STRUCTURE AND TRANSCRIPTION OF EUKARYOTIC tRNA GENES

Authors: **Stephen Jefferson Sharp**
Department of Microbiology and Molecular Genetics
California College of Medicine
University of California
Irvine, California

Jerome Schaack
Lynn Cooley
Deborah Johnson Burke
Dieter Söll
Department of Molecular Biophysics and Biochemistry
Yale University
New Haven, Connecticut

Referee: U. L. RajBhandary
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts

I. INTRODUCTION

The "major" function of tRNA is its involvement in the translation of mRNA into protein on the ribosome. However, specific tRNAs are also involved in other specialized cellular functions. The translational functions of tRNA include: (1) the initiation of peptide synthesis, (2) the elongation of polypeptide chains, and (3) the attachment of the growing polypeptide chain to the ribosome. While these functions of translation are mediated by the ribosome, the fidelity of protein synthesis is dependent on the esterification of the correct amino acid to the cognate tRNA, as well as on the specifity of codon-anticodon interactions between mRNA and tRNA. The tRNA structure, therefore, must contain all information necessary for specific interactions with components in the translation apparatus that interact with all tRNAs. The distinct members of the isoaccepting tRNA family must in addition embody structural information required for the homologous aminoacyl-tRNA synthetase reaction.

In recent years, considerable progress has been made in our understanding of tRNA structure.[1] The tertiary structure of tRNA is formed by folding the familiar "cloverleaf" between the D and T stems to form what has been termed the "L" structure. The integrity of the tRNA structure appears to depend on hydrogen bonds and, to a lesser extent, upon stacking interactions between bases from parts of the molecule that are remote in the sequence. The hydrogen bond interactions include those involved in the formation of each of the four common stems of the cloverleaf secondary structure as well as nine tertiary base-pairs which include base-pairing between the D and T loops.[1]

The degeneracy of the genetic code allows several nucleotide triplets to code for the same amino acid. The multiplicity of codons for a given amino acid is accompanied by a multiplicity of tRNAs having different primary structures which are charged with the same amino acid. A trivial relationship between the number of such isoaccepting tRNAs and codon degeneracy does not exist. In some cases isoaccepting tRNAs read the same codon whereas in others, several codons are read by the same tRNA.

Each cell therefore contains 50 to 100 chromatographically distinct tRNA species. The lengths of these vary between 76 and 90 nucleotides. Of about 400 tRNAs for which the primary sequences are known, approximately 200 have been purified from eukaryotic organisms with about 50 of these having an organellar origin, the remainder being coded for in the nucleus.[2]

While the genetic code is "universal", several coding exceptions occur within the mitochondrion.[3] Concomitant with use of the variant genetic code, tRNA structure and functions also are variant in the mitochondrion. Apart from mitochondrial tRNA, the structural features of tRNA are conserved throughout eubacteria (prokaryote) and eukaryotes.[4] Only limited information is available on tRNA (and tRNA gene) structure for the archaebacteria.[5]

The perspective of this paper is the regulation of eukaryotic tRNA gene transcription. The significance of these studies lies in an understanding of how the cell decodes mRNA by providing suitable correspondence between codon and anticodon, in order that protein synthesis proceeds optimally. For the eukaryotic cell the problem of tRNA biosynthesis is the same as for the prokaryotic cell; simply stated, the cell must ensure the synthesis of the correct tRNA in sufficient concentrations to respond to the codon complement of the mRNA being translated. An analysis of codon usage in several mRNA species from different organisms reveals a correlation between codon preferences and tRNA population.[6-8] It has been demonstrated that, in fact, the efficiency of translation is strongly influenced by the codon composition within an mRNA and that the rate of polypeptide elongation, therefore, varies dependent on the local context in which a particular codon is read.[9,10] This finding now gives a conceptual understanding to the translational function played by minor tRNA species and to isoacceptor tRNA abundance in general.

One possible level of control whereby the cell could coordinate the tRNA population to the specific translational requirements is at the transcriptional level. An understanding of such control mechanisms is now being sought by studying the basic mechanism of transcription of tRNA genes. An unraveling of the workings of the transcriptional process combined with an understanding of the function of tRNA gene organization and arrangement should provide a sound knowledge basis on which to answer this fundamental question.

II. STRUCTURE OF tRNA GENES

A. Identification of tRNA Genes

A DNA sequence that contains all the information to code for a complete tRNA structure is generally termed a tRNA gene. This must remain an operational definition, however, since the redundancy within most isoacceptor families makes it difficult to conclusively demonstrate in vivo activity of a particular locus. The gene/product relationship of tRNAs has allowed radioactively labeled tRNA and purified isoacceptor tRNA species to be used as probes to identify tRNA gene sequences in recombinant DNA libraries. Identification of DNA sequences by hybridization, however, does not address the functionality or completeness of the genomic sequences that hybridize. The problems of pseudogenes and of the structure of functional tRNA genes is discussed more fully below.

In vitro transcription of tRNA genes from such diverse organisms as *Drosophila melanogaster*, *Xenopus laevis*, *Saccharomyces cerevisiae*, and human reveal a commonality of the tRNA gene functional unit. Furthermore, the results of the in vitro transcription studies strongly imply that the tRNA complementary DNA sequences that have been cloned using hybridization methods for identification indeed have the potential of being functional tRNA genes. More direct evidence that tRNA complementary sequences of *D. melanogaster* are active tRNA genes comes from the studies of Tener and co-workers.[11,12] The level of $tRNA_{3b}^{Val}$ in the tRNA isolated from flies deficient in the major $tRNA_{3b}^{Val}$ loci (as determined by $tRNA_{3b}^{Val}$ hybridization) was shown to be reduced to approximately 50%. These studies demonstrate that tRNA gene loci which have been identified solely by *in situ* hybridization,

contain active tRNA genes that contribute to the cellular concentration of tRNA. The studies of Tener and co-workers also imply that a mechanism does not exist to compensate for the loss of $tRNA_{3b}^{Val}$ structural genes. Instead, in *D. melanogaster* the dosage of $tRNA_{3b}^{Val}$ genes appears to be compensated by increasing the expression of another isoacceptor, $tRNA_4^{Val}$.[12]

Both physical and genetic techniques have been used to demonstrate that *S. cerevisiae* $tRNA^{Tyr}$ is coded by eight unlinked genes. Physically, the DNA coding sequences have been localized by RNA-DNA hybridization on eight different EcoRI restriction fragments.[13] Genetically, tyrosine-inserting nonsense suppressors have been isolated at eight loci on six different chromosomes.[14] These genes have been studied individually by both genetic and biochemical methods and all eight genes have been cloned.[15,16] The association between the physical and genetic maps was established by identifying the cloned yeast DNA segments that corresponded to the specific tyrosine-inserting nonsense suppressor loci.[16] This correspondence was determined by incorporating the method of restriction enzyme site polymorphism using the considerable natural variation amongst laboratory yeast strains in the sizes of the $tRNA^{Tyr}$-hybridizing EcoRI and HindIII fragments. Since the size variants behave genetically as simple Mendelian traits, it was possible to associate particular suppressors with restriction fragments by demonstrating meiotic and mitotic linkage between the tyrosine-inserting suppressors and the genetic determinants of the naturally occurring size variant restriction fragment which contained its coding sequence.[16]

A *Schizosaccharomyces pombe* tRNA gene was isolated by using a functional selection scheme dependent on opal suppressor activity by transformation of an *S. cerevisiae* nonsense mutant with *Sc. pombe* DNA from a suppressor strain.[17] Genomic DNA of an opal suppressor strain of the fission yeast *Sc. pombe* was cloned into the BamHI site of the yeast plasmid vector pYRP17. The recombinant library was then transformed directly into an *S. cerevisiae* strain containing mutations complemented by the presence of plasmid. The yeast strain also contained a polar mutation in the *HIS*4A region of the *HIS*4 gene. Suppression of this mutation by the UGA-recognizing tRNA allowed translation of the downstream *HIS*4C region. In this manner the cloned sup3-e $tRNA^{Ser}$ gene of *Sc. pombe* was shown to be functional in vivo. The $tRNA^{Ser}$ gene has a dimeric tRNA gene structure. A 7 base pair (bp) spacer separated the $tRNA_{UGA}^{Ser}$ gene from an initiator $tRNA^{Met}$ gene. Each $tRNA^{Ser}$ gene belonging to this codon family (UCN) has this structure and to date three of four characterized initiator $tRNA^{Met}$ genes have this arrangement in the genome.[18] Transcription studies in vitro and in vivo using this dimeric tRNA gene[18] and a homologous dimeric tRNA gene (sup9-e)[18] demonstrate the synthesis of a dimeric tRNA precursor. The only other example of a dimeric tRNA gene arrangement is the $tRNA^{Arg}$-$tRNA^{Asp}$ gene of *S. cerevisiae* in which the two mature coding sequences are separated from each other by 10 bp and are transcribed in vitro as a dimeric precursor tRNA.[20]

B. The tRNA Exon

Sequences of tRNA genes from *S. cerevisiae*, *Sc. pombe*, *X. laevis*, *Caenorhabditis elegans*, *Bombyx mori*, *D. melanogaster*, *X. laevis*, rat, mouse, and human as well as from several other organisms have been determined.[21] These genes code for tRNAs corresponding to most of the 61 possible "sense" codons of the genetic code. This inventory of sequenced tRNA genes is extensive enough to allow general comments on tRNA gene structure: in contrast to *E. coli* tRNA genes, the 3′ terminal CCA sequence of mature tRNAs is not encoded in eukaryotic genes. This oligonucleotide sequence is formed posttranscriptionally by tRNA nucleotidyltransferase;[22] the eukaryotic tRNA-gene transcriptional unit is monomeric. (Two exceptions to this are the dimeric arrangements described above.) The arrangement of individual tRNA genes, therefore, appears not to be dependent upon concomitant organization with other transcriptional units. As will be described in following sections, tRNA genes are found in some cases in close proximity to other tRNA genes on either

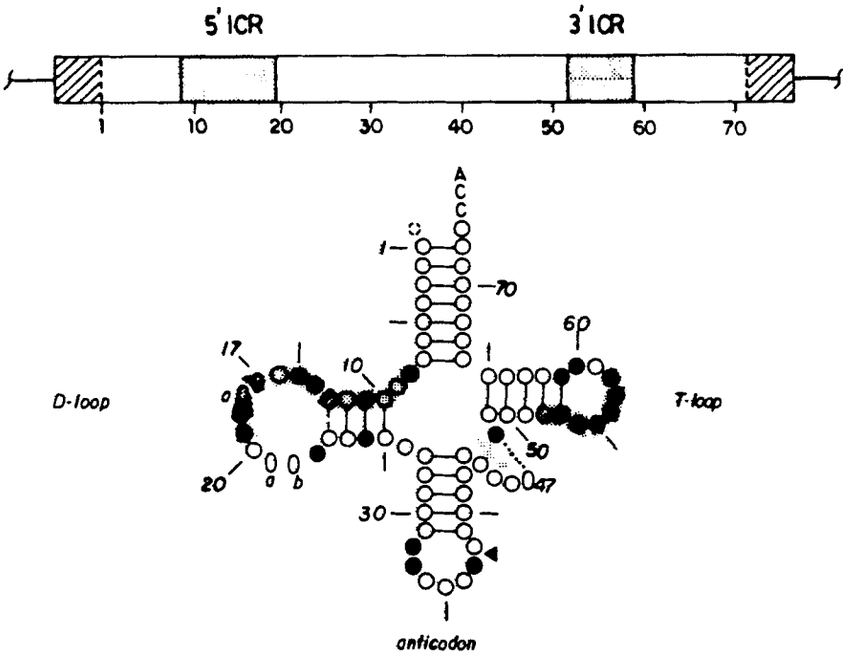

FIGURE 1. Representation of a tRNA gene. The boxed outline indicates the salient features of a tRNA gene showing tRNA gene coordinates. The sequences encoded by the 5′ and 3′ ends of the gene (cross-hatched) appear in the primary transcription product but are specifically removed to yield the mature-sized tRNA product. The position of the transcription ICRs are indicated (stippled) in the linear representation of the gene. These regions are also superimposed onto the "cloverleaf" representation of the tRNA transcript to indicate the tRNA structures encoded by the ICRs. The extra-arm encoding region has also been shown to have transcriptional importance. Eukaryote tDNAs do not encode the tRNA 3′ terminus CCA sequence. The closed circles represent nucleotides that are invariant or semi-invariant, in all eukaryotic tRNA sequences. Nucleotides represented by an elipse invariantly occur, and the length of the extra arm is invariant (dotted line). $tRNA^{His}$ contains an "extra" nucleotide on the 5′ terminus (dashed circle).

strands of the DNA duplex; to date, there is no specialized 5′ flanking sequence that is conserved for all tRNA genes of an organism. In fact, the general observation is that even within the set of genes for a single tRNA isoacceptor, little 5′ flanking sequence homology is displayed; similarly little sequence homology is held for 3′ flanking regions except that 3′-flanking sequences are AT-rich, and oligothymidylate stretches within the 3′-flanking sequences serve as termination signals for RNA polymerase III directed transcription.[23-25]

An intriguing difference exists between the sequence of rat $tRNA^{Asp26}$ and the corresponding gene.[26,27] The nucleotide sequence of the gene for $tRNA^{Asp}$ differs from that of the major rat liver $tRNA^{Asp}$ in two positions adjacent to the 5′ end of the anticodon; CT in the DNA but UC in the RNA. Of ten copies of this $tRNA^{Asp}$ gene, six display the nucleotide heterogeneity. Although four gene copies remain to be analyzed and may reveal the sequence displayed by $tRNA^{Asp}$, it seems probable that the genes containing the CT sequence indeed code for $tRNA^{Asp}$ and that a novel enzyme activity (or activities) is responsible for the posttranscriptional sequence change to UC. The envisaged reaction would be similar to the G to Q exchange performed by guanine insertion enzyme.[28-29] Coincidentally, $tRNA^{Asp}$ is a Q-containing tRNA and the UC sequence in question is 5′ adjacent to the encoded G base in $tRNA^{Asp}$ that is exchanged with Q.

The single *D. melanogaster* $tRNA^{His}$ species is similar to all other $tRNA^{His}$ species that

have been sequenced in that the 5′ stem of the acceptor arm is longer by one nucleotide than in all other tRNA species.[2] The 5′-terminal nucleotide is an unpaired guanylate residue.[30] Synthesis of the $tRNA^{His}$ has been examined in vitro from a *D. melanogaster* $tRNA^{His}$ gene, from region 48F of chromosome 2, which does not encode this residue;[31] instead, an adenylate residue is found at this position in the DNA sequence. Transcription reactions in vitro demonstrate that the 5′ terminal guanylate of $tRNA^{His}$ is added posttranscriptionally. This seems to be a common mechanism for eukaryotic $tRNA^{His}$ biosynthesis.[30,32]

C. tRNA Gene Introns

About 10 to 20% of eukaryotic tRNA genes appear to contain introns. Of the 300 to 400 tRNA genes in yeast, for example, perhaps 40 contain introns.[33] The introns of eukaryotic tRNA genes range in length from 8 to 60 nucleotides and in any family of isoacceptor tRNA genes, so far as is known, are homologous sequences; but between different tRNA gene families, the introns share little homology. Sequences at the boundaries of the introns are not conserved, but the location of the intron, one nucleotide to the 3′ side of the encoded anticodon (3′ of tRNA coordinate 37), is the same in all genes that have been sequenced. For most precursor tRNAs transcribed from genes containing an intron, the intervening sequence usually contains complementarity to the anticodon and the anticodon stem and loop. Notable exceptions to this rule are the *X. laevis* $tRNA^{Tyr}$ gene,[34,35] a $tRNA^{Trp}$ gene of *Dictyostelium discoideum*,[36] and a *Sc. pombe* $tRNA^{Lys}$ gene.[37] The function of the intervening sequence in the precursor tRNA will be discussed later (see Section V). Whether the intron affects the function of the tRNA gene is an especially intriguing problem, since most tRNA genes do not contain an intron.

To test its role in the expression of a yeast $tRNA^{Tyr}$ suppressor gene (SUP6), the intron was precisely deleted.[38] The altered gene gave phenotypic suppression of host-cell ochre mutations. This study demonstrated that the intervening sequence is not absolutely required for the expression of this gene. Recently, Raymond and Johnson have investigated intron function in vitro.[38] Deletion of 8, 10, 13, or 20 bp from the intron of a yeast $tRNA_3^{Leu}$ gene resulted in mutant templates having no impairment compared to wild-type, in their abilities to direct in vitro transcription. Insertion of an additional 10, 21, or 30 bp into the wild-type intron, at the naturally occurring HpaI site, also resulted in templates with unimpaired activity. Transcription was reduced from that of the wild-type gene, however, when an extra 103-bp segment was inserted into the HpaI site.[39] These results support the hypothesis that tRNA gene transcription is not sensitive to the structure of the intron and that transcription is relatively insensitive to the presence or absence of an intron. This is similarly implied from the finding that in *S. cerevisiae* only one of two closely-related species of $tRNA^{Ser}$ genes contains an intron.[40] The genes that code for the serine-inserting SUP-RL1 amber and SUQ5 (SUP16) ochre suppressors have been cloned and sequenced demonstrating the activity of both species. These two unlinked genes differ by only three base pairs in their coding regions and while the SUP-RL1 gene has a 19-bp intron the SUP16 gene has none.[40] As will be discussed later, the presence or absence of an intervening sequence in a tRNA gene transcript, however, can influence the rate of processing and some base modifications.

D. Flanking Sequences

For a few tRNA gene families 5′- and 3′-flanking sequences are highly conserved within members of the family. These families include the *D. melanogaster* genes for initiator $tRNA^{Met}$,[41] $tRNA^{Glu}$,[42] and $tRNA^{Gly}$,[43] human initiator $tRNA^{Met}$,[44] and rat $tRNA^{Asp}$.[27] The *D. discoideum* $tRNA^{Trp}$ gene is repeated on at least six different genomic DNA fragments bounded by EcoRI sites, and the 5′-flanking sequence of this gene is conserved, and in fact, repeated many times in the genome. With a few unique exceptions that will be discussed in a later section, sequence similarities that can be attributed to defining transcriptional control sequences, are not observed in regions 5′-flanking tRNA genes.

E. Associated Transposable Elements

Sigma is a yeast transposable element occurring 16 or 18 bp upstream from the 5′ end of the sequence coding for the mature tRNA. *Sigma* elements have been found adjacent to six different types of tRNA genes, encoding $tRNA^{Glu}$, $tRNA^{His}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Ser}$, and $tRNA^{Tyr}$.[45,46] In six of the seven analyzed cases the elements are in the same orientation relative to the tRNA gene. *Sigma* has 8-bp inverted repeats at its ends and is flanked by 5-bp direct repeats of the "target" sequence. Moreover, the absence of sequence homology among known and inferred *Sigma* "target" sequences suggests that the specificity of *Sigma* insertion depends on recognition of the adjacent tRNA-encoding region. While most tRNA genes in yeast are not associated with a *Sigma* element, it seems that all *Sigma* elements are associated with tRNA genes.[45,47] The $tRNA^{Tyr}$ gene at the SUP2 locus is preceded by *Sigma*. The demonstration of SUP2 suppressor activity shows that tRNA gene expression can occur in proximity to *Sigma* and offers a model system to examine the function of *Sigma* in tRNA gene expression.

The *D. discoideum* $tRNA^{Trp}$ gene, repeated on at least six different genomic subsegments, has a repetitive sequence in the region 5′-flanking the mature tRNA coding sequence. The repeated sequence is not an A/T-rich "satellite" segment and has a length greater than 100-bp.[36] Whether this sequence, which occurs many times in the genome, is transposable or is involved with $tRNA^{Trp}$ gene expression is not known.

In *D. melanogaster* an interesting polymorphism, in the 42A locus, exists between the two strains Oregon R and Canton S.[48] The cloned EcoRI fragment 12C (about 1300 bp), of Oregon R was 250 bp longer than the identical fragment cloned from the Canton S genome (EcoRI-17F). Furthermore, the cloned EcoRI-12E fragment, which neighbors EcoRI-12C, was 40 bp shorter in Oregon R than in Canton S. The EcoRI-12C fragment lies between two tRNA gene subclusters in the 42A region.[49,50] Of all the EcoRI fragments of a 94-kb segment that were tested, 12C (Oregon R) was the only probe that gave a smear of hybridization over the high-molecular-weight region of the chromosomal blot. The corresponding fragment, 17F, in Canton S, hybridized to approximately 10 discrete bands in addition to the approximately 1200-bp fragment of origin. These results suggest that the extra 250-bp sequence is highly repetitive in the genome, and that some of the 17F sequences are slightly repetitive.[48] Further analysis to test the association of these sequences with tRNA gene regions has not been performed.

F. Pseudogenes

One of the most interesting results of the sequencing of tRNA-hybridizing genomic regions is the finding of tRNA "pseudogenes" intermingled with "real" genes. By this terminology a "real" gene refers to a DNA sequence corresponding to one found in an RNA species or that can be folded into the shape of the familiar "cloverleaf" structure. Conversely, "pseudogene" means that these sequences usually cannot be folded into a "cloverleaf" configuration and a novel RNA has not been identified corresponding to this DNA sequence. Pseudogenes consist either of a partial sequence of a corresponding tRNA sequence or are DNA sequences displaying nucleotide heterogeneity within a corresponding tRNA sequence. Of the latter type, examples include genes from the initiator $tRNA^{Met}$ families of *D. melanogaster*,[41] *X. laevis*,[51,52] and human.[44] The *D. melanogaster* initiator $tRNA^{Met}$ gene contained on plasmid pPW591 has a single nucleotide G to T transversion when compared to the expected coding sequence at tRNA coordinate 30. This sequence encodes a tRNA-like structure that cannot form the last base-pair on the anticodon stem. A human initiator $tRNA^{Met}$ gene differs from the tRNA sequence by a similar transversion at tRNA coordinate 57, within the T loop. In *X. laevis* one of two initiator $tRNA^{Met}$ genes (B-copy), has a C to T transition at position 65; the A-copy contains the expected coding sequence. This position-65 polymorphism encodes a tRNA that would form a GU base-pair at the beginning of the T stem. Sequence heterogeneity has also been observed within the *D. melanogaster* gene

families for $tRNA_4^{Val}$[53] and $tRNA_5^{Lys}$.[54] In some instances,[54] nucleotide changes occur concomitantly with maintaining the potential stem structure of the putative tRNA product.

Pseudogenes for tRNAs have not been observed to date in the genomes of yeast species. Since many tRNA genes have now been characterized in *S. cerevisiae* it appears that tRNA pseudogenes may in fact be absent from this organism. An obvious difference in the organization of yeast tRNA genes and those of higher eukaryotes is their single dispersed nature. While it would be intriguing if this organization was the direct reason for the absence of pseudogenes, this does not appear to be the case since the initiator $tRNA^{Met}$ genes have a similar organization and yet within these families pseudogenes (see below) and coding region polymorphisms do occur.

A novel pseudogene arrangement has recently been discovered in *D. discoideum*.[55] Overlapping a region of the DNA that encodes a $tRNA^{Val}$, a cloverleaf-like structure representing a $tRNA^{His}$ pseudogene could be superimposed. The 5′ terminus sequence, GTTCG, of the $tRNA^{Val}$ gene serves to form the common tRNA-loop IV sequence of the $tRNA^{His}$ pseudogene. Several features of the $tRNA^{His}$-like encoded sequence suggest that this is a pseudogene. The $tRNA^{His}$ pseudogene does not encode several of the conserved nucleotides, found in all tRNAs, in the D loop and the anticodon-loop. As a result, an RNA having the sequence encoded by the pseudogene would lack the tertiary base pairing of tRNA.[55]

Of several *D. melanogaster* recombinant plasmids that hybridized to homologous initiator tRNA, one was found that did not contain the complete tRNA coding sequence.[41] DNA sequencing revealed several shorter regions to be homologous to parts of the initiator tRNA. The largest region of homology corresponds to initiator tRNA coordinates 7 through 39, which represents approximately 50% of an intact initiator tRNA sequence. This sequence may therefore be considered as a tRNA pseudogene. The *D. melanogaster* DNA insert of this plasmid hybridizes to more than 30 sites in the *D. melanogaster* genome and has a pattern of hybridization characteristic of a mobile DNA element. This is especially intriguing since the 18 kb of *D. melanogaster* DNA that flank this region in the parental clone[41,56] displayed hybridization kinetics of middle-repetitive DNA.[56,57] In this respect it appears that the *D. melanogaster* DNA segment represents a "fragmented" initiator tRNA gene that may have been brought about by repeated insertion and excision of a transposable element(s) into the tRNA gene coding region. Another *D. melanogaster* tRNA pseudogene has been observed in the $tRNA^{His}$ gene family.[58] This sequence is located on a *D. melanogaster* DNA fragment which contains a bona fide $tRNA^{His}$ gene in close proximity. Similarly to the initiator tRNA pseudogene, the fragment containing the $tRNA^{His}$ pseudogene was detected by hybridization using purified homologous tRNA.[59] The sequence of the $tRNA^{His}$ pseudogene differs from that of $tRNA^{His}$ in that the nucleotides between tRNA coordinates 38 and 45 have been replaced by a different sequence. The bona fide $tRNA^{His}$ gene does not contain an intron. The corresponding coding sequence of the pseudogene is of the same length as the bona fide $tRNA^{His}$ gene and also appears to have similar 3′-flanking sequences.

Similar observations have been made for DNA segments from the genomes of rat and mouse. A 3.3-kb EcoRI fragment of rat DNA contains sequences that code for $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Glu}$. This cluster is reiterated about ten times on the haploid DNA. Sequence analysis of six[27,68] copies reveals that five of the $tRNA^{Gly}$ sequences have deletions of seven nucleotides between residues 20 and 26. Three of the $tRNA^{Glu}$ sequences lack 14 nucleotides, 11 nucleotides from the 3′ end of the gene to three nucleotides beyond the end.

Screening a mouse recombinant DNA library using a synthetic 19-mer homologous to $tRNA^{Phe}$ resulted in the identification of a mouse $tRNA^{Phe}$ pseudogene.[61] Comparison of the cloned DNA sequence to the known $tRNA^{Phe}$ sequence revealed that 38 contiguous nucleotides of the $tRNA^{Phe}$ 3′ region from tRNA coordinates 39 to 76 including the terminal CCA was included in the cloned segment. This homology diverges at the sequence corresponding to the region encoding the $tRNA^{Phe}$ anticodon loop. A sequence homologous to the 5′ end of the $tRNA^{Phe}$ was not found within 100 bp from this point of divergence. The presence

of an encoded 3′ terminal CCA in the cloned fragment is unusual and led to the proposal that the information of the mature tRNA was reverse transcribed and integrated into the mouse genome.[61] Whether the formation of this pseudogene, by the proposed mechanism, reflects the presence of an intervening sequence in mouse precursor $tRNA^{Phe}$ is not known. A tRNA gene intron 3′ adjacent to tRNA coordinate 38, which would be required for this to occur, has not been observed.

All of the 12 fragment-class pseudogenes so far identified do not support RNA synthesis in vitro.[27,41] Whether these pseudogenes bind transcription factors in vitro has not been examined. While the presence of pseudogenes is reminiscent of features found in *Xenopus* 5S rRNA genes,[62] the inability to support transcription is not.[63] However, the location of tRNA pseudogenes intermingled with tRNA genes for the same isoacceptors poses the interesting question whether the pseudogenes are functional in terms of binding transcription factors, or merely "evolutionary remnants".[27,64]

III. ORGANIZATION OF tRNA GENES

A. Multigene Families

Transfer RNA genes are constituents of complex multigene families which are dispersed in the genome. Also, the gene for any particular tRNA isoacceptor is reiterated in the genome. The function of this redundancy is not known but may be related to a requirement at certain times for a peak rate of tRNA synthesis that exceeds the maximum possible transcription rate of a single template. Concurrent transcription of an appropriate number of templates would meet such a requirement. However, studies of tRNA gene location in the chromosomes indicate that this view is too simplistic. Not only are tRNA genes dispersed throughout the genome but also the genes for any particular isoacceptor are located at more than one chromosomal locus.

In considering possible control mechanisms for the expression of tRNA genes, information can be sought from studies of the organization and expression of other genes, the products of which are also involved in the protein synthetic apparatus. For example, it has been shown that for *Drosophila*[65] and *Xenopus*,[66] multiple copies of rRNA genes are present to enable an appropriate transcriptional response to the cellular demand for ribosome synthesis. The rDNA is organized in the chromosome in tandem array with the repeats being separated from each other by "spacer" DNA. Furthermore, to ensure sufficient ribosome synthesis in the *Xenopus* oocyte the rDNA undergoes massive amplification, which ensures the production of 28S, 18S, and 5.8S rRNAs. Alternatively, the 5S rRNA genes, localized separately from the rDNA[67] and occurring in tandem array, are transcribed by RNA polymerase III. In the *Xenopus* oocyte a haploid complement of about 20,000 copies of an ooctye-specific 5S DNA ensures the synthesis of 5S RNA.[68]

In the unrelated organisms *S. cerevisiae*,[69] *D. discoideum*,[70] and the fungus *Mucor racemosus*,[71] the genes for the four rRNAs are present in each repeating unit, even though 5S rRNA is synthesized by RNA polymerase III in the nucleoplasm and the other three encoded RNAs by RNA polymerase I in the nucleolus.[72] This is not a general mechanism for lower eukaryotes, since the 5S rRNA genes of *Sc. pombe*[73] and *N. crassa*[74] are not tandemly repeated, are dispersed within the genome, and are independent of other rRNA genes.

In higher eukaryotes, 5S rRNA genes are clustered, tandemly repeated, and also, occur separately from other gene families to the extent that the clusters appear to be uninterrupted. The functionality of such tandem clustering lies in the observation that 5S rRNA must be synthesized in sufficient amounts to maintain the protein synthetic apparatus. However, why eukaryotes use a completely separate gene mechanism to ensure the synthesis of the large rRNAs, and 5S rRNA, and tRNA is not obvious. Clustering appears to serve as a means to coordinate the synthesis of related gene products from independent transcription units. It

seems that as organismic complexity increases, so too does the reiteration or redundancy of these clusters.

B. tRNA Gene Complexity

Some shortcomings, in characterizing the presence of tRNA genes using hybridization, have been discussed in earlier sections and these include the inability to discriminate between bona fide tRNA genes, partial tRNA gene sequences, and polymorphic tRNA gene sequences (see Section II.F). The presence of pseudogenes in eukaryotic genomes leads, therefore, to inaccuracies in the determination of tRNA gene numbers such that an estimate of the number of bona fide tRNA genes is greater than actual. Other features of tRNA gene organization and arrangement also have been found to affect the ability to detect genes by hybridization. A major finding of the DNA sequence analysis of the *D. melanogaster* recombinant plasmid pCIT12 concerned the arrangement of the individual genes.[50] As referred to earlier, the eight tRNA genes on pCIT12, are irregularly spaced and arranged such that five genes are in one transcriptional direction and three in the other.[50] Because the transcription direction is different for various genes of the same isoacceptor, the tRNA genes are capable of forming inverted repeat structures in which the homology extends over the entire coding region of the tRNA genes. Structures with inverted repeat stems of 70 to 100 bp were observed by Yen and colleagues during electron microscopic analysis of heteroduplexes of pCIT12 and the vector DNA ColE1.[48] The sequence data explain the occurrence of these inverted repeat structures, most of them being formed by homologous tRNA genes having opposite polarity. The occurrence of inverted repeat structures in heteroduplex formation explains the difficulty or inability to detect the tRNA genes involved in their formation by hybridization with radioactive tRNA.[50] If similar tRNA gene arrangements occur with a reasonable frequency in the genome, one would expect, therefore, that the estimation of gene numbers to be less than actual. It is clear that exact determinations of tRNA gene numbers is not possible; however, the numbers that are obtained do appear to be a reasonable estimate in terms of the order of magnitude of the genomic tRNA gene complement.

Saturation hybridization analyses of *S. cerevisiae* genomic DNA displays on the order of 360 tRNA genes, which gives an average reiteration frequency of eight genes per tRNA species. In yeast, tRNA genes are not clustered but rather are dispersed throughout the genome.[33] The apparent overall distribution, in fact, appears to be random.

Cytological hybridization studies using unfractionated tRNA as a radioactive probe show that *D. melanogaster* contains at least 50 sites distributed throughout the genome[75,76] that code for tRNA. The X chromosome contains significantly fewer sites of hybridization than chromosomes 2 and 3, and chromosome 4, which lacks polytenization, has not shown sites of tRNA hybridization. The tRNA genes of *D. melanogaster* occur redundantly, having an average reiteration frequency of 12.5 for each of the approximately 60 different isoacceptor tRNA.[77] It appears that this redundancy is in the mitotic chromosomes and does not result from selective amplification of tRNA genes. At present, the location of the genes for about 25 individual tRNA species is known.[78,79] At these loci different, apparently unrelated tRNA species hybridize to the same sites on the genome and also particular species hybridize to different chromosomal regions. From such studies it has become clear that although tRNA genes are distributed throughout the genome there is marked clustering at particular loci.[76,78,79]

While the finding of as many as 18 tRNA genes located within one chromosomal locus, 42A, has so far been unique for tRNA gene organization in *D. melanogaster*,[48,50] the irregular clustered arrangement of these genes has turned out to be exemplary for *D. melanogaster* as well as other higher eukaryotes. Additional tRNA gene regions within the *D. melanogaster* genome have been analyzed by DNA sequencing and restriction enzyme mapping combined with hybridization. These DNA segments include the genes which code for $tRNA^{Gly}$,[43] $tRNA^{Glu}$,[42] $tRNA^{Leu}$ and $tRNA^{Ile}$,[80] $tRNA^{Lys}$,[54] and $tRNA^{Val}$, $tRNA^{Ser}$, and $tRNA^{Phe}$,[53] and $tRNA^{His}$.[58]

In *X. laevis* there is a large population of dispersed clusters of tDNAs.[81] For example, eight tRNA genes lie within a 3.18-kb segment of DNA that is tandemly repeated approximately 150 times per haploid genome;[34] *in situ* hybridization indicates that the repeating units probably form a large gene cluster on one chromosome (referred to in Reference 80). Each tRNA repeating unit contains two $tRNA^{Met}$ genes, the A-copy and the B-copy, along with one gene each for $tRNA^{Phe}$, $tRNA^{Tyr}$, $tRNA^{Asp}$, $tRNA^{Ala}$, $tRNA^{Leu}$, and $tRNA^{Lys}$. (This gene cluster is shown in Reference 92.) The tRNA coding regions are separated by large regions of noncoding or "spacer" DNA. DNA sequence homogeneity is maintained in both the coding and spacer DNA sequences, comparing each of the repeating units.[81]

Over 1000 tRNA genes are contained within the human haploid chromosomal complement, representing about 60 different genes of 10 to 20 copies each.[82] There is evidence here as well for a combination of dispersed unitary genes as well as dispersed clusters. So far, the initiator $tRNA^{Met}$ genes have been studied in most detail. The arrangement of this gene family in humans is similar to that of the initiator tRNA gene family of *D. melanogaster*.[41] The initiator tRNA genes are arranged individually and located within regions of high sequence conservation which are scattered throughout the genome.[44] Clustering with other initiator $tRNA^{Met}$ genes or with other tRNA genes is not observed.[44] A human tRNA gene cluster has been isolated and this cluster contains a copy of genes coding for $tRNA^{Lys}$, $tRNA^{Gln}$, and $tRNA^{Leu}$.[83] These genes are contained within a 1.6-kb fragment and are separated from each other by 0.4 to 0.5 kb. The cluster does not appear to be tandemly repeated since analysis of 8 kb on one side and 3 kb on the other of the 1.6-kb fragment did not reveal the presence of other tRNA genes. Recently, a human $tRNA^{Glu}$ gene was identified on a 2.4-kb DNA fragment which possibly also contains at least one other tRNA gene.[84] In the rat genome the tRNA gene reiteration frequency appears to be similar to that of humans with a complexity of 10 to 20 copies per isoacceptor.[85] Individual tRNA genes have been isolated and characterized from the genome of rat and in each instance these tRNA genes show evidence of being arranged in clusters.[27,60,85] One tRNA gene cluster, contained on a 3.3-kb EcoRI fragment, has a single copy of genes coding for $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Glu}$. This cluster appears to be part of a larger 13.5-kb repeating unit which has a copy number of about 10 per haploid genome.[27]

Overall, there does not appear to be a simple explanation for clustering of tRNA genes or for the dispersed arrangement generally displayed by tRNA gene families. Since each tRNA gene is a single transcriptional unit we are led to think in terms of dispersed gene clustering being a mechanism to coordinate the expression of a group of independent transcription units. Any mechanism for regulating transcription of such dispersed tRNA genes must at present remain conjecture.

C. Evolution and Maintenance

Dispersed multigene families, such as those for tRNA genes, pose an interesting evolutionary problem. One could speculate that if the individual members of a particular multigene family were subjected to natural selection, DNA sequence heterogeneity would quickly accumulate. However, since the several members of any given tRNA gene family are homologous, a mechanism must exist to maintain this gene sequence homogeneity. It has been proposed that natural selection within a multigene family in effect would be limited to a single copy gene.[86] Examination of the eight $tRNA^{Tyr}$ genes from *S. cerevisiae*, all which are functional genes (reviewed in Reference 87), shows that while the tRNA coding sequences (including the intervening sequences) of all eight genes are virtually identical, the flanking sequences are not. Specifically, these eight $tRNA^{Tyr}$ genes show only two polymorphisms: a C/T polymorphism within the intron and the presence of TA immediately 3′ of the tRNA coding region in five of the eight genes[87] (M. Smith, personal communication). It has been proposed that gene conversion is the only mechanism of homogenization which would result in conserving the sequence of the intron while not necessarily conserving

the flanking sequences.[86] Gene amplification can be excluded as a possibility in the example of the yeast $tRNA^{Tyr}$ genes because a recent gene amplification would have resulted in some flanking sequence duplication and an ancient amplification may have allowed the introns to diverge since these, apparently, are not essential for function of the tRNA gene per se.[86]

At the moment, it is not clear why gene conversion would not extend into flanking sequences; especially with regard to 3′-flanking sequences since these contain the functional sequences providing the signals for transcription termination. So while tRNA gene families that have extensive flanking-sequence homology (see above) may have been homogenized by a mechanism involving gene amplification, their occurrence implies that a stringent selection pressure operates on the flanking sequences and the intron, as well as on the exon for some tRNA gene families. While the regions containing the highly conserved initiator $tRNA^{Met}$ gene families of *D. melanogaster*[41] and human[44] are dispersed similarly to the $tRNA^{Tyr}$ gene family of *S. cerevisiae*, the highly conserved *D. melanogaster* $tRNA^{Gly}$ gene family is locally clustered.[43] These observations suggest that gene arrangement per se has little function in the homogenization process and also, pressures other than those immediately implied by the gene conversion argument must be operative. In this context it may be important to consider cellular usage of an individual tRNA gene as a function of chromosomal location. The pressures, therefore, for conserving the gene sequence and a particular flanking sequence may arise from mechanisms of transcriptional regulation.

A direct analysis of recombination between dispersed serine tRNA genes has been performed in *Sc. pombe*.[83] During meiosis in this yeast, transfer of genetic information occurs between tRNA genes of related sequence even when they are located on different chromosomes. This process of intergenic conversion was analyzed using nonsense suppressor alleles of these unlinked serine tRNA genes. In their wild-type form, two of the genes code for $tRNA_{UCA}^{Ser}$, the third for $tRNA_{UCG}^{Ser}$. A suppressor at one of these loci was inactivated by an intragenic second-site mutation and suppressor activity was again selected for at this locus. Since this event far exceeded the mutation rate at these loci, Munz and colleagues[89] suggest that intergenic conversion is likely to be the main mechanism responsible for the homogeneity of families of dispersed genes, and also of linked genes. This suppressor resulted from gene conversion events in which the nonhomologous $tRNA^{Ser}$ was clearly the donor. Such a mechanism by propagating or eliminating specific mutations would contribute to the concerted evolution of dispersed gene families.[89]

Evidence for amplification and subsequent conservative evolution of tRNA gene coding regions is provided from studies on the *D. melanogaster* $tRNA^{Glu}$ family.[42] This gene family can be accounted for if two ancestral genes each gave rise to gene doublets by duplication, and one of these gene pairs then gave rise in turn to a trio of genes as a result of unequal crossover.[42]

IV. TRANSCRIPTION OF tRNA GENES

A. DNA Control Signals

1. Intragenic Promotion

Transcription studies of eukaryotic tRNA genes have demonstrated that these genes, similar to *Xenopus* 5S rRNA genes,[90,91] contain intragenic control regions (ICRs) that direct their transcription. These regions have been characterized in a *D. melanogaster* $tRNA^{Arg}$ gene,[92] *Xenopus* $tRNA^{Met}$[93] and $tRNA^{Leu}$[94] genes, and in a *C. elegans* $tRNA^{Pro}$ gene.[95] The intragenic control regions of eukaryotic tRNA genes are contained within the sequences that in the tRNA code for the D loop (termed the D-control region, A box, or 5′ ICR) and the T loop (termed the T-control region, B box, or 3′ ICR). Thus, tRNA gene transcription is controlled by a noncontiguous promoter element comprised of two sequence blocks positioned within each half of the tRNA gene. The two ICRs code for sequences that are highly conserved in all tRNAs. Since these sequences are conserved throughout evolution (excluding mitochon-

dria), these eukaryotic sequences serve a critical function in the gene as well as in the tRNA itself.

Comparison of the sequences corresponding to the ICRs for all tRNAs and tRNA genes reveals a promoter consensus sequence (Table 1).[2,21] Based on 115 unique tRNA and tRNA gene sequences, a derivation of the promoter consensus sequences (noncoding strand) are shown in Figure 2.

Limited modification of these consensus sequences as shown by point mutations (see below), leads to a change in or loss of transcriptional activity. Therefore, it appears that these primary sequences are important features of a tRNA gene which impart transcriptional activity for RNA polymerase III-directed transcription. In direct proof of the consensus sequence concept *E. coli*[96] and chloroplast[97] tRNA genes that satisfy the requirements for homology to the consensus sequences support in vitro transcription by RNA polymerase III.

The 3′ ICR consensus sequence shows little variation among all tRNA genes (and tRNAs) whether, incidentally, prokaryotic or eukaryotic. This probably reflects selection, not only for transcriptional activity, but also for the T stem and T loop, encoded by this region, which are integral to the functions of the tRNA. The invariant nucleotides in the 3′ ICR are G_{53}, T_{55}, C_{56}, and A_{58}. Other positions show strong preference for a particular nucleotide. For instance, in position 54, a T is present in all tRNAs except eukaryotic $tRNA_i^{Met}$, and *B. mori* $tRNA^{Ala}$, which contain A in this position; in position 57, only purines occur; in positions 60 and 62, there is a strong preference for pyrimidines; and in position 61, a C is always present which base-pairs with G_{53} in the tRNA T stem. The constraints on the sequence of the 3′ ICR, however, do not necessarily correlate with the function of this region as a promoter element.

Mutational analyses have been performed on the *S. cerevisiae* $tRNA^{Tyr}$ gene,[98,99] the *Xenopus* initiator $tRNA^{Met}$ gene,[100] the *C. elegans* $tRNA^{Pro}$ gene,[100] and a *Sc. pombe* $tRNA^{Ser}$ opal suppressor gene.[19] For the *Xenopus* $tRNA^{Met}$ gene, nearly every GC and CG base pair in the tDNA has been mutagenized by C to T transitions. Several GC and CG base-pairs in the structural gene appear to be major promoter determinants because when mutated, transcription is reduced 3- to 20-fold. Most of these determinants occur between nucleotides 7 and 19, and 49 and 61, within the two ICRs for the $tRNA^{Met}$ gene.[100] Five of the thirty one-point mutations of the yeast $tRNA^{Tyr}$ gene were found to reduce template activity. All of these are within the ICRs of this gene.[100] Mutations in the *C. elegans* $tRNA^{Pro}$ gene 3′ ICR[88] revealed that nucleotides T_{54}, A_{58}, and G_{64} are critical to transcription activity, with mutations of other nucleotides in the 3′ ICR also significantly affecting transcription. These investigators suggest that for this $tRNA^{Pro}$ gene the 3′ boundary of the 3′ ICR extends as far as nucleotide G_{64}. From these studies, it is apparent that the effect on transcription of mutation of corresponding nucleotides in different tRNA genes varies widely. This is exemplified by the finding that changing T_{54} to A_{54} in $tDNA^{Pro}$ resulted in a level of transcription 20% that of the wild-type gene.[101] This is an intriguing result since $tRNA_i^{Met}$ and *B. mori* $tRNA^{Ala}$ isoacceptors have an A normally at this position. It has been suggested that the identity of the base present at a certain coordinate is only important in the context of the entire sequence of a particular tRNA gene, and may in part determine the strength of the internal promoter in which it resides.[86] The argument can be made that the importance of any single nucleotide in the ICR sequence depends on how close the rest of the sequence is to the consensus.

In contrast to the 3′ ICR, the 5′ ICR consensus sequences shows some variation with regard to sequence as well as to the number of nucleotides present; using the standard tRNA numbering system, genes vary as to whether they contain nucleotides at positions 17, 17:A, 20:A, and 20:B.[2,21] Also, the importance of the 5′ ICR sequence per se is arguable when the functional interchangability between the *Xenopus* 5S DNA 5′ ICR and the 5′ ICR of the *C. elegans* $tDNA^{Pro}$, is considered.[102] Less homology exists between the sequence of the 5S DNA 5′ ICR and the tDNA 5′ ICR consensus sequence, which suggests a certain "flexibility" in the sequence of the 5′ ICR. Integrating the proposed consensus sequence

Table 1

No.	Iso-acceptor organism	Source	tRNA Gene Nucleotide Position: 7 10 17a 20ab 25	52 62
			' ' '' ''' '	' '
001	Ala1	bm	GTAGCTCAGAT.GGT..AGAGC	GGATCGATACC
002	Ala2	bm	GTAGCTCAGAT.GGT..AGAGC	GGATCGATACC
003	Ala1	sc	GTGGCGTAGTC.GGT..AGCGC	GGTTCGATTCC
004	Ala1	tu	GTGGCGTAGTC.GGT..AGCGC	GGTTCGACTCC
005	Arg	bl	GTGGCCTAAT..GGAT.AACGC	GGTTCGAGTCC
006	Arg2	dm	GTGGCGCAAT..GGAT.AACGC	GGTTCGACTCC
007	Arg1	ma	GTGGCGCAAT..GGAT.AACGC	GGTTCGACTCC
008	Arg2	ma	GTGGCGCAAT..GGAT.AACGC	GGTTCGACTCC
009	Arg2	sc	GTGGCCCAAT..GGTC.ACGGC	GGTTCAAGTCC
010	Arg3A	sc	GTGGCGTAAT..GGC..AACGC	GGTTCGACCCC
011	Arg3B	sc	GTGGCGTAAT..GGC..AACGC	GGTTCGACCCC
012	Asn5	dm	GTGGCGCAATT.GGTT.AGCGC	GGTTCGAGTCC
013	Asn	ma	GTGGCGCAATC.GGTT.AGCGC	GGTTCGAGCCC
014	Asn	pt	GTAGCTCAGT..GGT..AGAGC	GGTTCGAATCC
015	Asp	bl	GTAGCGTAGT..GGC..AGCGT	AGTTCGATACT
016	Asp	eg	GTAGTATAGT..GGTA.AGTAT	GGTTCAATTCC
017	Asp	ll	CTAGTATAGT.GGTG.AGTAT	AGTTCGATACT
018	Asp	rh	TTAGTATAGT..GGTG.AGTAT	GGTTCGATTCC
019	Asp	rl	TTAGTATAGT..GGTG.AGTAT	GGTTCGAATCC
020	Asp	sc	ATAGTTTAAT..GGTC.AGAAT	GGTTCAATTCC
021	Asp	sp	TTAGTATAGG..GGT..AGTAC	GGTTCGAATCC
022	Cys	sc	ATGGCGCAGT..GGT..AGCGC	AGTTCGATCCT
023	Gln1	rl	ATGGTGTAAT..GGTT.AGCAC	AGTTCAAATCT
024	Glu	dm	ATTGTCTAGT..GGCT.AGGAT	GGTTCGATTCC
025	Glu4	dm	ATGGTCTAGT..GGCT.AGGAT	GGTTCGATTCC
026	Glu1	rl	ATGGTCTAGC..GGTT.AGGAT	GGTTCGACTCC
027	Glu3	sc	ATAGTGTAAC..GGCT.ATCAC	GGTTCGACTCC
028	Glu1	sp	GTGGTCCAAC..GGCT.AGGAT	GGTTCGACTCC
029	Gly1	bm	GTGGTTCAGT..GGT..AGAAT	GGTTCGATTCC
030	Gly2	bm	GTGGTGTAAT..GGTC.AGCAT	GGTTCGATTCC
031	Gly	dm	GTGGTTCAGT..GGT..AGAAT	GGTTCGATTCC
032	Gly1	hp	GTGGTTCAGT..GGT..AGAAT	GGTTCGATTCC
033	Gly2	hp	CTGGTGTAGT..GGT..ATCAT	GGTTCGATTCC
034	Gly	r	GTGGTATAGT..GGTG.AGCAT	GGTTCGATTCC
035	Gly	sc	GTGGTTTAGT..GGT..AAAAT	GGTTCGATTCC
036	Gly1	wg	GTGGTCTAGT..GGT..AGAAT	GGTTCGATTCC
037	His	dm	ATCGTCTAGT..GGTT.AGGAC	GGTTCGAATCC
038	His	m	ATCGTATAGG..GGTT.AGTAC	GGTTCGAATCC
039	His	sl	ATCGTATAGT..GGTT.AGTAC	GGTTCGAATCC
040	Ile	dm	TTAGCTCAGTT.GGTT.AGAGC	GGTTCGATCCC
041	Ile	tu	TTGGCCCAGTT.GGTT.AAGGC	AGTTCGATCCT
042	Leu1	an	GTGGCGGAATT.GGTA.GACGC	GGTTCGAGTCC
043	Leu2	an	CTGGCGAAATT.GGTA.GACGC	GGTTCAAGTCC
044	Leu	bl	GTGGCCGAGC..GGTCTAAGGC	GGTTCGAATCC
045	Leu	dm	ATGGCCGAGC..GGTCTAAGGC	GGTTCGAATCC
046	Leu	h	ATGGCCGAGC..GGTTCAAGGC	GGTTCGAATCC
047	Leu	mh	ATGGCCGAGT..GGTCTAAGGC	GGTTCGAATCC
048	Leu3	sc	TTGGCCGAGC..GGTCTAAGGC	AGTTCGAATCT
049	Leu	sc	TTGGCCGAGT..GGTTTAAGGC	GGTTCGAATCC
			' ' '' ''' '	' '
			' ' '' ''' '	' '
050	Leu*8	sp	ATGCCCGAGT..GGTGTAAGGG	AGTTCGAACCT
051	Leu	xl	ATGGCCGAGC..GGTCTAAGGC	GGTTCGAATCC
052	Lys2	dm	CTAGCTCAGTC.GGT..AGAGC	GGTTCGAGCCC
053	Lys5	dm	ATAGCTCAGTC.GGT..AGAGC	GGTTCAAGTCC

Table 1 (continued)

No.	Isoacceptor organism	Source	tRNA Gene Nucleotide Position 7 10 17a 20ab 25 52 62
			' ' '' ''' ' ' '
054	Lys1	ll	CTAGCTCAGTC.GGT..AGAGC GGTTCGAGCCC
055	Lys2	ll	CTAGCTCAGTC.GGT..AGAGC GGTTCGAGCCC
056	Lys3	ll	ATAGCTCAGTC.GGT..AGAGC GGTTCAAGTCC
057	Lys	mf	CTAGCTCAGTC.GGT.AGAGC GGTTCGAGCCC
058	Lys1	sc	TTGGCGCAATC.GGT..AGCGC GGTTCGAGCCC
059	Lys	sc	TTAGCTCAGTT.GGT..AGAGC GGTTCGAGCCC
060	Met	ma	TTAGCGCAGTA.GGT..AGCGC AGTTCGATCCT
061	Met3	sc	GTAGCTCAGTA.GGA..AGAGC AGTTCGAACCT
062	Met	ta	GTGGCTCAGCT.GGA..GGAGC GGTTCGATCCC
063	Meti	be	GTGGCGCAGC..GGA..AGCGT GGATCGAAACC
064	Meti	dm	GTGGCGCAGT..GGA..AGCGT GGATCGAAACC
065	Meti	ma	GTGGCGCAGC..GGA..AGCGT GGATCGAAACC
066	Meti	sc	GTGGCGCAGT..GGA..AGCGC GGATCGAAACC
067	Meti	sf	GTGGCGCAGT..GGA..AGCGT GGATCGAAACC
068	Meti	so	GTGGCGCAGT..GGA..AGCGT GGATCGAAACC
069	Meti	sp	GTAGGAGAGT..GGA..ACTCC GGATCGAAACC
070	Meti	tt	GTGGCGAAAT..GGA..ATCGC GGATCGAAACC
071	Meti	tu	TTGGCGCAGT..GGA..AGCGC GGATCGAAACC
072	Meti	wg	GTGGCGCAGC..GGA..AGCGT GGATCGAAACC
073	Phe1	bc	ATAGCTCAGTT.GGG..AGAGC GGTTCAATCCC
074	Phe	bg	ATAGCTCAGTT.GGG..AGAGC GGTTCAATCCC
075	Phe	bm	ATAGCTCAGTT.GGG..AGAGC GGTTCGATCCC
076	Phe	dm	ATAGCTCAGTT.GGG..AGAGC GGTTCAATCCC
077	Phe	eg	TTAGCTCAGTT.GGT..AGAGC GGTTCGATTCC
078	Phe	lp	ATAGCTCAGTT.GGG..AGAGC TGTTCGATCCA
079	Phe	ma	ATAGCTCAGTT.GGG..AGAGC GGTTCGATCCC
080	Phe	nc	TTAGCTCAGTT.GGG..AGAGC TGTTCGATCCA
081	Phe	sc	TTAGCTCAGTT.GGG..AGAGC TGTTCGATCCA
082	Phe	sp	ATGGTGTAGTT.GGG..AGCAT GGTTCGATCCC
083	Phe	wp	ATAGCTCAGTT.GGG..AGAGC TGTTCGATCCA
084	Pro	ce	ATGGTCTAGT..GGT..ATGAT GGTTCAATCCC
085	Pro1	rt	TTGGTCTAG...GGGT.ATGAT GGTTCAAATCC
086	Pro2	rt	TTGGTCTAG...GGGT.ATGAT GGTTCAAATCC
087	Pro	tu	GTGGTCTAGT..GGT..ATGAT GGTTCAATTCC
088	Ser**	bl	ATGATCCTCAGTGGTCCGGGGT GGTTCAATTCC
089	Ser1	rl	GTGGCCGAGT..GGTT.AAGGC GGTTCGAATCC
090	Ser3	rl	GTGGCCGAGT..GGTT.AAGGC GGTTCGAATCC
091	Ser1	sc	TTGGCCGAGT..GGTT.AAGGC GGTTCAAATCC
092	Ser2	sc	TTGGCCGAGT..GGTT.AAGGC GGTTCGAGTCC
093	Ser*	sc	ATGGCCGAGT..GGTT.AAGGC GGTTCAAATCC
094	Ser*	sc	ATGGCCGAGT..GGTT.AAGGC GGTTCAAATCC
095	Ser*3	sp	ATGTCCGAGT..GGTT.AAGGA GGTTCAAATCC
096	Thr1	sc	ATGGCCAAGTT.GGT..AAGGC GGTTCAAATCC
097	Trp	bl	GTGGCGCAAT..GGT..AGCGC TGTTCGAATCA
098	Trp	cc	GTGGCGCAAC..GGT..AGCGC TGTTCGAATCA
099	Trp	dd	TTAGCATAGT..GGTTTATTGT GGTTCAACTCC
100	Trp	sc	GTGGCTCAAT..GGT..AGAGC GGTTCAATTCC
101	Tyr	pp	ATGGCTGAGT..GGTT.AAAGC GGTTCGAATCC
102	Tyr	sc	GTAGCCAAGTT.GGTTTAAGGC CGTTCGACTCG
			' ' '' ''' ' ' '
			' ' '' ''' ' ' '
103	Tyr	sc	GTAGCCAAGTT.GGTTTAAGGC CGTTCGACTCG
104	Tyr	sp	ATGGTGTAGTT.GGTT.ATCAC AGTTCGATTCT
105	Tyr	tu	GTGGCCAAGTT.GGTTTAAGGC CGTTCGAATCG
106	Tyr	xl	ATAGCTCAGCT.GGT..AGAGC GGTTCGATTCC

Table 1 (continued)

No.	Isoacceptor organism	Source	tRNA Gene Nucleotide Position: 7 10 17a 20ab 25	52 62
107	Val4	dm	GTGGTGTAGC..GGTT.ATCAC	GGTTCGATCCC
108	Val1B	hp	GTAGTGTAGT..GGTT.ATCAC	GGTTCGAAACC
109	Val	ma	GTAGTGTAGT..GGTT.ATCAC	GGTTCGAAACC
110	Val	rh	GTAGTGTAGT..GGTT.ATCAC	GGTTCGAAACC
111	Val	rl	GTAGTGTAGT..GGTT.ATCAC	GGTTCGAAACC
112	Val1	sc	GTGGTCTAGTC.GGTT.ATGGC	AGTTCGATCCT
113	Val2A	sc	ATGGTCCAGT..GGTTCAAGAC	AGTTCGAACCT
114	Val2B	sc	ATAGTGTAGC..GGCT.ATCAC	AGTTCGATCCT
115	Val1	tu	GTGGTCTAGTT.GGTC.ATGGC	AGTTCGATCCT
CONSENSUS;			GTGGCNNAGT..GGT..AGNGC	GGTTCGANTCC
			7 10 17a 20ab 25	52 62
			5′-ICR	3′-ICR

Source of tRNA isoacceptor:

an	*Anacystis nidulans*	nc	*Neurospora crassa*
bn	bean	pp	*Paramecium primurelia*
bg	blue-green algae	pt	petunia
bc	bovine lens	r	rat
bl	bovine liver	rh	rat hepatoma
bm	*Bombyx mori*	rl	rat liver
cc	chicken cells	rt	reverse transcriptase primer
ce	*Caenorhabditis elegans*	sc	*Saccharomyces cerevisiae*
dd	*Dictyostelium discoidium*	sf	starfish
dm	*Drosophila melanogaster*	sl	sheep liver
eg	*Euglena gracilis*	so	*Scenedesmus obliquus*
h	human	sp	*Schizosaccharomyces pombe*
hp	human placenta	ta	*Tetrahymena acidophilum*
ll	rabbit liver	tt	*Tetrahymena thermophila*
lp	lupin	tu	*Torulopsis utilis*
ma	mammalian	wp	wheat/pea/barley
m	mouse	wg	wheat germ
mf	mouse fibroblast	xl	*Xenopus laevis*
mg	bovine mammary gland	*	nonsense suppressor
mh	morris hepatoma	**	UGA recognizing
ml	mouse leukemia		

Note: 115 unique tRNA and tDNA sequences were compiled[2-21] and the regions of the two major ICRs compared in order to derive ICR consensus sequences for eukaryotic tDNA. The spacing within the 5′ ICR is variant dependent upon the presence of nucleotides at positions 17, 17a, 20, 20a, and 20b, following the conventional tRNA numbering system after *S. cerevisiae* $tRNA^{Phe}$. Dots within the sequences indicate a contiguous region. Consensus within the extra-arm region has not been derived here.

and the 5′ ICR interchange experiments, we can derive the following more general consensus sequence (noncoding strand) for the 5′ ICR:

```
                    10        19
                    |         |
5′ ICR: 5′-TRRYNNARYGG-3′
```

Derivation of Consensus Sequences for tDNA ICRs

5' ICR

	7	8	9	10	11	12	13	14	15	16	17	a	18	19	20	a	b	21	22	23	24	25
G	53	0	71	112	1	38	18	0	93	2	1	0	115	115	12	5	0	3	65	34	78	1
A	36	0	41	1	0	10	6	114	21	3	2	0	0	0	16	3	0	112	31	32	36	1
T	19	115	1	1	39	31	40	1	0	92	27	1	0	0	79	42	11	0	17	9	0	31
C	7	0	3	1	75	36	51	0	1	16	11	0	0	0	8	10	3	0	2	40	1	82
e	0	0	0	0	0	0	0	0	0	2	74	114	0	0	0	55	101	0	0	0	0	0
	G	T	G	G	C	N	N	A	G	T	e	e	G	G	T	e	e	A	G	N	G	C

3' ICR

	52	53	54	55	56	57	58	59	60	61	62
G	92	115	0	0	0	93	0	0	15	0	3
A	14	0	12	0	0	22	115	54	18	0	6
T	6	0	103	115	0	0	0	44	65	0	14
C	3	0	0	0	115	0	0	12	32	115	92
	G	G	T	T	C	G	A	N	T	C	C

FIGURE 2. The nucleotide occurrence at each position within the 5' and 3' ICRs was computed from the collection of 115 sequences shown in Table 1.

where R = purine, N = any base, and Y = pyrimidine. (Note that not all sequences have a nucleotide at position 17.)

Mutations of the region encoding the D stem of two yeast $tRNA^{Leu}$ genes have been constructed by the use of synthetic oligonucleotides.[103-105] The nucleotides corresponding to positions 10, 11, and 12 are changed from GCC to AAA and/or those in positions 24, 25, and 26 are changed from GGC to TTT to preserve the base-pairing potential of the sequence. In the heterologous *Xenopus* germinal vesicle system, transcription of the $tRNA^{Leu}$ gene is not dramatically affected by those mutations; however, accurate excision of the intervening sequence occurs only in the double mutant which can form a stem structure.[104] The behavior of the mutants of the *SUP53* $tRNA^{Leu}$ gene in homologous yeast extract is different.[105] The AAA_{10-12} mutation reduces transcription tenfold while the TTT_{24-26} mutation has little effect on transcription. The double mutant is also very poorly transcribed. It appears that in the homologous system, the capacity to form a stem structure in the 5' ICR is unimportant to transcription while the sequence of the control region is critical.

In addition to transcription-down mutations in the internal control regions of the *Sc. pombe* $tRNA^{Ser}$ gene,[19] there is a transcription-down mutation (A_{48}) at the junction of the extra arm and T stem. Similarly, point mutations within the extra-arm and T-stem coding regions of the *C. elegans* $tRNA^{Pro}$ and *S. cerevisiae* $tRNA^{Tyr}$ genes reduced their respective transcriptional activities.[106,107] These data, in addition to extra-arm mutations of the yeast $tRNA^{Met}$ which affect transcription factor binding (see below), show that the extra-arm coding region

is either an extension of the 3′ ICR or is a separate control region.[95] 3′-Deletion mutants of the *D. melanogaster* $tRNA^{Arg}$ gene support near wild-type levels of transcription when the sequence up to A_{58} remains intact (pArg3.58). When the sequence between coordinates 51 and 58 is successively removed (pArg3.50), transcription efficiency decreases to a level supported solely by the 5′ half of the gene; the level of transcription of, for example, pArg3.40 is not lower than pArg3.50. This result implies that the extra-arm encoding region is not a separate control region but is a functional extension of the 3′ ICR.[92]

In the *Xenopus* $tRNA^{Met}$ gene, several additional GC and CG base-pairs in the region between the two ICRs also appear to contribute to promoter activity; their location suggests that a stem-loop structure in the DNA encoding the tRNA anticodon-arm is important for RNA polymerase III promoter function.[100] Specifically, mutations of the $tDNA^{Met}$ at position 29, changing G_{29} to A_{29}, or at position 41, changing C_{41} to T_{41}, led to a level of transcription approximately 10% that of the wild-type gene. Combining these mutations to form a mutant template containing A_{29} and T_{41} relieved, to some extent, the transcriptional defects of the single mutations, such that A_{29}/T_{41} transcribed to a level 50% that of the wild-type gene. In the 5-bp member anticodon-stem of the tRNA, nucleotides 29 and 41 serve as the third, or middle, base-paired set. The transcription results, therefore, imply that such an interaction is also important in transcription of the tDNA. While this model is appealing, transcription results using other mutant tRNA genes do not support it. Point mutations in three of the five nucleotides on the 5′ side and one on the 3′ side of the encoded anticodon-stem of yeast $tRNA^{Tyr}$ do not affect the template activity in vitro in an homologous transcription system,[99] these mutants include the mutation at tDNA position 29, changing A_{29} to T_{29}. Two mutations in the anticodon stem of the *Sc. pombe* $tRNA^{Ser}$ gene have no discernible effect on transcription of the gene either in vitro or in vivo.[18] Hybrid tRNA genes, composed of the 5′ ICR of one gene and the 3′ ICR of another (which conceivably, might not form anticodon stem-loop structures), support faithful and efficient transcription,[94] as do hybrid genes comprised of 5S RNA gene and tRNA gene control regions.[102] The basic consideration in such a model for tDNA promotion is whether DNA could form a structure wherein the A_{29}/T_{41} nucleotides would base-pair. To date, there is no direct evidence for the formation of an anticodon-like stem and loop structure in a tRNA gene. A further argument against this model is that several tRNA genes encode sequences that in the tRNA anticodon-stem form GU base-pairs; GT base-pairing is not possible in DNA. It is intriguing, however, that a GU base-pair at the tRNA 29-41 position does not occur in $tRNA_i^{Met}$, nor in any tRNA. A structural role in promoter activity for the anticodon-stem encoded region therefore may be limited to $tRNA_i^{Met}$. The anticodon-stem structure of mature $tRNA_i^{Met}$ has been shown to be unique among tRNAs.[108] Whether this function of initiator $tRNA^{Met}$ relates to possible structures in the DNA is not known. Mutagenesis of other tRNA genes in the anticodon-stem region should clarify this issue.

There is evidence for a function of the sequence between the ICRs in spacing the control regions for optimal interaction in transcription promotion.[93,109,110] The length of this region and its effects on transcription efficiency are of special interest since this region varies most among tRNA genes. As discussed in an earlier section, approximately 10% of tRNA genes encode intervening sequences, and furthermore, vary as to the length of their encoded extra-arm. Because of intron and extra-arm length heterogeneities, the distance between the two ICRs can range from 27 bp, for genes that do not encode an intervening sequence and encode a four-nucleotide extra-arm (for example, the genes for $tRNA^{Met}$, $tRNA^{Arg}$, $tRNA^{Pro}$, and $tRNA^{Ala}$), and 81, for a *D. melanogaster* $tRNA^{Leu}$ gene. (The length of this region is measured between and includes tRNA positions 26 and 52.) The extent to which the length of this region can be changed has been tested for several tRNA genes by the construction of insertion mutations. The relationship between the in vitro transcriptional activities of *D. melanogaster* $tDNA^{Arg}$,[110] *C. elegans* $tDNA^{Pro}$,[108] and yeast $tDNA_3^{Leu}$,[39] and the length of the region separating the 5′ ICR and the 3′ ICR is such that as the length increases, transcription

activity decreases. The transcriptional activity of the $tRNA^{Pro}$ gene insertion mutants were tested in the nucleus of *X. laevis* oocytes. An increase of 19 bp resulted in an apparent level of transcription approximately 20% that of the wild-type gene. This result is different to that obtained using the $tRNA^{Arg}$ gene where additional 62- and 77-bp fragments needed to be inserted for transcription to decrease to 25% and 17% the level of wild-type gene transcription, respectively, in cell-free extracts. Similarly, the yeast gene can tolerate insertion of an additional 30-bp fragment with no decrease in in vitro transcriptional activity. In this gene an additional 103-bp insert resulted in reduced transcription. The length between the 5′ ICR and the 3′ ICR of the yeast wild-type $tRNA_3^{Leu}$ gene is relatively long, normally 67 bp, because of an intron and a long extra-arm encoding region.

It seems that while tRNA gene transcription is dependent on two ICRs, nucleotides outside these sequences affect the levels of transcription initiation. These observations raise questions regarding the extent of the tRNA-coding sequence that is involved in the interaction with transcription components to form an actively transcribing gene. Specifically, two questions are raised: (1) are nucleotides outside the control regions required for direct interaction with transcription factors and RNA polymerase III or (2) are these nucleotides necessary to the extent that an interaction between the two control regions is facilitated?

2. Extragenic Modulation

While the transcription of eukaryotic tRNA genes is directed by control regions within the mature coding sequences, flanking sequences serve to modulate transcription. In vitro studies have identified both 5′-flanking sequence elements, which inhibit transcription in vitro, and other elements or regions which positively influence transcription. In addition, 3′-flanking sequence elements can dramatically alter transcription factor binding (see below). Analysis of the minimum sequence of a *D. melanogaster* $tRNA^{Arg}$ gene required to support transcription showed that while the 5′ half of the gene is sufficient to support a low level of transcription, the isolated 5′ ICR is not.[111] Therefore, sequence 5′ to the 5′ ICR is essential for transcription. This required sequence can extend into the 5′ flank of the coding region. The characteristics of this required sequence are unknown, since 5′-flanking sequences in general are not well conserved.

5′-Flanking elements which resemble RNA polymerase III termination signals have been shown to inhibit *in vitro* transcription of $tRNA_2^{Lys}$[112,113] and $tRNA_2^{Arg}$[114] genes from *D. melanogaster*. The sequence GGCAGTTTTTG is fairly well conserved in front of a number of $tRNA_2^{Lys}$ genes, although the position relative to the mature coding sequence varies.[50]

Deletion of this sequence, which begins at position −18, relative to the structural sequence from the $tRNA_2^{Lys}$ gene 2 and replacement by pBR322 sequence led to a dramatic increase in transcriptional efficiency in *X. laevis* germinal vesicle (GV) extract.[112] Moving this sequence by a single base-pair increments relatively closer to the mature coding sequence caused an increase in transcriptional efficiency.[113] This striking dependence on position suggests that this element is located in the most inhibitory position. In contrast to gene 2, $tRNA_2^{Lys}$ gene 4 has four consecutive T residues positioned from −23 to −20 and this gene is efficiently transcribed in GV extracts.

The *D. melanogaster* $tRNA_2^{Arg}$ gene, 17DArg, also has a 5′-flanking element with five consecutive T residues.[114] This sequence serves to inhibit 17DArg transcription in *Drosophila* KcO cell extracts but, in contrast to the element in the 5′ flank of $tRNA_2^{Lys}$ gene 2, it does not effectively inhibit transcription in GV extract or HeLa cell extract. The run of T residues in 17DArg occurs from position −25 to −21 relative to the mature coding region, while in gene 2 it occurs from −18 to −14.

The resemblance of the inhibitory elements in the 5′ flanks of $tRNA_2^{Lys}$ gene 2 and 17DArg to termination signals suggests that the inhibition may be caused by a termination-like event: that is, RNA polymerase III or associated factors could bind to the 5′ flank of the tRNA gene upstream of the inhibitory sequence and in translating toward the position

for transcription initiation, encounter the inhibitory element causing it to dissociate from the DNA. This explanation appears unlikely for two reasons. First, while transcription inhibition is as strong (in the case of gene 2) or stronger (in the case of 17DArg) in *Drosophila* KcO cell extract than in HeLa cell extract,[114] the *Drosophila* KcO cell RNA polymerase III transcription apparatus requires a stronger termination signal than does the HeLa cell apparatus.[115] Therefore, if the inhibition was brought about by a termination-like mechanism, inhibition should be greater in HeLa cell extract. A second reason comes from comparison of the 5′-flanking sequences of 17DArg and Dt59R (which encodes a *D. melanogaster* $tRNA_5^{Lys}$ gene). Dt59R has a stretch of five consecutive T residues from position −24 to −20 relative to the mature coding sequence, and yet this gene is transcribed at very high efficiency in *Drosophila* KcO cell extract.[54] The shift of this sequence by 1 bp relative to its position in 17DArg should not lead to such a drastic increase in transcriptional efficiency (for example, the effect of shifting the position of the inhibitory element in front of gene 2 by a single base pair led to a two- to threefold increase in transcriptional efficiency.[113]

The sequences of two *B. mori* tRNAs, $tRNA_1^{Ala}$ and $tRNA_2^{Ala}$, differ at the site of a single unmodified nucleotide at position 40.[116] While expression of $tRNA_1^{Ala}$ is silk gland specific, $tRNA_2^{Ala}$ expression is constitutive for several silkworm cell types.[116] The tRNA position 40 is outside the two ICRs and mutation solely at this position would not be expected to have a significant effect on $tRNA^{Ala}$ gene transcription. Seemingly, therefore, the different expression of these two $tRNA^{Ala}$ genes is dependent on signals in their flanking sequences. However, the involvement of other mechanisms in this differential control (for example, localization or arrangement of the tRNA genes in the genome) cannot be excluded.

Deletion of 5′-flanking sequence of a *B. mori* $tRNA_2^{Ala}$ gene to position −13 relative to the mature coding sequence led to a reduction in transcriptional efficiency to 16% that of the wild-type gene transcription in a *B. mori* extract.[117] These mutations led to little or no loss in transcriptional activity using a GV extract.[117] Further deletion analysis of this gene indicated that the sequence between coordinates −37 and −14 in the 5′ flank is required for wild-type transcription levels in *B. mori* extracts. Three regions of conserved sequence occur in the 35 bp immediately upstream of two $tRNA_2^{Ala}$ genes and one 5S RNA gene in *B. mori*.[118] Replacement sequences that resemble the conserved sequence led to transcriptional efficiencies approximating the wild-type gene.[113] Interestingly, the $tRNA_2^{Ala}$ gene has a run of five T residues from −21 to −17, and the conserved sequence has four consecutive T residues from −21 to −18. Whether the occurrence of the T residues in the $tRNA^{Ala}$ gene 5′ flank is coincidental or is integral to the function of the 5′ flank is not known. Also, if the T residues are functionally significant, it is not known how their presence can affect transcription positively for one gene and negatively for another.

The dependence of transcription of the *B. mori* $tRNA_2^{Ala}$ gene in homologous extracts on the wild-type 5′-flanking sequence compared to the insensitivity to the 5′-flanking sequence in GV extract led to the suggestion that 5′-flanking sequence dependence is specific for homologous systems.[117] However, since GV extract shows little positive 5′-flanking sequence dependence for *X. laevis* tRNA genes,[93,94] as well as genes from *D. melanogaster*,[92,112] *B. mori*,[120] *C. elegans*,[95] and *S. cerevisiae*,[39] the lack of 5′-flank dependence in GV extract appears to be a property of the transcription apparatus and not to be associated with the use of an homologous system.

Transcription inhibitory sequences have been identified in the 5′ flank of a variant *X. laevis* $tRNA_i^{Met}$ gene (B-copy; containing a sequence polymorphism at tRNA position 65). Since this sequence does not encode a correct $tRNA_i^{Met}$, transcription of this sequence may not be required. In accord with this notion, this gene is transcribed inefficiently in vitro,[121] and transcripts from this gene have not been found in vivo.[122] Transcriptional analysis of a series of mutants of this $tRNA_i^{Met}$ gene which contain deletions 3′ to 5′ in the 5′ flank (with the region encoding the primary transcript left intact) and a series of mutant genes deleted 5′ to 3′ in the 5′ flank indicated a surprisingly complex group of 5′-flank modulatory units

responsible for transcriptional repression.[123] The most inhibitory of these lies from −20 to −12, and is composed of alternating purines and pyrimidines. The second lies from −43 to −32. This inhibitory unit is composed of alternating purines and pyrimidines with one residue out of alternation. The second unit is not found at an optimally inhibitory location, but most drastically reduces transcription when moved to approximately the normal location of the first unit. The positional dependence of these units is striking: movement by only a few base-pairs relative to the coding sequence can change transcriptional efficiency dramatically. The replacement sequences were also found to affect transcription, but in a manner apparently dependent upon both the replacement sequence itself and the relationship of the replacement sequence to the remaining wild-type sequence. This analysis suggested that the conformation of the 5′-flanking DNA may be crucial for efficient transcription since, the two major inhibitory elements in this $tRNA^{Met}$ gene have the potential to assume Z-DNA conformation,[124] which could itself be a poor substrate for RNA polymerase III or could signal other proteins to bind and inhibit transcription.

Transcription of some tRNA genes in *Drosophila* KcO cell extract are positively modulated by wild-type 5′-flanking sequence. 5′-Deletion mutants of the *D. melanogaster* $tRNA_2^{Arg}$ gene contained on the plasmid pArg were examined for ability to support transcription in various extracts. While 5′ deletion up to position −8 (pArg5.−8) did not abolish transcription in GV extract or HeLa cell extract, it did in *Drosophila* KcO cell extract.[92] Transcription of pArg5.−8 in HeLa extracts, however, was reduced by 50% relative to that of pArg. The 5′-flanking sequence dependence of pArg for transcription has been more precisely mapped.[125] Deletion into the region between coordinates −60 and −36 led to increased transcriptional efficiency in both HeLa and *Drosophila* KcO cell extracts. Deletion to −33 led to little or no reduction in transcriptional efficiency, while deletion to −32 or beyond led to greater than 95% reduction in transcriptional efficiency in *Drosophila* KcO cell extract. Transcriptional efficiency in the HeLa cell extract was again reduced when the deletion extended to or beyond position −33. Therefore, the two extracts, one apparently insensitive and one very sensitive to the wild-type 5′ flank, are in fact dependent on exactly the same wild-type 5′-flanking sequence of pArg. It is only the extent of the relative response to this sequence which varies.[125]

A possible example of positive modulation of transcription by 5′-flanking sequence has been reported for a human $tRNA^{Glu}$ gene.[84] This gene is transcribed at extremely high efficiency by HeLa cell extract. The 5′ flank of this gene contains a sequence with the potential to form a tRNA-like structure and, while further study is needed, the possibility exists that this sequence positively regulates transcription.

Studies of the transcription of 5′-deletion mutant *S. cerevisiae* tRNA genes in homologous extracts have shown dependence on the presence of the wild-type 5′ flank, although to differing degrees for different genes.[39,126] Koski and colleagues deleted the 5′ flank of the SUP4-o $tRNA^{Tyr}$ gene to position −2 and showed that although there was a reduction in transcriptional efficiency, the gene was still active in vitro. Deletions to −27 or −15 also reduced transcriptional efficiency in vivo and in vitro. Shaw and Olson[127] have shown that deletion of the 5′-flanking sequence of the SUP4-o gene leaves it profoundly deficient in expression in vivo and in vitro. The comparison of these experiments is complicated by the fact that the activity observed could well depend on what new sequences are juxtaposed to the gene. This is underscored by the demonstration that the occurrence of three BamHI linkers at the deletion junction in one construct deleted to position −18 completely inhibited expression in vivo, while a single BamHI linker left the gene active.[127]

Deletion of the 5′ flank of a yeast $tRNA_3^{Leu}$ gene to position −22 permitted a high level of transcription when the introduced sequence was fairly A + T rich, and a somewhat lower level when it was G + C rich. Deletion to position −2 (which removed an element conserved between different genes) left the gene nearly transcriptionally inactive under standard assay conditions in homologous extracts.[39] The relative level of transcription of the $tRNA_3^{Leu}$ gene

with the 5′ flank deleted to position −2 was greatly increased when the tRNA gene and extract concentrations were reduced. These changes also altered the salt concentration dependence, but demonstrate that differences in assay conditions, which could potentially be brought about by differences in extract preparation, can play a major role in determining relative transcriptional efficiencies in vitro.[39]

Deletion of 5′-flanking sequence and replacement with vector sequence has been shown to affect the site as well as the efficiency of transcription initiation. Generally, alterations observed in the site(s) of initiation have been minor, such as changes in the ratio of initiation at two normally used sites.[93,123] However, more dramatic changes have also been observed. Initiation of transcription of a *Drosophila* $tRNA^{Arg}$ gene in *X. laevis* GVs or in GV extracts was found to occur at several sites when 5′-flanking sequences were deleted.[123] As the extent of the deleted sequence approached and entered the mature coding sequence, initiation sites farther upstream became more favored and transcripts therefore became longer. Initiation sites far upstream from the conserved T at position 8 were observed. The site of transcription initiation of a *D. melanogaster* $tRNA^{His}$ pseudogene in *Drosophila* KcO cell extract was changed to a site within the introduced sequence as well as a site downstream of the normal initiation site with deletion of 5′-flanking sequence, even when the deletion did not disrupt the normal initiation site.[58] This evidence suggests that the mechanism by which transcription is initiated is considerably more complex than the proposal that RNA polymerase III simply "measures back" from the ICRs.[102,128]

The yeast $tRNA^{Tyr}$ gene family provides a model system for assessing the effects of different flanking sequences on the expression of identical mature tRNA-coding regions. The *S. cerevisiae* mutation *SUP3*-o was found to depress sporulation, and indeed sporulation deficiency is exhibited to varying degrees by suppressor mutants of the eight loci for $tRNA^{Tyr}$ genes.[129] This sporulation deficiency of certain suppressor-bearing diploids appears to reflect the deleterious effect of efficient translation of ochre codons during sporulation. Among the eight suppressors, the *SUP3*-o mutation causes the most depression of sporulation. The sporulation deficiency observed in the diploids requires the presence of an active homozygous *SUP3*-o suppressor. Since *SUP3*-o alleles inactivated by second-site mutations are capable of sporulation, this implies that the wild-type gene product of the *SUP3* locus is not essential for sporulation. It has also been shown that an allelic, low-efficiency, *SUP3*-a-bearing strain sporulates; sporulation depression therefore appears to be specific to ochre suppression.[130] Homozygous *SUP11*-o diploids, however, exhibits no sporulation deficiency, suggesting that suppressor efficiency is an important component of the sporulation defect. DNA sequencing data unequivocally demonstrate that the eight mature $tRNA^{Tyr}$ coding regions are identical[88] (M. Smith, personal communication, 1984). The different levels of suppression in *SUP3*-o and *SUP11*-o strains therefore can be rationalized in terms of *SUP3*-o having a higherlevel of expression. The coding sequences of the two $tRNA^{Tyr}$ genes differ only in that *SUP3*-o contains the C/T polymorphism noted earlier (M. Smith, personal communication). The resulting U in the SUP3-o $tRNA^{Tyr}$ intervening sequence may lead to the precursor being more rapidly processed, compared to the precursor transcript of the *SUP11*-o gene°wever, to date there is no evidence that this is the case (C. Greer, personal communication, 1984). Since the ICRs of the two $tRNA^{Tyr}$ genes are identical, a tantalizing prospect is that their different levels of expression result from a modulatory function of flanking sequences. In this regard it is intriguing that the EcoRI-G fragment $tRNA^{Tyr}$ (*SUP11* locus) contains six thymidylate residues positioned between coordinates −19 and −14; a similarly positioned oligothymidylate stretch resulted in the negative modulation of transcription in vitro of a *D. melanogaster* $tRNA^{Lys}$ gene.[113] This may be coincidental, however, since the EcoRI-G $tRNA^{Tyr}$ supports transcription in an homologous cell-free extract (M. Smith, personal communication, 1984).

The three yeast $tRNA_{UCA}^{Ser}$ genes are expressed at different levels, as indicated by the efficiency of suppression by the corresponding SUP alleles,[131] even though the genes encode

identical tRNAs.[132] The order of the degree of suppression by the serine-inserting suppressors is SUP16 > SUP17 > SUP19, however, with SUP16 and SUP17, as class II suppressors, being relatively the same.[131] SUP19 tRNA also has six T residues, in this example, from coordinates −24 to −19 in its 5′-flanking sequence.[131] The occurrence of such a thymidylate stretch in both of the SUP11 and SUP19 tRNA genes may be coincidental to their low levels of in vivo expression. Mutation of these genes and subsequent transcriptional analyses, as has been performed using the SUP4-o gene,[127] should determine this.

B. Mechanism of tRNA Gene Transcription

1. Factor Involvement

Much of our knowledge concerning the DNA control signal requirements for eukaryotic tRNA gene transcription has been derived thorugh the use of extracts prepared from the following sources: *Xenopus* oocytes[133,134] and germinal vesicles;[135-137] human cell lines, KB and HeLa;[138,139] *Drosophila* KcO[140] and Schneider[141] cell lines; *B. mori* silk glands;[117] and *S. cerevisiae*.[126,142,143] The development of in vitro transcription systems was necessary because purified RNA polymerase III from mature *X. laevis* oocytes and human KB cells showed no transcriptional specificity with respect to purified 5S DNA or VA DNA.[144] In contrast, the 5S RNA genes and VA RNA genes in chromatin were accurately and selectively transcribed by purified RNA polymerase III, which led to the conclusion that RNA polymerase III and protein components contained within chromatin are necessary for faithful transcription.[145]

Initial fractionation of human KB cell cytoplasmic extract on phosphocellulose revealed that in addition to RNA polymerase III, two chromatographically distinct fractions were required for reconstitution of specific tRNA gene transcription.[146] Results using phosphocellulose fractions derived from *X. laevis* have shown that factors from *Xenopus* and human sources may be interchanged in vitro, suggesting universality in the function of these components.[147] Chromatography of *Drosophila* KcO cell extracts on CM-Sepharose separated two fractions and RNA polymerase III required to reconstitute tRNA gene transcription.[148] Promoter and sequence requirements of the transcription apparatus remain unchanged after fractionation of these components. Further fractionation of one of these fractions using phosphocellulose, DEAE-cellulose, and heparin-Ultrogel does not separate additional components required for specific transcription. *B. mori* transcription extracts chromatographed on phosphocellulose separate into two fractions (one containing at least RNA polymerase III) necessary to reconstitute transcription (K. Sprague, personal communication, 1984). Whole cell soluble *S. cerevisiae* extracts fractionated using heparin-Sepharose and DEAE-Sephadex or phosphocellulose and DEAE-cellulose, separate two fractions required for transcription.[149] These fractionation studies show that eukaryotic tRNA gene transcription involves a minimum of two transcription factors and RNA polymerase III.

Serum antibodies of patients with autoimmune diseases diagnosed as systemic lupus erythematosis have been shown in some instances to specifically immunoprecipitate ribonucleoproteins (RNPs).[150] The RNAs contained in the RNPs have been classified on the basis of the type of immune serum that precipitates them. Anti-La immune serum recognizes an RNP complex in uninfected mammalian cells which contains precursor forms of 5S RNA and certain tRNAs.[32] In addition, this RNP complex has been shown to associate with VA RNA transcribed in vitro.[152] Therefore, the possibility has been raised that the La antigen, which is associated with these RNA polymerase III transcripts, could be a transcription factor.[32] Also anti-La antibodies have been shown to inhibit in vitro transcription of 5S RNA and tRNA genes in Hela cell extracts (J. Gottesfeld, personal communication, 1984). Hela cell extracts depleted of these antigens by prior treatment with immune serum, however, will support VA RNA synthesis.[151] Purification of the La-antigen from Hela cell extracts by conventional and immunoaffinity chromatography resulted in co-fractionation of a required transcription factor activity (50,000 daltons) and an additional polypeptide (64,000

	5' ICR	3' ICR	Ref.
tDNA	TGGCnnAGTGG	GTTCGA	
Alu(CHO)	TGGCtcgagGT	GTTCaA	199
Alu(human)	TGGCtcAGgct	GTTCaA	200
EBER 1	TGcCctAGaGG	GTaCaA	153
EBER 11	TGcCctAGTGG	GgTCaA	153
Human 7SL	TGGCgcgtgcc	GTTCtg	201
Mouse 4.5S	TGGCgcAcgccGG	GTTCGA	202
rGH 1_Brepeat	TGGCtcAGTGG	GTTCaA	203
VA1	TGGtctgGTGG	GTTCGA	204
VA11	TaGCcggagGG	GTTCGA	204
	TRRYnnARYGG	GtTCRA	

FIGURE 3. Genes transcribed by RNA polymerase III are of two major types: Type 1, tRNA genes and tRNA-gene facsimiles, which apparently have the same or similar, mechanism to tRNA gene transcription; and Type 2, 5S rRNA genes. (To date, 5S RNA-gene facsimiles which do not include 5S DNA pseudogenes have not been encountered, so 5S DNA is the only example of Type 2 genes.) This figure compares the ICRs of several Type 1 genes to the major sequence components of tDNA ICRs demonstrating allowed variations from the ICR consensus sequences. From this comparison general ICR sequences for RNA polymerase III Type 1 transcription can be derived.

daltons).[152] Separation of these two proteins revealed that the 64,000-dalton protein is the component required for transcription reconstitution.[152] The 64,000-dalton protein has functional equivalence to the transcription factor present in the HeLa cell phosphocellulose C fraction.[146,152]

It appears that transcription of genes by RNA polymerase III (class III genes) involves a common set of transcription factors. Comparison of transcription control region sequences of these genes shows the 5′ and 3′ ICRs of the genes for tRNA, VA RNA,[153] EBER RNA,[154] and Alu RNA[155] to exhibit extensive sequence homologies (Figure 3). For *Xenopus* 5S RNA genes, however, only the 5′ ICR shares sequence homology with the corresponding 5′ ICR of other class III templates. The 5′ ICR of the somatic 5S RNA gene from *X. borealis* can be interchanged with the 5′ promoter element of *C. elegans* $tRNA^{Pro}$ gene.[102] Although *Xenopus* 5S RNA transcription requires the 5S RNA-specific factor TFIIIA,[134,156] fractionation of other eukaryotic extracts so far has not revealed other gene-specific transcription components.[146-148]

A general feature of all class III genes is that they are able to form stable transcription complexes[115,157,158] (see the following section). For tRNA genes this involves the binding of a transcription component(s) to the 3′ ICR of the gene which remains bound for many rounds of transcription initiation. In order to examine which of the separated transcription factors are required to form these stable transcription complexes, various fractions have been analyzed by using DNase-I protection[159,160] or template competition experiments.[148,150,158] In the yeast transcription system, a fraction derived from a whole cell extract contains at least one protein capable of stably binding to both intragenic control regions of tRNA genes.[159,160] A fraction derived from a yeast nuclear extract was found to protect only the region of the yeast *SUP53* tRNA gene containing the 3′ ICR.[105] A mutation in this gene, which resulted in the highly conserved C_{56} nucleotide being changed to G in the 3′ ICR, prevents the interaction of this factor(s) with the template, and decreases its competitive ability. Mutations in the 5′ ICR did not affect binding of the factor(s). Recently, a partially purified yeast

transcription component (called tau) was found to form a stable complex with the SUP4-o $tRNA^{Tyr}$ gene in the absence of another fraction required for reconstitution of transcription with RNA polymerase III.[149] Similarly, a protein fraction has been partially purified from HeLa cells that is required for in vitro transcription of the adenovirus VAI gene and the *B. mori* $tRNA_2^{Ala}$ gene.[160] This factor binds to the 3′ ICR of both of these genes and is necessary for formation of stable transcription complexes on these genes.[160]

Competition assays which utilized the separated transcription factors derived from human Hela cell extracts[158] showed that a fraction which contains the component designated **C**, is needed to form stable complexes with 5S RNA, VAI RNA, and tRNA gene templates. The binding of **C** with the 5S RNA gene appears to be preceded by its interaction with the 5S-specific factor **A**. The VAI template is able to stably interact with **C** in the absence of the other fraction (**B**) required for transcription. The tRNA gene, however, requires both **B** and **C** for the formation of a stable complex, but **B** does not remain stably bound and appears to be rapidly recycled. This is consistent with the mechanism described for stable complex formation using mutant tRNA gene deletion templates in an unfractionated *Drosophila* KcO cell extract.[110] Restriction site protection of a sequence adjacent to the 3′ ICR in the VAI gene was demonstrated in the presence of factor **C**.[158] Template competition experiments with either 5′ or 3′ ICRs of the tRNA gene suggests that the **C** component interacts directly with the 3′ ICR of the tRNA gene.

Based on transcriptional analysis of mutant tRNA genes and these fractionation studies which separate each of the transcription components, it appears that there are a minimum of two distinct factors in addition to RNA polymerase III which are required for accurate tRNA gene transcription. At least one of these factors interacts with the 3′ ICR and while it has been proposed that the other factor interacts with the 5′ ICR, this has yet to be demonstrated experimentally. In the yeast system, minimally, one transcription component interacts stably with the 3′ ICR of tRNA gene templates and does not require binding of factors to the 5′ ICR. The analogous human factor, however, interacts metastably and requires at least one additional factor to stabilize its binding to the template. Whether the difference in affinities of these factors from the two systems represents qualitative or quantitative differences between the factors of these systems or is a result of the differences in "promoter strengths" between the various tRNA gene substrates used is not yet clear.

The *Drosophila* KcO cell factor **C** has been found to be functionally equivalent to the Hela cell **C** factor, and can be reconstituted with human factor **B** to promote tRNA gene trascription. tRNA genes which are normally transcribed in the Hela cell system and are not transcribed in the KcO cell system due to their 5′-flanking sequences (see Section IV.A.2), are also not transcribed in the heterologous reconstituted KcO (factor **C**)/Hela cell (factor **B**) system. This suggests that extragenic modulation by 5′-flanking sequences of tRNA genes in the *Drosophila* system may be dictated, at least in part, by the factor **C** interactions[114,148] (and unpublished results). The *Drosophila* factor **B**, however, is not compatible with the human factor **C** and will not support transcription. Thus, although eukaryotic tRNA gene transcription may involve a general mechanism, interesting differences between transcription components of various systems may provide insight to different control mechanisms within these systems.

2. Stable Complex Formation

Bogenhagen and colleagues[157] demonstrated that *Xenopus* 5S RNA genes added to germinal vesicle extracts rapidly and stably sequester a limiting component, the 5S RNA gene-specific transcription factor TFIIIA. This factor binds to the intragenic control region, as shown by protection from DNase I. tRNA genes also rapidly and stably bind a limiting transcription component when added to transcriptionally active cell free extracts, as shown by the time-dependent inhibition of transcription of a reference tRNA gene added to transcriptionally active extract after the addition of a competitor gene. These assays do not require that the

gene (or part of a gene) to be examined support transcription, but rather examine the ability of the test gene to inhibit the transcription of another gene, the reference gene. The 3′ half of the gene was shown to be the most important region in transcription-competition assays.[161] The dependence of transcription factor binding on sequence was examined for a *Drosophila* $tRNA^{Arg}$ gene. Mutant genes deleted from either the 3′ or 5′ side were tested for ability to compete for limiting components in transcription-competition assays[162] or in preincubation-competition assays designed to test stable complex formation.[115] These assays demonstrated that the presence of the 3′ ICR was required for the gene to form stable complexes. While the 3′ ICR was shown to be the most important region for stable complex formation, other regions of the DNA throughout the coding sequence and in the flanking regions affect both the rate and stability of factor binding. In particular, the presence of the 5′ ICR is required for maximal rate and strength of stable complex formation. Deletions from the 5′ side of the gene which approached or removed part or all of the 5′ ICR (with replacement by the G + C-rich sequence leading from the BamHI site of PRR322) led to a substantial reduction in the rate of stable complex formation. This kinetic effect implied that recognition of the 5′ ICR region precedes factor binding to the 3′ ICR. Further, the reduction in competitive ability with deletion of the 5′ ICR implies a great interdependence between 5′ and 3′ ICR binding.

The formation of stable tRNA gene transcription complexes is insufficient to promote active transcription.[115] There is a lag phase which lasts considerably longer than the time required for stable complex formation and which, unlike stable complex formation, is temperature dependent between 24 and 30°C. This suggests that another step, perhaps involving a rearrangement of the complex of the tRNA gene and the bound factor(s), must occur before efficient initiation of transcription can occur.[115]

Studies on the abilities of *D. melanogaster* $tRNA^{Val}$ genes to form stable complexes in extracts of *Drosophila* Schneider cells yielded an interesting result.[141] The addition of a $tRNA_4^{Val}$ gene up to 15 min after the addition of a $tRNA_{3b}^{Val}$ gene totally inhibited transcription of the $tRNA_{3b}^{Val}$ gene. While the $tRNA_4^{Val}$ gene was found to be transcribed three to four times more efficiently than the $tRNA_{3b}^{Val}$ gene, this does not explain the variation in the rate of stable complex formation observed for the $tRNA_{3b}^{Val}$ gene. *D. melanogaster* $tRNA_2^{Arg}$ genes were found to compete at very similar levels despite tremendous variation in transcription efficiencies in *Drosophila* KcO cell extract.[114]

The role in stable complex formation of the spacer region between the two ICRs was examined by increasing the separation of the 5′ and 3′ ICRs.[110] A linear relationship was found between the spacing of the two control regions and the ability to form stable complexes in *Drosophila* KcO cell extract. Examination of the plot of these data suggests that when the two control regions are separated by approximately 400 bp or more, stable complex formation in *Drosophila* KcO cell extract no longer occurs, even though the separately cloned 3′-half gene, which is still present, forms stable complexes. However, transcription reactions performed in HeLa cell extracts do not display this relationship. Separation of the two control regions appears to reduce stable complex formation only to the level observed for the 3′-half gene (M. Nichols, unpublished results, 1984).

Dingermann et al.[110] have suggested a model of eukaryotic tRNA gene transcription based on the results from *Drosophila* KcO cell extract. They propose that two factors are required in addition to RNA polymerase III and that one factor binds the 5′ ICR and that this binding preferentially precedes binding of the 3′ ICR by the other factor. The binding of the 5′ ICR must then be sufficiently strong and have a lifetime long enough to prevent recognition of the 3′ ICR in a 15-min preincubation if the separation of the two regions is great enough to prevent interaction of the two factors when bound to their respective sites.

Support for the notions that one transcription factor binds to the 5′ ICR and the other to the 3′ ICR and that the interaction between the two factors enhances stable complex formation, comes from studies by Newman and colleagues.[105] A factor which binds and protects,

the 3′ ICR from DNase I digestion in a footprint assay, was separated from the other transcription components. Binding of this factor to the 3′ ICR was not affected by mutations in the 5′ ICR even though the ability to form stable transcription complexes was affected (see section on transcription factors). While the factor binding was not tested at close-to-limiting concentration (to determine if the binding to the wild-type genes and genes mutated in the 5′ ICR was truly identical), this analysis suggests that the isolated factor binds to the 3′ ICR and that the binding of the other factor to the 5′ ICR increases the strength of this binding. Footprinting experiments indicate that both ICRs are protected by *S. cerevisiae*[159] and HeLa[160] protein(s). A model significantly different from that of Dingermann and colleagues[110] has been proposed[158] based on results of experiments involving the ordered addition of transcription components to a number of different genes. In this model the order of transcription factor binding is dependent on a primary interaction with the 3′ ICR.

The effects of a number of point mutations in the *S. cerevisiae* SUP4-o $tRNA^{Tyr}$ gene on competitive ability have also been examined.[99] Twelve mutations in the 5′ ICR, the 3′ ICR, and the region encoding the extra arm were found to affect competitive ability. (The precise borders of the 3′ ICR have not been determined for any tRNA gene. It is possible that the extra-arm region represents an extension of the 3′ ICR generally, or at least, in this particular gene, or that it represents a different control region.) In general, those mutations which led to greater agreement with the consensus sequences for these regions increased competitive ability, and those which decreased homology with the consensus sequences reduced competitive ability.[99] Mutations in the 3′ ICR and extra-arm region generally had a greater effect on competitive ability than did mutations in the 5′ ICR, supporting the notion that the 3′ ICR is most important for competitive ability. Simple competition assays were used in this analysis, so differentiation between kinetic and thermodynamic effects of the mutations on factor binding were not made. Transcription of the mutant genes used tDNA and extract concentrations sufficient to yield half-maximal velocity with the wild-type gene, but the differences in transcriptional efficiencies observed were not nearly as dramatic as the differences in competitive abilities. The reason for the apparent transcriptional insensitivity of the extract used to mutations within the promoter regions was not examined. It may well be that the extract concentration used was such that there was little dependence on extract concentration (e.g., see the effect of extract concentration on transcription of 5′-deletion mutants in Reference 39), and that this masked the transcriptional effects of the mutations.

The 5′-flanking sequence can also affect the abilities of genes to compete for limiting transcription components, even though this competition is primarily due to the binding of transcription factors to the ICRs. Fowlkes and Shenk[153] showed that deletion of the 5′-flanking sequence of the adenovirus VAI RNA gene to position −33 or beyond caused a large decrease in the ability of this gene to compete for transcription factors. The presence of the wild-type 5′ flank of the *X. borealis* somatic 5S RNA gene, but not that of the oocyte 5S RNA gene, was required for maximal competitive ability.[163] Deletion of 5′-flanking sequence of a *D. melanogaster* $tRNA^{Arg}$ gene to between positions −60 and −36 led to a slight decrease in ability to compete in a stable complex formation assay in *Drosophila* KcO cell extract.[125] Deletion to −33 or beyond caused a slightly greater decrease. These deletion-mutant genes were much more drastically affected in transcription than competition, which suggests that it is the binding of RNA polymerase III rather than factor binding which is directly altered. This further suggests that RNA polymerase III plays a minor role in stable complex formation.

Deletion of the 3′-flanking sequence of this $tRNA^{Arg}$ gene led to a reduced competitive ability, compared to the wild-type gene pArg.[115,125,162] In *Drosophila* KcO cell extract, approximately 30 bp of the nontranscribed 3′-flanking sequence are required for maximal stable complex formation. This sequence is very A + T-rich, with runs of T-residues. Dependence on the 3′-flanking sequence was less significant in HeLa cell extracts. No loss in competitive ability was observed with deletion of the 3′ flank as long as the replacement

sequence included the element TTCTT. This element serves as a terminator at 25 to 50% efficiency in HeLa cell extract. When this element was replaced by TTCCC in a gene in which the entire 3′ flank, including the wild-type termination signal, had been deleted, the competitive ability in HeLa cell extract was drastically reduced. This evidence suggests that 3′-flank dependence occurs generally, and that there is a requirement for a termination-sequence-like run of T-residues. It also suggests that the degree of dependence on the 3′ flank varies between extracts, just as 5′-flank dependence varies. Interestingly, deletion of the 3′ flank, including the termination sequence, of an *X. borealis* somatic 5S RNA gene led to a great increase in competitive ability when assayed in an *X. laevis* GV extract.[163] It was suggested that the increased competitive ability was due to the tying up of transcription components in synthesizing the elongated RNAs, but it could be that there is a fundamental change in factor binding with deletion of the termination sequence.

The discovery that tRNA genes form stable transcription complexes helps to explain the observation that the addition of purified RNA polymerase III to chromatin depleted of RNA polymerase III greatly stimulates tRNA gene transcription, even though purified RNA polymerase III by itself cannot specifically transcribe tRNA genes.[141,144] This indicates that the transcription factors are associated in a stable manner with tRNA genes in vivo.

3. Chromatin Structure

The association of other proteins with tRNA genes in chromatin has also been studied through examinations of the sensitivity of tRNA encoding regions in chromatin to nuclease digestion. A study of chicken embryo $tRNA^{Lys}$, $tRNA^{Phe}$ and $tRNA_i^{Met}$ genes suggested the existence of a specific phase relationship of the nucleosomes: the structural sequence begins approximately 20 bp inside the nucleosome core.[164] However, more recent studies show that the nucleosomes, although regularly spaced on a 3.18-kb repeat fragment from *X. laevis*, are not uniquely phased in transcriptionally active tissues. Only in transcriptionally inactive erythrocytes were the nucleosomes phased with respect to the 3.18-kb repeat, but the phase relationship varies between the different tRNA genes in the repeat.[165] Similar results have been obtained from the nucleosome phasing on 5S RNA genes. In *X. laevis*[166] and in *D. melanogaster*[167] the nucleosomes within the 5S RNA gene clusters are nonrandom. In *D. melanogaster* the nucleosomes are positioned on the 5S RNA gene repeat in one of two possible phases. In one phase the middle of the gene is exposed while in the other, the 5′ end of the gene is exposed.[167]

For genes transcribed by RNA polymerase II (mRNA) DNase I sensitivity of chromatin has been used to differentiate whether specific genes are in an active or quiescent state. A study of the DNase I sensitivity of members of the gene families encoding $tRNA_4^{Glu}$ and $tRNA_i^{Met}$ in *D. melanogaster* chromatin revealed no DNase-I-resistant tRNA genes (M. Silberklang, personal communication, 1982). In *X. laevis* the oocyte 5S RNA genes are not transcribed in any known somatic cell (see below), and tRNA genes, which are transcribed in the hepatocyte, are inactive in the erythrocyte (discussed in Reference 168). Since the tRNA and oocyte 5S RNA genes are DNase I sensitive in both liver and erythrocyte nuclei it can be concluded that for the genes transcribed by RNA polymerase III there is no correspondence between DNase I sensitivity and expression.[168]

In *X. laevis* two classes of genes code for 5S RNA.[169] Investigation of this system has provided insight into the function of chromatin organization for genes transcribed by RNA polymerase III. The *Xenopus* somatic-type 5S RNA genes are present in about 400 copies per haploid genome and are expressed in both somatic cells and oocytes, while the approximately 20,000 oocyte-type genes are expressed only in oocytes and early embryos. Cloned oocyte or somatic 5S DNAs are transcribed in cell free extracts derived from either oocytes[136] or somatic cells.[133,139,163] Thus, the differential control of transcription is not retained with naked DNA.

It has been proposed that chromatin structure may be responsible for the differential

expression of the 5S RNA gene families in somatic cells.[157,170,171] The oocyte-type genes are not transcribed from chromatin or in nuclei isolated from somatic cells; only the somatic genes are expressed.[157,170] Oocyte-type genes, however, are transcribed from oocyte chromatin or from somatic chromatin washed with NaCl.[157,170] This latter procedure may cause the removal or structural rearrangement of chromosomal proteins allowing the oocyte-type genes to become accessible to transcription factors and RNA polymerase III since TFIIIA is required for the reconstitution of transcriptionally active 5S RNA gene chromatin.[170] Polynucleosomes reconstituted from 5S plasmid DNA and histones in the absence of TFIIIA are transcriptionally inert when incubated in extracts that efficiently transcribe 5S DNA and contain TFIIIA. The inactive state of the oocyte-type genes in somatic cells is retained in soluble chromatin prepared with either restriction endonucleases or micrococcal nuclease.[172,173] The level of chromatin structure responsible for the inactivity of the oocyte type genes in somatic cells occurs within chromatin fragments the size of individual gene units.[173] By analogy to the sizes of their transcriptional units and, therefore, from the similarity in their functional arrangement, the chromatin organization of tRNA genes may also be at the level of the individual gene.

V. MATURATION OF tRNA TRANSCRIPTS

Several recent reviews cover the details of eukaryotic and prokaryotic tRNA processing.[174-176] The aim of this section, therefore, will be to give an overview of this process and topresent novel aspects and new discoveries relating to the biosynthetic pathway of tRNA in the nucleus.

Eukaryotic tRNA genes are transcribed into precursor molecules, which are larger than mature-size tRNA. Transcription initiates at a purine nucleotide, usually within ten nucleotides upstream of the mature tRNA coding sequence. Transcription terminates within an oligothymidylate sequence relatively close to, but downstream of, the mature tRNA coding sequence. Precursors are subsequently cleaved into mature-size tRNA molecules by 5′- and 3′-processing nucleases and, when present, intervening sequences are excised by splicing enzymes; some bases are modified before excision of the intervening sequence. Further maturation of the transcript into a functional tRNA occurs by the addition of the 3′ terminal CCA and by an extensive variety of specific base modifications. A basic question still outstanding, however, is why tRNAs are transcribed as precursor molecules.

The formation of tRNA as a precursor molecule may reflect an evolutionary process; consider the *E. coli* multimeric transcription units from which embedded tRNA transcripts are processed. Eukaryotic precursor tRNAs, however, appear to have actual functional significance since the majority of genes transcribed by RNA polymerase III are not synthesized having 5′ and 3′ extra sequences as does tRNA; other RNA polymerase III transcripts of course do display "extra" 3′ uridylate sequences dependent upon RNA polymerase III termination. This function may involve the temporal ordering of the subsequent modifications and/or form the basis of biosynthetic regulatory mechanisms.

Precursor tRNAs with 5′-leader and 3′-trailer sequences do not leave the nucleus.[177] Injection of yeast precursor $tRNA^{Tyr}$ into enucleated *Xenopus* oocytes or into the oocyte's cytoplasm of nucleus, revealed that specific processing of the 5′ and 3′ ends and of the intervening sequence only occurs in the nucleus. The primary transcript of $tDNA^{Tyr}$ in *Xenopus* oocytes a precursor 108($\pm$4) nucleotides in length with a 5′ leader, intervening sequence, and 3′ trailer. In *Xenopus* oocytes the 5′ leader is removed in three stages (108 to 104 to 97 to 92 nucleotides), the last of which appeared to be accompanied by excision of the 3′ trailer and addition of the 3′ CCA. The mature size tRNA is 78 nucleotides.[177] Analysis of the in vivo *S. cerevisiae* transcripts of $tDNA^{Tyr}$, as well as $tDNA_2^{Ser}$ and $tDNA_{minor}^{Ser}$ have also been performed, using a Northern-hybridization procedure. In these results the largest RNA homologous to $tRNA^{Tyr}$ was 108 nucleotides, which appeared to process to 92

and 78 nucleotides. Heterogeneity in the 108 nucleotide presumptive initial transcript was not observed.[178] In general, therefore, there is very good agreement between the species detected in vivo in *S. cerevisiae* and those detected when cloned tDNA^{Tyr} is transcribed and processed by *Xenopus* oocytes or by extracts from *Xenopus* or yeast cells.

The three-stage trimming of the 5′ leader of the yeast tRNA^{Tyr} precursors has not generally been observed for transcripts produced in in vitro transcription reactions. An RNase P-like enzyme has been partially purified from *Sc. pombe*, which processes the 5′ termini of the pre-tRNA^{Tyr} of *E. coli* and various precursor transcripts produced in vitro, to the mature-tRNA 5′ terminus.[179] From these studies and analysis of transcript processing in in vitro transcription reactions, it appears that processing follows the order: endonucleolytic removal of the 5′ leader; endonucleolytic removal of the 3′ trailer; addition of the 3′ terminal CCA; site-specific base modifications; and, when present, excision of the intervening sequence, a two-step process, appears to be coupled to exit from the nucleus.[180,181] (The references cited in Section IV.A.1 discuss in some detail the effects of tRNA gene-mutations on the mechanism of processing of in vitro transcripts.)

The ability of the transcript to be processed appears to be dependent on its ability to form a tRNA-like conformation[52,182] (and see the above note). Mutations within tRNA genes that result in transcripts with potentially disrupted tRNA-secondary and -tertiary interactions lead to retardation or cessation in the rate of processing activities. While transcription in general is not affected by the presence of an intron, the structure of the resultant intervening sequence in the transcript can affect the rate of intervening sequence removal.[183] Recently, Swerdlow and Guthrie[184] compared the structures of yeast tRNA^{Tyr} and tRNA^{Phe} precursors, which contain intervening sequences, to their respective mature tRNAs, using enzymatic and chemical probes. They found that the folding of the precursor CCA terminus, acceptor stem, TΨC stem, variable loop, anticodon stem, and D stem, are identical to those of the cognate, mature tRNA. The TΨC and D loops appear to vary slightly in tertiary structure between mature and precursor species, and the precursors contain a helix involving the anticodon triplet and a complementary sequence in the intervening sequence. The stability of the helix is much greater for pre-tRNA^{Phe} than for pre-tRNA^{Tyr}, which is consistent with predictions from free-energy calculations.[184] Therefore, while complementarity between the intervening sequence and the anticodon stem and loop may not be essential, its occurrence may have a kinetic effect on excision of the intervening sequence from the precursor tRNA. In this regard it is worthwhile to point out that all of the insertion mutations of the yeast tRNA$_3^{Leu}$ gene, made within the intron, led to diminished processing of the respective precursor tRNAs.[39,185] These results confirm the view that recognition of universally conserved features of tRNA structure allow all tRNA precursors containing intervening sequences to be processed by a single splicing apparatus.[184]

A function relating the tRNA gene intron to transcriptional activity has not been found. Recently, however, Johnson and Abelson discovered that the intron of the yeast tRNA^{Tyr} gene is essential for correct modification of its tRNA product.[186] Specifically, precise deletion of the intron of the yeast SUP6-o tRNA^{Tyr} gene significantly reduced its suppressor activity relative to that of the unaltered gene. The anticodon sequence of the suppressor tRNA normally includes Ψ in the middle position. Removal of the intron from this gene resulted in failure to produce the Ψ modification in the anticodon. Furthermore, deletion of the intron caused a diminished amount of mature suppressor tRNA and a reduction in the level of suppression. This reduction appears to be due to the absence of Ψ in the anticodon. The most favorable mechanism for the pseudouridylation reaction is that the "Ψ-synthase" is dependent on the structure of the intervening sequence-containing pre-tRNA^{Tyr}. These authors further point out that other base modifications in the tRNA could also be affected by the intervening sequence.[186] Function of the sequence encoded by the tRNA gene intron is further argued by the lack of requirement on the part of the intervening sequence for a specific recognition sequence, necessary for the splicing activity. It is important to note,

however, that $tRNA^{Tyr}$ is the only yeast tRNA known to contain Ψ in the anticodon, and that the presence of intervening sequences in other tRNAs is not correlated with anticodon modifications in general. For example, the *S. cerevisiae* genes for $tRNA^{Phe}$, for which ten copies from six loci have been examined, all contain an intron.[187] Similarly, the *N. crassa* $tRNA^{Phe}$ gene contains an intron;[188] the *S. cerevisiae* and *N. crassa* have a conserved $tRNA^{Phe}$ sequence (91% homology — for collected tRNA-sequence works see Reference 2) and a well-conserved intron sequence (50% homology). The *Sc. pombe* $tRNA^{Phe}$ shares little sequence homology,[189] and the gene does not contain an intron,[37] yet these phenylalanine tRNAs have the same anticodon.

While the ultimate fate of the tRNA primary transcript is known, its immediate fate following transcription in terms of proteins and factor interactions is largely unknown. Several observations indicate that the biochemical pathway of the primary transcript across the nucleus involves a complex series of protein/nucleic acid interactions.

A mutant strain of *S. cerevisiae*, ts136, isolated by Hutchinson et al.,[190] was shown to accumulate precursor tRNAs.[191] This mutant carries a temperature-sensitive lesion in the *RNA*1 gene. The RNA1-gene product is presumed to participate in transport of RNA from the nucleus since mRNA, rRNA, and tRNA species accumulate. It seems that precursors to all of the tRNAs whose genes contain introns accumulate as a consequence of the rna1 mutation.[33] Using conditional loss-of-suppression as an assay, another tRNA-defective mutant has been identified in yeast.[192] The mutant, 201-1-5, which defines the *los*1 locus, conditionally loses the ability to suppress nonsense mutations. The mutation affects all eight tyrosine-inserting nonsense suppressors. The same mutation also causes the conditional accumulation of the yeast precursor tRNAs which contain intervening sequences. This mutation is "leaky", however, since 201-1-5 cells are not conditionally defective for growth. This trait, which is expected, is further characterized by the observation that *los*1-1 affects the suppression of some, but not all, nonsense mutations. Using a procedure to screen for loss-of-suppression mutants which do not require growth of mutants cells, Hopper and colleagues further isolated additional alleles of the *LOS*1 gene, which, similar to *los*1-1, are not conditionally defective for growth.[192]

Taken together these results on the *los*1 and *rna*1 mutants suggest that these mutant gene products affect a nuclear transport process. It is suggested that perhaps *RNA*1 specifies a nuclear pore component or some enzymatic or structural component of the transport process.[192] While 201-1-5 is not altered in the same gene as ts136, it is possible that the *los*1-1 mutation also affects the nuclear pore or, specifically, proteins required for the transport of tRNAs from the nucleus. It is not known as yet whether the *LOS*1 gene product is related specifically to the yeast-splicing activities. The discovery that the excision enzyme is membrane associated[180] raises this possibility and furthermore underscores the notion that a complex transport process is responsible for exit of the tRNA from the nucleus.

As discussed in an earlier section, there are several examples of wild-type tRNA genes that, in a sense, contain point mutations. Such a point mutation in the human initiator $tRNA^{Met}$ was sufficient to inactivate the nuclear escape of its transcripts;[193-195] the effect of this single G to U substitution at tRNA-position 57 clearly shows that the transport mechanism is critically dependent on a correct tRNA structure.[169] Zasloff was able to use this "polymorphic" tRNA to demonstrate saturability of tRNA exit from the nucleus, thereby proving that tRNA is indeed transported across the nuclear membrane.[196] A wider implication of this discovery is that transcripts resulting from such tRNA polymorphs and also from tRNA pseudogenes will be "trapped" in the nucleus. Since it is envisioned that many of these pseudogenes would support transcription, the function of their resultant transcripts remains to be investigated.

Transcription of a yeast $tRNA^{Tyr}$ gene in vitro using cell free extracts, has been observed to be stimulated by the addition of the homologous tyrosyl-tRNA synthetase.[197] Addition of the purified synthetase to semipurified, reconstituted, transcriptional components had no

effect on the level of $tDNA^{Tyr}$ promotion. The idea has been presented that while the synthetase appears not to be involved directly in the transcription complex, its presence modulates transcription, perhaps by rapidly removing precursor from the transcription complex.

An interesting discovery is that the La antigen (a 50,000-dalton protein from HeLa cells) specifically complexes with RNA polymerase III transcripts.[32,198] The most stable complexes between purified La protein and synthetic substrates were formed with RNAs that have three or four 3′ terminal uridylate residues.[198] This preferred terminal sequence is the same as the 3′ terminus of primary transcripts resulting from the RNA polymerase III transcription termination sequence. In vitro transcription kinetics suggest that the protein rapidly binds nascent transcripts. While the function(s) of La binding is as yet not known, its ubiquitous association with RNA polymerase III transcripts suggests that at least this interaction is not confined to only tRNA transcripts.[198] Since it appears that one of the last specifically nuclear processing steps involves endonucleolytic removal of the 3′ trailer, it is intriguing to speculate that the La antigen is integral in the passage of the RNA from the transcription complex to the nuclear membrane.

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