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Point mutations in the 5' ICR and anticodon region of a *Drosophila* tRNA^{Arg} gene decrease *in vitro* transcription

Thomas S.Stewart[1]*, Dieter Söll[1] and Stephen Sharp[2]

[1]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511, and [2]Department of Microbiology and Molecular Genetics, California College of Medicine, University of California, Irvine, CA 92717, USA

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ABSTRACT

We have examined the effects of various nucleotide substitutions in a Drosophila tRNA^{Arg} gene on in vitro transcription and stable transcription complex formation in Drosophila KcO and HeLa cell extracts. Substitutions in positions encoding the invariant G18 and G19 residues resulted in decreased transcription, however, the moderate decreases indicate that these nucleotides are not obligatory promoter recognition sites. An A21 to C21 mutation had no effect on transcription levels using homologous extract however, this mutant displayed decreased transcriptional abilities in HeLa cell extract. Nucleotide substitutions within the sequence encoding the anticodon led to a decrease in the transcription activity but not in the ability to form a stable transcription complex.

INTRODUCTION

Transcription of nuclear eukaryotic tRNA genes by RNA polymerase III is controlled by two DNA sequences within the genes themselves. By the ability of deletion and substitution mutants of tRNA genes to be transcribed, these intragenic control regions (ICR's) were defined as occupying those sections of the gene that encode part of the D stem and loop and TψCG stem and loop (1-3). The 5' ICR (also called A-block or D-control region) was localized to positions 8 to 19 in Xenopus tRNA genes (1,2) and positions 8 to 25 in a Drosophila tRNA^{Arg} gene (3), more recently further delimited to positions 8 and 22 (4). The 3' ICR (also called B-block or T-control region) was found to occupy positions 52 to 62 for Xenopus tRNA genes (1,2) and positions 50-58 for the Drosophila tRNA^{Arg} gene (3).

Experimental evidence to date indicates that the ICRs of tRNA genes form recognition and binding sequences for RNA polymerase III gene-specific transcription factors (5-9). By comparing a large number of eukaryotic tRNA gene sequences, consensus sequences for the ICRs have been defined (1-3,10). However, these consensus sequences may not necessarily be part of the tRNA gene promoter but may reflect sequences important to tRNA structure and function (11). Allison et al. (12) have suggested an hierarchy of relative importance of each position within tDNA ICRs, that incur optimal activity for transcription factor interaction. A possible mechanism for such an hierarchy

comes from an analogy to studies of promoters in E. coli (13) wherein specific recognition of base pairs in double-stranded DNA by a protein involves the recognition of at least pairs of specifically located sets of hydrogen bond donors and acceptor groups in the major and minor grooves of the DNA double helix (14). The referred to hierarchy (12), and the closeness of any given ICR sequence to the respective ICR consensus sequence (1-3,10), may be a reflection of the necessary positioning of hydrogen bond donor and acceptor groups. One implication of this is that a nucleotide change at a position in one tDNA may not equal the effect of the same change in another tDNA sequence. More importantly, however, a nucleotide change at a given position, while changing the DNA sequence, may not dramatically affect tDNA transcription because the arrangement of the necessary base pair donor or acceptor groups may not in fact be changed. Direct evidence for this proposal comes from experiments wherein different nucleotide substitutions were made in the 3' ICR of the C. elegans $tDNA^{Pro}$. Different base substitutions made at the same position resulted in templates having different transcription levels (15). For example, there was a four-fold difference in in vitro transcription levels between a G54 and an A54 mutation. It therefore becomes important to investigate particular ICR "sequences." Use of the site-directed mutagenesis methodology makes possible a definitive analysis of tDNA promoter consensus sequences and the benefit of this methodology is that the transcriptional function of any given position can be tested by substituting in turn, each of the remaining three bases.

Point-mutations in the $tDNA^{Arg}$ were constructed using synthetic oligodeoxyribonucleotides as site-specific mutagens. The Drosophila DNA was cloned into M13 DNA. The single strand phage DNA was isolated and used as template for primer-directed second-strand synthesis. Similar methodology has previously been applied to the construction of a point mutation in an E. coli suppressor $tRNA^{Tyr}$ gene (16). Difficulties encountered in the earlier application of this methodology (16) have now been overcome; for example, under the conditions presented here, hypothetical secondary structure in a cloned single stranded tDNA, was not a problem in the primer-extended synthesis. Also the use of two oligonucleotide primers, instead of one, has been found to circumvent problems related to displacement or degradation, of the primer directing mutagenesis which in the past has resulted in low yields of synthesized double-stranded DNA containing the mismatched nucleotides (17,18).

Of relevance to this study is the function of the invariant nucleotides G18 and G19 in tRNAs and whether these nucleotides constitute a component of the 5' ICR. Applying the technique of site-directed in vitro mutagenesis (19) point mutations at G18 and G19, have been constructed individually and in the double mutant form, in a truncated Drosophila $tRNA^{Arg}$ gene. There is an invariant A residue in tRNAs at position 21 and it is possible that this residue represents the 3' boundary of the 5' ICR for the $tRNA^{Arg}$ gene

(4). A point mutation was constructed at this position and its transcription characteristics in Drosophila KcO and HeLa cell extracts were examined. Also, because tRNA genes differ in their anticodon sequences as well as in the presence or absence of introns and sequences encoding the variable arm, each of which affects the spacing of the two ICRs, it is of interest to be able to generate mutations within all stem and loop encoding regions. We have therefore also tested this method of mutation and subsequent transcription analysis, by generating two point mutations in the encoded anticodon sequence of the $tRNA^{Arg}$ gene.

MATERIALS AND METHODS

Templates for in vitro Mutagenesis -- In order to simplify identification of constructed mutants by sequence analysis, a 3' deletion mutant of the Drosophila $tRNA^{Arg}$ gene (pArg) was used to prepare the single stranded template. Construction of this mutant, in pBR322, by nuclease Bal 31 digestions, is described elsewhere (3). Originally pArg3.85 (3' deletion of the gene with the last remaining nucleotide being at position 85 with position 1 being the first nucleotide of the mature coding sequence) was used, however, in view of the reported influence of the 3' flank on stable complex formation in Drosophila KcO extracts (4), it was decided to use the deletion mutant, pArg3.106, to prepare single stranded template for later constructions. The Hind III/EcoRI fragments were recloned into M13mp9 and the single stranded templates isolated using alkaline sucrose gradients as the final purification step.

In vitro Mutagenesis and Mutant Isolation -- Originally the procedures described by Zoller and Smith (19) were adopted using the appropriate oligonucleotides purchased from Biologicals. It was later found more efficient to use the two-primer modification of these methods (20) in which the M13 universal primer is added to facilitate synthesis 5' to the annealed oligonucleotide directing mutagenesis (corresponding to the 3' end of the $tRNA^{Arg}$ gene). This eliminated the requirement for closed circle purification by sucrose density gradient centrifugation. Each of the oligonucleotides was synthesized so that the "mismatch" occurred in the middle of the oligomers. For the C21 construction a 12-mer was used. The C18, T19 and C18T19 constructions used 13-mers. The T33A35, pArg3.85(sup) construction used a 15-mer.

The 5' phosphorylated mutant oligonucleotide (2pmoles) and M13 universal primer (1pmole) under standard sequencing conditions (19) were annealed to the single stranded template (0.05pmoles) in the presence of 0.05M NaCl by slow cooling from 85°C to 14°C. A mixture of the dNTPs (0.5mM final), ATP (0.5mM final), with E. coli DNA polymerase I (Klenow fragment, 2 units), and T_4 DNA ligase (2 units) was added and incubation continued for 15 h at 14°C after which time the extension/ligation was stopped by the addition of EDTA to 10mM, and aliquots were used to transfect compe-

tent E. coli JM101 cells (20). Plaques containing phage with mutant sequences were selected by hybridization using the respective 5' ^{32}P-labeled synthetic oligonucleotides as probes. It was found unnecessary to use a selective washing procedure (6) to identify the required mutant, because of the higher signal intensity displayed by the mutant plaques after the low stringency wash. By use of the appropriate single-lane dideoxy sequencing procedure, the M13 phage carrying the required mutation could be identified. DNA purified from the appropriate plaque was sequenced by the dideoxy procedure (19) at least through position -100 into the 5' flank of the tRNA^{Arg} gene.

In vitro Transcription Studies -- All the mutant tRNA^{Arg} genes, constructed in M13mp9, were recloned (as HindIII/EcoRI fragments) into pBR322. All transcription and competition assays reported here, except where specifically indicated, are for the mutant tRNA^{Arg} genes in the pBR322 vector.

Transcription assays using either Drosophila KcO or HeLa cell extracts, were performed as previously described (21). The total DNA in each 40 µl reaction was maintained at 1.2 µg by the addition of pBR322 DNA to minimize non-specific inhibition of transcription (22). Competition assays, with 15 min preincubation of the various competitors, were used to assay formation of stable transcription complexes (17). Competition assays use nonlimiting concentrations of template DNA in cell-free extracts which as prepared, contain limiting concentrations of transcription factors. The reference template used was the maxigene pArg26x36 (23) whose primary transcript was not processed in either extract used in this study. pArg26x36 is essentially pArg having a polylinker sequence (EcoRI/XhoI/BamHI) inserted between positions 26 and 36, giving rise to a gene 12 nucleotides longer than pArg. After isolation of the transcription products by phenol extraction and ethanol precipitation, the products were separated by electrophoresis on thin 8% polyacrylamide gels containing 8.3M urea. Results were quantitated by excision of the gel pieces containing the products followed by Cerenkov counting.

RESULTS

Using either the pArg3.85 or pArg3.106 truncated Drosophila tRNA^{Arg} genes cloned into an M13 vector for single stranded template isolation, six mutant tDNAs were constructed with the nucleotide substitutions shown in Figure 1. The C18 and T19 mutations, and the double mutant C18T19, were prepared using pArg3.106 as the parent truncated gene.

The T19 mutation was obtained in both truncated genes and results for both are presented to establish that the observed changes in transcriptional efficiencies were caused by the mutation and not by the loss of the 3' flank. It has previously been established (4) that nucleotide position 106 delimits 3' flank functionality of the tRNA^{Arg} gene with respect to the competitive ability of the gene in Drosophila KcO extracts. For

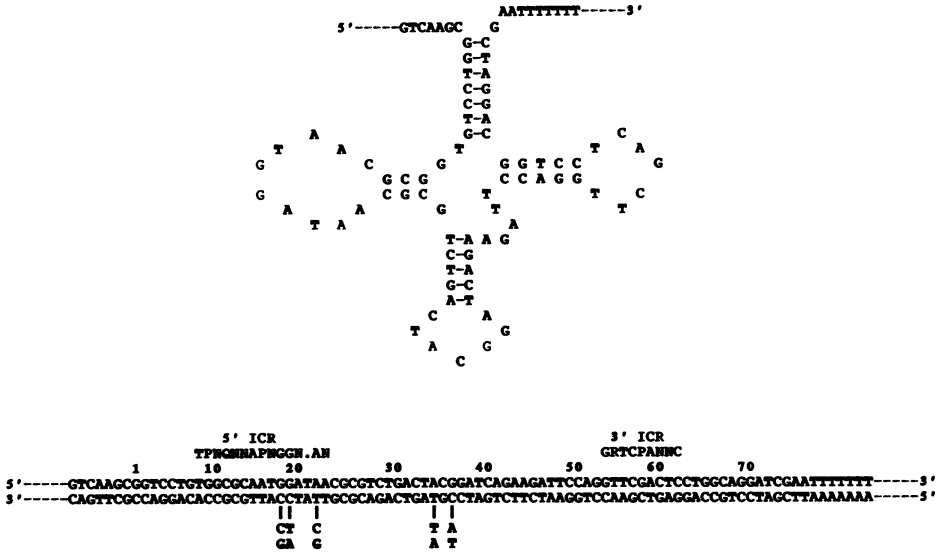

Figure 1. **Drosophila tDNA^{Arg} Gene** — The noncoding strand of Drosophila tDNA^{Arg} drawn in a tRNA like secondary structure with the 5' and 3' ICRs indicated (3,4). The positions of the mutations, constructed by the technique of in vitro mutagenesis, are shown in the linear representation of the gene. Mutants in which both G18 and G19 were replaced, individually and as the double mutant, were synthesized. The ICR consensus sequences are shown (1-3,10,15).

each mutation, two independent clones were isolated, initially in M13 mp9 then transferred to pBR322, and used in transcription analysis. In all cases, the duplicate clones gave identical results therefore analysis of only one example of each mutation is presented.

Transcription Studies using Drosophila KcO Extract — The mutant tDNAs were used to program transcription reactions using Drosophila KcO cell extract. The radioactive products were analyzed by polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 2A). The major product for each template is the precursor tRNA^{Arg} (ptRNA^{Arg}) which, in the case of the parent gene product is processed slowly to mature tRNA^{Arg} (via an intermediate product, probably the result of an RNase P-like cleavage). Processing of all mutant ptRNA^{Arg} products to mature tRNA^{Arg} was reduced, with the least amount of processing observed for those precursors generated from the gene containing the T19 mutation. This result and that for the C18 mutation are consistent with the proposal that altered tertiary structure in the precursor leads to a loss of recognition by the processing nucleases. However, the decreased processing of C21 and anticodon region (pArg3.85(sup) see below, Fig. 2A) mutants cannot be explained as easily by altered tertiary structure in the precursor.

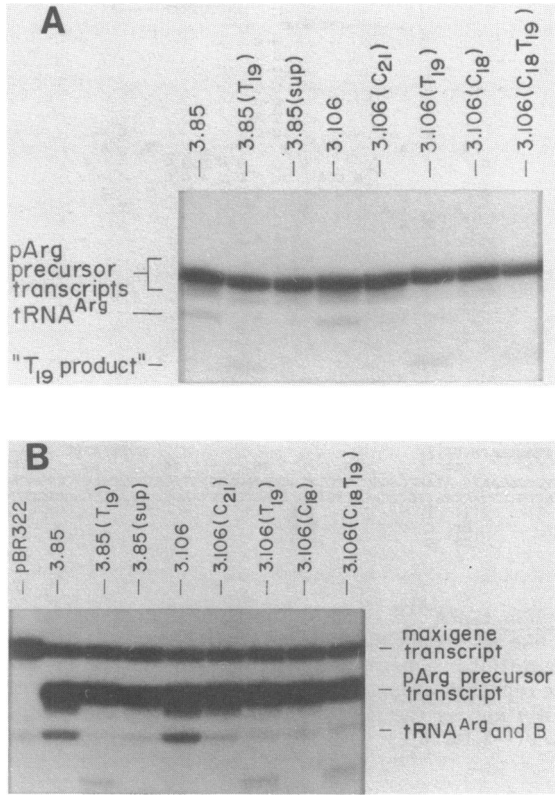

Figure 2. **Transcription (A) and Stable Complex Formation (B) of tDNA^{Arg} Mutants using Drosophila KcQ Cell Extract** -- Autoradiographs of polyacrylamide gel electrophoretic separation of ^{32}P-labelled RNAs synthesized from the respective tDNA^{Arg} mutants indicated above each lane. For the transcription assays (A), 0.6 µg of the mutant tDNA (as pBR322 clone) plus 0.6 µg of pBR322 DNA were added per reaction and transcription allowed to proceed for 90 minutes. For the stable complex assays (B), at time 0, 0.6 µg of competitor DNA, as noted above each lane, plus 0.3 µg pBR322 DNA were added to the incubation mix. At 15 min, 0.3 µg of the reference template (maxigene pArg26x36) was added and transcription continued for 90 minutes. Radioactive products were isolated and analyzed as described in Materials and Methods. tRNA^{Arg} represents mature-sized tRNA^{Arg} transcript produced from processing of the pArg precursor transcript; B is a transcription reaction background product resulting from in vitro guanylation of endogenous tRNA^{His} in cell-free extracts (26).

Except for the C21 mutant, all genes with mutations show decreased transcriptional efficiencies in the Drosophila cell-free system compared to the parent tDNA^{Arg} (see Table 1). The most notable decrease occurred for the double mutant C18T19 for which transcription dropped to 56% of parent levels (Table 1). The surprising result was that the pArg3.85(sup) mutant with nucleotide substitutions in positions encoding the

TABLE 1
RELATIVE TRANSCRIPTION AND COMPETITION ABILITIES[a]

	Drosophila KcO cell extract		HeLa cell extract	
GENE	Transcription[b]	Competition[c]	Transcription[b]	Competition[c]
3.85	102	90	100	98
3.85(T19)	86	92	80	94
3.85(sup)	75	90	84	99
3.106	100	100	100	100
3.106(C21)	100	102	86	95
3.106(T19)	82	96	87	93
3.106(C18)	77	94	97	98
3.106(C18T19)	56	82	80	87

(a) Each result represents the average of at least 3 experiments.
(b) Expressed as a percentage of wild-type gene (pArg) transcription.
(c) Amount of reference gene transcription in the presence of pBR322 DNA minus the amount of reference gene transcription in the presence of competitor DNA as a percentage of the amount of reference gene transcription in the presence of pBR322 DNA minus the amount of reference gene transcription in the presence of parent pArg3.106 truncated genes.

anticodon, T34A36, also gave significantly decreased transcription levels (75% of parent, Table 1). The C21 mutation had no effect on the level of transcription in the KcO cell free system. A KcO transcription product, estimated to be approximately 50 nucleotides in length, was observed for all mutants containing the T19 substitution (T19 product, Fig. 2A). The origins of this RNA were not investigated further but from pulse-chase experiment (see Fig. 4) "T19 product" does not appear to arise through processing of a large transcript.

The ability of each mutant $tRNA^{Arg}$ gene to form a stable transcription complex in KcO cell extract was examined by competition assays using the maxigene pArg26x36 as reference competitor. All mutant tDNAs were strong competitors compared to the pBR322 control. Quantitation of these levels confirmed the results of Schaack et al. (4) for the influence of the 3' flank on stable complex formation. All genes with deletions to position 85 gave higher levels of reference gene transcription (Fig. 2B) and thus were weaker competitors than those containing the additional 21 nucleotides of wild-type sequence in their 3' flank (pArg3.106). Except for the double mutant, C18T19, which was a weaker competitor, all the other mutations had little effect on competitive strengths as compared to the respective parent tDNA.

Transcription Using HeLa Cell Extract -- Transcription efficiencies (Fig. 3A) and competitive abilities (Fig. 3B) for the different mutants were determined also using HeLa cell extract. Similarities were evident in this heterologous system as in the KcO system; however, there were some notable differences. With the exception of the C18 mutant,

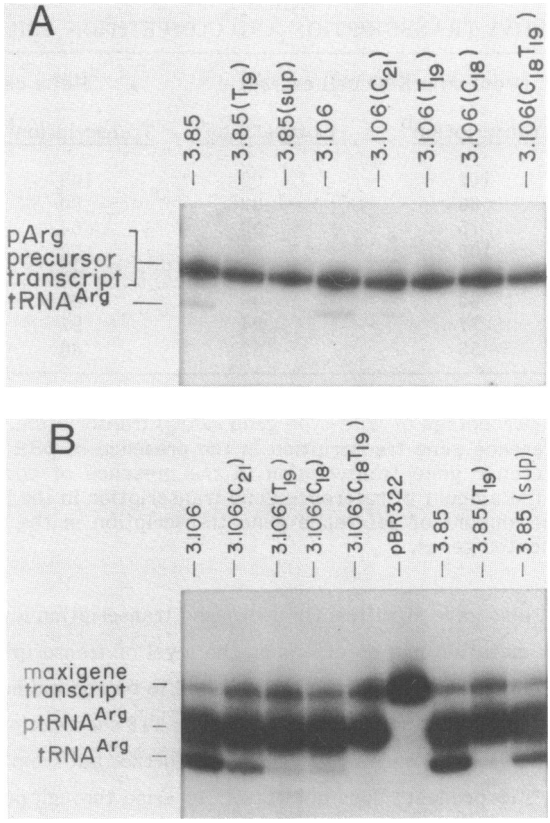

Figure 3. **(A) Transcription and (B) Stable Complex Formation of tDNA^{Arg} Mutants using HeLa Cell Extract** -- These experiments were performed as described in Fig. 2 except HeLa cell extract replaced the KcO extract. In the competition assays (B), the particular HeLa cell extract required only 0.05 µg competitor DNA to almost completely inhibit reference gene (0.3 µg) transcription.

all mutations led to decreased transcription levels, and again the double mutant showed the greatest decrease (Table 1). Although the C18 mutation did not affect transcription levels, it did amplify the effect of the T19 mutation (compare the levels of transcription for T19 and C18T19 mutations). The C21 mutation decreased the level of transcription in the HeLa cell extract whereas this mutation had no effect in the Drosophila KcO system.

Processing of the transcripts in the HeLa cell extract (Fig. 3A and 3B) follows the same pattern as in KcO extract. Reduced levels of processing are observed for the C21 and the anticodon mutations with barely detectable processing of transcripts containing

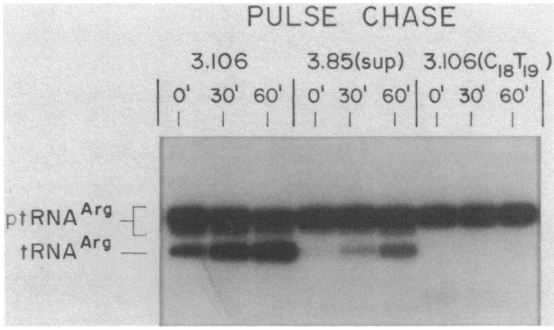

Figure 4. **Pulse Chase after Transcription of tDNA^{Arg} Templates using KcO Cell Extract** -- Transcription assays using tDNAs as noted above the lanes were allowed to proceed for 90 min. at which time nonradioactive GTP (to 2.5mM) and $MgCl_2$ (to 2.5mM) were added. Aliquots were removed at the time intervals indicated above the lanes and the radioactive products analyzed as described in Fig. 2.

nucleotide substitutions for G18 or G19 and no processing of the transcript from the C18T19 mutant.

All mutant tDNAs form stable transcription complexes efficiently as judged by the levels of reference gene (pArg26x36) transcription in the presence of either pBR322 DNA or the various mutant tDNAs (Fig. 3B). 3' flank dependence was not observed for the HeLa system, again confirming the observation of Schaack et al., (4). The T19 mutant showed a slight decrease in competitive ability whereas the double mutant displayed the lowest level of stable transcription complex formation. A product corresponding to the "T19 product" which was observed in the Drosophila system was not detected using HeLa cell extracts.

Stability of Transcripts -- In view of the decreased rates of processing of the mutant ptRNA^{Arg} transcription products, it was important to establish that the decreased levels of transcript production were the result of depressed transcription, and not due to preferential degradation. A pulse-chase experiment was performed for pArg3.106, pArg3.106 C18T19 and pArg3.85 (sup) in which a 25-fold excess non-labeled GTP (over α^{32}P-labeled GTP) was added to each reaction at 30 min (Drosophila KcO extract) and aliquots removed over the next 60 min; total radioactivity in the form of transcription products remained essentially constant over the 60 min following addition of non-radioactive GTP. Processing was only observed for the primary transcript of the mutant pArg3.85(sup) which had nucleotide substitutions in the anticodon (Fig. 4). The rate of processing of the pArg3.85(sup) transcript was reduced compared to the parent pArg3.106 processing rate. Processing of the C18T19 mutant transcript was not detected. The T19

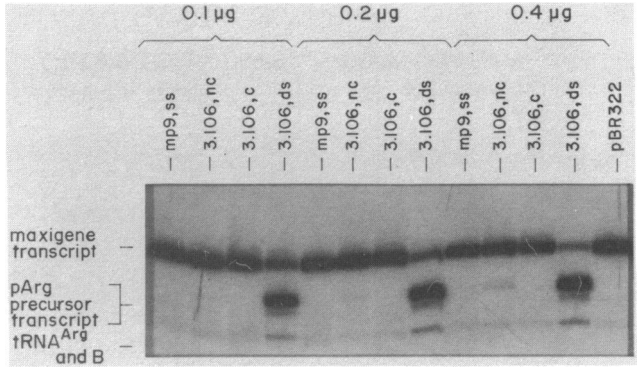

Figure 5. **Competition using Single Stranded Forms of the tDNA^{Arg} in KcO Cell Extract** -- The noncoding (nc) and coding (c) single-stranded tDNA were provided by the single-stranded phage form of the tRNA^{Arg} gene cloned into M13mp9 and M13mp10, respectively. The double-stranded (ds) form was the M13mp9 RF clone (rather than with pBR322). At time 0, the amounts of competitor DNA indicated above the lane were added, with the appropriate amount of pBR322 carrier DNA (1.2 µg 40 µl final DNA concentration), and, after 15 min., 0.3 µg of reference template (maxigene pArg26x36) was added and the incubation was continued for a further 90 min after maxigene addition.

product (Fig. 2A) was degraded in the chase experiment and therefore appears to be a primary transcription product and not a degradation product of the C18T19 transcript (Fig.4).

Competition Assays Using Single Stranded tDNA^{Arg} — To determine if either single strand of the tRNA^{Arg} gene could form a stable transcription complex and thus compete for limited transcription components in the Drosophila KcO extract, competition assays (using pArg26x36 as reference) were performed using different concentrations of either strand of the pArg3.106 truncated gene (in the form of the M13 mp9 and mp10 single stranded phage for the noncoding and coding strands respectively). Single stranded M13 mp9 was used as the control and pBR322 DNA as the example of a noncompetitor. As shown in Fig. 5, there was no competition by either the noncoding or the coding strands for transcription components, the levels of reference gene transcription being identical at each concentration of the respective single stranded DNAs (slight transcription inhibition for all higher concentrations of added single strand). Only the double-stranded form of the tRNA^{Arg} gene formed the transcription complex and sequestered limiting transcription components.

DISCUSSION

This study was initiated to test the feasibility of using oligonucleotide site-directed mutagenesis to generate a complete mutant array within the ICRs of a tRNA^{Arg} gene.

While this technique is proving successful in a growing number of instances, there have been technical considerations that impose limits on the ease and potential of generating many different mutations within a DNA sequence of interest. Without considering aspects of using M13 phage, briefly these limitations are: (i) possible displacement of the mismatched oligonucleotide during the elongation reaction; (ii) the inefficiency of the synthesized second strand in the ligation reaction; (iii) the subsequent need to isolate covalently closed circles from the reaction using alkaline sucrose gradients; and (iv) a low percentage of resultant phage contain the desired mutation, therefore necessitating a large number of phage DNAs to be screened. In the present study use of the two-primer method (20) was found to alleviate these problems, and because of the subsequent relative increase in the number of phage DNAs that contained the desired mutations, less phage needed to be screened and only one hybridization procedure was required to identify the "positive" DNAs.

We have substituted several bases in different positions in a Drosophila $tDNA^{Arg}$, by one other base. For the Drosophila transcription system, the 5' ICR of $tDNA^{Arg}$ does not appear to extend to position A21 since a C21 mutation had no effect on transcription levels or stable complex formation. Using deletion mutants, the extent of the 5' ICR in the $tRNA^{Arg}$ gene was recently mapped to within position 22 (4). While the transcriptional activity of this mutant tDNA was unaffected in homologous transcription reactions, its transcription levels were reduced using HeLa cell extracts. These templates may offer a means to help identify the nature of the observed differences in the transcription factors of the Drosophila and HeLa systems (9).

Results from the G18 and G19 nucleotide substitutions indicate that their invariant occurrence in tRNA sequences results from their requirement in tRNA function and not because of an obligatory promoter function. While these residues contribute to factor recognition, or factor binding to the 5' ICR, as assayed by competition analysis, a dramatic decrease in the level of transcription for the mutant tDNA having both these G residues substituted was not observed. This result is not consistent with an observed 10-fold decrease in in vitro transcription for a C19 substitution in a yeast $tRNA^{Leu}$ gene (27). Similarly, an A18A19 construction in the $tRNA^{Met}$ gene of X. laevis resulted in 30% transcription compared to parent gene levels in oocytes (27). However, an A19 mutation in a yeast suppressor $tRNA^{Tyr}$ gene had no effect on in vitro transcription using a yeast cell extract (12). These apparent inconsistencies may be explained by the "tolerance" level of the substituted base in the ICR in each transcription system, and therefore these results support the nucleotide hierarchy proposal (12).

From the experiments which defined tDNA ICRs (1-3,10), it was expected that transcription would be independent of the anticodon sequence. However, nucleotide substitutions in the $tDNA^{Arg}$ anticodon region, to produce a tDNA gene that would

encode a $tRNA_{UGA}^{Arg}$ affected the transcription abilities of this gene in KcO and HeLa cell extracts. Mutations in a $tRNA^{Met}$ gene in the region encoding the anticodon stem, which affects the hypothetical structure of this region, severely reduced transcription efficiencies (27). However, similar mutational changes in yeast $tDNA^{Leu}$ did not have the same effect (28). Two sets of nucleotides within the $tDNA^{Arg}$ anticodon loop can hypothetically form base pairs (C32G37 and T33A36) in the $tRNA_{UGA}^{Arg}$, and while this base-pairing, or at least the potential change in tRNA structure, may explain the reduced level of processing of the resulting transcript. A mechanism to explain why mutation in this region should decrease in vitro transcription, needs further consideration. A further point to note is that pArg3.85(sup) DNA directs stable complex formation almost at wild-type levels in HeLa and KcO cell extracts. That is, mutations within the anticodon region do not appear to affect the rate nor the binding itself, of factor to the 3' ICR. Since point mutations within the $tDNA^{Arg}$ 5' ICR decrease stable complex formation concomitant with a decrease in in vitro transcription ability, one implication is that mutations within the 5' ICR (and the 3' ICR) are affecting one transcription function whereas mutations within the anticodon region are affecting another separate transcription function. Studies of transcription factor binding to tRNA genes indicate that a single transcription factor interacts with the 5' ICR and 3' ICR (6-8). Since two transcription factors as well as RNA polymerase III are required for tRNA gene transcription (5), perhaps at least one function of the second transcription factor (TFIIIB) involves an interaction within the region encoding the anticodon. While the occurrence of such an interaction is speculative, it may explain the present results as well as the decreased in vitro transcription observed for a Xenopus $tRNA^{Met}$ gene which contained point mutations in the anticodon stem encoding region (27).

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*Present address: School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, 2033 N.S.W., Australia

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