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Transcription Factor Binding Is Limited by the 5′-Flanking Regions of a *Drosophila* tRNA^{His} Gene and a tRNA^{His} Pseudogene

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We determined the sequence of a *Drosophila* tRNA gene cluster containing a tRNA^{His} gene and a tRNA^{His} pseudogene in close proximity on the same DNA strand. The pseudogene contains eight consecutive base pairs different from the region of the bona fide gene which codes for the 3′ portion of the anticodon stem of tRNA^{His}. The tRNA^{His} gene is transcribed efficiently in *Drosophila* Kc cell extract, whereas the pseudogene is not. The pseudogene is also a much poorer competitor than the real gene in a stable transcription complex formation assay, even though the sequence alteration in the pseudogene does not affect the sequence or spacing of the putative internal transcription control regions. Recombinant clones were constructed in which the 5′-flanking regions are exchanged. The transcription efficiencies and competitive abilities of the recombinant clones resemble those of the genes from which the 5′ flank was derived; for example, the tRNA^{His} pseudogene with the 5′-flanking sequence of the tRNA^{His} gene is now efficiently transcribed. Deletion analysis of the pseudogene 5′ flank failed to uncover an inhibitory element. Deletion analysis of the real gene showed very high dependence on the presence of the wild-type 5′-flanking sequence for factor binding to the internal control regions and stable complex formation. The 5′-flanking sequence of a *Drosophila* tRNA^{Arg} gene active in the *Drosophila* Kc cell extract does not restore transcriptional activity or stable complex formation. The tRNA^{His} gene and pseudogene behave atypically in HeLa cell extract. Both genes compete for HeLa transcription factors, but neither of them is efficiently transcribed. Removal of the 5′-flanking sequences of each gene and replacement with various sequences, including the tRNA^{Arg} gene 5′ flank, does not allow increased transcription in HeLa cell extract.

The formation of eucaryotic tRNA precursors by RNA polymerase III and specific transcription factors (3, 15, 31, 36, 41) is regulated by two internal control regions (4, 6, 14, 16, 20, 38, 40). Transcription of tRNA genes is also affected by elements in the 5′-flanking sequence. Sequences responsible for transcriptional repression have been identified in the 5′-flanking regions of *Drosophila* tRNA^{Lys} (9, 10) and tRNA^{Arg} (12) genes. Conversely, the wild-type 5′-flanking sequence of some genes is required for active transcription in *Drosophila* Kc cell and *Bombyx mori* cell transcription systems, and deletion of these sequences leads to a loss of template activity (13, 22, 34, 38, 44). The transcription of *Drosophila* tRNA genes examined to date in HeLa cell extract is relatively independent of 5′-flanking sequence modulation and does not require the presence of the wild-type 5′ flank (34). Sequences that affect the ability of a *Drosophila* tRNA^{Arg} gene to bind transcription factors at the internal control regions are present in the 3′- and 5′-flanking regions (34, 35). Deletion of 5′-flanking sequence leads to a small decrease in the ability to compete with a reference template for transcription factors. The binding of transcription factors is dependent on the presence of the wild-type 3′-flanking sequence to about 34 nucleotides downstream of the mature coding sequence.

The selection of the initiation site for 5S RNA gene transcription has been suggested to occur by measuring back from the 5′ internal control region (5, 32). The results of a recent study on the in vivo transcription of *Xenopus* oocytes of 5′ deletion mutants of a *Drosophila* tRNA^{Arg} gene suggest that a measurement function is also responsible for the selection of the initiation nucleotide in tRNA gene transcription (40). The stringent selection of the initiation site is altered when part or all of the D-control region is deleted.

In this paper we report the sequence of a *Drosophila* tRNA gene cluster containing a tRNA^{His} gene and pseudogene. We have examined the effect of the 5′-flanking regions of these genes on transcription and stable complex formation. The 5′-flanking sequence of the tRNA^{His} pseudogene, when present with the mature coding sequence of either the tRNA^{His} gene or pseudogene, dramatically limits transcriptional activity. The pseudogene 5′-flanking sequence does not contain an inhibitory sequence; rather, it is nonpositive. The 5′-flanking sequence of the tRNA^{His} gene is specifically required for optimal activity of either gene; a variety of other sequences, including the 5′-flanking sequence of a *Drosophila* tRNA^{Arg} gene, was found to be incompatible.

MATERIALS AND METHODS

Plasmid DNA. The plasmid p48FHis consists of a 1.1-kilobase fragment (*Hin*dIII-*Bam*HI) of *Drosophila* DNA inserted into pBR322 between the *Bam*HI and *Hin*dIII sites (8). The plasmid was cut with either *Bam*HI or *Hin*dIII and digested with BAL 31 (24) to subclone the tRNA^{His} gene and the tRNA^{His} pseudogene contained on the insert. After fill-in synthesis with *Escherichia coli* DNA polymerase I (Klenow fragment), the DNA was ligated to either the *Bam*HI or *Hin*dIII linker to restore the original end. Inserts were excised by digestion with *Bam*HI and *Hin*dIII, fractionated by polyacrylamide gel electrophoresis, and ligated to the large fragment from a *Bam*HI-*Hin*dIII digestion of pBR322 DNA. The resulting plasmids were transformed into *E. coli*

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HB101, and covalently closed circular plasmid DNA was prepared by standard methods (7). Deletion mutations in the 5'-flanking region of the $tRNA^{His}$ pseudogene subclone (pψHis) were constructed by BAL 31 digestion from the *Bam*HI site as described above, except that *Eco*RI linkers were used and the fragments were cloned between the *Hin*dIII and *Eco*RI sites of pBR322. Recombinant clones in which the 5'-flanking sequences of the two genes are switched were constructed by using the *Ava*II site located within the mature coding sequence. The *Bam*HI-*Ava*II fragment (containing the 5'-flanking sequence and part of the mature coding sequence) and the *Ava*II-*Hin*dIII fragment (containing remainder of the mature tRNA coding region and the 3'-flanking sequence) were isolated from both subcloned genes. The *Bam*HI-*Ava*II fragment containing the $tRNA^{His}$ gene 5'-flanking sequence was ligated with the *Ava*II-*Hin*dIII fragment containing the $tRNA^{His}$ pseudogene (and vice versa) into pBR322 (*Bam*HI-*Hin*dIII). The 5'-flanking sequences of the pseudogene 5'-flanking sequence deletion mutants were switched onto the $tRNA^{His}$ gene in a similar manner by using the internal *Ava*II site. In this case, the recombinant genes were cloned between the *Eco*RI and *Hin*dIII site of pBR322. The 5' deletion mutants of the $tRNA^{His}$ pseudogene are named by a number, for example, pψHis5.−9. The unit of the number indicates that the deletion is of the 5'-flanking sequence, and the number after the decimal point indicates the last remaining nucleotide from the *Drosophila* DNA. The recombinant clones containing the deleted pseudogene 5'-flanking sequences and the $tRNA^{His}$ gene are designated similarly and followed by an *s* (switched). Recombinant genes containing the 5'-flanking sequence of a *Drosophila* $tRNA^{Arg}$ gene (43) and the mature coding sequence of either the $tRNA^{His}$ gene or the pseudogene were constructed as follows. The 3' deletion mutant pArg3.4 contains the 5'-flanking sequence of a $tRNA^{Arg}$ gene through nucleotide +4 of the mature coding sequence plus the *Eco*RI linker. The *Hin*dIII-*Eco*RI fragment containing the pArg3.4 insert was ligated to the (gene-containing) *Eco*RI-*Hin*dIII fragments of pHis5.−1, pHis5.8, pψHis5.−1, and pψHis5.8 in the *Hin*dIII site of pBR322. DNA sequencing was by the method of Maxam and Gilbert (26).

In vitro transcription of tRNA genes. Plasmid DNA was transcribed in S-100 extracts prepared (13) from *Drosophila* Kc cells or HeLa cells. Transcription in *Drosophila* Kc cell extract was as previously described (13). HeLa cell extract transcription reactions were in 40 μl with 10 μl of extract, 500 μM each ATP, CTP, and UTP, 100 μM [α-^{32}P]GTP (0.75 to 1.25 Ci/mmol), 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid–KOH (pH 8.0), 95 mM KCl, 3 mM $MgCl_2$, 3 mM dithiothreitol, and 8 mM creatin phsophate. RNA transcripts were resolved on thin polyacrylamide gels (33) and quantitated by measuring the Cerenkov radiation of excised gel pieces. 5'-Terminal nucleotides were determined by nearest neighbor analysis and confirmed by fingerprinting analysis (for transcripts of pHis only). The ability of the cloned genes to stably sequester transcription factors was determined in the competition assay involving two different tRNA genes as described by Schaack et al. (35). Plasmid DNA (0.1 to 0.6 μg) containing the gene to be tested was incubated with either *Drosophila* Kc cell or HeLa cell extract under transcription reaction conditions (13) for 15 min before the addition of a reference template (pArg26×36) (14) (0.3 or 0.6 μg). The total concentration of DNA in each reaction was kept constant at 1.2 μg by the addition of pBR322 DNA. Transcription was terminated 90 min after the addition of the reference template. The amount of reference template transcription was quantitated by measuring Cerenkov radiation of excised gel pieces containing the single pArg26×36 transcript band. Competition strength is calculated as the percentage of competed maxigene transcription compared with competition by pBR322.

RESULTS

Structure of $tRNA^{His}$ genes. The sequence of the $tRNA^{His}$ genes contained on the plasmid p48FHis (8) is shown in Fig. 1. The first gene, His, corresponds to the $tRNA^{His}$ sequence (2), except for the 5'-terminal nucleotide (8). The second gene, ψHis, contains a region of 8 consecutive base pairs (bp) that differ from those in His and is therefore considered a pseudogene. The 3'-terminal CCA sequence of tRNA is not encoded. Transcription of ψHis would result in an RNA that is unable to form the normal $tRNA^{His}$ secondary structure in the anticodon stem (Fig. 2). The 3'-flanking regions of the two genes from the 3' end of the mature coding sequence to the transcription terminator sequence are unusually long (35 bp) for a tRNA gene and highly homologous; however, the 5'-flanking sequences are not homologous. Computer analysis (37) of the DNA sequence revealed that the 5'-flanking sequence of the pseudogene contains regions of homology to regions within the mature coding sequence (Fig. 1).

The two genes on plasmid p48FHis were subcloned separately by using BAL 31 (see above). The plasmid pHis contains the fragment from positions 1 through 370 (Fig. 1). Plasmid pHis-t contains a fragment (positions 1 through 178) that lacks the termination sequence. The pψHis clone contains the $tRNA^{His}$ pseudogene (coordinates 228 through 530).

In vitro transcription of $tRNA^{His}$ genes. The initiation site for transcription of the $tRNA^{His}$ gene was previously reported to be at position −5 (8) based on electrophoretic mobility and nearest neighbor analysis, which identified pppApU as the 5' end of the primary transcript. During the course of the present work, the $tRNA^{His}$ gene primary transcript was subjected to fingerprint analysis (data not shown). The initiation nucleotide was found to be the A at position −13 (Fig. 1).

The plasmid p48FHis is efficiently transcribed in *Drosophila* Kc cell extract (Fig. 3). The individually subcloned genes were tested for the ability to support transcription in vitro. The clone containing only the $tRNA^{His}$ gene is more efficiently transcribed than the parent clone containing both genes at saturating concentrations (Fig. 3). Transcription of a $tRNA^{His}$ gene lacking the termination sequence in clone pHis-t terminates at different oligothymidylate stretches upstream of the *Eco*RI site in the vector pBR322. The primary transcripts of $tRNA^{His}$ from the clone pHis-t therefore include 3'-trailer sequences 85, 140, or 175 bases in length. The pseudogene is very poorly transcribed (Fig. 3). Neither of the genes is efficiently transcribed in HeLa cell extract (data not shown).

Recombinant clones in which the 5'-flanking sequences of the two genes are switched were constructed to determine whether the low transcriptional activity of the pseudogene is due to the alteration in the mature coding sequence or to the 5'-flanking sequence. Transcription efficiencies of the recombinant clones correspond to those of the parent clone with the same 5'-flanking sequence (Fig. 3). The pseudogene with the 5'-flanking sequence of the $tRNA^{His}$ gene (p5'His/ψHis) is transcribed very efficiently, whereas the $tRNA^{His}$ gene with the 5'-flanking sequence of the pseudo-

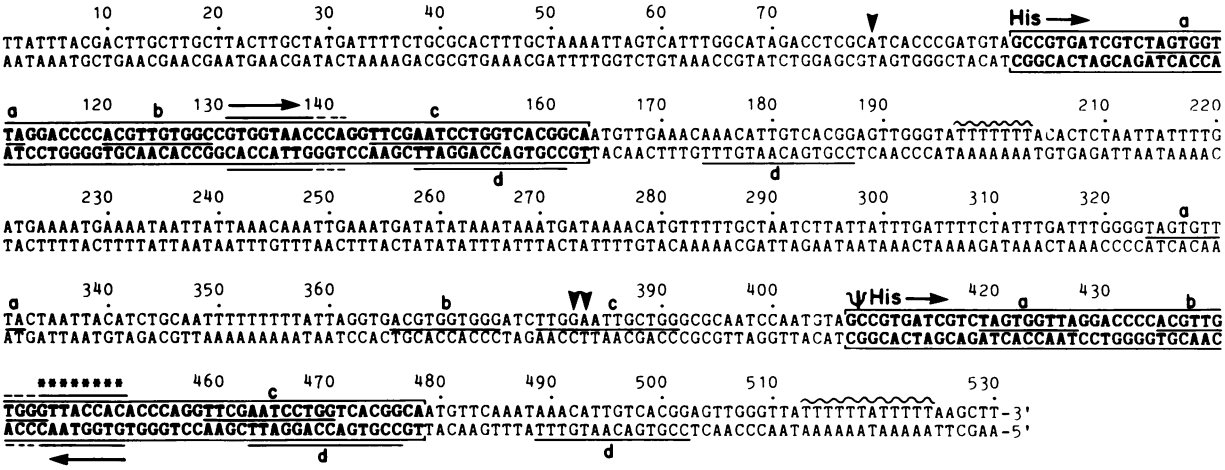

FIG. 1. Sequence of the tRNA^{His} gene and pseudogene contained on plasmid p48FHis. The tRNA^{His} gene (His) and pseudogene (ψHis) sequences are boxed. Transcription initiation sites are indicated with arrowheads, and transcription termination signals are indicated by wavy lines. Regions of homology between sequences in the 5′-flanking sequence of the pseudogene and sequences in each mature coding sequence are shown with a line between the DNA strands and are labeled a, b, and c. The 8-bp alteration in the pseudogene is indicated with asterisks. An inverted repeat of 8 to 11 bp is indicated in each gene by lines on either side of the DNA strands and arrows.

gene (p5′ψHis/His) is transcribed very poorly. The primary transcript of the pseudogene is not processed to mature-sized tRNA (Fig. 3).

The pseudogene is a poor competitor in stable complex formation. The tRNA^{His} gene and pseudogene were tested for their ability to compete for transcription factors in forming stable transcription complexes (35). Examination of the DNA concentration dependence (Fig. 4) revealed that nearly maximal competitive strength for each gene was obtained upon addition of 0.2 μg of competitor DNA. Fifty

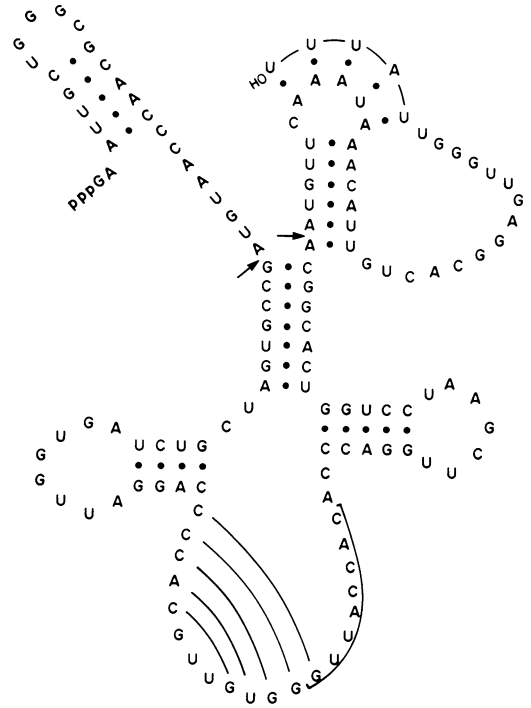

FIG. 2. Possible secondary structure of the tRNA^{His} pseudogene primary transcript. The putative sites for 5′- and 3′-flanking sequence processing are indicated with arrows. The RNA is drawn with the amino acid acceptor stem, D-stem and loop, and T-stem and loop in the same configuration as the tRNA^{His} cloverleaf structure (2). The 8-bp alteration is indicated with a bar. Possible hydrogen bonding in the anticodon stem and loop region is shown with lines.

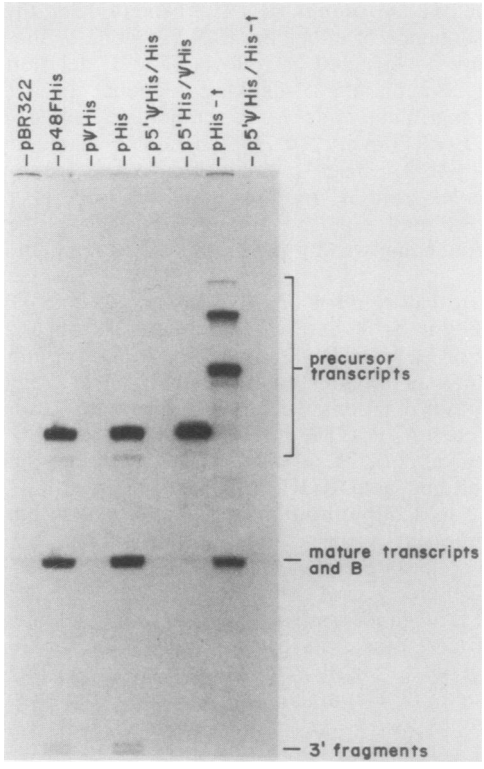

FIG. 3. Transcription of the tRNA^{His} gene, the pseudogene, and recombinant tRNA genes derived from them. A 0.5-μg sample of the DNA indicated was added per standard transcription reaction. Incubation was for 90 min. B indicates the background band that is formed by 5′-guanylation of tRNA^{His} in the presence of [α-^{32}P]GTP (8).

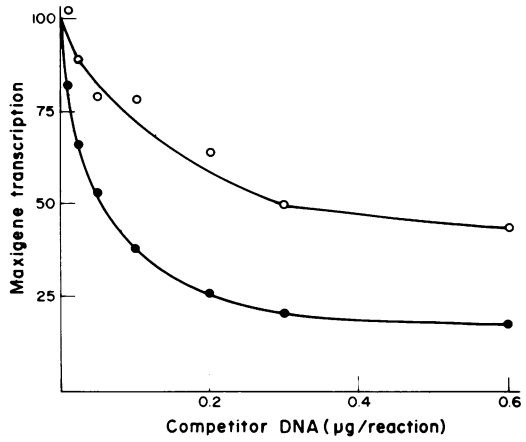

FIG. 4. Stable complex formation by pHis and pψHis in *Drosophila* Kc cell extract. A 0.0- to 0.6-μg sample of pHis (●) or pψHis (○) was added to the transcription reaction 15 min before the addition of 0.3 μg of reference template (maxigene). Maxigene transcription was quantitated and is plotted as the percentage of the uncompeted reaction.

percent inhibition of reference template transcription was obtained with 0.06 μg of pHis or 0.3 μg of pψHis. The tRNA^{His} gene and pseudogene (together on the parent clone and recloned separately) and the recombinant genes with the 5'-flanking sequences exchanged were tested for competitive strength (Fig. 5). The tRNA^{His} gene competes better when the pseudogene is not present (compare p48FHis and pHis). This difference in competition strength is not due to the slight difference in gene concentration, nor is it due to difference of insert size in the plasmids. Competition for transcription factors is reduced for the tRNA^{His} gene lacking the termination signal (pHis-t). The pseudogene is a poor competitor.

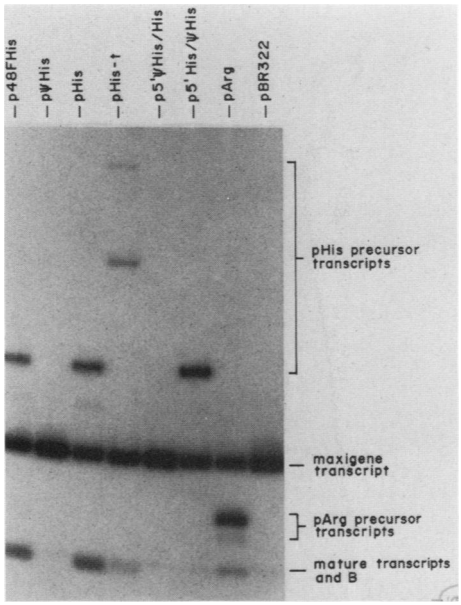

FIG. 5. Stable complex formation by the tRNA^{His} gene, pseudogene, and recombinant clones in *Drosophila* Kc cell extract. A 0.2-μg sample of the template indicated and 0.4 μg of pBR322 were incubated in a transcription reaction for 15 min before the addition of 0.6 μg of reference template (maxigene). B indicates the background band that is formed by 5'-guanylation of tRNA^{His} in the presence of [α-^{32}P]GTP (8).

Competition by the genes in *Drosophila* Kc cell extract is dramatically affected by the 5'-flanking sequence. The pseudogene cloned with the 5'-flanking sequence of the tRNA^{His} gene competes almost as well as pHis (Table 1). Conversely, competition by the tRNA^{His} gene is greatly reduced by the presence of the 5'-flanking sequence of the pseudogene. The tRNA^{His} gene and pseudogene compete with similar efficiencies in HeLa cell extract (data not shown).

Pseudogene deletion mutants. The 5'-flanking sequence of the pseudogene contains sequences (a and c in Fig. 1) that are homologous to the putative D- and T-control regions in the mature coding sequence as well as an oligothymidylate stretch reminiscent of inhibitory elements found in the 5'-flanking sequences of *Drosophila* tRNA^{Lys} (10) and tRNA^{Arg} (12) genes. We thought that deletion of some or all of these regions would result in an increase in transcription or stable complex formation (or both), as observed for the tRNA^{Lys} gene. The extent of each deletion is indicated in Fig. 6. Each deletion template was tested for the ability to support RNA synthesis and to stably bind transcription factors. Very little transcription activity was recovered compared to the level of transcription of either pHis or the recombinant clone containing the pseudogene and the pHis 5'-flanking sequence (p5'His/ψHis) (Fig. 7A). (Note that the lanes marked pHis and p5'His/ψHis in Fig. 7B contain 1/10 of a transcription reaction.) The small increases in activity were observed for templates with deletions extending inside nucleotide −33. Deletions to nucleotides −14, −12, −9, and −1 showed a severalfold increase over pψHis transcription; however, the activity of these templates does not approach the activity of pHis.

TABLE 1. Transcription and competition on *Drosophila* Kc cell extract by the tRNA^{His} gene and pseudogene and recombinant clones[a]

5' Flank	His		ψHis	
	Transcription efficiency[b] (%)	Competitive strength[c] (%)	Transcription efficiency (%)	Competitive strength (%)
His	100	100	100	81
ψHis	<1	46	<1	31
ψHis−82	<1	48	<1	35
ψHis−63	<1	37	<1	29
ψHis−34	<1	37	<1	19
ψHis−33	<1	37	<1	25
ψHis−33B[d]			<1	33
ψHis−32	<1	38	<1	19
ψHis−26	<1	46	<1	21
ψHis−17	<1	40	<1	17
ψHis−14	<1	40	<1	23
ψHis−12	<1	48	<1	27
ψHis−9	<1	37	<1	23
ψHis−1	<1	29	<1	21
ψHis+8	<1	19	<1	33
His−45	100	96		
Arg	<1	13	<1	6

[a] For each gene tested, the mature coding sequence is listed at the top of the column and the 5' flanking region is listed at the left.

[b] Transcription efficiencies are given as a percentage of pHis transcription.

[c] Relative competitive strengths are given as a percentage of competed maxigene transcription (with pHis competition normalized to 100%).

[d] The 5'-flanking sequence of deletion mutant pψHis5.−33B is replaced with pBR322 sequence leading from the *Bam*HI site instead of with pBR322 sequence leading from the *Eco*RI site as for the other pseudogene deletion mutants.

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His TACGACTTGCTTGCTTACTTGCTATGATTTTCTGCGCACTTTGCTAAAATTAGTCATTTGGCATAGACCTCGCATCACCCGATGTA GCCGTGATCGTC
ψHis GGGTAGTGTTTACTAATTACATCTGCAATTTTTTTTTATTAGGTGACGTGGTGGGATCTTGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
          -80       -70       -60       -50       -40       -30       -20       -10         1        10

-82 ttccAGTGTTTACTAATTACATCTGCAATTTTTTTTTATTAGGTGACGTGGTGGGATCTTGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
-63 gccctttcgtcttcaagaattccTGCAATTTTTTTTTATTAGGTGACGTGGTGGGATCTTGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
-34 ttaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccGGGATCTTGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
-33 attaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccGGATCTTGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
-32 cattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccGATCTTGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
-26 tcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccGGAATTGCTGGGCGCAATCCAATGTA GCCGTGATCGTC
-17 ccattattatcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccGGGCGCAATCCAATGTA GCCGTGATCGTC
-14 aaaccattattatcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccCGCAATCCAATGTA GCCGTGATCGTC
-12 agaaaccattattatcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccCAATCCAATGTA GCCGTGATCGTC
- 9 ctaagaaaccattattatcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccTCCAATGTA GCCGTGATCGTC
- 1 cctgacgtctaagaaaccattattatcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaagaattccA GCCGTGATCGTC
+ 8 aagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaatAggcgtatcacgaggccctttcgtcttcaa gaattccTCGTC

Arg/His5.-1 TGTGTACAAATAATATTCGAGTTTGACTTTCTACTTGTTTGAACTGTTACACTCGCACGTCAAGCGGTCggaattccA GCCGTGATCGTC
Arg/His5.+8 GATAACAATGTGTACAAATAATATTCGAGTTTGACTTTCTACTTGTTTGAACTGTTACACTCGCACGTCAAGCGGTCg gaattccTCGTC
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FIG. 6. 5′-Flanking sequence deletion mutants of the tRNA^{His} pseudogene. The first two lines show the noncoding strand of the 5′-flanking sequence of the tRNA^{His} gene (His) and pseudogene (ψHis). Colons between these lines indicate homologous nucleotides. The coordinates relative to the mature coding sequence are shown. The underlined sequences in the pseudogene 5′-flanking sequence correspond to the regions of homology noted in Fig. 1. The lines marked −82 through +8 show the sequences of the 5′-flanking sequences of the deletion mutants. The remaining *Drosophila* DNA is in uppercase, boldface letters; the pBR322 sequence is in lowercase letters. The number to the left of each line indicates the position of the last remaining *Drosophila* nucleotide. Transcription initiation nucleotides are boxed. The last two lines are the sequence of the 5′ flank of the tRNA^{Arg}-tRNA^{His} hybrid genes. The tRNA^{Arg} sequence is in uppercase letters, the *Eco*RI linker is in lowercase letters, and the tRNA^{His} sequence is in uppercase boldface letters.

Each of the deleted 5′-flanking sequences of the pseudogene was recloned with the tRNA^{His} gene. The transcription activity of these recombinant genes closely mirrors that of the pseudogene deletion mutant from which the 5′-flanking sequence was obtained (Fig. 7B). Again, transcription activity was low and did not approach levels obtained for either gene with the tRNA^{His} gene 5′-flanking region. (Note that the lanes marked pHis and p5′His/ψHis in Fig. 7B contain 1/10 of a transcription reaction.)

The 5′-flanking sequence deletion mutants were also tested for the ability to bind transcription factors (Table 1). No recovery of competitive ability was observed with successive deletion of the pseudogene 5′-flanking sequence. The tRNA^{His} gene with the pseudogene 5′-flanking sequence competes slightly better than the intact pseudogene, indicating that the presence of the eight bp alteration in the mature coding sequence does affect competitive ability. Similarly, the clones containing the pseudogene 5′-flanking sequence deletions and the tRNA^{His} gene all compete slightly better than the pseudogene 5′-flanking sequence deletion mutants. It is important to note that the tRNA^{His} gene, which is a good competitor in the presence of its wild-type 5′-flanking sequence, competes at a greatly reduced level when the 5′-flanking sequence is replaced by pBR322 sequence (see pψHis5.−1/His).

Transcription of the pseudogene 5′-flanking sequence deletion mutant templates (or the 5′-deletion mutant templates with the tRNA^{His} mature coding sequence) reveals an inter-

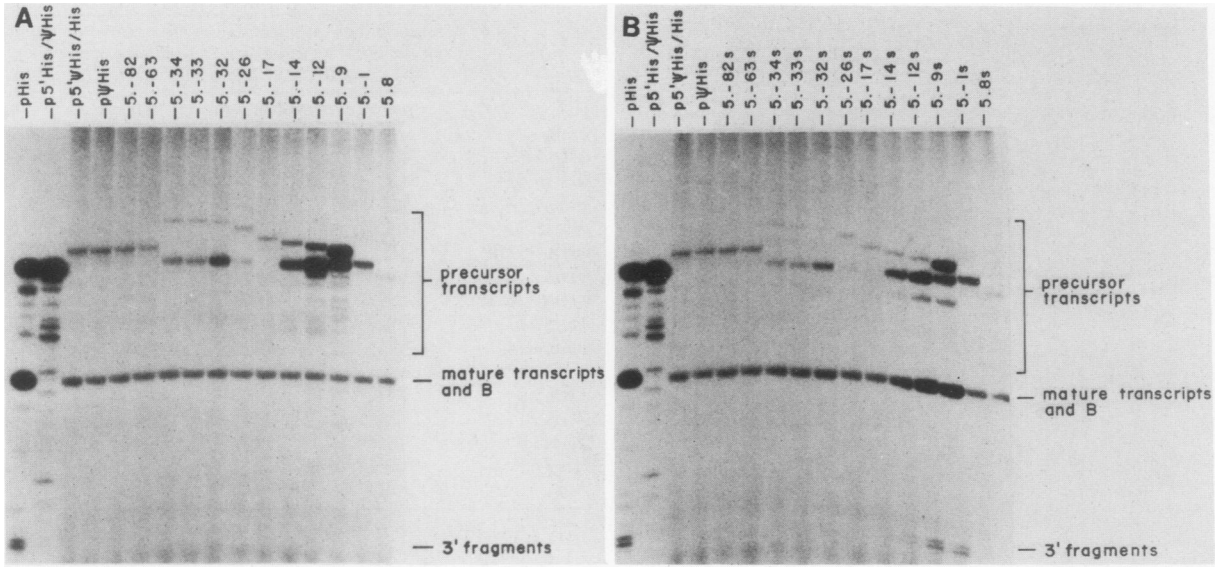

FIG. 7. Transcription of the tRNA^{His} pseudogene 5′-flanking sequence deletion mutants in *Drosophila* Kc cell extract. (A) tRNA^{His} pseudogene 5′-flanking sequence deletion mutants. (B) tRNA^{His} gene with pseudogene 5′-flanking sequence deletions. The *s* following the clone designation is for switched. A 0.5-μg sample of the template indicated above each lane was transcribed. The lanes marked pHis and p5′His/ψHis contain 1/10 of the transcription reaction. Autoradiography was overnight with intensification. B indicates the background band that is formed by 5′-guanylation of tRNA^{His} in the presence of [α-^{32}P]GTP (8).

esting pattern of initiation sites. Templates with deletions extending to nucleotide −82 or −63 appear to utilize the same initiation nucleotide as does the pseudogene with its intact 5' flanking sequence. Transcription initiation for templates pψHis5.−34 to 5.−9 occurs at two sites (Fig. 7). The site farthest upstream from the mature coding sequence is 15 bp upstream of the pBR322-*Drosophila* fusion in the pBR322 sequence introduced by the cloning procedure (Fig. 6). The RNA transcript that initiates at this site is progressively smaller, with increasing deletion of the 5' flanking sequence. This initiation site is also utilized in transcription of the template with deletion to nucleotide −1. Transcription initiation in HeLa cell extract, although very weak, occurs nearly exclusively at this upstream site introduced in the pBR322 sequence (data not shown). The second initiation sites for genes pψHis5.−14 and pψHis5.−12 appear to be at −13 and −11, respectively. For pψHis5.−9, the second initiation site appears to be at −14 (5 bp upstream of the pBR322-*Drosophila* fusion) according to nearest neighbor analysis (data not shown) and electrophoretic mobility. The pattern of initiation efficiency at this site is affected by the upstream sequence in what may be a periodic fashion. The deletion mutant pψHis5.−32 initiation efficiency is greater than the efficiency for the mutant templates with deletion to −33 and −26. Initiation efficiency is increased for pψHis5.−14, pψHis5.−12, and pψHis5.−9 to a much greater extent than for pψHis5.−32. An RNA polymerase III-binding site introduced in the vector sequence, which is in register on the DNA helix with the downstream initiation site (and probably an internal control region) for pψHis5.−32, would again be in register for deletion mutant pψHis5.−12, where the binding site is two turns of the helix closer to the initiation site. Such a binding site would be exactly out of register (one and one-half turns closer) for initiation from deletion mutant pψHis5.−17, which does not support transcription from the inside initiation site. A prediction from this model is that pψHis5.−22 would initiate with an efficiency intermediate between those of pψHis5.−32 and pψHis5.−12.

Can a tRNA^{Arg} gene 5'-flanking sequence replace the tRNA^{His} gene 5' flank? The tRNA^{His} gene and pseudogene mature coding sequences were cloned with the 5'-flanking sequence of a tRNA^{Arg} gene (38), which transcribes efficiently in vitro in both *Drosophila* Kc and HeLa cell extracts. Two deletions (5.−1 and 5.+8) of both the tRNA^{His} gene and pseudogene were recombined with the tRNA^{Arg} gene 5' flank (pArg3.4). The constructions resulted in genes with insertions of 13 to 5 bp between the pArg 5'-flanking sequence and the 5' terminus of the tRNA^{His} mature coding sequence (Fig. 6). The level of transcription and the competitive strength of the hybrid genes in both *Drosophila* Kc cell extract (Fig. 8 and Table 1) and HeLa cell extract (data not shown) are virtually unchanged from those of the tRNA^{His} deletion mutants from which the hybrids were derived. The transcription initiation sites are slightly altered. It cannot be ruled out that different transcriptional properties would be observed by changing the relative positions of the tRNA^{Arg} gene 5'-flanking sequence and the tRNA^{His} gene and pseudogene sequences. However, the lack of significant change in the transcriptional properties of the hybrid genes suggests that the tRNA^{Arg} gene 5'-flanking sequence is not compatible with the tRNA^{His} mature coding sequence in forming an active tRNA gene.

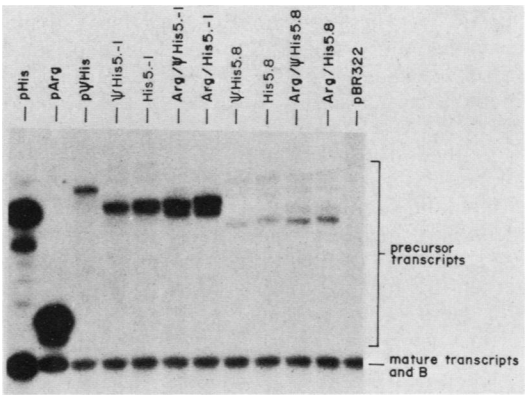

FIG. 8. Transcription of tRNA^{Arg}-tRNA^{His} hybrid genes in *Drosophila* Kc cell extract. A 0.5-μg sample of the template indicated above each lane was transcribed. The lanes marked pHis and pArg contain 1/10 of the transcription reaction. Refer to Fig. 6 for the sequence of the hybrid genes. Autoradiography was overnight with intensification. B indicates the background band that is formed by 5'-guanylation of tRNA^{His} in the presence of [α-^{32}P]GTP (8).

DISCUSSION

Structure of histidine tRNA genes. The structure of the tRNA^{His} gene is strikingly different from other *Drosophila* tRNA genes. The transcription termination signal is 35 bp downstream of the mature coding sequence instead of adjacent to, or within about 10 bp of, the 3' end of the mature coding sequence. As suggested for eucaryotic tRNA processing (17), the 3' trailer of the tRNA^{His} gene transcript is processed by a single endonucleolytic cleavage in *Drosophila* cell-free extract (D. Frendewey, T. Dingermann, L. Cooley, and D. Söll, J. Biol. Chem., in press). The transcription initiation site at coordinate −13 is unusually far upstream of the mature coding sequence compared with other *Drosophila* tRNA genes. The mature coding sequence of the pseudogene contains an 8-bp alteration in the anticodon stem which does not affect either the sequence or spacing of the two internal control regions. This change may have arisen by an 8- to 11-bp inversion (Fig. 1). The 3'-flanking sequences of the two genes are nearly identical. The 5'-flanking sequences are not homologous.

The 5'-flanking sequence of the tRNA^{His} gene is specifically required for transcription of this gene. Transcription of the tRNA^{His} gene, the pseudogene, and various mutant constructions revealed that the 5'-flanking sequence of the tRNA^{His} gene is specifically required for both the ability to form stable transcription complexes and for transcription initiation in *Drosophila* Kc cell extract. This sequence is contained within 45 nucleotides upstream of the 5' terminus of the mature coding region (Table 1). The mechanism by which the pseudogene 5'-flanking sequence limits transcription is distinct from the inhibition of other *Drosophila* tRNA genes (10, 12) and of a *Xenopus* tRNA^{Met} gene (19), where specific inhibitory sequences are found in the 5'-flanking sequence. Deletion of part or all of the 5'-flanking sequence of the tRNA^{His} pseudogene does not result in a significant increase in either transcription or competition activity. Considering the lack of an inhibitory sequence and the requirement for a specific 5'-flanking sequence in the tRNA^{His} gene, it appears that the pseudogene 5'-flanking sequence is nonpositive rather than negative.

The importance of the 5'-flanking sequence of the tRNA^{His} gene to both the ability to compete for transcription factors and the transcriptional level in *Drosophila* Kc cell extract is unprecedented in tRNA gene transcription. Careful study of a *Drosophila* tRNA^{Arg} gene (34) and a *Bombyx*

mori $tRNA^{Ala}$ gene (22) showed that the 5′-flanking sequence immediately adjacent to the 5′ end of the mature coding sequence is essential for efficient transcription in their homologous systems. Deletion of the 5′-flanking sequence of $tRNA^{Arg}$, however, has little effect on that gene's ability to bind transcription factors (34). Stable complex formation still occurs, although inefficiently, when the wild-type 5′-flanking sequence is removed (Table 1). Therefore, stable complex formation appears to occur both through 5′-flanking sequence-dependent and -independent pathways.

Whether the high degree of control of the *Drosophila* $tRNA^{His}$ gene in vitro reflects the existence of similar control in vivo is unknown. The gene product is unusual among tRNA species in that its amino acid acceptor stem is one nucleotide longer on the 5′ end. This suggests that $tRNA^{His}$ may have another function besides a role in ribosomal protein synthesis, possibly as a control element in cellular metabolism.

HeLa cell transcription factor incompatiblity with the *Drosophila* $tRNA^{His}$ gene. The transcription efficiencies in the HeLa cell extract of the $tRNA^{His}$ gene and pseudogene and of all 5′-flanking sequence deletion mutants are very low. This is surprising in light of the observation that the HeLa cell extract efficiently transcribes all other tRNA genes examined to date. Even genes with 5′-flanking sequences, which are inhibitory to transcription in HeLa cell extract, are transcribed efficiently when the 5′-flanking sequence is deleted. The sequence of human $tRNA^{His}$ (29) is 83% homologous to the sequence of *Drosophila* $tRNA^{His}$ (2). This could indicate that the human transcription system can recognize the *Drosophila* gene. In fact, the *Drosophila* $tRNA^{His}$ gene and pseudogene both compete in HeLa cell extract. Why then are they not transcribed?

In an attempt to answer this question, separated transcription factors from both *Drosophila* Kc and HeLa cell extracts were reconstituted to see whether one of the HeLa components is unable to interact efficiently with the $tRNA^{His}$ gene. The heterologous reconstituted transcription system with HeLa cell-derived factor B (36) and *Drosophila* Kc cell factor C and RNA polymerase III (3) were able to promote accurate transcription of *Drosophila* $tRNA^{Lys}$ and $tRNA^{Arg}$ genes (Burke, unpublished results). However the $tRNA^{His}$ gene was not transcribed efficiently compared with these templates. Factor C from HeLa has been shown to interact with the T-control region of tRNA genes (15, 23). By analogy, the B factor may interact with the D-control region of the gene. Although the *Drosophila* factor B is incompatible with the HeLa transcription system and will not promote efficient transcription when reconstituted with HeLa factor C and RNA polymerase III (Burke, unpublished results), a slight increase in transcription of the $tRNA^{His}$ gene in certain preparations of HeLa extract is observed with the addition of *Drosophila* factor B (Schaack, unpublished results). No transcription is observed under similar conditions with the pseudogene as the template. These results suggest that, although the human C factor may interact stably with the $tRNA^{His}$ gene, the human factor B may be unable to recognize the $tRNA^{His}$ gene and thus prevent efficient transcription. Since the D-control regions of the human and *Drosophila* $tRNA^{His}$ genes (inferred from the tRNA sequences) are highly homologous, the possibility exists that the *Drosophila* factor B interacts with the D-control region and RNA polymerase III such that a specific 5′-flanking sequence is required.

The unusually high degree of 5′-flanking sequence dependence for transcription of the *Drosophila* $tRNA^{His}$ gene coupled with the sequence similarity of *Drosophila* and human $tRNA^{His}$ and the inability of HeLa cell extract to transcribe the *Drosophila* $tRNA^{His}$ gene suggest that human $tRNA^{His}$ genes will also display 5′-flanking sequence dependence in HeLa cell extract. The two organisms may have evolved $tRNA^{His}$ gene 5′-flanking sequences that are not mutually recognized by the two transcription systems. In light of the atypical structure of the *Drosophila* gene (positions of the initiation and termination sites), it will be interesting to learn whether the structure of a human $tRNA^{His}$ gene is also atypical among human tRNA genes.

Implications for transcription initiation. The two patterns of initiation sites in the transcription of the $tRNA^{His}$ pseudogene deletion mutants, one constant with respect to the introduced vector sequence and one nearly constant with respect to the mature coding sequence (Fig. 7), suggest two different mechanisms for transcription initiation. The upstream initiation site suggests that the choice of the initiation nucleotide is governed by the upstream flanking sequence (containing an RNA polymerase III-binding site) and not by an internal control region. This is in agreement with the proposal that the initial interaction of the transcription apparatus is with the 5′ flank in tRNA gene transcription (34). Initial binding in the 5′-flanking sequence implies that RNA polymerase III, perhaps in a complex with transcription factors, is capable of "scanning" the DNA and can bind unstably with low specificity at many positions. Indeed, purified RNA polymerase III, or RNA polymerase III depleted of transcription factors, when incubated under transcription reaction conditions with purified templates, initiates "random" transcription (3, 27). The upstream initiation site is utilized most efficiently when it is positioned in the range of the normal pseudogene initiation site (Fig. 7). This implies that the efficiency with which this site is used is affected by the internal control regions.

The inside initiation site appears to be chosen by a mechanism that resembles measuring back from an internal control region (5, 40). It remains relatively constant with respect to the mature coding sequence at a position that appears to be characteristic for the $tRNA^{His}$ gene. The efficiency of initiation at this site is dependent on the upstream sequence, possibly in a periodic fashion (see above). As stated above, the B factor from *Drosophila*, which may interact with the D-control region, recovers a small amount of $tRNA^{His}$ transcription in HeLa cell extract. This suggests that the control region most important for initiation is the D-control region as suggested for a *C. elegans* $tRNA^{Pro}$ gene (5) and a *Drosophila* $tRNA^{Arg}$ gene (40).

The pseudogene: fossil or functional? To date, only a few tRNA pseudogenes have been reported, in contrast to many complete tRNA genes. Are the pseudogenes evolutionary intermediates bound for homogenization (45) or deletion? Have they become functional in their own right? The $tRNA^{Tyr}$ pseudogenes in the *E. coli tyrT* locus may be an example of the latter possibility. They appear to be involved in a complex transcription pattern (30). The locus contains two complete copies of a $tRNA^{Tyr}$ gene and additional copies of the 3′-distal 19 bp of the gene, each associated with a 180-bp repeat unit. Readthrough transcription to the termination site in the second repeat following the $tRNA^{Tyr}$ genes produces a primary transcript with the capability of encoding, in addition to two tRNA molecules, a small basic protein (1). A human $tRNA^{Glu}$ gene that is very efficiently transcribed has a sequence in its 5′-flanking sequence which has the potential to form a tRNA-like secondary structure (18).

The "pseudogene" in the 5'-flanking region, which is not homologous to a tRNA sequence, may be related to the high transcriptional activity of the $tRNA^{Glu}$ gene.

Other tRNA pseudogenes have been found for which no function has been postulated. An initiator $tRNA^{Met}$ pseudogene in *Drosophila* (inactive in vitro) consists of four dispersed regions of sequence homology (39). The DNA fragment containing the pseudogene fragments has properties of mobile DNA, suggesting that the pseudogene was created by repeated insertion and excision of a transposable element. In the rat genome there is a cluster of tRNA genes, repeated about 10 times, which encodes $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Glu}$ (25, 42). Some of the clusters contain pseudogenes for $tRNA^{Gly}$ and $tRNA^{Glu}$ (also inactive in vitro), which contain 7- to 11-bp deletions in the coding region. The $tRNA^{Asp}$ gene sequence is highly conserved, suggesting that this gene is essential to rat cells (42). A mouse pseudogene for $tRNA^{Phe}$ corresponds to 38 nucleotides at the 3' end of $tRNA^{Phe}$, including CCA (28). The presence of CCA in the pseudogene a suggests a flow of information from RNA to DNA. A similar mechanism has been suggested in the formation of pseudogenes for tubulin (46), immunoglobulin (21), and U1 RNA (11).

The $tRNA^{His}$ pseudogene may have arisen by a duplication event that resulted in a gene lacking the specifically required 5'-flanking sequence. Such a gene, inactive transcriptionally, may have sustained the sequence inversion within the mature coding sequence. The regions of homology to the $tRNA^{His}$ gene in the spacer region between the $tRNA^{His}$ gene and the pseudogene may be remnants of another tRNA gene fragmented by DNA rearrangement. It is also possible that the pseudogene, as it exists now, has evolved a function in the cell. This can only be evaluated after determining whether the pseudogene is expressed in vivo.

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