tyrosine-inserting suppressor tRNA gene, the integrity of the B-block sequence is essential for both in vivo suppression activity and in vitro transcription of the gene in Xenopus and yeast (43) extracts. Our results, in conjunction with the tRNA gene footprint data of Klemenz et al. (56) and the tRNA evidence, lead us to conclude that transcription of tRNA genes by yeast RNA polymerase III is

enhanced by the presence of an intact B-block sequence.

The question of whether the yeast transcription apparatus is sensitive to the spacing of the A and B-blocks may give some insight into the structure of an active tRNA gene. Because we observe active use of the gene when the size of the spacer region between the two promoter elements is varied over a range of 120 bp, it initially would seem that positioning of the B-block relative to the 5'-end of the transcription unit is not a crucial aspect of gene structure. Indeed, this may be the case. However, it is also possible that all modified forms of the gene tested have, when active, a structure such that the A-B spacing is not perturbed by these modifications. Inspection of possible intramolecular base-pairing patterns for the IVS deletion (Fig. 3) and insertion (Fig. 5) mutants shows that they all leave intact the base-paired stem regions of the D-, anticodon and GTψC loops. If these intrastrand base-pairs form in an active tRNA gene, the deleted or added nucleotides could all be accomodated in an appendage, similar to the so called "extra-arm" of some tRNAs, which would not alter the linear separation of the A and B blocks. Computer generated alternatives (37) for the secondary structures assigned the ALU 1 and ALU 2 insertion mutants (Fig. 3) have been considered and likely exist in the transcription products of these genes as evidenced by the difference in electrophoretic mobilities of the full-length transcripts from the two genes (Fig. 3A, lanes a and b; Fig. 5A, lanes c and d). In these alternatives, the sequence transcribed in the ALU 1 form of the gene invades the tRNA cloverleaf for base-pairing partners and eliminates both the D- and anticodon loops. If this also occured at the DNA level, the A block would be in a much more extensively base-paired region but the number of intramolecular base-pairs between the A and B regions would change by only 1. Additional support for the hypothesis that tRNA biosynthesis is not sensitive to IVS structure can be found in the study of Wallace _et al_. (55) which demonstrates that a plasmid bearing a $tRNA^{Tyr}$ gene with the IVS precisely deleted can give phenotypic suppression of host cell ochre mutations.

The processing of pre-$tRNA^{Leu}_3$ transcripts to a mature tRNA structure has been demonstrated to take place in _Xenopus_ oocyte (24, 38), Hela cell (6) and yeast extracts (24). They were found to be unaffected by the 21 bp enlargement of the IVS reported previously (24). The processing of transcripts from other expanded forms of the gene by yeast extracts is seen in Figure 3A. End-trimming and $-CCA_{OH}$ addition results in the conversion of band 4 RNA to band 3. These reactions proceed with the normal pre-tRNA molecules as well as the 10, 21, and 30 bp enlarged forms of the gene, but not with

either orientation of the 103 bp insert. Similar results are seen when the large genes are transcribed in *Xenopus* extracts (Fig. 5A). This block in end-processing may be due to a steric inhibition of the processing enzymes by the large IVS. Alternatively, the presence of the extra nucleotides may perturb the position of 3'-proximal sequences or the base-pairing patterns in the D-stem both of which are crucial for end-processing in higher eukaryotes (43, 57, 58). Splicing of the IVS takes place with transcripts bearing the 10, 21, and 30 bp enlargements to produce a mature tRNA albeit with a progressive reduction in efficiency. Maturation of the 5' and 3' ends of pre-tRNA molecules with 8, 10, 13, and 20 nucleotides deleted from the IVS also proceeds efficiently (Fig. 2A,5A). We conclude that the sequence in the IVS does not play a direct role as a recognition element for the nucleases responsible for end-maturation. Removal of the abbreviated IVS and ligation of ½-molecules to form mature tRNA is observed in yeast extracts with all deletion mutants except the Δ20 form where the excision reaction is blocked and form 2A precursor accumulates (Fig. 2A). In this transcript, a C residue replaces the A normally found at the 3'-proximal splice junction. The DNA sequences of other yeast tRNA genes with an IVS show the nucleotide at this position to be either A or U (36) thus the resistance of the Δ20 transcripts to splicing endonuclease may reflect a base-selection property of the enzyme. It is also possible that the additional reduction in size or loss of other nucleotides from the nuclease-sensitive Δ13 transcripts are responsible for the loss of recognition by the splicing activity. We think this unlikely as no consensus sequence is found in this region of yeast pre-tRNA molecules formed *in vivo* (36), and the splicing-system is known to function with other pre-tRNAs of similar size and secondary structure [c.f. pre-$tRNA^{Ser}_{UCG}$ (36)]. Colby *et al.* (59) have demonstrated that splicing of yeast pre-$tRNA^{Tyr}_{SUP4}$ is reduced about 7 fold in a point mutant which changes the nucleotide adjacent to the 5'-side of the 5'-proximal splice junction from A to G. This shows the yeast splicing system is sensitive to the bases present at the splice junction. However, it was not apparent from this study whether the decrease in splicing efficiency resulted from a change in excision as observed in our study, or the subsequent ligation.

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