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The minimum intragenic sequences required for promotion of eukaryotic tRNA gene transcription

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ABSTRACT

Transcription of eukaryotic tRNA genes is controlled by two intragenic regions, the D-control region (which in the tRNA codes for the D-stem and -loop) and the T-control region (which in the tRNA codes for the TΨC loop). To determine whether these sequences alone are sufficient to promote tRNA gene transcription *in vitro*, the two control regions of a *Drosophilia* tRNA^{Arg} gene were cloned separately from the context of the parental DNA (these constructions are called tRNA minigenes). The tRNA minigene that contains both intragenic control regions supports *in vitro* RNA synthesis in *Xenopus laevis* oocyte and HeLa cell transcription systems. The mutant which has deletions to nucleotide 7 within the mature tRNA coding region, pArg5.7, and minigenes derived from it do not support RNA synthesis in a *Drosophilia* Kc cell transcription system. *Xenopus* and Hela extracts transcribe pArg5.7 albeit at reduced levels compared to the wild-type gene. The tRNA minigene that contained only the D-control region was not able to support RNA synthesis in any of these three transcription systems. A mutant tRNA gene comprising the 3' half of the tRNA^{Arg} gene similarly was not able to support RNA synthesis.

These experiments show that the DNA sequence from nucleotides 7-58, which contains both intragenic control regions of the tRNA gene, possesses sufficient information to initiate specific transcription by RNA polymerase III in *Xenopus* and HeLa systems. The transcription efficiency of this tRNA minigene however is reduced to about 20% the transcription level of the wild type tRNA gene. This lowered level of transcriptional efficiency results from deleting the ends of the native tRNA gene and its adjacent flanking sequences. The affects of deleting 5' sequences are most pronounced in the *Drosophilia* transcription system.

INTRODUCTION

Intragenic control regions appear to be a general feature of genes transcribed by RNA polymerase III and have been identified in 5S RNA, tRNA, adenovirus virus-associated (VA) RNA and human Alu family genes (1-9). The boundaries of the internal control regions in tRNA genes were determined qualitatively by the ability of series of 5' and 3' deletion mutants to support RNA synthesis *in vitro* (3-6). In this way tRNA gene transcription control sequences were found to be the regions that code for the D-stem

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and D-loop (D-control region) and the T-stem and T-loop (T-control region) (3-6).

The 5' and 3' deletion mutant tRNA genes, which were used in the analysis of the intragenic control region of a *Drosophila* $tRNA^{Arg}$ gene (4), retain one or the other end of the gene and its adjacent flanking sequence. These additional tRNA gene sequences (now deleted and replaced by foreign DNA) may supplement the transcriptional function of the intragenic control regions. To determine whether the intragenic control regions alone are sufficient to direct transcription initiation, we have cloned tRNA gene residues 7-26 and also 7-58 without any additional tRNA gene sequences. These sub-clones are referred to as tRNA minigenes and each was examined for its ability to support RNA synthesis *in vitro*. These regions were selected for testing since the 5' deletion mutant pArg5.7, which has a deletion up to position 7 within the mature tRNA coding sequence can support *in vitro* transcription. Similarly the 3' deletion mutants pArg3.26 and pArg3.58, which have deletions up to positions 26 and 58 respectively can support RNA synthesis in some *in vitro* transcription systems (4).

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs, Beverly, MA; *E. coli* DNA polymerase (Klenow fragment) and proteinase K from Boehringer, Mannheim; nuclease BAL-31 from BRL Inc., Gaithersburg, MD; T4 polynucleotide kinase and S1 nuclease from P-L Biochemicals, Milwaukee, WI; RNase T2 and pancreatic RNase A from Sigma, St. Louis, MO; molecular recombination linkers EcoRI (GGAATTCC) and BamHI (CCGGATCCGG) from Collaborative Research, Waltham, MA. [α-^{32}P] Ribonucleoside triphosphates were obtained from Amersham, Amersham, U.K. [γ-^{32}P] ATP was prepared by the procedure of Walseth and Johnson (10).

Construction of $tRNA^{Arg}$ Gene Deletion Mutants pArg5.7 and pArg5.36. The plasmid pArg (pYH48) consists of a 508 bp HindIII *Drosophila* DNA fragment carrying the gene for the major $tRNA^{Arg}$ species inserted into the HindIII site of pBR322. To construct 5' deletion mutations, covalently closed circular pArg DNA (50 µg) was linearized using BamHI, the mixture was phenol extracted and DNA collected by ethanol precipitation. The DNA was digested in a 400 µl reaction with BAL-31 (9 units) (11). After incubation at 30°C, aliquots (80 µl) were removed at 4, 6, 8, 10 and 12 min and sequentially added to a mixture (400 µl) of 88% (v/v) phenol saturated with

0.3 M sodium acetate (pH 7.0), 10mM EDTA. After phenol and chloroform extraction, the DNA was collected by ethanol precipitation and subjected to fill-in synthesis using E. coli DNA polymerase (Klenow fragment). The resulting DNA was ligated to 20-fold molar excess of polynucleotide kinase-[32P]-phosphorylated BamHI linker and then redigested using BamHI. Inserts were liberated from the original vector using EcoRI then fractionated by polyacrylamide gel electrophoresis (12). Selected size classes of BAL-31-digested DNA were ligated into the EcoRI/BamHI sites of pBR322 and transformed into E. coli strain HB101.

Construction of $tRNA^{Arg}$ Gene Deletion Mutants pArg3.58 and pArg3.26. To construct 3' deletion mutations, 35 µg pArg was linearized using EcoRI and subsequently digested at 30°C (final volume of 200 µl) with 5 units of BAL-31 for 1, 2, 3, 4 and 5 min. Samples were treated as described above except EcoRI linker was used and the deletion fragments were liberated by subsequent digestion with BamHI.

Deletion mutations were selected with respect to insert size by restriction enzyme analysis of DNA prepared from mini-lysates. All deletion tDNAs were completely characterized by DNA sequence analysis (13). The nomenclature of 5' and 3' deletion mutations is as follows: 5' deletion clones are described as pArg5. and 3' deletion clones as pArg3.; the second number in the designation (e.g. pArg5.7) indicates the extent of deletion, the number being the first (5' deletion) or the last (3' deletion) nucleotide that remains from the wild type Drosophila tRNAArg sequence.

Construction of $tRNA^{Arg}$ Minigenes. Approximately 5 µg of the 5' deletion template pArg5.7 was digested using BamHI then labeled using [γ-32P] ATP and T4 polynucleotide kinase. The plasmid was digested using HhaI and the labeled BamHI/HhaI fragment of the Drosophila tRNA gene was purified by polyacrylamide gel electrophoresis (12). This fragment will represent the 5' terminus of the minigenes. EcoRI/HhaI Drosophila tRNA gene fragments were similarly isolated from each of the 3' deletion templates pArg3.26 and pArg3.58. These two fragments respectively will represent the 3' terminus of a short minigene and a long minigene. The gene 5' fragment was ligated to either of the gene 3' fragments through their HhaI sites and each of the newly formed gene fragments were ligated into the BamHI/EcoRI sites of pBR322 and transformed into E. coli host HB101.

In Vitro Transcription of tRNA Genes and Analysis of RNA Products. Covalently closed circular plasmid DNA was transcribed in extracts prepared from X. laevis oocytes (14), HeLa cells (15), and Drosophila Kc cells (16).

The standard in vitro reaction (40 µl) contained 0.3 µg of DNA, 20 µl cell extract and 2 µCi [α-^{32}P]-GTP in a buffer containing 30 mM Hepes-KOH, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$, 8 mM creatine phosphate, 6 units/ml creatine phosphokinase, 100 µM GTP and 500 µM each of ATP, CTP and UTP. Reactions were incubated at 25°C for 90 min; 40 µl of a solution (preincubated at 37°C for 30 min) containing 1 mg/ml proteinase K, 10 mM Tris-HCl, pH 8.0, 0.1% (w/v) sodium dodecyl sulphate was added and the mixture incubated at 37°C for 15-20 min. After adding 120 µl 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, the solution was phenol extracted and RNA recovered by ethanol precipitation. The dried RNA sample was resuspended in 2 µl of electrophoresis loading buffer (80% formamide, 2 mM EDTA, 0.3% xylene cyanol FF and 0.3% bromophenol blue). RNA transcripts were identified by gel electrophoresis using 8% polyacrylamide, 8.3 M urea thin-gels.

To determine 5' terminus nucleotides of primary transcripts transcription reactions were performed in the presence of either [α-^{32}P]-GTP, -UTP, -CTP or -ATP. After electrophoresis of RNA transcripts the autoradiograph was used to guide excision of radioactive bands. RNAs were eluted by soaking the gel piece (approximately 0.5 cm x 0.5 cm) overnight at 37°C in 200 µl of gel elution buffer (10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1 mM EDTA, 1% phenol); 5 µg carrier tRNA (E. coli) was added and after ethanol precipitation RNA was resuspended in 5-10 µl 20 mM ammonium acetate, pH 4.5 and digested using 0.5 units RNase T2 and 0.1 µg pancreatic RNase at 37°C for 2-3 hours. Nucleoside 5'-triphosphates 3'-monophosphates (primary transcript 5' termini) were identified by thin layer chromatography on PEI-cellulose plates (Macherey-Nagel, Polygram CEL 300 PEI) in 0.75 M potassium phosphate, pH 3.5. Unlabeled guanosine 5'-tetraphosphate, adenosine 5'-tetraphosphate, GTP and ATP were used as markers and located by ultraviolet absorption.

S1 Nuclease Protection Mapping. Transcripts formed from transcription of pArg were hybridized to 5' ^{32}P labeled HinfI/HinfI fragment (938 bp) terminating at the HinfI site within the gene at position 48, and extending several hundred nucleotides upstream of the gene. The same HinfI site was used to generate a suitable 5' labeled fragment from the DNA of pArg5.7. The DNA of pArg3.58 was 5' labeled at the recombinant-generated EcoRI site (position 64); a suitable fragment for S1 nuclease mapping was generated after cleavage with BamHI. Mapping experiments were performed using slight modifications to the usual procedure (17,18). Labeled DNA (100-200ng) and RNA transcript combined from two transcription reactions were each resus-

pended in 5 µl hybridization buffer (80% formamide, 0.4 M NaCl, 0.04 M Pipes, pH 6.8, 1 mM EDTA). DNA was denatured at 100°C for 5 min and RNA at 80°C for 90 seconds. After denaturation DNA and RNA samples were immediately added together and heated at 80° C for 1 min. Hybridization was performed for 15 hours at 42°C. The hybridization mixture was then diluted using 10 volumes of 60 mM sodium acetate, pH 4.6, 100 mM NaCl, 2 mM $ZnCl_2$ and S1 nuclease was added to a concentration of 1500 units/ml. Digestion was performed at 25°C for 60 min then 0°C for 30 min. The reaction was subsequently phenol extracted and DNA recovered by ethanol precipitation. Protected DNA was characterized on an 8% polyacrylamide thin-gel using Maxam-Gilbert sequencing reactions as size markers.

RESULTS

Construction of tRNA Genes that Contain Only the Intragenic Control Regions. To determine whether the intragenic control regions alone were sufficient to promote transcription truncated native tRNA genes (minigenes) were required in which the ends of the tRNA gene and adjacent flanking sequences were replaced by plasmid DNA. The construction of two tRNAArg minigenes is outlined in Fig. 1.

The DNA sequences of the parental tRNAArg gene, the derived deletion mutants and the resultant minigenes is presented in Figure 2. The shorter minigene, pArg7/26, only contains the sequence that codes for the D-stem and -loop of the mature tRNAArg (D-control region). The longer minigene, pArg7/58, contains the sequences that code for the D-stem and -loop, the anticodon-stem and -loop and the 5' half of the T-stem and -loop (T-control region). Potential initiation and termination sequences for the tRNA minigenes are provided by the adjoining pBR322 sequences (4).

The Intragenic Control Regions Direct Limited tRNA Synthesis. The ability of DNA from pArg7/26 and pArg7/58 to support RNA synthesis was examined in extracts derived from Xenopus whole oocytes (see Fig. 3), HeLa, and Drosophila Kc cells and compared to that of the DNA from the parental clones. Since the normal termination site of pArg in the cloned pArg3.26 and pArg3.58 has been replaced by a new one located approximately 40 bp from the tDNA/ pBR322 fusion site, the primary transcript of the pArg3.26 DNA (oocyte and Drosophila systems) is 73 nucleotides long, while that of pArg3.58 DNA is even longer (105 nucleotides) (Fig. 3). The short minigene pArg7/26 is not transcribed while the minigene pArg7/58 gives rise to a 105 nucleotide long RNA in oocyte and HeLa extracts.

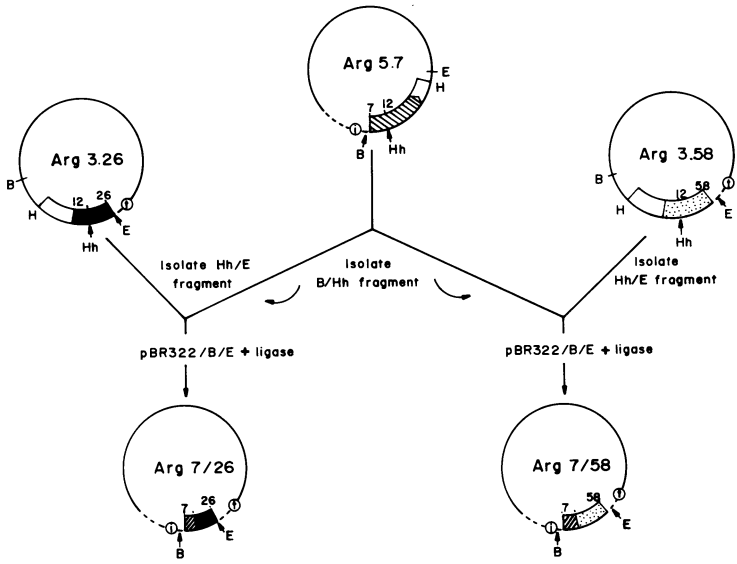

Figure 1. Scheme for the Construction of Drosophila Arg tRNA Minigenes. The plasmids Arg5.7, Arg3.26 and Arg3.58 are 5'- or 3'-deletion clones. DNA fragments were isolated from these templates and ligated together and to vector DNA as shown. The Drosophila DNA insert contained within pBR322 (narrow line) is indicated by the boxed region. The dashed line represents the area in which the BAL-31 generated deletions were made. The orientation and position of the tRNA gene is indicated by the broad arrow or I represents potential initiation sites and t represents a potential termination sequence. Shading has been used to indicate the fragments that have been ligated together. B, BamH1; E, EcoR1; Hh, Hha1; H, HindIII; pBR322/B/E, the isolated large fragment of pBR322 double digested with BamH1 and EcoR1.

In the oocyte transcription system the primary transcript of pArg initiates with G coded for at position -7. However in the Drosophila and HeLa systems transcription initiates at -7(G) as well as -4(A). Figure 4 demonstrates this result obtained using transcripts from the HeLa system. The deletion template pArg 3.58, which is transcribed in the three transcription systems, maintains the respective initiation start sites of the wild type pArg primary transcripts. (Similarly, the deletion template pArg3.26, which is transcribed in only the oocyte and Drosophila systems, maintains the respective wild type gene start sites). In the template

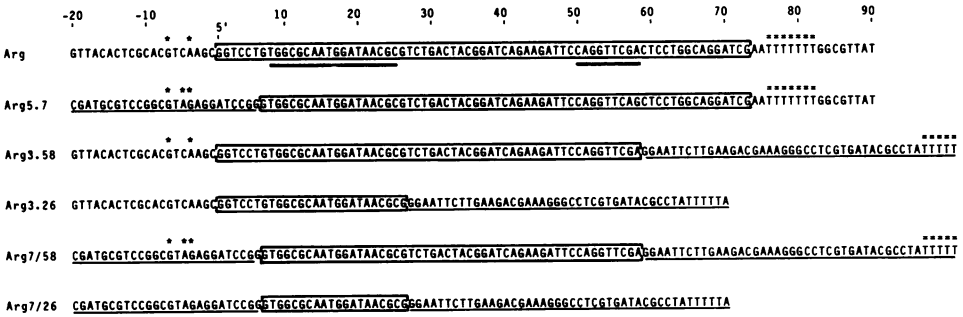

Figure 2. DNA Sequences of the Non-coding Strand of Wild-Type and Mutant Arg tRNA Genes. The bold underline indicates the two intragenic control regions. The boxed sequences indicate the mature Arg tRNA coding region and the sections of this sequence that have been subcloned. pBR322 and linker sequences are underlined. The * indicates transcription start sites and = indicates transcription termination sequences.

pArg5.7 the native pArg 5' flanking sequences are deleted. Therefore the initiation start sites for this mutant gene are selected from within a different 5' flanking sequence than that for the wild type gene as well as for the 3' deletion templates. pArg5.7 DNA can be transcribed only in the oocyte and HeLa extracts. In the oocyte extract the start site is -7(G) whereas in the HeLa system the transcripts start at positions corresponding to coordinates -4(A), -5(G) and -7(G). The RNA initiation sites (from HeLa cell experiments) are summarized in Figure 2.

Transcription Efficiencies of Mutant tDNAs in Homologous and Heterologous Extracts. It is conceivable that the transcription of the mutant tRNA genes may be different dependent on the use of homologous or heterologous extracts. For this reason the transcriptional activities of the minigenes were tested in cell-free extracts derived from Xenopus oocytes, Drosophila Kc cells and HeLa cells. In order to compare quantitatively the template efficiency of the various tRNA genes, we measured the formation of RNA transcripts/gene/hour in each cell-free extract (Table 1).

The DNA of clones pArg5.7, pArg 3.58 and pArg3.26 were transcribed in the Xenopus extract with a reduced efficiency compared to that of pArg. The pArg7/58 mutant while supporting RNA synthesis the level was lower than that for either pArg5.7 or pArg3.58 (Table 1). The minigene pArg7/26 did not support RNA synthesis in any extract. The results obtained in the Drosophila extract show that the native 5' flanking sequences are crucial for transcription in this system since pArg5.7 and both minigenes derived

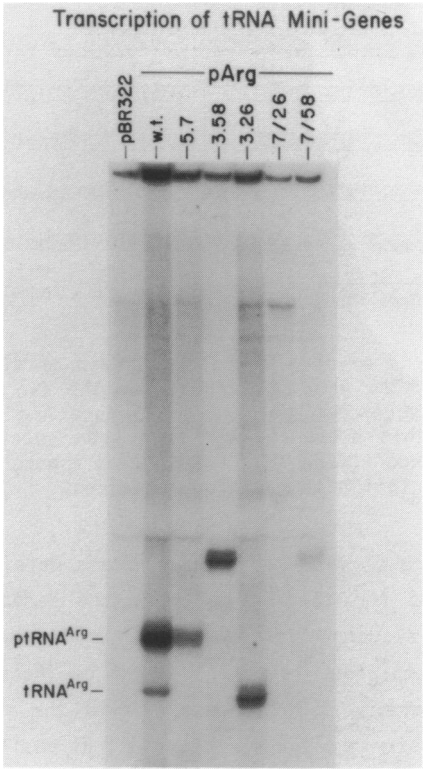

Figure 3. Transcription of tRNA Mini-Genes. Autoradiograph of a polyacrylamide gel electrophoretic separation of ^{32}P-labeled RNAs which have been synthesized in an X. laevis oocyte extract programmed with either a plasmid containing a wild type Arg tRNA gene or plasmids lacking portions of the wild type Arg tRNA sequence. pArg tRNA corresponds to precursor Arg tRNA which is 84-86 nucleotides in length and Arg tRNA is mature sized Arg tRNA which is 73 nucleotides long.

from it were not transcribed (Table 1). In the Drosophila extract the amount of transcription of pArg3.26 DNA is very low; even the transcription level of pArg3.58 is reduced compared to that of the wild type gene. In the HeLa cell extract pArg5.7 and pArg3.58 were transcribed relatively efficiently and the derived minigene pArg7/58 also supported RNA synthesis (Table 1). The mutants pArg3.26 and pArg7/26 did not support transcription in the HeLa extract. In all extracts the 5' deletion mutant pArg5.36, which contains only the T-control region, does not support RNA synthesis (Table 1).

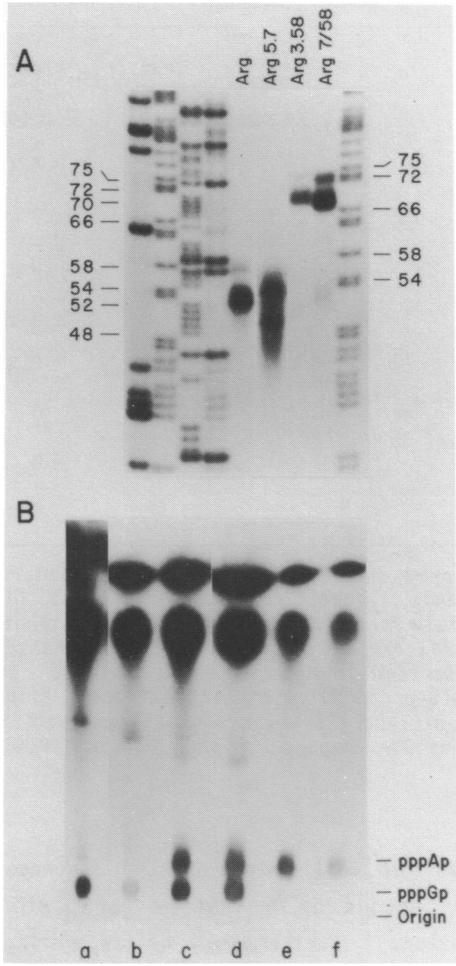

Figure 4. 5'-End Analysis of RNA transcripts. A. S1 nuclease protection mapping: The Maxam and Gilbert sequencing reactions used as size markers are of the EcoRI/BamH1 insert liberated from pArg DNA and 5' labeled at the EcoRI site. The DNA protection fragments for pArg and pArg5.7 are labeled at the nucleotide corresponding to position 48 within the mature tRNA sequence; those for pArg3.58 and pArg7/58 are labeled at the nucleotide corresponding to position 64. B. 5'-end pppNp's were determined as described in Materials and Methods. a-d, analysis of transcripts formed in the presence of [α-^{32}P]-GTP; e & f, analysis of transcripts formed in the presence of [α-^{32}P]-ATP. The primary transcripts were formed from a & e, pArg; b & f, pArg3.58; c, pArg5.7; d, pArg7/58.

Table 1. Template Efficiencies of Mutant tRNA^{Arg} Genes

tDNA	Transcripts/gene/hour[a]		
	Xenopus oocyte	Drosophila Kc	HeLa
pArg	4	6 (6,300 cpm)	6
pArg5.7	1.7	0	4
pArg5.36	0	0	0
pArg3.58	2.5	3	2.4
pArg3.26	2.5	0.2	0
pArg7/26	0	0	0
pArg7/58	0.8	0	1.5

[a] Determined by Cerenkov counting of excised RNA bands, using a counting efficiency of 40% and a specific activity of 0.5 Ci/mmole [^{32}P]GTP. Transcript RNAs contain the following GMP composition: ptRNA^{Arg}, 25; Arg5.7, 27; Arg3.58, 31; Arg3.26, 23; Arg7/58, 33. Transcription reactions were performed as described in Materials and Methods. These results were obtained using a single preparation of each extract. Although variation in efficiencies between different preparations is observed the relative efficiencies of each mutant DNA compared to pArg is maintained.

DISCUSSION

We have examined the ability of the DNA sequences comprising the intragenic control regions of a tRNA^{Arg} gene, to direct specific transcription initiation. Furthermore, by separating the intragenic control regions from all other Drosophila DNA sequences (tRNA minigenes) the contribution to transcription activity of sequences adjacent to the control regions were determined. While the wild type tRNA^{Arg} gene supported efficient RNA synthesis in extracts derived from Drosophila Kc cells, Xenopus oocytes and HeLa cells, mutant forms of this gene displayed different transcription capabilities in each of the systems.

The transcriptional analysis of the two tRNA^{Arg} minigene DNAs revealed that the DNA sequence from nucleotides 7-58 (pArg7/58), which contains both intragenic control regions of the tRNA gene, directs specific initiation of transcription in the Xenopus oocyte and HeLa systems. However pArg7/58 does not contain sufficient information to initiate specific transcription by RNA polymerase III in an homologous Drosophila Kc cell

transcription system. The inability of the Drosophila extract to transcribe pArg7/58 is not due to an unavailability within the 5' adjacent sequence of a potential site for initiation. The tRNA genes and mutant genes that transcribed in the Drosophila extract displayed the same selection in the initiation nucleotides as the HeLa extract. Thus the inability of the Drosophila extract to transcribe pArg7/58 resides in the removal of the Drosophila DNA sequence 5' and 3' adjacent to the region defined by nucleotides 7-58.

Importantly, although pArg7/58 was transcribed in both the Xenopus and HeLa transcription systems, the level of transcription attained was far less than that of the wild type $tRNA^{Arg}$ gene (see Table 1). Since the deletion mutant tRNA genes used to construct the pArg7/58 minigene directed specific initiation in both systems, the reduction in transcription efficiency does not appear to be due to an inability to select an initiation site. As in the Drosophila transcription systems the Xenopus and HeLa systems are also affected by the collective removal of sequences 5' and 3' adjacent to the 7-58 coding region.

The importance of the 5' adjacent sequences are emphasized by the failure of the pArg7/26 minigene to direct transcription in all extracts. The deletion mutant pArg3.26 used to construct pArg7/26, directs specific initiation of transcription in the Xenopus and Drosophila systems. The failure of pArg7/26 as a transcription template reflects the importance of the sequence 5' adjacent to the D-control region (nucleotides 8-25; ref. 4).

Previously we had observed transcription of 5' deletion mutant $tRNA^{Arg}$ genes in Drosophila extracts to a level approximately 5-10% of the level of wild type $tRNA^{Arg}$ gene transcription (4). Subsequent deletion of 5' sequences up to and including nucleotide at position 8 in the mature tRNA coding region, resulted in DNAs no longer able to support RNA synthesis in Xenopus, HeLa and Drosophila transcription systems (4). In the present study the Drosophila transcription system failed to transcribe a mutant $tRNA^{Arg}$ gene which had sequences deleted 5' adjacent to the D-control region (pArg5.7). Compared to the transcriptional levels of the wild type $tRNA^{Arg}$ gene pArg5.7 transcription in Xenopus and HeLa systems, was also reduced. Therefore it seems that the inability of the Drosophila system to transcribe the 5' deletion mutant, pArg5.7, is not necessarily characteristic of the use of homologous versus heterologous systems as has been described for a Bombyx mori homologous transcription

system (19). Instead, the lesser ability of the HeLa and Xenopus systems and the variability in the Drosophila extract, to transcribe tRNA genes with altered 5' flanking sequences, appears to be a more general characteristic of in vitro transcription.

Although the 5' flanking sequences and the region coding for the 5' arm of the acceptor stem of the tRNA do not contain sequences "essential" for in vitro transcription these regions have been noted to be important in the transcription of a eukaryotic tRNA gene (18,21). A comparison of the transcription efficiencies of pArg, pArg3.58 and pArg7/58 shows that the mechanism for the effect of the 5' regions on transcription is brought about by the presence of not only the T-control region but also sequences 3' of position 58. This region includes the sequences that in the tRNA code for the 3' half of the T-loop and -stem and the 3' arm of the amino acid acceptor stem. In the gene sequence this region would also include the transcription termination sequence.

The in vitro analyses have clearly defined the function of two intragenic control elements within a eukaryotic tRNA gene (3-6). It is conceivable that these elements (promoter consensus sequences; 3-6) function as recognition sequences for tRNA gene transcription factor(s). The actual binding of these factor(s) within a tRNA gene although directed by the promoter consensus sequences might be influenced by interacting with sequences throughout the entire tRNA gene sequence. From the present studies these sequences would include those coding for the 5' and 3' arms of the tRNA acceptor stem as well as 5' and 3' flanking regions. A direct interaction between the 5' and 3' arms of the acceptor stem coding regions during transcription, does not seem likely from the results of previous studies (3-6) and this study.

The transcription activities of more eukaryotic tRNA genes need to be determined. Knowledge of the nucleotides within a specific sequence context that contribute to tRNA gene promoter strength may explain differences observed in the transcription abilities of different resected tRNA genes. The separated halves of the Xenopus initiator $tRNA^{Met}$ (3,22) and $tRNA^{Leu}$ (5) genes do not support RNA synthesis in Xenopus transcription systems in vivo or in vitro. The 5' control region (e.g. pArg3.26) of the Drosophila $tRNA^{Arg}$ gene is transcribed in the Xenopus and Drosophila systems in vitro. Therefore the inability of pArg7/26 to support RNA synthesis in the Xenopus system suggests that the 5' flanking and 5' stem regions of the $tRNA^{Arg}$ gene permits the 5' element of the pArg gene to have a stronger

promoter than the corresponding regions of the Xenopus initiator $tRNA^{Met}$ and $tRNA^{Leu}$ genes. As a comparison only it is worthwhile to note that the Drosophila initiator $tRNA^{Met}$ gene (20) has a transcription efficiency approximately 10% that of pArg in the Drosophila transcription system (unpublished results). This transcriptional level is equal to the level of pArg7/58 transcription in the Xenopus oocyte extract. This observation is consistent with the failure of a Drosophila initiator $tRNA^{Met}$ pseudogene to support RNA synthesis in vitro (20); the pseudogene has homology to Drosophila initiator $tRNA^{Met}$ between nucleotides 7-39 and thus is analogous to an in vivo generated Met 7/39 'minigene'.

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