MOLECULAR AND CELLULAR BIOLOGY, Jan. 1986, p. 105–115
0270-7306/86/010105-11$02.00/0

Vol. 6, No. 1

Modulation of Transcriptional Activity and Stable Complex Formation by 5′-Flanking Regions of Mouse tRNA^{His} Genes

MARY J. MORRY† AND JOHN D. HARDING*

Department of Biological Sciences, Columbia University, New York, New York 10027

Received 7 May 1985/Accepted 10 October 1985

We determined the nucleotide sequences of three mouse tRNA^{His} genes and a tRNA^{Gly} gene present in two different λ clones. One λ clone contained two tRNA^{His} genes 600 base pairs (bp) apart in opposite orientations. The other clone contained a tRNA^{His} and a tRNA^{Gly} gene 569 bp apart in the same orientation. The coding regions of the three tRNA^{His} genes were identical to sequenced mammalian tRNA^{His} if posttranscriptional modifications are not considered. Notably, the three tRNA^{His} genes and a fourth gene previously sequenced by us contained within the flanking regions, various amounts of short, conserved 5′ leader sequences and 3′ trailer sequences directly abutting the coding regions. Otherwise the flanking regions were not homologous. Deletion mutants of one of the tRNA^{His} genes were constructed which contained 228, 99, 9, and 3 bp of the wild-type 5′-flanking region, respectively. Deletion of 5′-flanking sequences from positions −9 to −4 reduced transcriptional activity substantially (ca. fivefold) in a HeLa cell S-100 lysate. This effect was independent of the vector sequences in the deletion clone, implying that the region from −4 to −9 of the intact gene contains a positive modulatory element for transcription in vitro. The deletion mutant containing 3 bp of wild-type 5′-flanking sequence also had a greatly reduced ability to inhibit the transcription of a second tRNA gene in a competition assay. Thus, the normal 5′-flanking region influences the ability of the gene to form stable complexes with transcription factors. These data further indicate that a mammalian transcription extract is sensitive to 5′-flanking-region effects if a suitable tRNA gene is assayed.

Mammalian tRNA genes are reiterated and dispersed in the genome. Individual members of some mammalian tRNA gene families vary in respect to coding- or flanking-region sequences or both (26, 37, 41, 45). Similar types of variation are also seen in tRNA genes isolated from amphibians (6), insects (e.g., see references 1, 10, 20), and yeasts (reviewed in reference 16). To obtain a detailed understanding of the mechanisms by which tRNA gene expression is regulated, it is necessary to determine the effects of intrafamilial sequence variation on gene activity.

The efficiency of transcription, as measured by in vitro assays, is one level at which sequence variation can modulate tRNA gene activity. Eucaryotic tRNA genes contain highly conserved sequences within the coding regions that are absolutely required for transcription (4, 14, 19, 42). These "internal" control regions appear to be sites of interaction of specific transcription factors with the gene (12, 13, 24, 43, 47). In addition, sequences in the 5′-flanking regions of certain *Xenopus* (18), *Drosophila* (8, 10, 11, 38), *Bombyx mori* (23), and yeast (22, 44) tRNA genes can modulate transcription either positively or negatively. Thus, major questions in regard to any specific tRNA gene family are whether modulatory sequences are present in the flanking regions and the mechanisms by which these sequences affect gene activity.

To address these issues for mammalian tRNA genes, we isolated and sequenced four members of the mouse tRNA^{His} gene family. We show that the flanking regions of the mouse tRNA^{His} genes contain different amounts of short conserved 5′ leader and 3′ trailer sequences directly abutting the coding regions. Part of the conserved 5′ leader sequence is a positive regulatory element for transcription in vitro and promotes stable interaction of the gene with transcription factors present in cell extracts.

MATERIALS AND METHODS

Isolation of λ clones containing tRNA^{His} genes. A recombinant DNA library containing DBA-2 mouse genomic DNA cloned in phage λ Charon 4A (17) was plated, and the plaques were transferred to cellulose nitrate filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Benton and Davis (2). Filters were hybridized with 5×10^6 cpm of ^{32}P-labeled tRNA^{His}-specific probe as described in reference 27. After hybridization, the filters were washed for 1 h at 68°C in 4× SET–0.1% sodium dodecyl sulfate–0.1% sodium PP_i and for 1 h at 68°C in 2× SET–0.1% sodium dodecyl sulfate–0.1% sodium PP_i. (1× SET is 0.15 M NaCl, 2 mM EDTA, 30 mM Tris hydrochloride, pH 7.5.) The filters were autoradiographed with Kodak XS-5 film and an intensifying screen. Phage plaques which gave a signal on duplicate filters in the first screening were picked, and the phage were plaque purified.

Hybridization probes. A tRNA^{His}-specific probe was prepared from the tRNA^{His} coding region of phage λMt1 (17). This probe contains 51 base pairs (bp) of the tRNA^{His} coding region cloned into the *Eco*RI site of pBR322. It was labeled with ^{32}P by nick translation and used as a probe to detect tRNA^{His} coding sequences in λ and M13 phages.

Total mouse liver tRNA was labeled with ^{125}I as described in reference 17.

DNA sequence analysis. All DNA sequences were determined from M13 subclones derived from λ clones. Phage λMt5 and λMt6 DNAs were digested with various restriction enzymes (see Fig. 1 legend), and the resulting fragments made blunt ended, if necessary, by incubating 600 ng of DNA in 30 μl of 20 mM Tris hydrochloride (pH 7.5)–10 mM

* Corresponding author.
† Present address: Laboratory of Molecular Parasitology, The Rockefeller University, New York, NY 10021.

$MgCl_2$–10 mM dithiothreitol containing 33 μM each deoxynucleoside triphosphate and 0.1 U of DNA polymerase I (large fragment) at 37°C for 30 min. The target fragments were ligated with *Hin*cII-cut M13 mp7 DNA (29) essentially as described in reference 17, transfected into $CaCl_2$-treated *Escherichia coli* JM103, and plated as described in reference 29. M13 plaques were screened as described above for λ phage except that sonicated *E. coli* DNA carrier was not used in the hybridization and each filter was hybridized with 2.5×10^6 cpm of ^{32}P-labeled $tRNA^{His}$-specific probe. White plaques corresponding to autoradiographic signals were picked, and the mouse DNA inserts were sequenced by the dideoxy chain termination method, using 8 or 6% thin sequencing gels (35, 36).

Isolation of M13 subclones for transcription analysis. Clone nomenclature is as follows, e.g., for the clone m5A-228R: m = M13 vector, 5A = the $tRNA^{His}$ gene designated *Mt5A* (see Fig. 1 and 2), −228 = the last 5′-flanking residue of mouse DNA in the clone, R = coding strand of the tRNA gene inserted into the noncoding strand of the vector (as defined in reference 29). Clones m5A-228R (termed Av 1 in Fig. 1), m5A-99R, m5A-9R, and m5A-3R (termed A30 in Fig. 1) were isolated during DNA sequencing from *Ava*I, *Sma*I, *Hpa*II, and *Alu*I digests of λMt5 DNA, respectively. Clone m5B-34R was isolated during DNA sequencing from an *Hha*I digest of λMt5 DNA. Clone m5B-1R was isolated from a *Hpa*II-*Hha*I double digest of λMt5 DNA, the 5′ flank being anomalously replaced by a foreign DNA sequence. Clone m6-175R was isolated from a *Sau* 96-*Dde*I double digest, and clone m6-9R was isolated from an *Hpa*II digest of λMt6 DNA.

In vitro transcription. HeLa cell S-100 transcription extract was prepared essentially as described in reference 49. The final step involved dialysis in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9)–100 mM KCl–2 mM $MgCl_2$–2mM dithiothreitol–20% glycerol. The dialyzed extract was aliquoted and stored at −80°C.

HeLa cell nuclear extract was prepared as follows. Nuclei obtained during the preparation of the S-100 extract were suspended in a volume of hypotonic buffer equal to the original packed volume of HeLa cells. Hypotonic buffer is 10 mM Tris hydrochloride (pH 7.9), 10 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol. Saturated ammonium sulfate solution (0.1 volume) was added dropwise to the nuclei, and the solution was incubated on ice for 20 min with occasional gentle shaking. One packed cell volume of hypotonic buffer and glycerol (1:1, vol/vol) was added, and the suspension was centrifuged at 100,000 × *g* for 2 h. The supernatant was removed, dialyzed as for the S-100 lysate, and stored at −80°C.

A typical 15-μl transcription reaction mixture contained 7.5 μl of HeLa cell S-100, 5 μl of HeLa cell nuclear extract, 500 μM ATP, CTP, and UTP, 25 μM GTP, 10 μCi of [α-^{32}P]GTP (410 Ci/mmol), and 4 mM creatine phosphate. DNA template solution (1 μl) (typically containing 0.2 μg of M13 clone DNA in 10 mM Tris hydrochloride [pH 7.4]–0.1 mM EDTA) was added, and the reaction mixture was incubated for 1 h at 30°C. Stop buffer (1 volume) (8 M urea, 150 mM NaCl, 20 mM Tris hydrochloride [pH 8], 10 mM EDTA, 0.5% sodium dodecyl sulfate, 100 μg of yeast RNA per ml) and gel buffer (1 volume) (7 M urea, 15% glycerol, 1× TBE buffer [35], 5% dimethyl sulfoxide, 0.03% xylene cyanole) were added, and the sample was incubated at 70°C for 5 min. Samples were run on 8% thin polyacrylamide–8 M urea gels (35) which were dried and autoradiographed.

RESULTS

Isolation and sequence analysis of mouse $tRNA^{His}$ genes. From a mouse recombinant DNA library, we plaque purified 12 λ phage which hybridized with a $tRNA^{His}$-specific probe. The λ clones can be divided into three groups based on *Eco*RI (and other restriction enzyme) digestion patterns. Of the 12 clones, 9 contained a 10-kilobase *Eco*RI mouse DNA insert and, in addition, identical *Xba*I and *Bam*HI fragments. One of these phage, termed λMt5, was further characterized. One of the 12 λ clones, termed λMt6, contained two *Eco*RI mouse DNA fragments, 2.2 and 4.3 kilobases in size, respectively. The remaining two λ clones were identical to phage λMt1, which contains a single $tRNA^{His}$ gene (17).

Two noncontiguous sequences, termed Mt5A and Mt5B, were obtained from phage λMt5 (Fig. 1, 2, and 3). The Mt5A and Mt5B sequences comprise 956 and 799 bp, respectively (Fig. 2 and 3). Restriction mapping experiments based on known sites in the sequences established that the Mt5A and Mt5B sequences are separated by ca. 250 bp of unsequenced DNA (Fig. 1). Each sequence contains a single $tRNA^{His}$ gene. The two $tRNA^{His}$ coding regions are present in opposite orientations, ca. 600 bp apart.

We derived a 1,373-bp sequence from phage λMt6 which contains a $tRNA^{Gly}$ and a $tRNA^{His}$ gene, 569 bp apart in the same orientation (Fig. 1 and 4).

On the basis of the sequences shown in Fig. 2, 3, and 4, we analyzed λMt5 and λMt6 DNA by Southern blotting (46), using both mouse tRNA- and $tRNA^{His}$-specific probes. These experiments indicated that the two λ clones contain no additional unsequenced tRNA genes (data not shown).

The tRNA coding regions. The three $tRNA^{His}$ coding regions present in the Mt5A, Mt5B, and Mt6 sequences can encode a $tRNA^{His}$ identical to sequenced mouse $tRNA^{His}$ (34) if posttranscriptional modifications are not considered. The $tRNA^{His}$ coding region of phage λMt1 differs at a single position in the D loop (17).

The $tRNA^{His}$ genes do not encode the 3′-terminal CCA residues of the tRNA, as is usual for eucaryotic tRNA genes. In addition, cytoplasmic $tRNA^{His}$ contains a 5′-terminal G residue (modified to methyl-G in mouse $tRNA^{His}$ [34]) that is not encoded in the DNA of λMt1, λMt5, or λMt6. Cooley et al. (7) have shown that extracts of *Drosophila* Kc cells add a G residue to the 5′ end of *Drosophila* $tRNA^{His}$ transcribed in vitro. The mouse gene sequences suggest that the 5′-terminal G residue of mammalian $tRNA^{His}$ is also added post-transcriptionally.

The $tDNA^{Gly}$ encoded by λMt6 does not correspond to any sequenced $tRNA^{Gly}$ listed in a recent compilation (15). It is identical to the $tRNA^{Gly}$ coding region of two other sequenced mouse genes (21, 26).

Comparison of $tRNA^{His}$ gene flanking regions. Inspection of the sequences shown in Fig. 2, 3, and 4 indicates that the $tRNA^{His}$ genes contain short homologous 5′ leader and 3′ trailer sequences contiguous to the coding regions. No other obvious flanking region homologies are evident.

The proximal 5′-flanking regions of the $tRNA^{His}$ genes are compared in Fig. 5A. The first 13 bp of the Mt5A 5′ flank are homologous to the first 14 bp of the Mt5B sequence, the latter containing one additional C residue. Mt5B and Mt6 share a common 13-bp leader sequence containing two mismatches. The proximal 5′-flanking region of the Mt1 gene is less homologous to those of the other three genes. It is most similar to the Mt6 sequence, with five of the first seven bp homologous.

A comparison of the proximal 3′-flanking residues of the

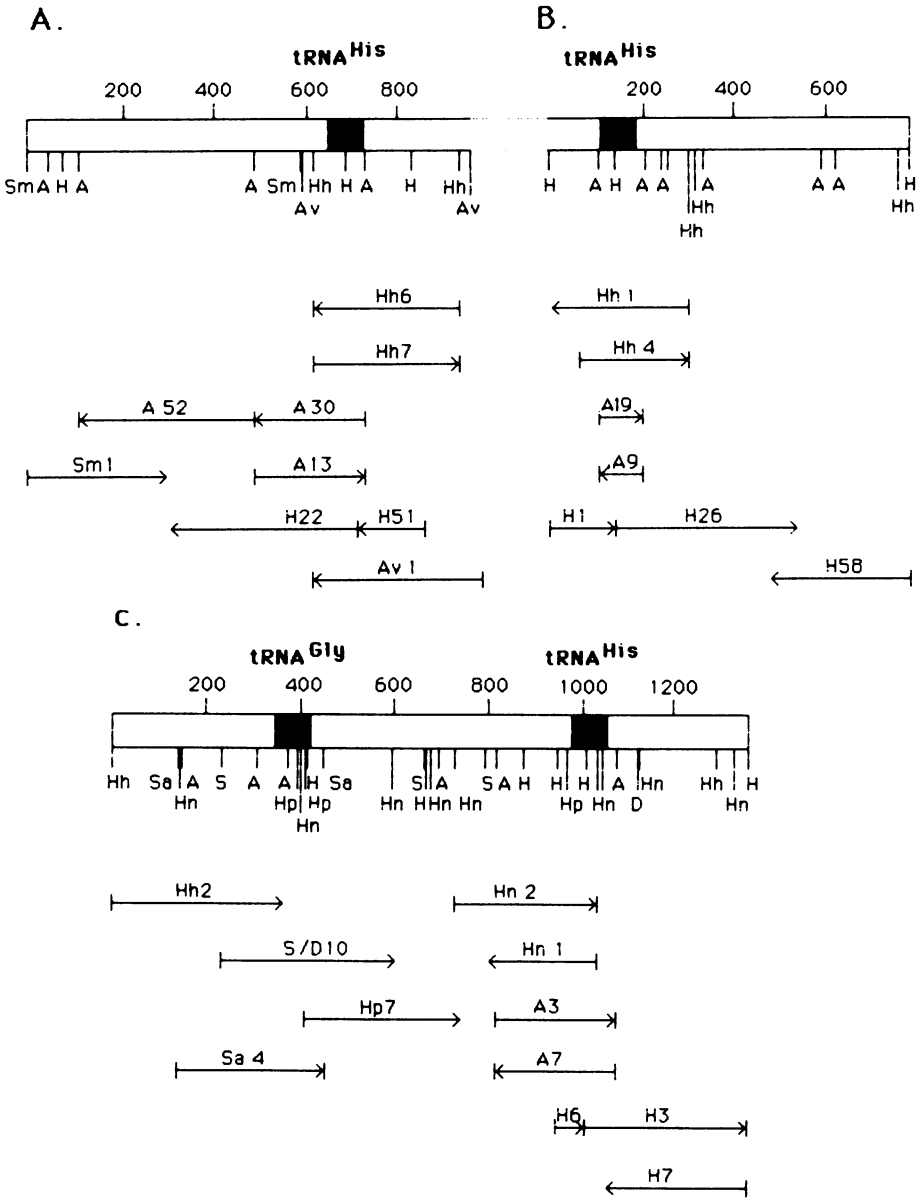

FIG. 1. M13 subclones used to sequence the tRNA genes in clones λMt5 and λMt6. (A and B) The bar represents the sequenced region of clone λMt5. The dotted lines mark ca. 250 bp of unsequenced DNA identified by restriction mapping. The tRNA^{His} coding regions are filled in. The leftward (5′) gene is termed Mt5A and the rightward gene is termed Mt5B. (C) The bar represents the sequenced region of clone λMt6. The tRNA^{Gly} and tRNA^{His} coding regions are filled in. The labeled arrows below the bars represent the sequenced regions of M13 subclones derived from λMt5 and λMt6 DNAs, respectively. The beginning of the mouse DNA insert relative to the M13 sequencing primer is indicated by a vertical line; the arrowhead denotes the end of the sequence read from the gel. An arrowhead followed by a vertical line indicates that the entire mouse DNA insert of the M13 subclone was sequenced. Arrows pointing to the right indicate that DNA corresponding to the coding strand (as defined by the tRNA^{His} gene in the particular sequence) was sequenced; arrows pointing to the left indicate that the noncoding strand was sequenced. The first letter of each M13 subclone designation denotes the restriction digest of the lambda phage from which it was isolated: A, *Alu*I; Av, *Ava*I; D, *Dde*I; H, *Hae*III; Hn, *Hin*fI; Hh, *Hha*I; Hp, *Hpa*II; S, *Sau*96I; Sa, *Sau*3A; Sm, *Sma*I.

tRNA^{His} genes is shown in Fig. 5B. The Mt5A and Mt5B genes share a homologous 29-bp sequence with one mismatch. The homology ends just before the putative transcription termination site, consisting of a string of four or five consecutive T residues in the two genes. There is no obvious homology among the 3′-flanking regions of the Mt6 and Mt1 genes and any of the other tRNA^{His} genes.

Effects of 5′-flanking sequences on transcription in vitro. To analyze the effects of 5′-flanking region sequences on tRNA^{His} gene transcription, we prepared several deletion mutants of the tRNA^{His} genes. Each clone was transcribed in a modified HeLa cell S-100 lysate as described in Materials and Methods. Addition of HeLa cell nuclear extract to the basic S-100 lysate substantially increases the transcriptional activity of the lysate (R. Rooney and J. Harding, unpublished data). In preliminary experiments (data not shown), we established that each tRNA gene is transcribed by RNA

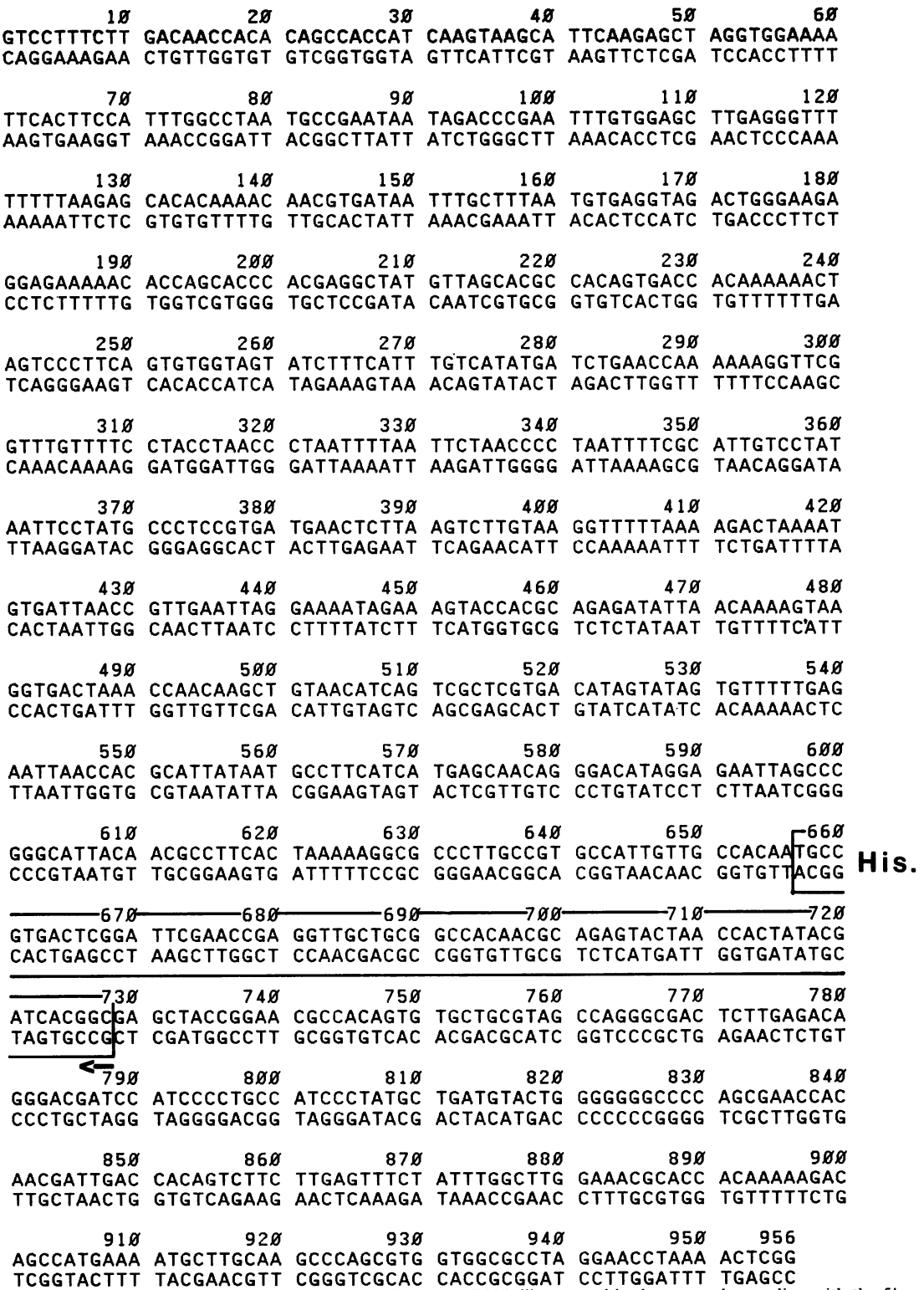

FIG. 2. Sequence of the A region of clone λMt5. The coding (non-tRNA-like) strand is shown on the top line with the 5′ end to the left. The $tRNA^{His}$ coding region is boxed. The arrow denotes the direction of transcription.

polymerase III and characterized the optimal range of DNA concentrations that yielded maximal transcription. Optima are nearly the same for all M13 clones (400 ng of DNA per 25-μl reaction).

Figure 6 shows the transcripts obtained from each M13 clone. (See Materials and Methods for an explanation of clone nomenclature.) Based on parallel electrophoresis of a DNA sequencing ladder, all of the transcripts in the figure are larger than mature tRNA. Thus, during a 60-min incubation in this extract, little or no processing occurs. Compar-

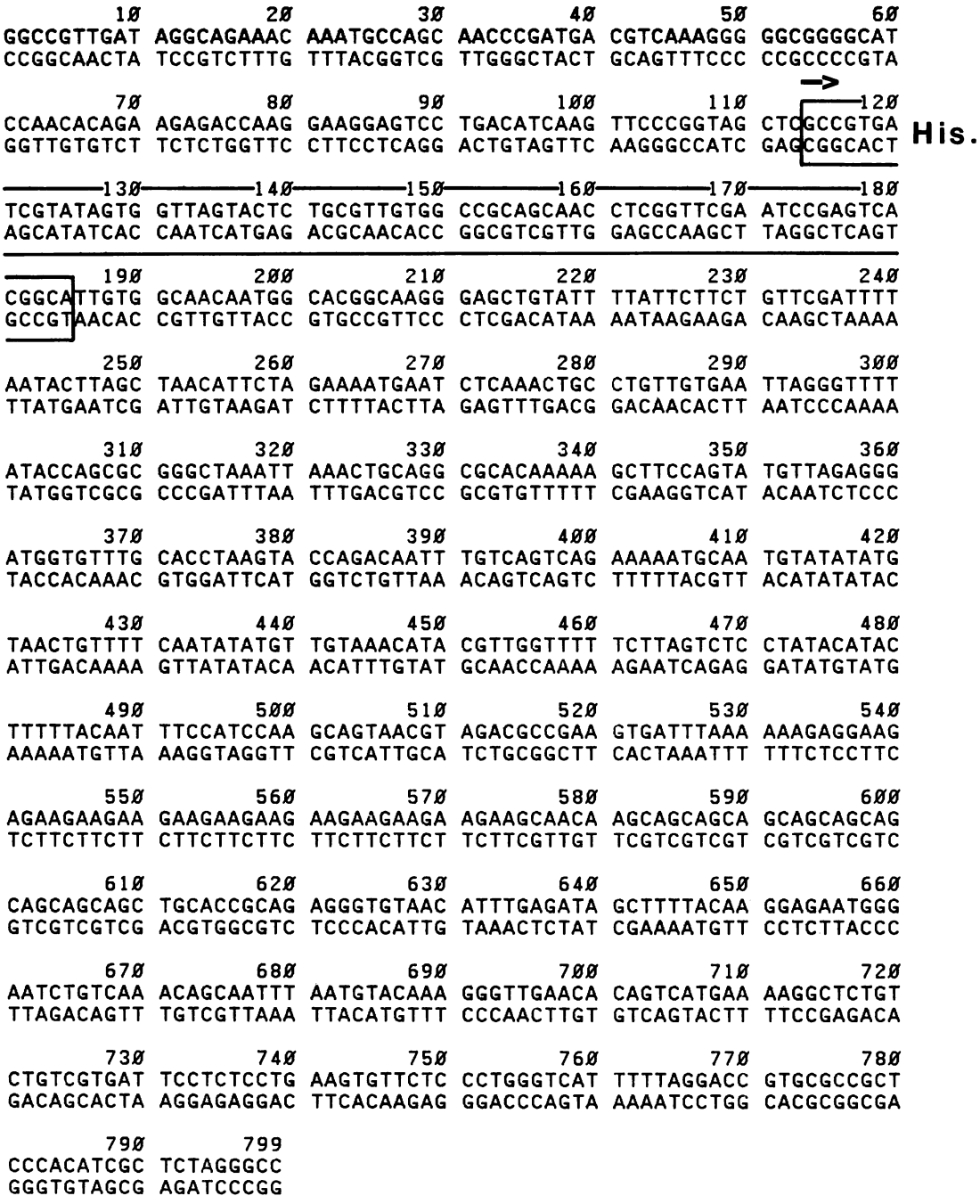

FIG. 3. Sequence of the B region of clone λMt5. The noncoding (tRNA-like) strand is shown on the top line with the 5′ end to the left. The tRNA^{His} coding region is boxed. The arrow denotes the direction of transcription.

ison of the intensities of the autoradiographic bands in lanes b, c, f, h, j, and k indicates that all four tRNA^{His} genes (Mt5A, Mt5B, Mt6, and Mt1) are transcribed with qualitatively similar efficiencies when they contain 28 to 228 residues of their original 5′-flanking-region DNA. The M13 vector does not yield transcripts in the size range of the unprocessed tRNA^{His} gene transcripts (lane a). Variable and minor amounts of larger transcripts are sometimes copied from the vector.

Comparison of lanes d and e with lanes b and c of Fig. 6 indicates that the conserved 5′ leader sequence of the Mt5A gene contains sequences that markedly affect transcription in vitro. When the original 5′-flanking residues between positions −99 and −10 are replaced by M13 vector sequences, a modest reduction in transcription occurs (compare lanes c and d). A similar result is seen for the Mt6 gene (lanes h and i). Further deletion of 5′-flanking residues of the Mt5A gene to position −3 (lane e) causes a much more severe reduction in transcription and synthesis of a more heterogeneous set of transcripts. Scans of autoradiographs obtained from six independent experiments of the type shown in Fig. 6 indicate that the average relative transcriptional efficiencies of clones −228R, −9R, and −3R are 1, 0.85, and 0.23, respectively. The slightly greater activity of

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        1Ø          2Ø          3Ø          4Ø          5Ø          6Ø
CGTGGAGATA ACATTCAGAC CAAAAACTAA TCACTTAAAA TATGAAAATG TAAAGTGCAA
GCACCTCTAT TGTAAGTCTG GTTTTTGATT AGTGAATTTT ATACTTTTAC ATTTCACGTT

        7Ø          8Ø          9Ø          1ØØ         11Ø         12Ø
TCTTTTCTAC ATTTGTACTA AAGAACGTCA CACATTAAAA AACATCCACA GCAGAGCCTT
AGAAAAGATG TAAACATGAT TTCTTGCAGT GTGTAATTTT TTGTAGGTGT CGTCTCGGAA

        13Ø         14Ø         15Ø         16Ø         17Ø         18Ø
TTACAGGATC ACAGGATTCC AGAAGCTCCC TTCAAACGAT AAAAAGAATT AATTTTCTTT
AATGTCCTAG TGTCCTAAGG TCTTCGAGGG AAGTTTGCTA TTTTTCTTAA TTAAAAGAAA

        19Ø         2ØØ         21Ø         22Ø         23Ø         24Ø
AAACTTGGCT ATCCTAGAGT GTTTGTCTGT CATCTCTCTC CGTCTGTCTT ACAGGTCCCC
TTTGAACCGA TAGGATCTCA CAAACAGACA GTAGAGAGAG GCAGACAGAA TGTCCAGGGG

        25Ø         26Ø         27Ø         28Ø         29Ø         3ØØ
CCCGCCCCCG TCTTAATTTT TCTTCAATCA AAACAAATTT GCAAGAAAAG AAACAAAAAA
GGGCGGGGGC AGAATTAAAA AGAAGTTAGT TTTGTTTAAA CGTTCTTTTC TTTGTTTTTT

        31Ø         32Ø         33Ø         34Ø         35Ø         36Ø
CTTTCTGTCA TAGCTTTCAT TAAGATATCC AAACAGAACA ATCGCTAACC TGCGTTGGTG
GAAAGACAGT ATCGAAAGTA ATTCTATAGG TTTGTCTTGT TAGCGATTGG ACGCAACCAC

        37Ø         38Ø         39Ø         4ØØ         41Ø         42Ø
GTATAGTGGT GAGCATAGCT GCCTTCCAAG CAGTTGACCC GGGTTCGATT CCCGGCCAAC
CATATCACCA CTCGTATCGA CGGAAGGTTC GTCAACTGGG CCCAAGCTAA GGGCCGGTTG

        43Ø         44Ø         45Ø         46Ø         47Ø         48Ø
GCATAAGGTT GTCTTTTTAC GTTTAGAAAT CCGAAGATCT CTGATCCCTG GATCTAACTC
CGTATTCCAA CAGAAAAATG CAAATCTTTA GGCTTCTAGA GACTAGGGAC CTAGATTGAG

        49Ø         5ØØ         51Ø         52Ø         53Ø         54Ø
CACCGAGTTT CTTCTCAAGT TTATGTTATT CCTATCTGTT AGTACAGCGA ATGGCGTCAC
GTGGCTCAAA GAAGAGTTCA AATACAATAA GGATAGACAA TCATGTCGCT TACCGCAGTG

        55Ø         56Ø         57Ø         58Ø         59Ø         6ØØ
AGACTTGTAG GATTATCCTT TTGGGATTGT TCAGGAAAGT ATCTAAAATG GAGTCTGGCT
TCTGAACATC CTAATAGGAA AACCCTAACA AGTCCTTTCA TAGATTTTAC CTCAGACCGA

        61Ø         62Ø         63Ø         64Ø         65Ø         66Ø
CTTTGAGACT TTAGAAGCAG CCCCGCCTTC GGTTCGGGAG GTTTTGTGAG TGGAACCAGG
GAAACTCTGA AATCTTCGTC GGGGCGGAAG CCAAGCCCTC CAAAACACTC ACCTTGGTCC

        67Ø         68Ø         69Ø         7ØØ         71Ø         72Ø
AAACAGGCCC CTGGAGAAGG ACTCCTTCAC AGCTCTGCTC CTTTTCTGGT TGCACCTTCT
TTTGTCCGGG GACCTCTTCC TGAGGAAGTG TCGAGACGAG GAAAAGACCA ACGTGGAAGA

        73Ø         74Ø         75Ø         76Ø         77Ø         78Ø
GGTAACAAAA CGTTGATTCC GTGGCTTGTT TCTTTCCAGA GAAACAACAT AGGTCTGCTG
CCATTGTTTT GCAACTAAGG CACCGAACAA AGAAAGGTCT CTTTGTTGTA TCCAGACGAC

        79Ø         8ØØ         81Ø         82Ø         83Ø         84Ø
GTGCGGCGGT TTCTTATCCC AGGTCCATGA AATCCCAGAA GCAAGGAGCT GGTGCTCGTG
CACGCCGCCA AAGAATAGGG TCCAGGTACT TTAGGGTCTT CGTTCCTCGA CCACGAGCAC

        85Ø         86Ø         87Ø         88Ø         89Ø         9ØØ
TTAGTATTGC GGAAGCACCA GGGTTTCAAA GCCAGACTTT TGATATTGCG GCCATATAGA
AATCATAACG CCTTCGTGGT CCCAAAGTTT CGGTCTGAAA ACTATAACGC CGGTATATCT

        91Ø         92Ø         93Ø         94Ø         95Ø         96Ø
CTATACTAAA TAAATCAGGC AACTCTGGAA GGCTACTCTC ATTCAAAGAC CATTAAATTG
GATATGATTT ATTTAGTCCG TTGAGACCTT CCGATGAGAG TAAGTTTCTG GTAATTTAAC
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Gly.

FIG. 4. Sequence of clone λMt6. The noncoding (tRNA-like) strand is shown on the top line with the 5′ end to the left. The $tRNA^{Gly}$ and $tRNA^{His}$ coding regions are boxed. Arrows denote the direction of transcription.

the −99 clone relative to the −228 clone in Fig. 6 does not occur reproducibly.

Comparison of lanes f and g and lanes k and l in Fig. 6 suggests that the 5′-flanking regions of the Mt5B and Mt1 genes also influence transcription. Clone m5B-1R (lane g) is transcribed to a lesser extent than clone m5B-34R (lane f). Likewise, the loss of the 5′-flanking region plus 5 bp of the coding region greatly reduces transcription of the Mt1 gene (compare lanes k and l).

While the results of Fig. 6 suggest that the conserved 5′

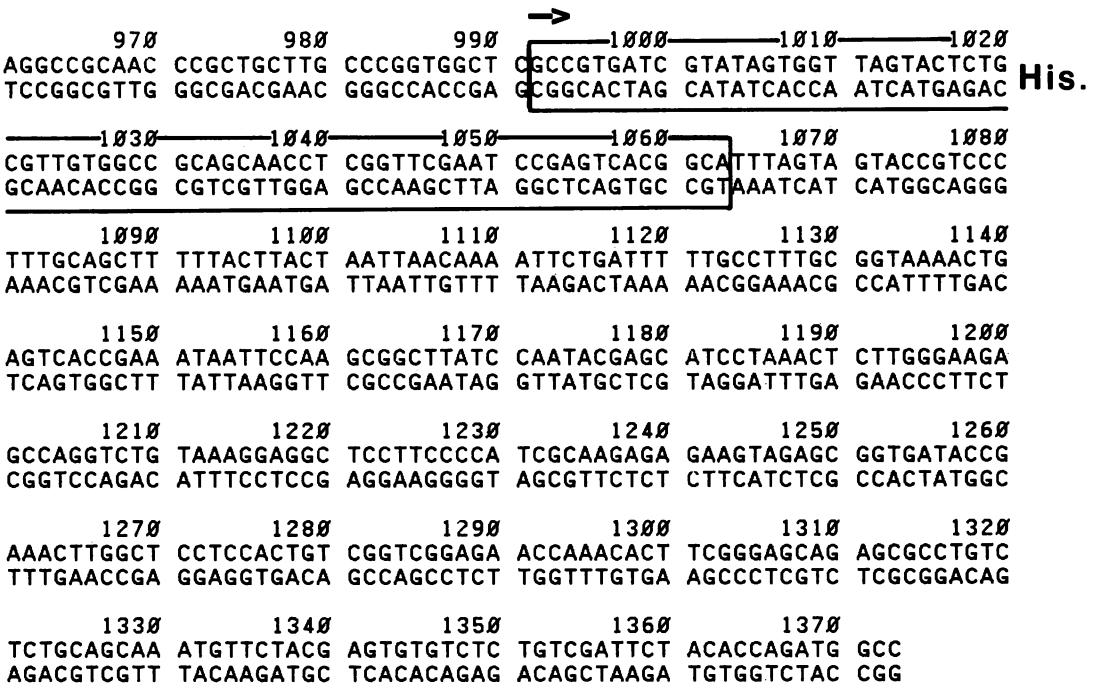

FIG. 4. *Continued.*

leader sequence of the Mt5A gene influences transcription, it is also possible that the M13 vector contains a sequence which, when moved a critical distance from the coding region, inhibits transcription. To test for possible vector effects on transcription, we constructed two additional sets of clones which contain the same mouse DNA inserts as those transcribed in Fig. 6, but with different vector sequences flanking the mouse DNA inserts. One set of clones contains the inserts of clones m5A-228R, m5A-99R, m5A-9R, and m5A-3R in the opposite (L) orientation in M13. The second set of clones contains the mouse DNA sequences inserted into the *Bam*HI site of pBR322. When transcribed as in Fig. 6, these clones yielded the same result. Replacement of normal mouse DNA sequences between positions −9 and −4 with vector sequences greatly reduced transcription (data not shown).

Effect of 5′-flanking region on formation of stable transcription complexes. To determine whether the 5′-flanking resi-

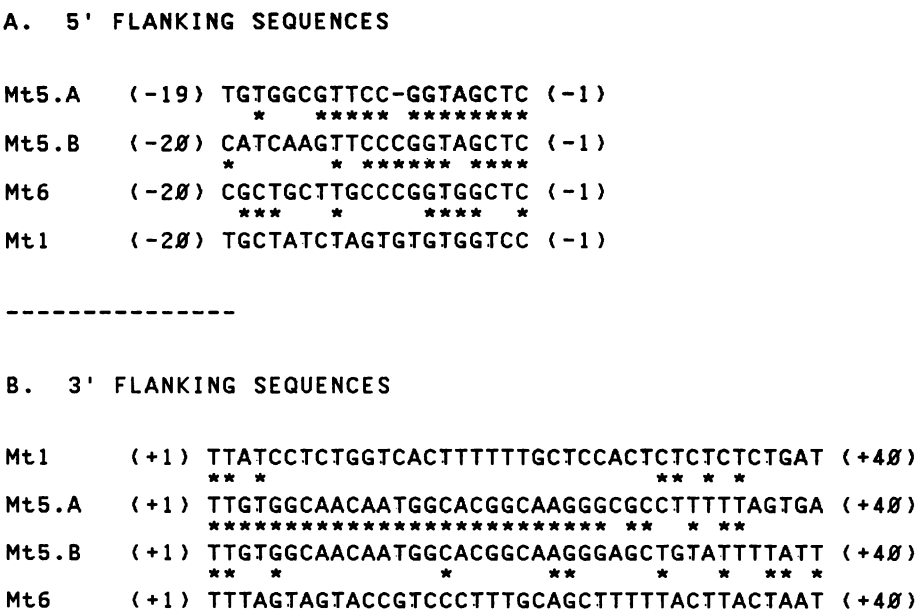

FIG. 5. Comparison of proximal 5′- and 3′-flanking regions of tRNA^{His} genes. In panel A, (−1) indicates the first residue 5′ to the coding region. In panel B, (+1) indicates the first residue 3′ to the coding region. The noncoding DNA strand is shown.

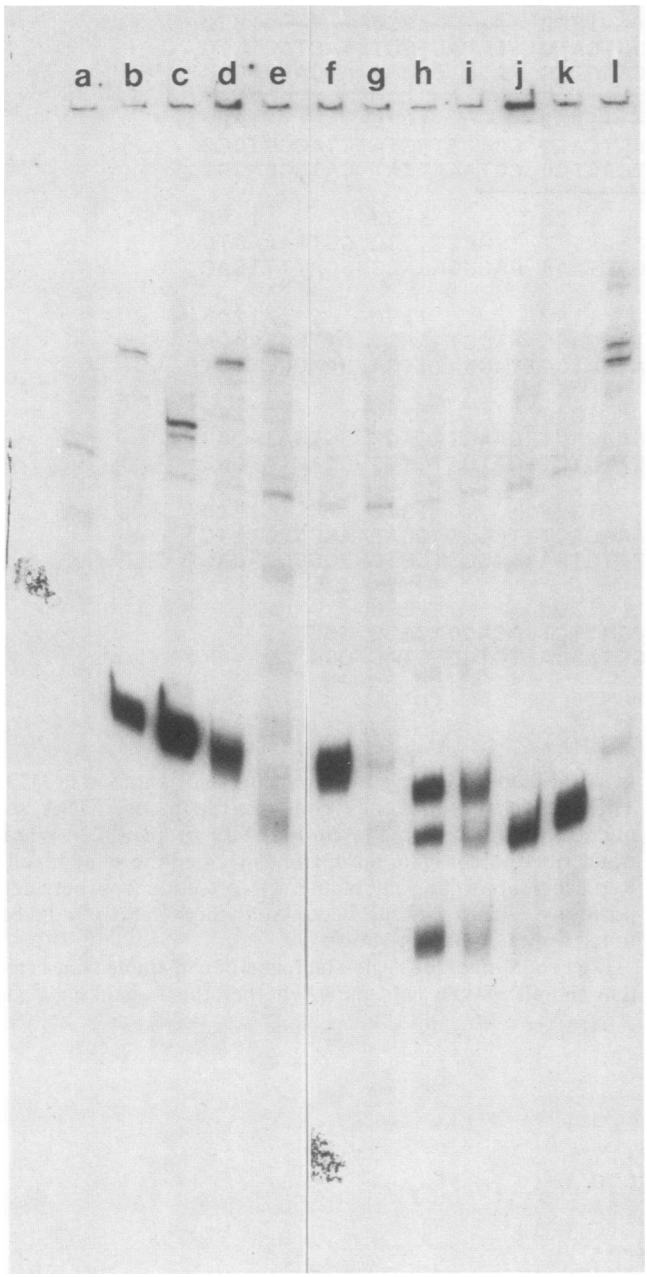

FIG. 6. In vitro transcription analysis of M13 clones containing $tRNA^{His}$ coding sequences. Each DNA (400 ng) was transcribed in 25 μl of a standard transcription reaction mixture for 60 min at 30°C. An autoradiograph of the gel is shown. Lanes: a, M13 mp7; b, m5A-228R; c, m5A-99R; d, m5A-9R; e, m5A-3R; f, m5B-34R; g, m5B-1R; h, m6-73R; i, m6-9R; j, m1-167L; k, m1-28R; l, m1+6R. Lanes a through e and f through l are from the same gel. Irrelevant lanes between lanes e and f were deleted from the figure.

dues critical for efficient transcription also influence formation of stable transcription complexes in the HeLa cell extract, we performed a competition experiment in which two genes are added sequentially to the same transcription reaction (39). As a reference template, we used a mouse $tRNA^{Gly}$ gene (26), the transcripts of which are readily distinguishable from the $tRNA^{His}$ gene transcripts on a polyacrylamide gel (compare Fig. 7, lanes b and f). Neither

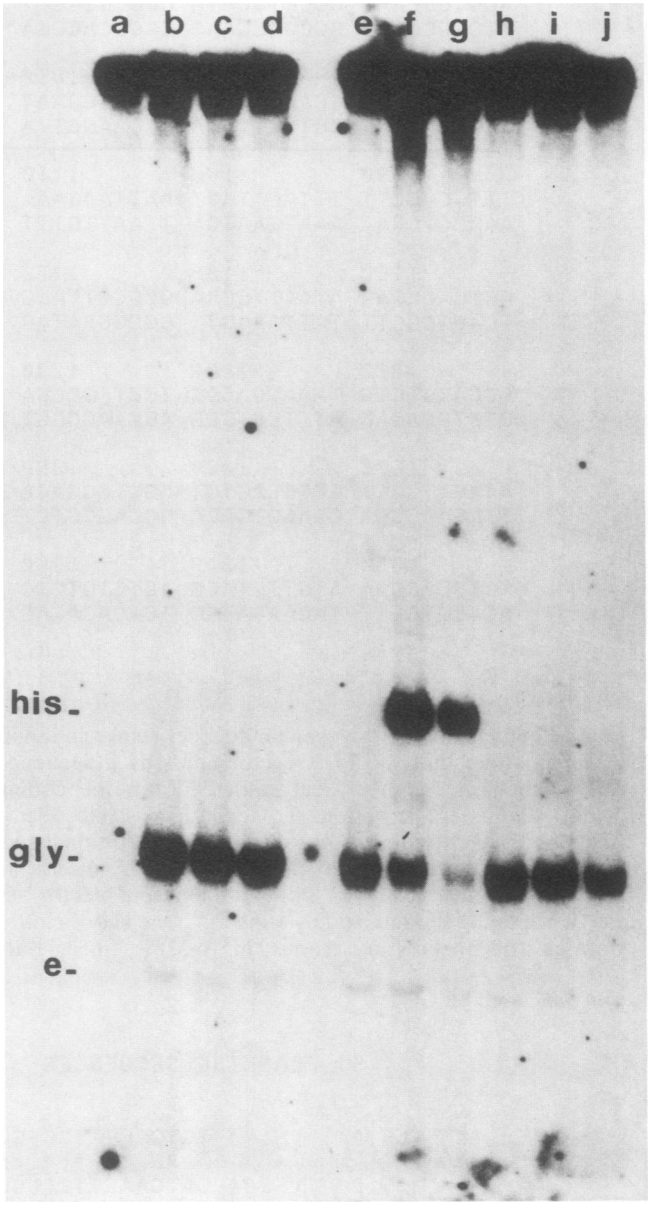

FIG. 7. Competition between $tRNA^{His}$ genes and a $tRNA^{Gly}$ gene in vitro. The $tRNA^{Gly}$ gene is contained in the M13 clone Nar8 (see reference 26). Each reaction mixture contained 300 ng of total DNA added to 10 μl of standard transcription mix. M13 mp7 DNA was added as necessary to yield this total DNA concentration. Lanes a through d, Transcription of the $tRNA^{Gly}$ gene alone: a, 38 ng; b, 75 ng; c, 150 ng; d, 300 ng of Nar8 DNA, respectively. Lanes e through j, Competition experiments. A given amount of $tRNA^{His}$ gene DNA and M13 mp7 DNA (total DNA concentration, 225 ng) were incubated for 15 min at 30°C in 10 μl of a standard transcription reaction. $tRNA^{Gly}$ gene DNA (75 ng) was then added, and incubation was continued for 60 min at 30°C. Lanes: e, no $tRNA^{His}$ gene DNA; f, 150 ng of m5A-228R; g, 225 ng of m5A-228R; h, no $tRNA^{His}$ gene DNA; i, 150 ng of m5A-3R DNA; j, 225 ng of m5A-3R DNA. An autoradiograph of the gel is shown. On the left of the figure, his marks the position of $tRNA^{His}$ gene transcripts, gly marks $tRNA^{Gly}$ gene transcripts, and e marks an endogenous band labeled in the lysate. Neither $tRNA^{His}$ nor $tRNA^{Gly}$ transcripts were processed in these reactions.

the tRNA^{Gly} nor the tRNA^{His} gene transcripts are processed appreciably in these reactions.

Before performing the actual competition experiments, we determined the lowest amount of tRNA^{Gly} gene DNA that would yield a strong signal on the autoradiograph. In the presence of M13 DNA, 75 ng of tRNA^{Gly} gene DNA yielded a maximal amount of transcript which was not increased appreciably by additional tRNA^{Gly} gene template (Fig. 7, lanes a through d). The rather low and sharp concentration optimum in the presence of inert vector DNA (compare lanes a and b) is characteristic of S-100 extracts and has also been noted by others (43).

For the competition experiments in lanes e through j of Fig. 7, a given tRNA^{His} gene DNA and M13 DNA were first incubated with the transcription lysate, followed by tRNA^{Gly} gene DNA. Preincubation of 225 ng of clone m5A-228R DNA significantly reduced the transcription of the tRNA^{Gly} gene (compare lanes e and g), indicating that stable transcription complexes had been formed on the tRNA^{His} gene before addition of the tRNA^{Gly} gene. In contrast, preincubation of clone m5A-3R DNA in the lysate affects the transcription of the tRNA^{Gly} gene to a much lesser extent (compare lanes h through j). Thus, a tRNA^{His} gene which contains only three residues of the 5′-flanking region has a greatly reduced ability to form stable complexes with transcription factors present in the S-100 lysate.

DISCUSSION

Effects of 5′-flanking sequences on transcription. Several invertebrate tRNA genes contain 5′-flanking sequences that stimulate transcription in homologous extracts (8, 11, 22, 23, 38, 44). However, in some cases, the 5′-flanking dependence of transcription appears to be reduced in mammalian cell extracts (3, 11, 32, 38, 43). These observations have therefore raised questions regarding the sensitivity of mammalian cell extracts to 5′-flanking-region effects and the existence of modulatory sequences in the 5′ flanks of mammalian tRNA genes. We show here that a mouse tRNA^{His} gene contains sequences located between positions −4 and −9 in the 5′-flanking region that have a marked positive influence on transcription in a HeLa cell extract. Thus, at least some mammalian tRNA genes are similar to invertebrate genes in regard to 5′-flanking-sequence requirements for efficient transcription in vitro. Furthermore, the HeLa cell extract clearly is sensitive to 5′-flanking effects if a suitable tRNA gene is assayed.

From the experiments reported in this paper we cannot infer whether the entire −4 to −9 region of the Mt5A tRNA^{His} gene is necessary for modulation of transcription. Inspection of the sequences of the M13 and pBR322 clones we constructed suggests the potential importance of residues at two particular positions. All actively transcribed mouse tRNA^{His} gene clones (representing all four sequenced genes) contain a G residue on the noncoding strand at position −4. None of the poorly transcribed clones (such as m5A-3R) contains a G residue at this position. Another important position may be at −6, which is always a pyrimidine in transcribed clones and, with one exception, a purine in poorly transcribed clones. The importance of these two consensus positions may also explain why a *Drosophila* tRNA^{His} gene is transcribed with much lower efficiency in a HeLa cell extract than in a *Drosophila* cell extract (3, 8). Although the *Drosophila* gene contains a proximal 5′-flanking region which is similar in some respects to that of the mouse tRNA^{His} genes, it lacks a G at position −4 and a pyrimidine at −6. Alternatively, other sequences as well as differences in human and *Drosophila* transcription factors (3) may influence transcription of the *Drosophila* gene in the HeLa cell extract.

A major question raised by our data is the mechanism by which the proximal 5′-flanking region of the tRNA^{His} gene influences transcription. There is currently no evidence for factors that bind solely to the 5′ flanks of eucaryotic tRNA genes, although this possibility has not been ruled out definitively. Rather, it is more likely that the 5′-flanking region influences the interaction of the gene either with factors that bind to the internal control regions or with RNA polymerase III. The competition experiment shown in Fig. 7 demonstrates that the 5′-flanking sequence influences stable complex formation with transcription factors. *Drosophila* and HeLa cell transcription extracts contain at least two components essential for formation of stable complexes in vitro (3, 24). Transcription factor C probably interacts with the 3′ internal control element (or B block) of a tRNA gene. Its association with the gene is stabilized by factor B (3, 24). The resulting complex can then be transcribed by RNA polymerase III. Burke and Soll (3) have suggested that the 5′-flanking region of a *Drosophila* tRNA^{His} gene, which is required for efficient transcription in a *Drosophila* extract, probably influences at least the interaction of factor B with the factor C-gene complex. By analogy, the −4 to −9 region of the mouse tRNA^{His} gene may also be involved in the interaction of the B factor with the gene, perhaps serving as part of the binding site, as is suggested by the apparent sequence requirements at specific positions, as noted above. The −4 to −9 region may also influence some aspect of interaction of RNA polymerase III with the gene, as is suggested by the heterogeneous array of transcripts obtained from the mutants containing only 3 bp of wild-type flanking-region sequence. These hypotheses are directly testable by construction of specific point mutations in the wild-type 5′-flanking sequence and transcription analyses with fractionated extracts.

The members of several tRNA gene families present in yeasts (22, 30, 31, 40, 48), *Drosophila melanogaster* (1, 9, 20, 33, 50), and rats (41) also contain variable amounts of short, conserved 5′ leader and 3′ trailer sequences contiguous with the coding region. In most cases the influence of these sequences on transcription has not been analyzed. However, the transcriptional properties of yeast tRNA^{Leu} genes are strikingly similar to those of the mouse tRNA^{His} genes. The yeast genes contain a conserved 5′ leader that is a positive modulator of in vitro transcription (22, 32). Components present in yeast transcription extracts protect the 5′ leader from DNase digestion (47). The 5′ leaders of the yeast and mouse genes are, however, completely different in sequence, showing that different flanking-sequence motifs can influence transcription. These results suggest that the combination of a particular 5′-flanking sequence with a particular tRNA coding sequence to produce a more active transcription unit may be a common (although not universal) feature of eucaryotic tRNA gene organization.

Sequence organization and evolution of the mouse tRNA^{His} gene family. It is not known whether all of the members of a mammalian tRNA gene family are expressed equally in vivo. To answer this question it will be necessary to isolate and characterize an entire gene family. In this paper and in reference 17, we report the sequences of four mouse tRNA^{His} genes. A number of observations suggest that these genes are representative, and perhaps make up a majority, of the genes encoding tRNA^{His} in mouse cells.

Histidine is specified by the CAU and CAC codons, both of which can be read by the GUG anticodon present in tRNA encoded by the four sequenced genes. Mouse and human $tRNA^{His}$ isolated from tissue culture cell lysates by immunoprecipitation with anti-Jo-1 antibody is identical in sequence to the tDNA sequences of the Mt5 and Mt6 genes if posttranscriptional modifications are not considered (34). Anti-Jo-1 antibody is isolated from the sera of autoimmune patients having polymyositis and probably recognizes histidyl-tRNA synthetase. The antibody immunoprecipitates all of the $tRNA^{His}$ charging activity from a HeLa cell lysate (28). Thus, if there is a mammalian $tRNA^{His}$ not immunoprecipitated by the antibody (and therefore not sequenced), it must either be present in very small amounts in tissue culture cells or not be recognized by the antigenic $tRNA^{His}$ synthetase. The available evidence therefore suggests that all mouse $tRNA^{His}$ molecules have essentially the same primary structure, as encoded by the genes we sequenced. This structure can probably be modified in various ways, as evidenced by the identification of multiple $tRNA^{His}$ isoacceptors by reverse-phase chromatography (see, e.g., reference 25).

From the Clarke-Carbon equation (5), we calculate a 78% probability that our genomic DNA library contains any given unique sequence. We screened the library rigorously for $tRNA^{His}$ genes. Eighty signals from the initial screening with $tRNA^{His}$-specific probe were characterized. Most of these were false-positives that did not yield hybridizing phage upon a secondary screening. A total of 12 phage, 9 of which are very similar, if not identical, to λMt5, were finally plaque purified. The $tRNA^{His}$-specific probe obtained from phage λMt1 contained one mismatched residue (near its 5′ end) relative to the $tRNA^{His}$ coding regions of λMt5 and λMt6. Therefore, it is likely that other minor sequence variants would have been detected in the screening since the probe identified the Mt5 and Mt6 sequences. The probe might not have hybridized with highly mutated pseudogenes or gene fragments.

An issue that remains unresolved is the precise number of $tRNA^{His}$ genes in the mouse genome. In genomic Southern blots, a $tRNA^{His}$-specific probe hybridizes only to *Eco*RI fragments of genomic DNA having the same size as those present in phages λMt1, λMt5, and λMt6 (J. Harding, unpublished data). We have not sequenced the multiple copies of the phage λMt5 sequence obtained from the DNA library and thus have not yet determined whether this sequence is reiterated in the genome.

Notably, the $tRNA^{His}$ gene sequences exhibit a hierarchy in respect to sequence homology. The Mt6 sequence lacks the 3′ trailer and part of the 5′ leader relative to the Mt5A and Mt5B sequences. The Mt1 gene has the least homologous leader, no homologous trailer, and a single variant residue in the coding region. The evolution of this pattern of sequence variation can perhaps be explained by two hypotheses. First, the sequence differences may reflect the relative times during evolution at which the various genes duplicated and diverged if it is assumed that there is little or no selective pressure for maintaining many of the flanking-region residues. Thus, the duplication event that produced the Mt5A and Mt5B sequences may have occurred more recently than the duplication that produced the Mt5 and Mt1 (or Mt6) sequences. An alternative explanation is that similarities in flanking-region homologies are related to the relative efficiency of gene conversion, which can homogenize related sequences and has been shown to occur in the $tRNA^{Ser}$ genes of *Schizosaccharomyces pombe* (30).

ACKNOWLEDGMENTS

This work was supported by grants 1-802 from the March of Dimes Birth Defects Foundation and Public Health Service grant GM26884 from the National Institutes of Health.

We wish to thank Jang Han for preparing the $tRNA^{His}$-specific probe and Robert Rooney for providing transcription extracts.

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