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The 5'-flanking sequence of yeast tRNA^{Leu3} genes enhances the rate of transcription from stable pre-initiation complexes

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ABSTRACT

A conserved sequence that enhances transcription is present at or near the 5'-end of genes which code for several abundant yeast tRNAs, one of which is tDNA^{Leu3}. Mutants with alterations in this region have been compared to the parent tDNA^{Leu3} and a yeast tDNA^{Ser} that normally lacks the conserved sequence. The apparent K_M of the transcription apparatus is insensitive to 5'-flanking sequence variation. Replacement of the normal 5'-flanking sequence does result in 3-18 fold reductions in V_{MAX} and a 7-13 fold loss of transcription in tests with competitor genes. A second template exclusion assay revealed that sequences upstream of the conserved region effect a 2 fold change in the ability of the genes to stably sequester a component(s) of the obligatory pre-initiation complex while positions -22 to +9 have little or no effect.

INTRODUCTION

The transcription of tRNA genes in eukaryotes is promoted by two non-contiguous sequence blocks located within the DNA segments encoding mature tRNAs (cf ref. 1 for review). These internal control regions (ICRs) are each about 10 bp long and encode portions of the dihydro U and TΨ arms of the tRNA. Consensus sequences have been derived for each of the two regions (2) and mutations which reduce homology to these decrease the extent of RNA polymerase III transcription (3-6). Further, spacing between the two ICRs can also modulate their ability to interact with components of the transcription apparatus (7,8).

Coding sequences outside the well defined ICRs also influence the extent of transcription of a tRNA gene. These include bases in the extra arm (9,10) and the anticodon stem (4). Non-coding sequences flanking tRNA genes have also been found to alter the extent of transcription. Strong positive effects are attributed to the 5'-flanking sequences of some tRNA genes when transcribed in cell-free systems from *Bombyx, Drosophila,* or *S.*

cerevisiae (3,8,11-15). The positive modulation in yeast can also be detected *in vivo* by measuring the strength of a suppressor activity (16). The 5'-flanking sequences of some tRNA genes inhibit their transcription (17,18). Also, a number of cases have now been documented where vector DNA, used to replace normal 5'-flanking sequence, has negatively affected transcription to varying degrees (8,14,15). Sequences flanking the 3'-end of tRNA genes have been shown to stabilize the interactions with transcription factors which form stable complexes with tDNA prior to RNA polymerase III involvement (13,19-20).

The *S. cerevisiae* $tRNA^{Leu3}$ gene family encodes the most abundant leucine-inserting tRNA in yeast (21). All genes of this type which have been examined have a conserved pentadecanucleotide contiguous with the 5'-end of the mature tRNA sequence which enhances transcription (8). DNA sequences from other *S. cerevisiae* genomic tDNAs were searched for the presence of the conserved sequence identified in $tRNA^{Leu3}$ genes (16). The analysis shows that several different *S. cerevisiae* tRNA genes have segments very similar to that of $tRNA^{Leu3}$ in both position and sequence while many did not. The genes which show the best fit to the canonical sequence, $tDNA^{Leu}$, $tDNA^{Tyr}$, $tDNA^{Arg}$ and $tDNA^{Glu}$, all code for abundant tRNAs in *S. cerevisiae* (21). We therefore postulate this sequence may be one mechanism by which yeast cells adjust tRNA biosynthesis to match demand created by codon use preferences. One segment of the conserved pentadecanucleotide, TCAACA, which is located 16-18 bases upstream from several of the tDNA sequences, is very similar to the terminal repeat of a putative transposable element termed *sigma* (22) found upstream from several yeast tRNA coding sequences. The consistent association of the *sigma* sequence with abundant tRNA genes suggests a possible functional interaction.

We have investigated the mechanism of how this sequence modulates transcription by measuring both kinetic parameters and the ability to form stable pre-initiation complexes for normal and mutant genes. The various forms of the $tRNA^{Leu3}$ gene have also been compared to a $tRNA^{Ser}$ gene which normally lacks the conserved sequence (16,23). We find that the conserved 5'-flanking sequence does not measurably affect the ability of the gene to stably sequester transcription factors but does enhance the rate at which transcripts are formed from the complexes.

METHODS

DNA

Plasmids containing the parental $tDNA^{Leu3}$, also referred to as LEU3 or unmodified, and the Δ5'-22,-12,-2,+9, and +11 mutations have been described in detail (14). All clones used in this study are oriented such that transcription proceeds clockwise from the EcoR1 site relative to the normal pBR322 representation. The LEU3Δ20 mutant, a 20 bp deletion in the intervening sequence of $tDNA^{Leu3}$, has also been described (8). The $tDNA^{Ser}$, also referred to as SER or SERSUQ5 is an ochre suppressor allele of SUQ5 on a 1.1 kb fragment of yeast DNA isolated and characterized by Olson et al. as pPM16 (23). All plasmid DNA was isolated by CsCl gradient centrifugation (9) and/or Sephacryl S1000 chromatography (Raymond et al., submitted for publication).

Transcriptions and Factor Binding Assays

To determine apparent Km values, plasmid templates were transcribed in 30µl reaction mixtures optimized for time, temperature, ionic strength, $MgCl_2$, and amount of yeast nuclear extract. The reaction conditions were 20mM HEPES-KOH (pH 7.9), 100mM KCl, 5mM 2-mercaptoethanol, 0.1mM EDTA, 1.5mM DTT, 5mM $MgCl_2$, 0.6mM each ATP, CTP, and GTP, and 25µM α-32P-UTP (6-8 Ci/mM), 10% glycerol, and 60 µg of yeast protein from an $(NH_4)_2SO_4$ fraction of a crude nuclear extract (25). The amount of template DNA was varied from 0.21nM (6 fmol) to 1.9nM (54 fmol) in increments of 6-9 fmol. The total amount of DNA in each reaction was kept constant by the addition of pBR322. Reactions were initiated by addition of extract, incubated at 20° for 30 minutes, and then terminated by the addition of 70 µl of 100mM NaOAc (pH 5.5), 0.4% SDS, and 1 mg/ml tRNA. The accumulation of transcripts was determined to be linear during this time period, the very short lag phase being within the range of experimental error. Each mixture was extracted first with an equal volume of a 1:1 mixture of phenol and $CHCl_3$, then $CHCl_3$ alone followed by ethanol precipitation of the aqueous phase. The resulting pellet was vacuum dried, dissolved in 10 µl of 80% formamide, and electrophoresed on 0.05x34x40 cm 10% polyacrylamide gels containing 8M urea. Radioactive bands corresponding to the tRNA transcripts were located by fluorography, excised, and ^{32}P measured in 5 ml of Biofluor (NEN) with a liquid scintillation counter.

For the determination of initial rates of transcription,

reactions were performed under the same conditions as described above except DNA concentration remained constant using 100 ng each of test and control templates. Aliquots were removed at 5 min intervals to 25 minutes and processed as described previously. Initial rates of transcription for the unmodifed gene and various 5'-flanking deletion mutants were calculated relative to either LEU3Δ20 or SER, each of which produce tRNA products that are smaller and therefore resolvable from the test templates. Further, us of the $tRNA^{Ser}$ gene may allow us to determine whether any gene specific factors are involved with $tRNA^{Leu3}$ synthesis.

The strength of LEU3 and the 5'-flanking deletion mutants ability to form a stable complex was measured using a second template exclusion assay (26,27). These assays were done in a mix maintaining all components at concentrations described for K_M determinatons. Competitor DNA was varied from 0 to 1.3nM and pBR322 DNA was added to maintain a constant DNA concentration for all reactions. Competitor DNA, pBR322 DNA, and yeast transcription extract were mixed in a volume of 22 µl and incubated at 20° for 5 minutes at which time 3 µl of a reference template containing either LEU3Δ20 or SER was introduced yielding a final concentration of 0.9nM. This mix was incubated an additional 5 min then 5 µl of a ribonucleoside triphosphate mixture was added such that their final concentrations were identical to those described previously. Transcription was then allowed to proceed for 30 min. Reactions were stopped, processed, and radioactivity in transcripts determined as before.

RESULTS

Promoter Sequences of the Genes

Several mutants of the $tRNA^{Leu3}$ gene with deletions in their 5'-flanking and tRNA coding sequences have previously been shown to be less effective as templates than the unmodified form for transcription in yeast extracts (8,14-16). The sequence and spacing of the ICRs and 5'-flanking regions of these genes are shown in Figure 1. The same information is also presented for a yeast $tRNA^{Ser}$ (23) used in some of the experiments. Consensus sequences for the ICRs are derived from broad comparisons of eukaryotic tRNA gene sequences (2). The 5'-flanking consensus sequence has been identified only in a subset of yeast nuclear tDNAs (16).

The unmodified $tRNA^{Leu3}$ gene used has 1 base in the A-block and 2 in the B-block which do not conform to the consensus

5'FLANKING	A	SPACING	B	NAME
CCGCGCTCGCTTTCAACAAATAAGT	TGGCCGAGcG	73bp	GaGTTCGAATCt	UNMODIFIED
SAME	SAME	53bp	SAME	LEU3Δ20
ATTCGCTCGCTTTCAACAAATAAGT	SAME	73bp	SAME	-22
TCTTCAAGAATTcCAACAAATAAGT	SAME	73bp	SAME	-12
GCCCTTTCGTCTTCAAgAAtTccGT	SAME	73bp	SAME	-2
GTATCACGAGGCcCtttggtcttca	cGGCCGAGcGG	73bp	SAME	+9
GGCGTATCACgaggccCtttcgtcT	TtcCCGAGcGG	73bp	SAME	+11
TTTCTAACACTacCAgattATtttg	TGGCCGAGTGG	42bp	GGTTCaAATCC	SER
YYTCAACAAATAAGT	TGGCNNAGTGG	33bp	GGTTCGANNCC	CONSENSUS

Figure 1: DNA Sequences and Spatial Relationships of the Internal Control Regions and 5'-Flanking Sequences of the tRNA Genes. Consensus sequences for the ICRs of eukaryotic tRNA genes are from Ciliberto et al. (2) and 5'-flanking sequence of *S. cerevisiae* tDNA is derived in reference 16. Bases which differ from the consensus sequences are in small case. The numbers represent the spacing between the A and B blocks of the ICRs. Complete sequences have been presented for the LEU3 mutants (14) and SERSUQ5 (23).

sequences (Fig. 1). The $tRNA^{Ser}$ gene has only a single divergent position, an A for G substitution, in the B-block. Also, except for the LEU3Δ20 mutant, the spacing of the A and B-blocks is much closer to the optimum in the serine inserting tRNA gene (7). For these reasons, one would expect that the $tRNA^{Ser}$ gene would be a better template for transcription by RNA polymerase III (1). However, in transcription reactions in which $tDNA^{Ser}$ and $tDNA^{Leu3}$ are added simultaneously, $tRNA^{Leu}$ accumulates as much as 3.3 (±0.19) times more rapidly than $tRNA^{Ser}$ (data not shown). Figure 1 indicates that the 5'-flanking sequence of the $tDNA^{Ser}$, even though it is A+T rich, conforms only weakly to the canonical 5'-flanking sequence associated with the genes coding for abundant tRNAs in *S. cerevisiae* (16). We have determined 5'-flanking sequence to -382 for $tDNA^{Leu3}$ and -110 for $tDNA^{Ser}$ (unpublished). Attempts to align these sequences with each other and with repetitive elements found in the vicinity of other yeast tRNA genes did not reveal any significant homologies(29-31). We have compared the template activity of the $tDNA^{Ser}$ gene with unmodified and mutant forms of $tDNA^{Leu3}$ to determine what aspect(s) of the transcription process is affected.

Determination of Apparent K_M and V_{MAX} Values

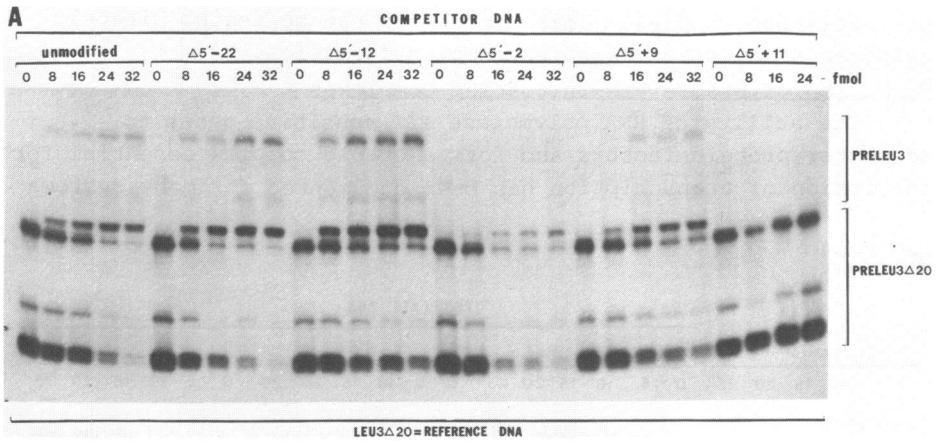
The concentrations of template DNA required to half-saturate the transcription reactions were determined by measuring the

TEMPLATE	APPARENT K_M(nM)	V_{MAX}(fmol/30 min)
UNMODIFIED	0.42(±0.05)	8.5(±5.0)
-22	1.02(±0.85)	8.9(±6.7)
-12	0.73(±0.41)	12.7(±8.8)
-2	0.99	0.69
+9	0.55	2.6
+11	ND	ND
SER	0.43(±0.26)	8.5(±2.7)
LEU3Δ20	0.44	13.3

Table I: Apparent K_M and V_{MAX} values for Various Template tDNAs. Reactions as described in Methods were started by addition of DNA to a premixed cocktail which included all other components. Total DNA in each reaction was kept constant by inclusion of pBR322. Reactions were incubated for 30 min. at 20° then terminated and processed as described in Methods. Apparent K_M and V_{Max} values are calculated from 2-5 separate determinations in which template concentrations were varied from 0.21 to 1.60 nM.

extent of transcript accumulation from each template as a function of gene concentration. The total amount of DNA was kept constant by addition of vector. Apparent K_M values were calculated from linear regression analysis of measurements made at template concentrations from 0.21-1.6nM. Significant variability in data from this type of assay was seen and has been noted previously (27). We have included only those experiments with r-values ≥0.9. Also, a significant inhibition of transcription was observed with the stronger templates at the highest DNA concentration used, therefore this point was omitted from all data sets. The data, tabulated in Table I, show that the templates all require similar concentrations for half-saturation of the system. Although the standard deviations are rather high, there is no significant increase in the apparent K_M values for the templates with alterations in either the 5'-flanking sequence or the first 9 bases of $tRNA^{Leu3}$. The +9 form substitutes a C for the highly conserved U8 in the A-block (Fig. 1) with no significant increase in K_M. The +11 template restores U8 but changes the -GG- at positions 9 and 10 to -TC- (Fig. 1) and diminishes the amount of transcription such that statistically meaningful data cannot be obtained.

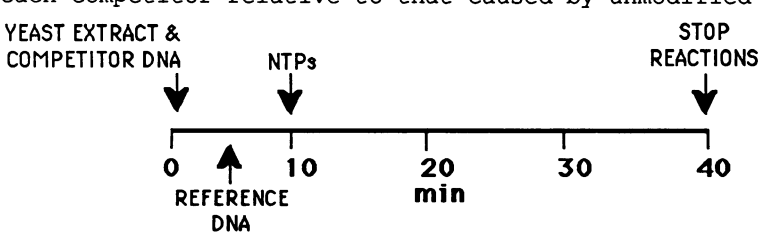
Calculations of V_{MAX} values from the same data show that there is at least a 10 fold drop resulting from deletion of the



B

RELATIVE INHIBITION OF	COMPETITOR unmodified	-22	-12	-2	+9	+11
LEU3Δ20	-100-	63	53	67	61	18
SER	75	57	51	54	56	1

Figure 2: Formation of Stable Pre-Initiation Complexes by 5'-Flanking Mutants. Stable complex formation was measured using a second template exclusion assay as described in Methods. Competitor DNA was varied from 0 to 1.3nM (final concentration) with pBR322 being added to maintain a constant amount of DNA during the first preincubation. After a 5 min preincubation, a reference template, either LEU3Δ20 or SER, was added to 0.9nM (final concentration) and incubation continued for an additional 5 min, at which time ribonucleoside triphosphates were added and transcription allowed to proceed for 30 min. The time line shown below is a diagrammatic representation of the reaction sequence. Panel A is an autoradiogram from an experiment using LEU3Δ20 as a reference template. Panel B is a summary of data from 3 such experiments with each of the reference templates. The numbers represent the per cent inhibition of the reference template by each competitor relative to that caused by unmodified $tDNA^{Leu3}$.

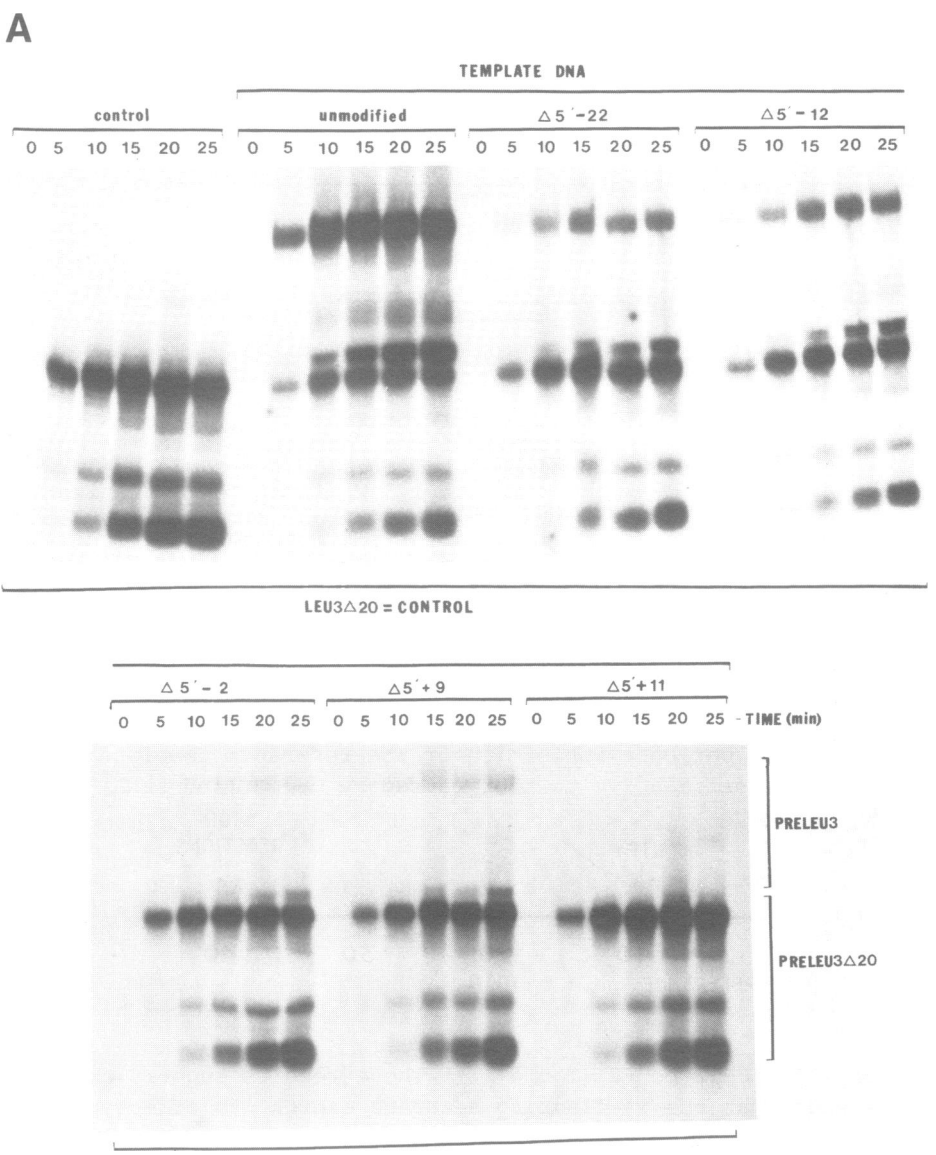


sequence from -12 to -2 (Table I). These results support the conclusion that the 5'-flanking sequence exerts its effect during transcription *per se* rather than in assembly of the

preinitiation complex. This hypothesis can be tested directly using an assay which measures complex formation.

Formation of Stable Pre-Initiation Complexes

The ability of RNA polymerase III sensitive genes to sequester protein factors and form a stable complex essential for initiation of transcription has been documented for many systems



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COMPETITOR	RELATIVE INITIAL RATES LEU3Δ20	COMPETITOR	RATIO
none	5.89	-	-
unmodified	2.60	5.56	2.14
-22	3.36	0.94	0.28
-12	2.84	1.14	0.40
-2	3.02	0.47	0.16
+9	3.39	0.67	0.20
+11	5.18	0.17	0.03
	SER	**COMPETITOR**	**RATIO**
none	5.86	-	-
unmodified	5.65	5.36	0.95
-22	5.94	1.65	0.28
-12	5.31	2.07	0.39
-2	4.83	0.43	0.09
+9	3.94	0.57	0.14
+11	5.00	0.38	0.08

Figure 3: Initial Rates of Transcription of 5'-Flanking Sequence Mutants. Transcription reactions were performed with mixtures of an internal control gene, either LEU3Δ20 or SER, and each of the various 5'-flanking sequence configurations. Reactions have equivalent mass amounts of each template added simultaneously to the complete transcription cocktail. Aliquots from each reaction were withdrawn at 5 min intervals and processed as described in Methods. Panel A is an autoradiogram of the transcription products from one series of experiments using LEU3Δ20 as reference template. Panel B is a summary of the ratios of the rates of $tRNA^{Leu}$ formation from the various 5'-flanking region mutants relative to that from a reference gene, either LEU3Δ20 or SER.

(1). We have used a second template exclusion assay (26-27) to determine whether the 5'-flanking sequence may affect the ability of yeast tRNA genes to form these pre-inititaion complexes. The order of addition of the various reaction components is shown on a timeline in the legend to Figure 2. Comparable experiments were done using either LEU3Δ20 or SER as the second (reference) template. The autoradiogram in Figure 2A depicts the products from one such reaction series. The data from three experiments is summarized in Figure 2B as the ratio of the slopes from linear regression analysis of inhibition of reference template transcription by each competitor DNA relative to that seen with unmodified $tDNA^{Leu3}$.

The results show that the competitive strength of the LEU3 gene is slightly greater when measured against the LEU3Δ20 reference than the SER. The effectiveness of the competitor gene is reduced by about one-third by altering the sequence upstream

of -22 when measured against either the LEU3Δ20 or SER (Fig.2B). Alterations between -22 and +9 do not alter the ability to inhibit transcription of either reference. As seen in K_M determinations, the +11 form has virtually completely lost the capacity to bind the factor(s) essential for RNA polymerase III activity.

Initial Rates of Transcription

The rate of accumulation of tRNA molecules from each of the templates was measured by mixing two tDNAs prior to adding them to a transcription cocktail. The reactions were done using equivalent mass amounts of each template, sufficient to saturate the transcription system. Transcripts from the template pairs are resolved by polyacrylamide gel electrophoresis (Fig.3A) and the radioactivity appearing as unspliced precursor RNA from each tDNA quantitated by liquid scintillation counting. Slopes taken from linear regression plots of the data points between 5 and 20 minutes of each reaction were used to determine initial rates of transcription (Fig.3B). Under the conditions used in these experiments, the accumulation of transcripts is linear during this time. Whether the LEU3Δ20 or SER gene is used as reference template, substitution of plasmid DNA for the normal 5'-flanking sequence reduces the rate of transcription of the $tDNA^{Leu3}$ genes. Removal of the 5'-flanking sequence from the unmodified gene, which has about 380 bp of yeast DNA upstream of the tRNA coding region, to -22 or -12 bp results in a 3-6 fold loss of activity (Fig.3B). Further removal to the -2 position reduces transcription an additional 2-4 fold. Continuing to +9 in the coding sequence has no further effect, while alterations of two additional bases causes another 2-5 fold loss of template activity.

A comparison of the competition between the two reference templates reveals a modest difference in the competitive strengths of the genes with 5'-flanking sequence intact when compared with the LEU3Δ20 or SER. Under these conditions, the rate of transcription of the -22 and -12 forms of $tDNA^{Leu3}$ is about 2 fold less with the cognate gene than in the presence of $tDNA^{Ser}$. It is also apparent from comparing the ability of the competitor to inhibit the signal from the reference template that all forms except the +11 gene significantly diminish the signal from the LEU3Δ20 while SER transcription is only slightly impaired by the $tDNA^{Leu}$ competitors.

DISCUSSION

We have previously shown that replacement of 5'-flanking sequence of $tDNA^{Leu3}$ with vector DNA reduces the template activity of the adjacent gene 8-10 fold. The effect is seen both *in vitro* and *in vivo* (14-16). We have now attempted to identify what aspect of the transcription process is affected by the 5'-flanking sequence. Apparent K_M values for the various forms of the genes were determined (Table I). The values found are in accord with those measured in a *Drosophila* cell free system using homologous $tRNA^{Ser}$ genes (24) and also with equilibrium binding constants measured for yeast SUP4-$tRNA^{Tyr}$ with partially purified TFIIIC (6). With the *caveat* that the standard deviations are rather large, the values presented in Table I indicate that replacement of normal 5'-flanking sequence and up to 8 bp of the coding region does not significantly alter the apparent affinity of the transcription apparatus for the DNA. We do,however, note a dramatic increase in apparent K_M when the 5'-proximal sequence of the A-block of the ICR is altered (Fig.1, +11). Baker et al.(6) have measured 2-5 fold reductions in TFIIIC binding to $tRNA^{Ser}$ or $tRNA^{Tyr}$ genes when the A-block sequences are altered.

Formation of a stable complex between a large protein, TFIIIC (C factor or tau), and tRNA genes is known to be an obligatory step in assembling an active transcription complex (1). This protein initiates stable complex formation by binding to the B and A blocks of the ICR. Footprint analyses of TFIIIC-DNA complexes using DNaseI often show protection in the 5'-flanking region of tDNA as well as the two ICR sequences (29). At least one additional factor, TFIIIB, is also required for a stable transcription complex (30). We have used a second template exclusion assay to measure the ability of the mutant genes to form stable complexes prior to initiation of transcription (Figure 2). In these experiments, the unmodified gene has 380 bp of 5'-flanking yeast sequence. Removal of all of this up to the conserved pentadecanucleotide reduces the ability of the DNA to form the stable pre-initiation complex by 1/3 to 1/2 (Fig 2B). Further changes including the conserved flanking sequence and the first 8 bp of the coding region do not have any additional effect. This result is similar to that observed with *Drosophila* and *Bombyx* genes where little (13) or no (19) effect on stable complex formation is seen when normal 5'-flanking sequences are replaced. A recent report indicates sequences

upstream of a human $tRNA^{Val}$ gene affect the extent but not the stability of complex formation (28). In the $tDNA^{Leu3}$ gene, replacement of the next two bases, -GG- by -TC-, in the A-block of the ICR reduces the ability to form the complex to a level unmeasurable in these experiments (Fig. 2A and B). Essentially identical results are seen with either reference gene. The $tRNA^{Ser}$ gene used was chosen because it does not have a 5'-flanking sequence which conforms to the consensus (16) nor does this allele have a copy of the repetitive element *sigma* found a fixed distance upstream of several different tRNA genes including other alleles of this serine isoacceptor (22,23,31). Sequence homology comparisons of the 5'-flanking regions of the LEU3 and SER genes with *delta* (32) and *tau* (33), repetitive elements suggested to have effects on template activity of tRNA genes (34), also fail to reveal any significant identities. Our results indicate that sequences upstream of the conserved region affect assembly of pre-inititation complexes at least with respect to the limiting component in this yeast nuclear extract. The pentadecanucleotide region does not have a measureable influence on the process.

The initial rate of transcription from the modified $tRNA^{Leu3}$ genes was also measured using either a $tRNA^{Leu3}$ derivative or a $tRNA^{Ser}$ gene as an internal standard. The ratio of transcripts produced from each template is altered in two steps (Fig. 3). Under these conditions, mutations which remove 5'-flanking sequence to positions -22 or -12 reduce the rate of transcription 3-6 fold. Effects of distal upstream sequence on tDNA transcription have also been noted in *Drosophila* and *Bombyx* extracts (13, 19). Additional removal to -2 or +9 results in a further 2-4 fold reduction. Experiments with either $tDNA^{Leu}$ or $tDNA^{Ser}$ controls show an overall 8-10 fold reduction in transcription rate resulting from substitution of 5'-flanking sequence. However, the $tDNA^{Leu}$ templates do not inhibit expression from the SER gene as they do the LEU3Δ20 reference, regardless of 5'-flanking sequence configuration. This suggests the possibility that some gene specific component(s) in this unfractioned system may be limiting in these reactions. Such an activity has recently been identified for the yeast ribosomal protein YL3 which can specifically stimulate yeast 5S RNA synthesis (35).

In summary, the template exclusion assays indicate that 5'-flanking sequence upstream of the conserved region affects formation of stable pre-initiation complexes, however, there is

no measurable affect of any upstream sequence on the apparent K_M of the tDNA in active transcription complexes. The conserved pentadecanucleotide influences the rate of RNA synthesis probably by altering the rate of transcription initiation.

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