ABSTRACT  Two Drosophila tRNA\textsuperscript{5s} genes with identical coding sequences were shown to transcribe with very different efficiencies in nuclear extracts from Xenopus oocytes. Injection of cloned genes for Xenopus 5S RNA (2), Xenopus initiator tRNA (3), and yeast tRNA (4) into the Xenopus oocyte nucleus results in the correct transcription of these genes into the mature RNA. The use of nuclear extracts from Xenopus oocytes has allowed in vitro transcription of Xenopus 5S RNA (5) or Drosophila, yeast, and Bombyx tRNA genes (6–9). Such in vitro experiments have defined initiation and termination sites for RNA transcription in Xenopus 5S RNA (10), Drosophila tRNA\textsuperscript{Lys} (11), and Bombyx tRNA\textsuperscript{Ala} (8, 9).

Comparison of the DNA sequences of several Xenopus 5S RNA genes revealed a conserved nucleotide sequence in the 5'-flanking region of the mature 5S RNA coding sequence (12). However, the results of RNA transcription experiments using 5S genes with shortened 5'-leader regions suggested that these conserved sequences are not important for initiation of 5S gene transcription (13). Experiments with enzymatically truncated genes had an unexpected result: the sole requirement for proper transcription of 5S or 5S-size RNA is the maintenance of the gene segment between nucleotides +50 and +80 of the mature 5S RNA coding sequence (14, 15).

Sequence analysis of various eukaryotic tRNA genes (7, 16–19) revealed no obvious common "promoter-like" sequence in the 5'-flanking regions. Moreover, a tRNA gene that has only 10 nucleotides upstream of the RNA initiation site transcribes well (8, 9). However, because the protein initiation factors required for 5S DNA transcription are different from those for tRNA genes (20), the DNA regions controlling transcription may not be the same for 5S and tRNA genes. This is also suggested by recent experiments showing that both the anterior and posterior portions of a Xenopus tRNA\textsuperscript{Met} gene unit are required for its proper transcription (21).

Analysis of Drosophila tRNA genes gave the nucleotide sequences of four genes coding for tRNA\textsuperscript{Lys} (ref 19; this report).

A sequence comparison (22) of the 5'-flanking regions of these genes revealed a highly conserved deca nucleotide sequence, G-T-C-A-G-T-T-T-T-A, located around position −20. Apart from the mature coding region, this was the only sequence conservation observed in these genes. Surprisingly, the individual tRNA\textsuperscript{Lys} genes differed widely in their ability to serve as templates for in vitro transcription. In this study we took two of these genes with different template efficiency and exchanged their 5'-flanking sequences, leaving the mature coding sequence intact. In vitro transcription of the resulting DNAs revealed two control regions for this process located in the mature coding region and the 5'-flanking regions.

MATERIALS AND METHODS

Source of Clones. Gene 2 plasmid is a pBR322 hybrid plasmid containing a 0.45-kilobase (kb) Drosophila DNA fragment (the HindIII/EcoRI fragment F of pGT12) of known sequence (19). Gene 4 plasmid, a pBR322 hybrid containing a 0.5-kb Drosophila DNA HindIII fragment, was kindly given to us by J. Loewenberg and P. Wensink.

Preparation of Recombinant Plasmids. Drosophila DNA inserts were excised from gene 2 and gene 4 plasmids by digestion with HindIII/EcoRI and HindIII, respectively. Both DNA fragments contain a single Hpa II cleavage site, located at position +3 of the mature tRNA\textsuperscript{Lys} coding sequence (see Fig. 1). Thus, cleavage with Hpa II allowed a convenient separation of the region coding for the mature tRNA (and 5'-flanking sequences) from their corresponding 5'-flanking sequences. For construction of the plasmids described in the text, the 5'-fragment of gene 2 was ligated (23) to the 5'-fragment of gene 4 and vice versa. These "recombinant" genes were then cloned into the HindIII or HindIII/EcoRI sites of pBR322. In a similar manner the plasmids Δ2 and Δ4 were constructed by ligating the 5'-fragments of genes 2 or 4 to a Hpa II/HindIII fragment of pBR322 (nucleotides 3901–29 in Δ2, nucleotides 29–161 in Δ4) and cloning into pBR322 as above (see Fig. 2). After transformation into Escherichia coli HB101 (24), recombinant plasmids were selected by hybridization to Drosophila 5S\textsuperscript{32P} tRNA\textsuperscript{Lys} (25). DNA sequence analysis of all the recombinant plasmids confirmed the expected identity of the insert.

Transcription of DNA. Xenopus germinal vesicle extracts and ribonucleoside [α\textsuperscript{32P}]triphosphates were used to transcribe the DNA of the recombinant plasmids (11). The RNA transcripts were analyzed by polyacrylamide gel electrophoresis. After autoradiography the amounts of precursor tRNA and of tRNA formed were quantitated by excision of the gel pieces and determination of their radioactivity. In other experiments the autoradiographs were scanned on a densitometer. 55 DNA (molar ratio to TDNA, 1:70) was added to these experiments to provide an internal standard. A standard 20-μl reaction mixture yielded 9000 ± 1800 cpm after incubation for 30 min at 22°C. Primary transcripts were isolated as described (11).

Abbreviation: kb, kilobase.
DNA Sequence Analysis. The chemical method of Maxam and Gilbert (26) was used. Each nucleotide was determined at least twice, and in many cases both strands of the DNA were analyzed.

RESULTS

Experimental Strategy and DNA Sequence of Normal and Altered tRNA Genes. The sequences of gene 2 and gene 4, aligned according to the mature tRNA^Lys^ coding sequence, are presented in Fig. 1. Gene 2 originated from a tRNA gene cluster located in region 42A of the Drosophila chromosome. Its sequence has been determined (19). Gene 4 is a tRNA^Lys^ gene from a different chromosomal region; its precise location is not yet determined.

A Hpa II cleavage site at position +3 of tRNA^Lys^ coding sequence was used to split the DNA enzymatically in that position and to exchange the leader sequences to generate genes 2-4 and 4-2 which maintain the mature tRNA^Lys^ coding sequence. The 3'-terminal fragments (containing almost all of the coding region for the tRNA) from the same endonuclease digests were isolated and ligated to defined Hpa II fragments of pBR322. This yielded the truncated genes Δ2 and Δ4 which lack a single C residue (between positions +2 and +4 of the mature coding sequence) in the amino acid acceptor stem of the tRNA. The construction of these plasmids is shown schematically in Fig. 2. The detailed nucleotide sequence of the coding region and the immediate flanking sequences are shown in Fig. 3.

Transcription Studies. The purified DNAs from these plasmids were transcribed in the Xenopus germinal vesicle system. The transcription products were separated by polyacrylamide gel electrophoresis (Fig. 4) and their identity was established by nucleotide sequence analysis. Because the transcription system also contains the processing nucleases, the major isolated products are the precursor tRNA and mature tRNA. Therefore, in order to quantitate the transcription efficiency, their combined amounts were measured. The inclusion of Notophthalmus viridescens 55 DNA in the transcription reactions provided an internal standard. Although the efficiencies varied somewhat with different germinal vesicle preparations, the template efficiency of gene 4 was much higher than that of gene 2 (Table 1). This result was not dependent upon the choice of plasmid vector or upon the orientation of the DNA insert within the plasmid because the excised linear DNA fragments alone could serve as efficient templates (35% compared to the data in Table 1).

In order to analyze the basis for the differential transcription, the "reconstructed" plasmids were transcribed. The experiments revealed two striking facts. (i) The 5'-flanking sequences are crucial in transcription regulation. The gene 2-4 resembles the inefficient gene 2 but, more importantly, the inefficient gene (gene 2) becomes well transcribed (a 14-fold increase) when the 5'-flanking sequences of gene 4 precede it. The efficient transcription of gene 4-2, inserted into pBR322 in the opposite orientation of gene 4 (Fig. 2), provides additional proof that the insert orientation does not affect template activity.

(ii) The template efficiency of the truncated gene Δ4 remained approximately at the level of the normal gene, and the template efficiency of the truncated gene Δ2 increased 10-fold.

![Fig. 2. Schematic representation of normal and altered tRNA^Lys^ genes. Heavy lines represent vector pBR322 DNA; narrow lines represent Drosophila DNA; broken heavy lines denote additional pBR322 DNA fragments. Relevant restriction endonuclease cleavage sites are indicated: †, EcoRI; †, HindIII; †, Hpa II. The arrows denote direction of tRNA^Lys^ transcription. The numbers indicate the length of the fragments (in base pairs).](image-url)
Thus, it appears that the mature coding region contains the control region crucial for transcription, unless one assumes that such a region is located downstream from the 3'-end. This appears unlikely because transcription termination occurs only a few nucleotides past the 3'-end of the coding region. In any case, if the 3'-flanking region is important, it need not exceed 50 base pairs in length for efficient transcription as shown in studies with a different tRNA\textsuperscript{Lys} gene. The 5'-flanking sequences, however, appear to exert a negative effect on the transcriptional control. It is interesting to note that the deletion of a single C residue in positions +2 to +4 of truncated genes \(\Delta 2\) and \(\Delta 4\) results in the production of a more stable precursor (Fig. 4).

By using affinity chromatography as described earlier the ptRNA\textsuperscript{Lys} band (Fig. 4) was shown to represent the primary transcript. The transcripts of all genes (Fig. 4) were initiated with pppA. Sequence analysis of the RNAs elucidated the actual position of RNA initiation indicated in Fig. 3.

Although we have not determined unambiguously the sequence of the 3'-terminal oligonucleotide, knowledge of the RNA initiation site and of the chain length of the RNAs (estimated from gel analysis (Fig. 4)) gives a strong indication that transcription termination occurs in the oligothymidylicate stretches of the anticoding strand.

**DISCUSSION**

We interpret our results as implicating a two-step model of tRNA gene transcription. The mature coding region (internal control region) determines whether transcription will proceed. The 5'-flanking region (external control region) modulates this transcription. This resembles to some extent the finding of a control region for 5S transcription within the 5S gene (14, 15). In contrast to the 5S situation, however, we found that the 5'-flanking sequences of our tRNA genes can repress the transcription dictated by the internal control region. Because, by necessity, we are using a crude transcription system, it is difficult to measure absolute efficiencies.

What are the sequences of these control regions? Comparison of the sequences of mature tRNAs reveals common nucleotides in several specific positions. Such conserved residues occur in the D loop and TV loop, and there is also a semi-invariant U preceding the anticodon. To test whether such an array of widely separated bases has significance for transcription regulation would require carefully selected in vivo or in vitro made...
"mutant" tRNA genes. In initial experiments, DNA fragments comprising only half of the mature coding sequence of Xenopus tRNA\textsuperscript{Met} do not support transcription of tRNA sequences (21). The conclusion that the internal control region spans both tRNA halves may not be universal because separated halves of the Schizosaccharomyces pombe tRNA\textsuperscript{the} gene serve as effective templates for the transcription in vitro of tRNA sequences (unpublished results).

The most striking feature of the 5′-flanking regions is the undecanucleotide sequence G-T-C-A-G-T-T-T-T-A-T which is conserved in four Drosophila tRNA\textsubscript{Lys} genes (22). The importance of this conserved sequence for tRNA\textsubscript{Lys} gene transcription is not clear. The conserved sequence is not directly involved in promoting RNA polymerase III activity because the 5′-flanking region of gene 4 did not direct any observable transcription in vitro when separately recloned (data not shown) nor did its deletion significantly affect transcription. The external control region may be a site of interaction with DNA or RNA sequences or with proteins to bring about the modulation effect.

The length of the 5′-flanking sequences might affect tRNA gene transcription. The negative modulation was seen only in the case of gene 2 which has a 240-base-pair-long 5′-flanking sequence. Therefore we cannot rule out the possibility that an external negative control region has been included in the selection of the efficiently transcribed tRNA clones with short 5′-flanking regions. Several Drosophila, Bombyx, and Xenopus tRNA genes with very short 5′-flanking sequences are transcribed as well as a Drosophila tRNA\textsuperscript{A+6} gene which contains 288 nucleotides of Drosophila DNA preceding the start of the mature tRNA coding sequence (11). Therefore, the length of 5′-flanking sequences of Drosophila tRNA genes contained within a recombinant plasmid does not by itself affect the efficiency of in vitro transcription.

The exact site of transcription initiation of genes 2 and 4 have been shown to differ slightly in their precise location relative to the 5′ terminus of the mature tRNA\textsubscript{Lys} (Fig. 3). The recombinant genes 4-2 and 2-4 maintain the initiation site specified by the 5′-flanking region present. The initiation of transcription of truncated genes Δ2 and Δ4 occurs at A residues whose distance from the 5′-end of the mature tRNA differs in the two genes. The lack of a "counting" mechanism for determining the exact initiation site is also observed for Xenopus 5S RNA genes and may prove to be a general feature for transcription by RNA polymerase III (10). It is also evident from examination of Fig. 3 that the initiation of transcription of these tRNA genes occurs very near the 5′-terminus of the mature tRNA sequence. In contrast to some E. coli precursor tRNAs, the eukaryotic tRNA precursors analyzed to date have only short leader sequences (8, 9, 11).

It is interesting to note that the disruption of the tRNA sequence by construction of the truncated genes Δ2 and Δ4 affects the subsequent processing of the RNA transcript. Thus, although not essential for efficient transcription to proceed, the integrity of the mature tRNA sequence appears critical for subsequent processing of tRNA precursors.

It would be desirable to know whether the same differential expression of these two tRNA\textsubscript{Lys} genes takes place in vitro. Although the Xenopus germinal vesicle system has been shown to give specific transcription of tRNA genes from various eukaryotes, it may be possible that the effect described occurs only during oogenesis and not during other developmental stages. The direct experiment—the isolation and sequence analysis of tRNA\textsuperscript{Lys} precursors formed in vitro—is unfortunately not feasible because of the many (about 22) tRNA\textsubscript{Lys} genes encoded in the haploid Drosophila genome (27).

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