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APPROVAL SHEET

Title of Thesis: "Characterization of two cysteine tRNA genes from Xenopus laevis"

Name of Candidate: Shirley S. Lee
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Thesis and Abstract Approved:

Margaret J. Johnston
Committee Chairperson — 01/07/85 Date

Committee Member — 01/07/85 Date

Ronald C. Peterson
Committee Member — 01/07/85 Date

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Shirley S. Lee

Shirley S. Lee
Department of Biochemistry
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Thesis: Characterization of Two Cysteine Transfer RNA Genes from Xenopus laevis

Shirley Syu-Ling Lee, Master of Science, 1984

Thesis directed by : Ronald C. Peterson, Ph.D.
Department of Biochemistry

A DNA fraction enriched in X. laevis tRNA genes was separated from its genomic DNA by CsCl density gradient centrifugation in the presence of actinomycin D. The presence of tRNA genes in this fraction was detected by Southern blot analysis after digestion with restriction endonuclease Eco RI and hybridization with kinase labeled tRNA. The DNA fragments were separated by size on 1% agarose gel in which DNA fragments 1600-1800 bp long were eluted from the gel and cloned into the plasmid pBR 325. One clone which hybridized to kinase labeled tRNA was identified and shown to produce a tRNA size product when transcribed in vitro using Xenopus S-100 cell extract. The nucleotide sequence of this fragment was analyzed by dideoxynucleotide chain termination method after constructing a set of deletions and recloning into the plasmid pUC 8. The DNA fragment is 1737 bp long and contains two cysteine tRNA genes (anticodon 5'-GCA-3') that are oriented in the same direction. The sequences coding for the mature tRNA of the two genes are 95.8% homologous

resulting from a 3 bp difference. The two tRNA genes are on individual transcription units and can be transcribed independently. They do not contain intervening sequences and are separated by a 452 bp spacer DNA. It has been reported that a 3.18 kb tandemly repeated DNA fragment of X. laevis contains a cluster of eight tRNA genes. We have determined that this 1.74 kb DNA fragment is included in a tandemly repeated DNA fragment of X. laevis, approximately 1.85 kb long. Therefore, it contains approximately 96% of this tandem repeat. This indicates that a tandem repeat of X. laevis may contain as few as two tRNA genes.

CHARACTERIZATION OF TWO

CYSTEINE TRANSFER RNA GENES FROM *XENOPUS LAEVIS*

by

Shirley Syu-Ling Lee

Thesis submitted to the Faculty of the Department of Biochemistry
Graduate Program of the Uniformed Services University of
the Health Sciences in partial fulfillment of the
requirements for the degree of
Master of Science 1984

DEDICATION

In memory of my father-- Mr. Macklin W. Lee

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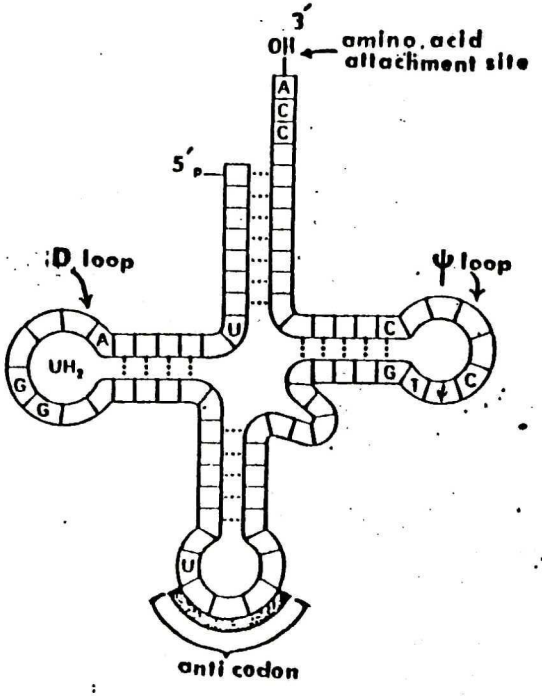
Introduction

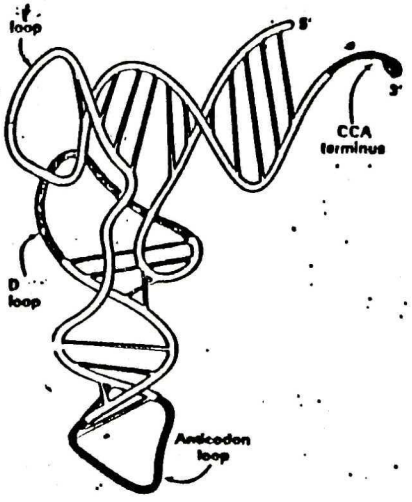
Transfer RNAs are small but major constituents in every cell. A tRNA consists of a single strand of 75 to 93 ribonucleotides corresponding to a molecular weight of 23,000 to 30,000 daltons. Its role is to act as an intermediary between the mRNA and the protein. Each of the 20 amino acids found in proteins has one or more corresponding tRNAs to bind it and carry it to the ribosome where it serves as an adaptor for translation of mRNA into protein. Transfer RNAs may have some other functions also. For example, in E. coli, attentuation of the tryptophan biosynthetic operon depends on the level of charged $tRNA^{trp}$. Cell wall synthesis in Staphylococi involves a $tRNA^{gly}$ species. Some viral RNA genomes contain tRNA-like structures at their 3' ends and some specific tRNAs can be used as primers for reverse transcriptase. (52)

The generalized structure of a tRNA consists of four base-paired segments and three non-hydrogen bonded loops. They are the amino-acid acceptor (AA) stem, the dihydrouridine (D) stem, the anticodon (AC) stem, and the TψCG stem. Extending from the D stem, the AC stem and the ψ stem are the D loop, the AC loop and the ψ loop, respectively. (Fig. 1) The number of bases are specific for each stem. The AA stem has seven, the D stem three or four, and the AC and ψ stems five base pairs each. The D loop contains six to eight nucleotides, the AC and ψ loops each contain seven nucleotides. The most variability is found in the "variable" loop (V loop). The V loop may contain three to five nucleotides or as many as 21 nucleotides creating a fifth double-helical stem of 3 to 7 base pairs and a loop of 3 to 5 residues. All tRNAs have a unique basic two dimensional 'cloverleaf' structure and contain nucleotides CCA at their 3'

Figure 1

The secondary and tertiary structures of a transfer RNA. The bases shown are those conserved among all tRNA. CCA at the 3' terminal is added post transcriptionally and is found in all mature tRNA.


3'
OH
amino acid attachment site
A
C
C
5'
P
D loop
ψ loop
A
U
C
UH2
G
G
G
T
C
U
anti codon


T loop
5'
3'
CCA terminus
D loop
Anticodon loop

ends. Base modifications are commonly found in in all tRNAs. The most common base modifications are methylation, acetylation, pseudouridylation, and ribose methylation. (83)

In recent years the genes coding for nuclear tRNA in higher organisms have been extensively studied due to the availability of DNA cloning and sequencing techniques. Studies include the organization and arrangement of tRNA genes in their genomes, their function and their regulation of gene expression. The total number of nuclear tRNA genes found in various species always exceeds the number of different tRNA species indicating that each tRNA gene is likely to be repeated. However, they are more highly repeated in eukaryotic cells than in prokaryotic cells. E. coli contains about 60 tRNA genes and therefore each gene species probably exists in only a few copies. (5) In contrast, S. cerevisiae contains about 360 tRNA genes per haploid genome where a specific tRNA species may range from 1 to 11 copies. Drosophilia contains 60 or so tRNA genes with each tRNA species repeated about 12 fold. Xenopus laevis has 7800 tRNA genes per haploid genome and each species may repeat about 200 fold on the average. (50)

Although the number of tRNA genes increases with the complexity of the organism, the number of tRNA species found remains the same throughout evolution. The structural aspects of tRNA gene repetition may be explained by the functional needs of an organism. For example, high levels of fibroin, containing amino acids glycine, alanine and serine, are produced by the posterior silk gland of Bombyx mori and therefore high level of $tRNA^{gly}$, $tRNA^{ala}$ and $tRNA^{ser}$ are found in these glands. (8) Likewise, in X. laevis, the oocyte requires about 40 ng of tRNA to be

synthesized within a year. (13) Since a single copy gene could not produce the required amounts of the product, it might be necessary for these genes to exist in multiple copies. No tRNA gene amplification has been observed at the present time.

The arrangement of tRNA genes varies greatly throughout evolution. In E. coli, most tRNA genes are clustered in polycistronic transcription units and are processed from large precursor molecules. Some tRNA genes are on individual transcription units and some are found in between rRNA genes. (2) In yeast, most tRNA genes are scattered throughout the genome. Analysis of 175 different cloned yeast DNA fragments containing tRNA sequences showed that most of the 360 genes are not tightly clustered. (4) For example, single genes for $tRNA^{met}_3$ (63) tRNA $^{glu}_3$ (20,21) and $tRNA^{val}_1$ (3) from a separate DNA fragment of 3-7 kilobases contain no other tRNA genes. The $tRNA^{phe}$ genes are found on at least ten different restriction fragments of DNA ranging from 3-20 kb. (92) However, not all tRNA genes in yeast are scattered throughout the genome. In S. cerevisiae, single copies of $tRNA^{arg}_3$ and $tRNA^{asp}$ are only separated by spacer DNA of 10 nucleotides. These tRNA genes are co-transcribed in Xenopus extracts as a dimeric precursor with intragenic spacer which is removed in a series of discrete nucleolytic steps to yield mature tRNAs. (73) A similar arrangement was found with $tRNA^{ser}$-$tRNA^{met}$ in S. pombe. (56)

In Drosophila, in situ hybridization of labelled total tRNAs reveals about 140 loci distributed throughout three autosomes and the X chromosome. (88) The tRNA arrangement in Drosophila is very diverse. Several fragments of the DNA from these loci have been cloned and the overlapping clones show that a 94 kb DNA fragment contains 18 tRNA genes.

(27, 38, 98, 99) The central region of this DNA fragment, ~ 46 kb long, contains eight $tRNA^{asn}$ genes, four $tRNA^{arg}_2$ genes, five $tRNA^{lys}_2$ genes and one $tRNA^{ile}$ gene. These genes are irregularly spaced and are transcribed from both DNA strands. Another DNA fragment, 3.4 kb long, was isolated to contain a cluster of five $tRNA^{ile}$ genes which are also irregularly spaced, distributed over both DNA strands and are intermingled with two $tRNA^{leu}$ genes. (66) However, two $tRNA^{met}$ genes are found neither tightly clustered nor mixed with other tRNA genes. They occur as single copies within repeated DNA segments of 415 bp that are separated from each other by many kilobases of DNA. (78) Also, single copies of a $tRNA^{gly}$ genes of D. melanogaster are found to occur on each 1.1-2.0 kb unit of two direct repeats that are separated only by about 0.9 kb of DNA. (33)

Little is known about the mammalian tRNA genes. Analysis of five clones containing rat tRNA genes showed that the tRNA genes are distributed in small clusters spanning 1-2 kb and interpersed with large regions (8-20 kb) that do not code for tRNA. The clusters contain a variety of tRNA species. (48) A tRNA gene cluster within a 2.1 kb fragment of rat DNA was found to contain single genes for $tRNA^{asp}$, $tRNA^{gly}$, and $tRNA^{glu}$ separated by DNA segments of about 450 base pairs. (75) Another rat DNA fragment, 11.9 kb long, contains a cluster of three $tRNA^{pro}$ and three $tRNA^{lys}$ genes.(76)

A 1.8 kb DNA sequence containing mouse $tRNA^{asp}$, $tRNA^{gly}$, and $tRNA^{glu}$, separated by spacer sequence of 587 bp and 436 bp respectively, was found to have sequences homologous to that of the rat 2.1 kb sequence described above. The coding regions of $tRNA^{asp}$ and $tRNA^{gly}$ are identical and only two nucleotides on the $tRNA^{glu}$ differ. Another mouse clone contains only

one $tRNA^{asp}$ gene with an identical coding region as the one found in the cluster. (51)

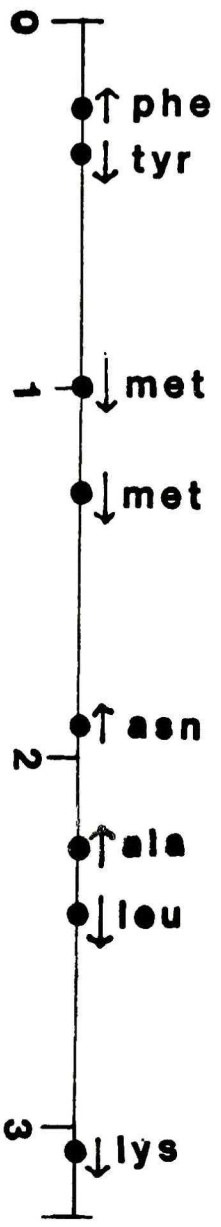
The organization of tRNA genes in the human genome resembles that of yeast. Four cloned $tRNA^{met}_i$ genes are found at some 11-13 scattered locations in the human genome. (69) Another clone shows $tRNA^{lys}$, $tRNA^{gln}$ and $tRNA^{leu}$ are within 1.5 kb of each other. (40)

Studies of X. laevis tRNA genes show yet another kind of gene organization. X. laevis tRNA genes can be separated from other genomic DNA by buoyont density centrifugation. A tandemly repeated 3.18 kb DNA fragment has been cloned and found to contain a cluster of 8 tRNA genes. (14) This DNA fragment is repeated about 150 times at a single chromosomal locus. (15) The genes are separated by different lengths of spacer sequences. (12) The two $tRNA^{met}$ genes within this DNA fragment are separated by only a 0.35 kb spacer. (15) Some tRNA genes are oriented in different polarity. (Fig. 2)

Many tRNA genes contain intervening sequences (introns) that are not found in the mature tRNA. These intervening sequences are transcribed as part of tRNA precursors by RNA polymerase III and then excised by an endonuclease from the precursor. The remaining two halves are then joined together by an RNA ligase to give the mature tRNA. (41,42) Transfer RNA genes containing intervening sequences are reported in $tRNA^{tyr}$ and $tRNA^{phe}$ of yeast, (28,92) $tRNA^{leu}$ of Drosphila, (66) $tRNA^{tyr}$ genes of X. laevis (61) and $tRNA^{lys}_2$ and $tRNA^{lys}_4$ of chicken. (97) Transfer RNA genes from mammals examined so far do not contain intervening sequences. All intervening sequences are found in the precursor tRNAs and therefore are fully transcribed.

Figure 2

A 3.18 kb repeating unit of X. laevis that comprises 8 tRNA genes. Distances are given in kilobases. Arrows indicates the polarity of the genes.


0
phe
tyr
1
met
met
asn
2
ala
leu
3
lys

The sizes of the intervening sequences vary from 13 to 31 bases and they are all located one nucleotide 3' to the anticodon. (61,97) Only the secondary structure of the anticodon stem and loop region are altered. The amino-acid acceptor, and the dihydrouridine and the TψCG stems and loops are conserved as in the standard cloverleaf model of tRNA. The anticodon stem is intact but is augmented by a second helical region of variable length. The second region is sometimes separated from the anticodon stem by a few unpaired bases. This usually includes the anticodon that pairs with 1 to 3 complementary nucleotides in the intervening sequence thereby protecting the anticodon from S1 digestion. However, a $tRNA^{lys}$ genes from S. pombe is found to contain an 8 bp intervening sequence which cannot base-pair with the anticodon, yet is still spliced correctly in vitro. (25)

The function of the intervening sequences in tRNA genes is not well defined. In S. cerevisiae, a $tRNA^{tyr}$ gene intron is reported to be essential for correct pseudouridine modification of the middle base of the anticodon loop. (39) However, comparison of the anticodon loop and stem structures of $tRNA^{phe}_{UUC}$ species from S. cerevisiae and S. pombe reveals that all five modified bases are identical. Since $tRNA^{phe}$ of S. pombe does not contain an intervening sequence, it is unlikely that the intervening sequence is essential for such modification. (25) In S. cerevisiae, the mature $tRNA^{ser}_{UCG}$ and $tRNA^{ser}_{UCA}$ coding sequences show 96% homology reflecting a 3 bp difference while an intervening sequence is present in the $tRNA^{ser}_{UCG}$ gene but absent from three $tRNA^{ser}_{UCA}$ genes. (6,64)

It has been suggested that intervening sequences might help to suppress crossing over between identical genes arranged in tandem order. (90)

For example, $tRNA^{tyr}$ and $tRNA^{phe}$ genes of X. laevis are very closely related with over 70% homology but only the $tRNA^{tyr}$ gene contains an intervening sequence. (61) Recombination of these genes is presumably hindered by the intervening sequence. The diverse spacer sequences and the different polarity of identical tRNA genes may also contribute to the suppression of crossing over between identical genes in tandem repeats. (47)

In eukaryotes, tRNA genes are highly conserved even among distantly related species. For example, the $tRNA^{phe}$ of X. laevis contains the same primary sequence as $tRNA^{phe}$ of rabbit, calf liver and human placenta. (61) The $tRNA^{lys}_2$ sequences are identical in Drosophila and mammals (87) while $tRNA^{gly}_1$ sequences of D. melanogaster and human plencenta are 94.6% homologous. (33) The homologies suggest that these genes are derived from a common ancestral gene.

Some tRNA genes are extensively disrupted so that only short regions of the coding sequence remain intact. Such incomplete or pseudogenes are found in a 1.15 kb DNA fragment of D. melanogaster containing four regions that display sequence homology to $tRNA^{met}_i$. (78) A 3.2 DNA fragment of rat liver contains $tRNA^{glu}$ and $tRNA^{gly}$ pseudogenes in addition to genes for $tRNA^{leu}_{CUG}$ and $tRNA^{asp}_{GAC}$. (53) Pseudogenes may have arisen by the repeated insertion and exision of a transposable element into intact tRNA genes.

Most tRNAs in eukaryotes are transcribed as precursors that are then processed by nucleases to yield mature tRNAs. The precursor tRNAs are also modified at specific sites by a set of modification enzymes. Studies of precursors performed by either injecting plasmids containing

tRNA genes into X. laevis germinal vesicles or using eukaryotic cell free extracts reveal that all precursors contain a 5' leader and a 3' trailer sequence. (45,30,59,82,60) Since tRNA genes do not contain sequences coding for the CCA end of the 3'end of the mature tRNA, they must be added postranscriptionally.

Studies performed with $tRNA^{tyr}$ from yeast and $tRNA^{leu}$ from Caenorhabditis elegans (60) show that the first steps which occur after transcription are the stepwise removal of the 5' and 3' trailer sequences with simultaneous modifications of some bases, and addition of the CCA end in the nucleus. The last step is the excision of the intervening sequences which may occur in the nucleoplasm or in the nuclear membrane. The mature tRNA is eventually transported into the cytoplasm where protein sythesis takes place. All eukaroytic tRNAs analyzed thus far are on single transcription units. Multimeric precursors have not yet been found in the higher eukaryotes. Two dimeric precursors are found in S. cerevisiae and S. pombe when transcribed in Xenopus germinal vesicle extract. (73,56)

RNA polymerase III is responsible for the transcription of small RNAs such as tRNA, 5S RNA, and some viral RNAs. It has a molecular weight of approximately 700,000 and is composed of at least 10 subunits. (67) The initiation site of this RNA polymerase III on eukaryotic tRNA genes occurs within 20 base upstream from the mature tRNA coding sequence. Transcription initiates with a purine nucleoside triphosphate at a site on the DNA preceded by a pyrimidine nucleotide (non coding strand). However, no sequence homology has been detected at these regions within or among species.

The termination sites for RNA polymerase III appear to be within a cluster of four to six T residues at the 3' ends. The RNA polymerase seems to pause within this T tract whereupon termination of the sequence occurs. Experiments supporting this include the detection of precursors with a variable number of U residues at their 3' ends corresponding to the number of T residues of a particular T tract. (26, 30, 44, 82) A sequence of the type TTNTTT can also permit termination of the transcription at these sites. (24) Mutants of yeast $tRNA^{tyr}$ containing a run of five to six T residues within the coding region result in premature termination *in vitro* yielding a product containing five to six U residues at their 3' ends. (43) Termination sites for tRNA genes usually occur within a few nucleotides of the 3' end of the tRNA coding region but are sometimes found further downstream. For example, a tract of four thymidines occurs 18 bp after a *B. mori* $tRNA^{ala}_2$ gene (26,30) and 75 bp after a *X. laevis* $tRNA^{lys}$ gene (52) and transcription terminates efficiently at these sites yielding primary transcripts of 98 and 157 nucleotides respectively.

The promoters for tRNA genes in prokaryotes are known to be located just upstream of the transcription initiation sites, where the regions are both conserved in sequence and position. (84) However, early studies on cloned tRNA genes using restriction enzymes or exonucleases showed that the essential elements of the eukaryotic promoters lie within the transcribed part of the mature tRNA genes. (46,16) More detailed studies show that in *X. laevis* $tRNA^{met}$ and $tRNA^{leu}$ genes, *C. elegan* $tRNA^{pro}$ gene and *Drosophila* $tRNA^{arg}$ gene, the promoters are split into two essential elements that coincide with sequences conserved among eukaryotic and many prokayotic tRNAs. (35,24,11,79) These two regions, termed the A and B

blocks (24), are composed of nucleotides 8-19 and 52-62 respectively, and are located in the D and the TψCG stem and loop regions of the cloverleaf structure. Deletion mutants of Drosophila $tRNA^{arg}$ gene show that one promotor control region is bounded by nucleotides 50 to 58 in the B block. (77) The location of the A and B blocks appears to be important for the transcription of tRNA genes. The distance between the A block and the initiation point is usually restricted to 10-16 base pairs. The distance between the A and B block varies from 31 to >74 base pairs due to the variability of the length of the variable loop and presence of intervening sequences in some tRNA genes. (56)

Analysis of the transcriptional control regions of other genes that are transcribed by RNA polymerase III show that the same transcriptional elements involved in the transcription of tRNA genes are present in adenovirus VA I and VA II, the Esptein-Barr virus EBER 1 and EBER 2 RNA genes, and Alu-related sequences. (74) It has been suggested that the A and B block function by interacting with one or more transcription factors rather than directly with the RNA polymerase.

Some of these nucleotides which appear to be promoter determinants are among the highly conserved nucleotides (T_8, A_{14}, G_{18} and G_{19} from the A block and G_{53}, T_{55}, C_{56}, A_{58} and C_{61} from the B block) found in both eukaryotic and prokaryotic tRNAs. However, not all conserved nucleotides are promoter determinants. In X. laevis, a study where nearly every G-C and C-G bp in the $tRNA_i^{met}$ gene has been mutagenized by bisulfite shows that nucleotides G_7, G_{10}, C_{11}, G_{12}, C_{13} and G_{19} from the A block and nucleotides G_{49}, G_{52}, C_{56} and C_{61} from the B block appear to be promoter determinants. Highly conserved nucleotides C_{48}, G_{57}, and G_{62} seem to

have no effect on the transcription but a nonconserved nucleotide, C_{68}, on the aminoacyl stem appears to reduce transcription activity also. (22) Other studies have shown that T_8 from the A block (77,91) and G_{45}, C_{56} and possibly G_{52} are also crucial for promoter activity. (43,9) Mutations in some G-C and C-G bp in the anticodon region of the $tRNA_1^{met}$ gene which destablized the anticodon stem and loop structure appear to reduce the transcription activity. Hence, the stem and loop structure of the anticodon region may play an important role in the RNA polymerase III promoter function or in the formation of a stable transcriptional complex. (22)

A study of promoters using mutants of yeast SUP4 $tRNA^{tyr}$ genes show that mutations in five conserved nucleotides in the A and B blocks reduce transcription significantly. When used in a competition assay for a transcriptional component present in a limiting amount in the yeast cell extract, eleven mutants are found to affect the promoter activity. Nine out of these eleven mutations are in the A and B block and only two mutations, at positions 45 and 46, are outside of these blocks. Mutations at position 56 and 57 cause drastic reduction whereas mutations at position 15, 52, and 54 cause moderate or slight reduction in template activity. In contrast to the X.laevis system, mutations at position 27, 29, 30 and 40 which disrupt the $tRNA^{tyr}$ anticodon stem do not seem to have any effect on the transcription. This finding implies that a stem and loop structure of the anticodon region is not necessary in the yeast system. (1)

One model proposes that the DNA sequences in the B block exist in an intrastrand stem and loop conformation and that the primary interaction required for transcription is the binding of transcription factors to the B block sequences. This binding then promotes a tertiary inter-

action between sequences involving some of the conserved nucleotides of the D and TψCG loops, resulting in formation of a tRNA-like structure in the DNA. According to this model, only a few nucleotides in the A and B blocks are necessary for the binding of the transcription factors, but some sequences in the B block are needed as regions of dyad symmetry while other conserved nucleotides of both blocks are required for mutual tertiary interactions. The sequences between the A and the B blocks appear to space the control region so that the tRNA secondary structure can be formed during transcription initiation. (31) Experiments supporting this model include the findings that no transcription products are formed when either the 5' or 3' half of a X. laevis $tRNA_i^{met}$ gene are deleted. The 3' half was able to inhibit transcription of a reference gene in the cell extract by competing for transcription factors. (46) In vitro transcription was severely reduced when nucleotide C_{56} was substituted either by a G or a T which weakens the proposed G_{19}-C_{56} tertiary interaction in the yeast $tRNA^{tyr}$ gene. (43) Substitution at the G_{57} position, which did not participate in tertiary interaction, did not affect transcription of a human $tRNA_1^{met}$ gene. (100) The model also predicts that deletions in the tRNA gene which result in chimeric tRNA genes that are unable to form a ψ stem should be poorly transcribed even if it contains all the conserved nucleotides of the B block. This hypothesis is supported by the fact that a 4 bp deletion from the 5' portion of the ψ stem of a C. elegan $tRNA^{pro}$ gene which eliminated the ψ stem except for an invariant G_{53}-C_{61} base pairing, reduces transcription of this gene by a factor of 100. (10)

Another model (19) also proposes that at least two transcription factors other than RNA polymerase III are involved in transcription of tRNA genes. They are an A factor, δ, that binds to the D control region and a B

factor, τ, that bind to the TψCG control region. Since the δ factor cannot be competed for in the transcription reaction, it may be exchanged relatively rapidly on the tRNA gene. The τ factor is required to form a stable transcription complex in vitro and this factor remains bound to the tRNA gene for many rounds of transcription. (70) Therefore, if the TψCG region is deleted the tRNA gene will not compete for transcription factors. (70,79,23,46) The δ factor interacts concomitantly with the τ factor, binding to the D control region as the primary recognition event in the formation of a stable complex. In the formation of this complex, the τ factor would then bind stabily to the TψCG control region. When the TψCG control region is deleted or moved more than 400 bp away relative to the D control region, no stable complex is formed. (19)

An experiment supporting this model was done using Drosophila $tRNA^{arg}$ deletion mutants that contained either the 5' or 3' control regions rejoined by an Xho I linker. Different lengths of DNA fragments ranging from 12 to 1530 nucleotides were inserted into the cloning site and the genes were assayed for transcription efficiency and competition for transcription factors. It was observed that transcription was still efficient when the two control regions were separated by 12-17 nucleotides. Stable complexes were detectable when these regions were separated by 200-400 bp, but beyond that the competitive strength of the mutant genes decreased dramatically. Also, the mutants used generated tRNA transcripts that had little resemblance to a tRNA. Although, many of these mutant tRNA genes could hypothetically form double stranded helices, no structure within the spacer region of the mutants was common to the wild type tRNA genes. Therefore, it was not likely that a direct tertiary interaction between the D and TψCG control regions of these mutants was required for

their transcription and that the tRNA-like structure of the DNA was probably not essential for tRNA gene transcription. (19)

However, both models consider only the intragenic sequences that are essential for transcription initiation in the eukaryotic genes. It has been known that the 5' flanking sequences also play an important role in the transcription of tRNA genes. For example, deletion of all but 14 bp of the 5' flanking region of a B. mori $tRNA^{ala}$ gene abolished tRNA gene transcription in homologous cell free extract. (87) Transcription of a D. melanogaster $tRNA^{arg}$ gene was reduced when all but 8 bp of its 5' flanking sequences was replaced by a plasmid or synthetic linker DNA even when initiation still occurred at the normal initiation site. (18) The 5' flanking sequences may have negative influence on tRNA gene expression also. For instance, the transcription of several D. melanogaster $tRNA^{lys}_{2}$ genes that contain a highly conserved 11 bp tract on the 5' flanking region was inhibited when this tract was located from position -13 to -23 but not when it was moved closer to or further away from the tRNA gene. (16,71) A sequence located at the -12 to -20 position in $tRNA^{met}_{1B}$ gene of X. laevis was able to form Z-DNA and when a synthetic DNA fragment capable of forming Z-DNA structure was inserted preceded the coding region, the expression of the gene was inhibited. (34)

We wished to learn more about the organization and arrangement of tRNA genes in eukaryotes by studying the tRNA genes in X. laevis. It has been shown that the ratio of tRNA gene to rRNA gene in a haploid Xenopus genome is approximately 14, 7800 - 9600 tDNA copies (13) to 610 rDNA copies. (5). This ratio reflects an unusual transcriptional requirments during oogenesis. The oocyte formation begins about six to eight weeks after

fertilization of the ovum but takes about a year for it to mature. At the early stages of oocyte development, tRNA is synthesized vigorously and stored in a cytoplasmic 42 S ribonucleoprotein storage particle. (13) Rapid rRNA synsthesis occurs over a more restricted period late in oocyte development and results in the large content of rRNA in the egg relative to tRNA. (7) However, the presence of 40 ng of tRNA in a single cell is still very high. Assuming closest packing of the RNA polymerases of 150 Å on chromosomal DNA, it is calculated that in order to accumulate 40 ng of tRNA in a single cell in about a year, a rate of polymerization of 30 nucleotides/second for RNA polymerase III without tRNA turnover, and a reiteration of 4×10^4 tRNA cistrons in the tetraploid oocyte nucleus is required. (13)

At the present time, the arrangement and organization of tRNA genes in X. laevis has been studied in only one DNA fragment at the genomic level, namely the 3.18 kb DNA fragment described earlier. We wished to study tRNA genes in other DNA fragments in X. laevis and hoped to better understand tRNA gene organization and perhaps their functions in eukaryotic cells. We have isolated and sequenced a DNA fragment, 1737 bp long, containing two cysteine tRNA genes.

EXPERIMENTAL PROCEDURES

A. MATERIALS

The restriction endonuleases, DNA polymerase I (*E. coli*), Bal 31 exonuclease, Exonuclease III, ddNTP, M13 15-base primer, agarose, Low-melting agarose and NACS PREPAC™ columns were purchased from Bethesda Research Laboratory. Calf intestinal alkaline phosphatase, pancreatic RNAase A, and the antibiotics were supplied by Sigma. The reverse transcriptase was a gift from D. Beard. (37) T_4 DNA ligase were purchased from Collaborate Research, yeast extract and Bacto typtone were obtained from DIFCO and agar was purchased from GIBCO. Unlabeled nucleoside triphosphates were supplied by either BRL or Pharmacia PL Biochemicals, α-and γ-[^{32}P] ATP from New England Nuclear, and α-[^{32}P] GTP from ICN. *X. laevis* S-100 cell extract was prepared using procedures according to S. Y. Ng, et. al. and B. S. Shastry, et. al. (62,80)

B. SOLUTIONS AND MEDIUM

L-broth (LB):	1% Bacto tryptone, 1% yeast extract, 10 mM Tris-Cl (pH 7.5), 1 mM $MgSO_4$
2X YT broth:	1.6% Bacto tryptone, 1.0% yeast extract, 8 mM NaCl
2X YT top agar:	Same as 2X YT plus 0.75-1.0% agar
1X YT agar:	0.8% Bacto tryptone, 0.5% yeast extract, 42.5 mM NaCl, 1.5% agar
SOB media:	2.0% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM $MgCl_2.6H_2O$, 10 mM $MgSO_4.7H_2O$
SOC media:	Same as SOB media with the addition of 20 mM glucose
FSB media:	10 mM potassium acetate (pH 7.0), 100 mM KCl, 45 mm $MnCl_2.\ 4H_2O$, 10 mM $CaCl_2.\ 2\ H_2O$, 3 mM $HACoCl_3$, 10% glycerol adjusted to a final pH of 6.4 with 0.1N HCl
LM plates:	1% Bacto tryptone, 0.5% yeast extract, 10 mM $MgSO_4.7H_2O$, 1.5% agar
Triton X-100 mix:	0.5% Triton X-100, 62.5 mM Na_2EDTA, 0.5 M Tris-Cl(pH 8.0)
PEG solution:	30% polyethylene glycol, 1.5 M NaCl
CsCl solution:	CsCl in 10 mM Tris-Cl (pH 7.5), 1 mM Na_2EDTA; refractive index = 1.392
TNE buffer:	100 mM Tris-Cl (pH 8.0), 1 M NaCl, 10 mM EDTA
E-Buffer:	0.02 M Tris-Cl, 0.1 M NaOAc, 1.0 mM Na_2EDTA adjusted to pH 7.8
1X TBE buffer:	75 mM Tris-Cl, 50 mM boric acid, 1.5 mM EDTA
10X SSC buffer:	1.5 M NaCl, 0.15 M Sodium Citrate
0.2% DENHARDT:	1% each of BSA, polyvinyl pyrrolidone, Ficoll 400
2X SET:	0.3 M NaCl, 0.1 M Tris-Cl, 2 mM EDTA, pH 7.9

C. METHODS

Preparation of $CaCl_2$ treated HB-101 or JM-83 E. coli cells

A 2 ml overnight culture of HB-101 or JM-83 was grown at 37°C with constant shaking at 225 rpm. A 0.5 ml aliquot of the fresh overnight culture was inoculated into a 200 ml L-broth (1% Bacto tryptone, 1% yeast extract, 10 mM Tris-HCl(pH 7.5), 1mM $MgSO_4$) and was grown until it reached an optical density at 550 nm of 0.5 or 5 x 10^7 cells/ml. The cells were distributed into four 50 ml sterile screw-cap centrifuge tubes and incubated on ice for 10 min followed by centrifugation at 3,000 rpm for 10 min at 4°C. The pellets were washed with 10 mM Tris-HCl (pH 7.5) in 10 mM NaCl and centrifuged again at 3,000 rpm for 10 min. The cells were resuspended in 20 ml of sterile 75mM $CaCl_2$ and incubated on ice for 15 minutes. The $CaCl_2$ treated cells were divided into 4 equal volumes, quickly frozen in a dry ice/EtOH bath and stored at -70°C until used.

Transformation of DNA using $CaCl_2$ treated HB-101 or JM-83 E. coli cells

The $CaCl_2$ treated HB-101 or JM-83 cells were thawed on ice. For DNA in a volume of 200 ul or less, 200 ul of the cell suspension was added. For DNA in volume greater than 200 ul, 400 ul of the cell suspension was added. The mixture was incubated on ice for 30 minutes and then "heat pulsed" at 37°C for 5 minutes without aggitation. A 1 ml 2X YT broth (1.6% Bacto tryptone, 1.0% yeast extract, 85 mM NaCl) was added to the cell suspension and incubated at 37°C for 30-60 min with gentle shaking. The desired aliquot was added 2.5 ml of 2X YT top agar (0.75-1.0% agar) at 46°C and plated on an 1X YT agar (0.8% Bacto tryptone, 0.5%

yeast extract, 42.5 mM NaCl, 1.5% agar) plate containing an appropriate antibiotic. The plates were incubated overnight at 37°C.

Preparation of Frozen DH-1 E. coli Cells for Transformation Use (32)

A 2 ml overnight culture was grown in SOB media (2.0% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2.6H_2O$, 10 mM $MgSO_4.7H_2O$) at 37°C with constant shaking at 275 rpm. 0.5 ml of the overnight culture was incubated with 100 ml of SOB media and was grown to an optical density at 550 nm of 0.5. The cells were transferred into 2-50 ml sterile conical polypropylene tubes and incubated on ice for 15 min, pelleted by centrifugation in IEC Floor model at 2000 rpm for 15 min at 4°C. The pellets were resuspended in 33 ml of sterile FSB [10 mM potassium acetate (pH 7.0), 100 mM KCl, 45 mM $MnCl_2.4H_2O$, 10 mM $CaCl_2.2H_2O$, 3 mM $HACoCl_3$ (hexamine cobalt III chloride), and 10% restilled glycerol adjusted to a final pH of 6.4 with 0.1 N HCl]. The cells were incubated on ice at 4°C for 15 minutes and centrifuged again at 2,000 rpm for 15 min. The pellet was resuspended in 8ml of FSB, and 280µl of 2.5M dithiothreitol in 40 mM potassium acetate, pH 6.0 was added before incubating the mixture on ice for for 5 min. Aliquots (425µl) were pipetted into 1.5 ml sterile Eppendorf tubes and quickly frozen in a dry ice/EtOH bath and stored at -70°C until used.

Transformation of plasmid in DH-1 E. coli Cells (32)

DH-1 frozen cells were thawed on ice. A 200 ul aliquot was transferred into a chilled 16 x 125 mm sterile polystyrene screw-cap culture tube (Falcon 3033). The DNA solution (<10µl volume) was added to the cells and incubated on ice for 30 min followed by "heat pulsing" at 42°C

for exactly 2.5 min without aggitation. After incubating the mixture on ice for 1-2 min, 800ul of SOC media (same as SOB media plus 20mM glucose) was added. The cells were incubated at 37°C with shaking at 225 rpm for 1 hr. Aliquots were removed and added to 2.5 ml of SOB top agar and plated on LM plates (1% Bacto tryptone, 0.5% yeast extract, 10mM NaCl, 10mM $MgSO_4.7H_2O$, 1.5% agar) containing the appropriate antibiotic and incubated overnight at 37°C.

Plasmid Boiling Preparation For Minilysates (36)

A 5 ml overnight culture was grown in a sterile 16 x 100 mm borosilicate glass culture tube in an appropriate media (LB or LM for plasmids in HB 101, 2X YT for plasmids in JM-83, and LM for plasmids in DH-1) and incubated at 37°C with shaking at 225 rpm. The cells were collected by centrifugation at 3000 rpm in a SS-34 rotor for 10-15 min at 4°C. The pellet was resuspended in 350 ul of cold 8% glucose, 5% Triton X-100 100, 50 mM Na_2EDTA, 50 mM Tris-HCl, pH 8.0, incubated on ice for 15 min, and transferred to a 1.5 ml Eppendorf tube. Cold lysozyme solution (25 ul, 10 mg/ml in 50 mM Tris-Cl, pH 8.0) was added to the tube and incubated on ice for 5 min. The suspension was heated in boiling water for 1 min and centrifuged immediately at 12,000g for 10 minutes at room temperature. The pellet was removed and discarded. The supernatant was extracted with phenol, and precipitated in 95% ethanol at -20°C for 10-20 minutes. The DNA was collected and redissolved in 50 ul of TE buffer containing 0.1 ug/ml RNase A.

Isolation of Plasmid DNA on CsCl Gradient (96)

A 2 ml overnight culture was grown at 37°C, 225 rpm as described

previously. 600 ml of media (M9/casamino acid supplement for plasmids in HB-101 cells, 2X YT for plasmids in JM-83 cells, and SOB for plasmids in DH-1 cells) containing appropriate antibiotics was inoculated with 1.0 ml of overnight culture and incubated at 37°C with shaking at 225 rpm until the cell culture reached an optical density of 0.5 at 600 nm. The plasmids were amplified by addition of chloramphencol to a final concentration of 250 ug/ml and incubated at 37°C at 250 rpm overnight.

The cells were collected by centrifugation at 4000 rpm in a GS-3 rotor for 20 min at 4°C. The pellet was resuspended in 7.5 ml of cold 25% sucrose, 50 mM Tris-HCl, pH 8.0. 40 mg lysozyme in 2.0 ml 0.25 Tris-HCl, pH 8.0 was added and the suspension was incubated on ice for 45-60 min. At the end of the incubation 2.0 ml of 0.5 M Na_2EDTA, pH 8.0 was added and incubated for 5 min on ice, 5 min at 37°C and 5 min on ice. 10 ml of cold Triton X-100 mix (0.5% Triton X-100, 62.5 mM Na_2EDTA, 0.5 M Tris-HCl, pH 8.0) was added and incubated for 5 min. on ice, 5 min at 37°C and 5 min on ice.

The solution was transferred into Ti-60 tubes and centrifuged for 1 hr at 30,000 rpm at 4°C in a Ti-60 rotor. The DNA was precipitated by addition of 11 ml of cold PEG solution (30% polyethylene glycol, 1.5 M NaCl) to the supernatant and incubated on ice for 30-60 min, followed by centrifugation in a HB-4 or SS-34 rotor at 9,000 rpm for 15 minutes. The precipitate was collected and resuspended in 5 ml of CsCl solution (refractive index = 1.392 made in 10 mM Tris-HCl, pH 7.6, 1 mM Na_2EDTA).

When the DNA was completely dissolved, an additional 19.5 ml of CsCl solution and 0.5 ml of 1% ethidium bromide was added. The solution was centrifuged in a VTi-50 vertical rotor at 45,000 rpm overnight at

10°C. The plasmid (lower) band was carefully removed from the CsCl gradient, transferred into a Beckman 15 ml Quick-Seal™ tube, and centrifuged in a Ti-50 or Ti-75 rotor at 33,000 rpm for a minimum of 40 hrs at 10°C. The plasmid band was removed and EtBr was extracted with H_2O saturated n-butanol. The volume was measured and 2 volume of H_2O was added prior to precipitation with 2 volumes of 95% ethanol overnight at -20°C. The DNA was collected by centrifugation in HB-4 rotor at 9,000 rpm for 30 min. at 4°C and dissolved in 200 ul of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The DNA concentration was determined by making a 1/200 dilution of a sample and measure its absorbance at 260 nm. The DNA concentration was adjusted to 1 ug/ml, assuming 20mg/ml of the DNA gave an absorbance reading of 1.0 at 260 nm.

In Vitro Transcription of tRNA genes in Xenopus S-100 Extract (62)

All transcription reactions were in a final volume of 50 ul and included the following components: 40 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 10% glycerol, 20 ul of Xenopus S-100 cell free extract, 0.5 mM each of ATP, CTP, UTP, 0.025 mM of GTP, 20 uCi α-^{32}P GTP (650 Ci/mmole), and template DNA at 10 ug/ml. The reactions were incubated at 30°C for 2 hrs and terminated by addition of 1 ul 0.25 M EDTA, 2.5 ul 10% SDS, 1 ul 20 mg/ml carrier tRNA and 3 ul 3 M NaOAc. The mixture was extracted with phenol and the RNA was precipitated with ethanol. The precipitates were collected and redissolved in 10 ul of buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA. Each sample was prepared for electrophoresis by addition of 10 ul formamide solution (98% formamide, 10 mM EDTA, 0.5% bromophenol blue, and 0.5% xylene cyanol, and then loaded on 8% polyacrylamide gel (15 x 17 cm) containing 8 M urea in 1.5 X TBE (75

mM Tris, 50 mM boric acid, 1.5 mM EDTA) buffer and electrophoresed at a constant voltage of 300-350V.

Dephosphorylation of DNA using Calf Intestinal Alkaline Phosphatase

The terminal 5' phosphates were removed from DNA by treatment with calf intestinal alkaline phosphatase (C.I.P.). The DNA to be dephosphorylated was digested with the restriction enzyme(s) of choice, phenol extracted and ethanol precipated. The precipitate was redissolved in 10 ul TE buffer and then added to a reaction mix containing 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, 2 ul C.I.P (0.0176 u/ul) in a final volume of 20 ul. After incubation at 37°C for 30 min, an additional 1 ul C.I.P. was added to the mixture and incubated at 37°C for another 30 min. The reaction was terminated by adding 5 ul TNE (100 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM EDTA), 2.5 ul 10% SDS, diluted to a final volume of 50 ul. The mixture was incubated at 68°C for 15 min followed by phenol extraction and ethanol precipitation. The DNA was collected and redissolved 10 ul of TE buffer.

Ligation of DNA Molecules

The T_4 DNA ligase from T_4 infected E. coli catalyzes the formation of a phosphodiester bond between adjacent 3'-OH and 5'-P termini in DNA. (95) Ligation of DNA molecules was carried out for 12-16 hrs at 4°C in a 10 ul reaction mix containing 66 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.2-1.0 ug of DNA, and 1 unit of T_4 ligase (Weiss unit).

α-[^{32}P]dATP Labeling of a DNA Fragment using Reverse Transcriptase

Reverse transcriptase consists of two activities: 5'->3' polymerase activity using DNA or RNA as templates and a processive 5'->3' ribo-exonuclease that is specific for RNA-DNA hybrids. (93) In this reaction, it was used for labeling the termini of DNA fragments with protruding 5'-ends.

The DNA fragment to be labeled was digested with a restriction endonuclease that gives an end with 5' overhangs. The DNA was extracted with phenol, precipitated with ethanol, and dissolved in a final volume of 10 ul with the following components: 50 mM Tris-Cl (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, 0.1 mM each dCTP, dGTP, dTTP, 1 mM DTT, 0.1 mg/ml BSA, 50 uCi α^{32}-P dATP (3000 mCi/mmol) and 1.5 units of reverse transcriptase. After incubation at 37°C for 30 minutes, 1 ul of 1 mM dATP and 1.5 units of the reverse transcriptase were added and incubated for another 15 min at 37°C. The reaction was terminated by the addition of 1 ul 0.25 M EDTA, 1 ul 10% SDS, 5 ul 3M NaOAc and 31 ul of H_2O. The mixture was extracted once with phenol and DNA was precipitated by addition of 3 vol 95% EtOH. The DNA was collected by centrifugation and redissolved in the buffer of choice.

Partial Restriction Enzyme Digestion For Restriction Enzymes Mapping (84)

DNA labeled at the 3'end using reverse transcriptase or 5' end using polynucleotide kinase was redissolved in no more than 8 ul of H_2O. One ug of a carrier DNA, 1 ul of 10X restriction buffer and 1-2 units of restriction enzyme of choice was added to a final volume of 10 ul and incubated at the appropriate temperature. Aliquots (1.8 ul) were removed

at 2 min, 5 min, 10 min, 15 min, 30 min and added to 1 ul of 0.5M EDTA, 2 ul of 50% glycerol containing 0.5% each of bromophenol blue and xylene cyanol. One microliter from each sample (may be combined) was electrophoresed on a 1% agarose vertical gel in 1X E buffer until the bromophenol blue migrated two thirds of the length of the gel. An autoradiogram was obtained by exposing the gel overnight to XAR-5 film with a Lighting Plus™ intensifying screen. The above procedure was carried out using a collection of restriction enzymes. The restriction enzyme maps were constructed by analyzing the size of the restriction fragments resulting from the partial restriction enzyme digestions.

Construction of a Set of Deletions in a DNA Fragment using Bal 31 Exonuclease (49)

Approximately 35 ug of purified plasmid DNA was linearized by restriction endonuclease of choice, extracted with an equal volume of phenol and ethanol precipitated. The precipitate was collected and redissolved in a reaction mix containing 20 mM Tris-HCl(pH 8.0), 12 mM $CaCl_2$, 12 mM $MgCl_2$, 200 mM NaCl, 1 mM EDTA, 0.1 mg/ml BSA, 4 units (8 ug DNA/unit) of Bal 31 exonuclease in a final volume of 100 ul and incubated at 30°C. Aliquots were removed at two minute intervals and stopped by addition of EGTA to 27 mM. A portion of each sample was analyzed on 1% agarose gel and the size of the DNA fragments were calculated. The samples containing the desired deletions were pooled and precipitated in 95% ethanol.

Labeling the 5' Ends of DNA with T_4 Polynucleotide Kinase (57,54)

T_4 polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to a 5'-OH terminus in DNA or RNA. One to 50 pmoles of 5'

dephosphorylated DNA were added to a reaction containing 1 ul 10X kinase buffer (0.5M Tris-Cl (pH 7.6), 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA), 68 pmol γ-^{32}P ATP (200 uCi,2900 mCi/mmole) and 10 units of T_4 polynucleotide kinase in a final volume of 10 ul. The reaction mix was incubated at 37°C for 30 minutes. At the end of the incubation, 2 ul of 10 mM ATP, 1 ul of 10X kinase buffer, 10 units of T_4 polynucleotide kinase, and 6 ul of H_2O was added to bring the final volume to 20 ul. The reaction mix was incubated for an additional 30 minutes at 37°C. 1 ul of 10X kinase buffer was added to the mixture and diluted to a final volume of 30 ul with H_2O. The reaction was terminated by submerging the tube in powdered dry ice and stored at -20°C until used.

Elution of DNA Fragments from Agarose Gel

a) Electro-elution (81,58)

The desired DNA band was excised from the agarose gel and transferred into a dialysis bag of appropriate size that contained 0.5 X E buffer (0.02 M Tris-HCl, 0.1 M NaOAc, 1.0 mM Na_2EDTA adjusted to pH 7.8). The bag was submerged in a horizontal gel apparatus filled with 0.5 X TBE buffer and electrophoresed either overnight at 25 V, or 3-5 hrs at 75 V. The current was reversed for 2 min prior to the removal of the bag. The sample was loaded onto a NACS PREPAC™ column, and eluted with 800 ul 2 M NaCl, 10 mM Tris-Cl (pH 7.6), 1 mM EDTA (2 M NaCl/TE). The DNA was precipitated in 2 vols of 95% ethanol and collected by centrifugation. The precipitate was redissolved in TE buffer.

b) Elution of DNA from Low-Melting Agarose (89)

A desired DNA band was excised from a 1.2 to 2% low-melting agarose

gel and its weight was determined. Assuming the entire weight of the gel slice was due to water, 2 M NaCl/TE was added and adjusted the salt concentration to either 0.2 M NaCl (DNA fragments < 1000 bp) or 0.5 M NaCl (DNA fragments > 1000 bp), and the agarose concentration to 0.1 to 0.2%. The gel slice was then dissolved by heating in 68°C water bath, loaded onto a NACS PREPAC™ column, and eluted with 800 ul of 2 M NaCl/TE buffer. The DNA was precipitated in two volumes of 95% EtOH and then redissolved in 10 mM TE buffer.

Construction of a Single Stranded DNA Region in pUC 8 or pUC 9 for Dideoxysequencing

Restriction endonuleases Eco RI and Hind III were used to linearized pUC 8 and pUC 9, respectively. 5-10 ug of DNA was digested with the appropriate restriction enzyme in 50 mM Tris-Cl (pH 7.6), 10 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 14-16 units of restriction enzyme in a final volume of 15 ul. For crude DNA, 1 ul of 1 mg/ml RNAse A was also included in the reaction mix above. The reaction mixture was incubated at 37°C for 3 hrs. The buffer was adjusted with 6 ul 0.6 M Tris-Cl (pH 8.0), 0.77 M NaCl, 50 mM $MgCl_2$, 0.1 M DTT and 75 units of Exo III was added before bringing the final volume to 60.5 ul. The reaction mixture was incubated for 2 hr, terminated by adding 15 ul 0.25 M EDTA and 8 ul 3M NaOAc, phenol extracted and precipitated in 2.5 volume of ethanol. The precipitate was collected and redissolved in 32 ul of H_2O for the dideoxysequencing reaction. The DNA concentration should be approximately 0.07 to 0.15 ug/ul.

DNA Sequencing with Dideoynucleoside Triphosphates as Chain Terminators (68)

The DNA treated with Exo III described above was used as the tem-

plate in the dideoxysequencing reaction. The template-primer annealing reaction consisting of 8 ul of the DNA solution (0.07-0.15 ug/ul), 4 ng of the 15 bp primer (5'-AGTCACGACGTTGTA-3') in 7 mM Tris-Cl, pH 7.5, 7 mM $MgCl_2$, 50 mM NaCl was heated at 90-95°C for 5 min in a 0.5 ml Eppendorf tube. The mixture was cooled slowly to room temperature (approximately 45 min) before the addition of 1 ul of 0.1 M DTT, 2.5 ul of α^{32}-P dATP (800Ci/mmole) and 1 ul of large fragment DNA polymerase I ('Klenow', 1 unit/ul). The solution was mixed and centrifuged at 12,000g for 5 sec and 3 ul aliquots were added to 4 separate 0.5 ml Eppendorf tubes in a final volume of 5 ul containing the following components: tube 1, 25 mM each dTTP, dCTP, dGTP, 0.1 mM ddATP, 3.5 mM Tris-Cl, (pH 7.5), 3.5 mM $MgCl_2$, and 25 mM NaCl; tube 2, 25 mM each of dTTP, dGTP, 3.2 mM dCTP, 0.05 mM ddCTP, 4.6 mM Tris-Cl(pH 7.5), 4.6 mM $MgCl_2$, and 32.8 mM NaCl; tube 3, 25 mM each of dTTP, dCTP, 3.2 mM dGTP, 0.1 mM ddGTP, 4.6 mM Tris-Cl (pH 7.5), 4.6 mM $MgCl_2$, and 32.8 mM NaCl; tube 4, 25 mM each of dCTP, dGTP, 1.6 mM dTTP, 0.2 mM ddTTP, 4.6 mM Tris-Cl(pH 7.5), 4.6 mM $MgCl_2$, and 32.8 mM NaCl. The reaction mix was incubated for 5 min at 30°C followed by 10 min. at 42°C. A chase solution of 0.5 mM dATP (1 ul) was added to each tube and incubation continued for an additional 15 min at 42°C. The reaction was terminated by the addition of 10 ul formamide solution containing 98% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples (2 ul) were loaded on 8% polyacrylamide gel (33 x 39 cm) containing 8 M urea in 1.5 X TBE (75mM Tris, 50mM boric acid, 1.5mM EDTA) buffer and electrophoresed at a constant voltage of 2000V.

Preparation of a Radioactive DNA Probe for Hybridization Reaction (65)

The DNA was nick translated using DNAase I and E. coli DNA poly-

merase I. The reaction consisting of 90 uCi of α^{32}-P dATP (650 Ci/mmole), 0.4 mM each dCTP, dGTP, dTTP, 0.2 ug/ul DNA, 100 ug DNAse I, 11 units E. coli DNA polymerase I in a total volume of 20 ul was incubated at 12°C. Aliquots were removed at 60 min, 90 min, 120 min and added to 25 ul of 10 mM EDTA. A portion of the diluted samples was spotted on a PEI cellulose strip and developed in 1 N HCl and was cut in half. The top and bottom halves of each strip were counted in a scintillation counter and the percentage of incorporated radioactivity in the DNA was determined from the ratio of incorporated counts to the total counts. When the percentage of incorporation reached 50-60%, the reaction was terminated by adding 1 ul of 0.25 M EDTA and 2 ul of 10% SDS, followed by phenol extraction. An equal volume of 4 M NH_4OAc was added to the aqueous layer and the DNA was precipitated with 2 volumes of 95% EtOH at -70°C for 20 minutes. The mixture was brought to room temperature with gentle shaking and centrifuged at 12,000 g for 10 min. The pellet was redissolved in 10 ul of TE buffer and precipitated again by adding 10 ul of 4M NH_4OAc and 40 ul of 95% ethanol, at -70°C for 20 min. The DNA was collected and redissolved in 40 ul TE buffer and stored at 4°C until used. An aliquot was removed and denatured by heating at 100°C prior to hybridization.

Transfer and Hybridization of DNA from Agarose Gels (86)

DNA was first fractionated by electrophoresis on an agarose gel. The gel was then incubated in 0.25 M HCl for 5 min, 0.5 M NaOH, 1.5 M NaCl for 30 min (2 changes), and 1 M Tris-Cl (pH 7.5), 1.5 M NaCl for 30 min (2 changes). The gel was rinsed with water between each solution change. The DNA on the gel was transferred onto a nitrocellulose

filter through capillary motion by placing the filter on the gel and sandwiched it between Whatman 3MM paper in 10X SSC buffer (1.5 M NaCl, 0.15 M sodium citrate) as shown in Maniatis, T., et. al. (55) DNA transfer was usually completed in about 14-16 hrs. The nitrocellulose paper was removed from the gel and dried at 80°C for 2 hrs. The nitrocellulose filter containing the DNA to be hybridized was placed inside a heat-sealable plastic bag and prehybridized at 68°C for 1 hr in a 10 ml solution containing the following components: 4X SSC (0.6 M NaCl, 0.6 M sodium citrate), 0.2% Denhardt [1% each of BSA, polyvinyl pyrrolidone, Ficoll 400 (17)], 0.1% SDS, and 50 ug/ml sonicated salmon DNA. The prehybridization solution was discarded at the end of the incubation and replaced with 10 ml of the fresh solution mixed with ^{32}-P labeled denatured DNA probe. The filter was hybridized by incubation at 68°C overnight and washed 3 times in 250 ml 2X SET (0.3 M NaCl, 0.1 M Tris-Cl, 2 mM EDTA, pH 7.9) at 30 min intervals, 68°C. The filter was air dried and an autoradiogram was obtained by exposing to XAR-5 film.

RESULTS

Isolation and Identification of a DNA Fragment Containing tRNA Genes

A DNA fraction containing X. laevis tRNA genes was isolated from a CsCl/actinomycin D gradient and was digested with restriction endonuclease Eco RI. The DNA fragments were separated on a 1% agarose gel and the presence of tRNA genes were detected by Southern blot analysis using a labeled tRNA probe. DNA fragments of 1600-1800 base pairs long containing tRNA genes were exised and eluted from the gel and cloned into the Eco RI site of pBR 325, thereupon, disrupting a gene for chloramphenicol resistance. The recombinant DNAs were transformed into $CaCl_2$ treated HB-101 E. coli cells, and screened for colonies harboring plasmids with inserts (ampicillin^R, tetracyclin^R and chloramphenicol^S). The selected colonies were analyzed again for tRNA genes by Southern transfer using labeled tRNA. Positive clones were selected and the recombinant DNA fragments were purified in CsCl gradient.[1] To ensure that the selected DNA fragments contained active tRNA genes, in vitro transcription was employed using X. laevis ovary S-100 cell extract. Fig. 3 shows the products of transcription reactions using various DNA templates. One plasmid containing a DNA fragment identified as D37 (lane E) gave the most intense band for 4S RNA products, thereby making it a good candidate for a DNA fragment containing an active tRNA gene(s). Therefore, this clone was chosen for further studies. A diagram of the recombinant DNA pBR325 D37 is shown in Fig 4.

[1]The initial DNA isolation and cloning was done by Dr. Ronald C. Peterson and Stephen White prior to my joining the project.

Figure 3

Transcription of tRNA genes: Autoradiogram of (^{32}P) RNA products made by in vitro transcription in Xenopus S-100 extract using ptM_{10} (pBR 322 containing $tRNA^{met}$, lane 1) or various clones of pBR 325 containing unknown tRNA genes (lane 2,3,4,5,6) as template. pBR 325 D37 is shown on lane 5. All reaction mixtures contained 0.5 ug of DNA template, 0.5 mM each of ATP, CTP,UTP, 0.025 mM GTP, 20 uCi [^{32}P] GTP, 20 ul of Xenopus S-100 extract, 40 mM Tris-Cl (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, and 10% glycerol in a final volume of 50 ul. The reaction mixture was incubated at 30°C for 2 hrs. The RNA products were separated on 8% polyacrylamide gel in 1 x TBE buffer (50 mM Tris, 50 mM boric acid, 1.0 mM EDTA at pH 8.3), 7 M urea, electrophoresed at constant voltage (2000 V) and exposed to XAR-5 film for 7 hrs with Lighting Plus screen at -70°C.

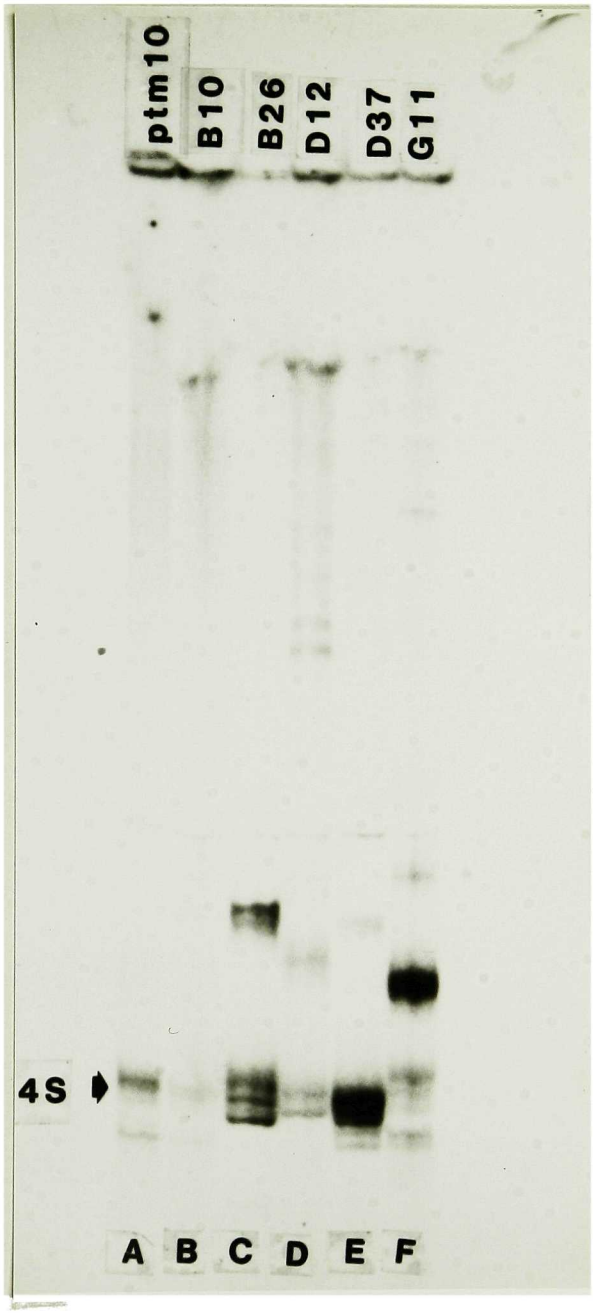
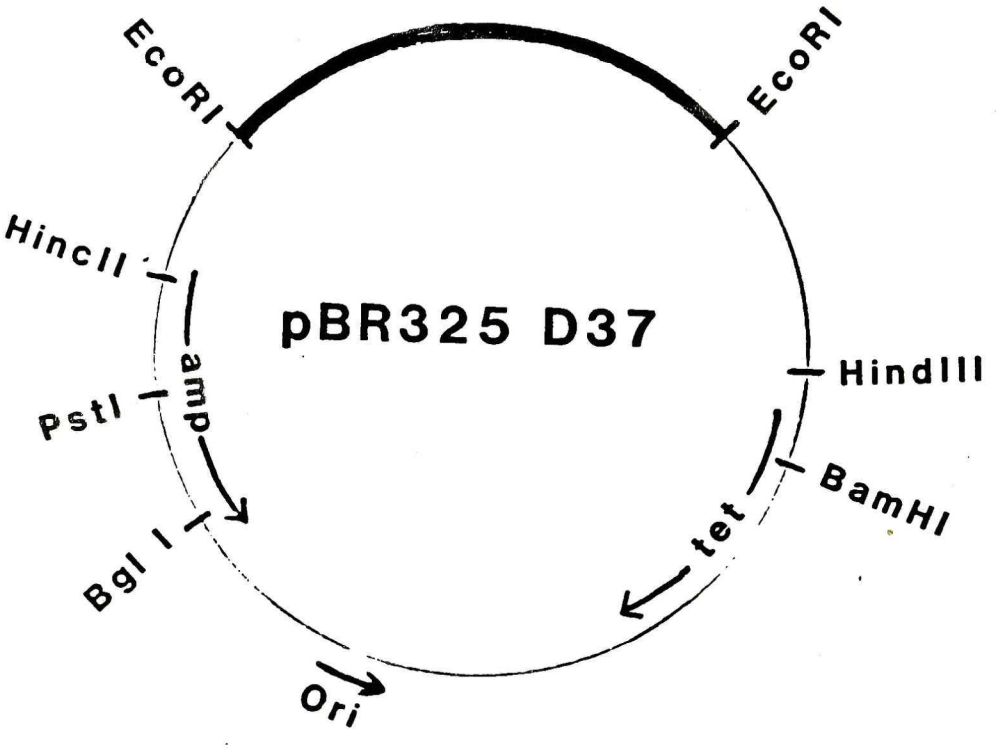

ptm10
B10
B26
D12
D37
G11
4S
A
B
C
D
E
F

Figure 4

pBR 325 D37: Plasmid pBR 325 that contains a DNA fragment identified as D37 at its Eco RI site. pBR 325 is a derivative of pBR 322. It has a single restriction endonuclease site for Eco RI, Hind III, Bam HI, Pst I and also carries resistance to three antibiotics: ampicillin, tetracycline and chloramphenicol. It is 5995 bp long corresponding to a molecular weight of 3.7×10^6 dalton.


EcoRI
EcoRI
HincII
pBR325 D37
HindIII
PstI
amp
BamHI
tet
Bgl I
Ori

Subcloning D37 into pUC 9

We wished to sequence this DNA fragment by constructing a set of deletions of the insert D37 using an appropriate vector. pUC8 or pUC9 (94, Fig. 5) were chosen because they contain a cluster of single restriction enzyme sites as well as a DNA sequence complementary to a 15-base pair primer that is adjacent to these restriction enzyme sites. Therefore, a DNA inserted into these plasmids at these sites can be sequenced by the dideoxy chain termination method. The strategy taken for subcloning D37 from pBR 325 into pUC 9 is shown in Fig. 6.

Isolation of a particular DNA fragment was not necessary in this recloning procedure because digestion of pBR325 D37 with both Eco RI and Hind III resulted in three DNA fragments, from which only the desired DNA fragment (D37) contained Eco RI termini that were complementary to that of pUC 9. Removal of the 5' terminal phosphate from pUC 9 after Eco RI digestion, decreased the probability for the plasmids to self anneal or ligate to each other. Since the genetic markers on pBR 325 were different from pUC 9, E. coli cells transformed by these plasmids could be selected on media containing appropriate antibiotics. The possible ligations were shown in Fig. 6; pUC 9 carries resistance only to ampicillin and insertion at its Eco RI site would inactivate the ß -lactamase gene. Therefore, in the presence of X-gal and IPTG, E. coli JM 83 (lac Z M 15) cells transformed with pUC 9 plasmids contain insert(s) would appear as white colonies. Transformants of fragment 2 ligated together would not contain resistance to antibiotics and would not be able to grow in the presence of ampicillin or tetracyclin. Fragment 2 ligated to fragment 3, however, would result as pBR 325 and

Figure 5

pUC 8 and pUC 9: Plasmids pUC 8 and pUC 9 contain the Pvu II/Eco RI fragment from pBR 322 carrying resistance to ampicillin and the Hae II fragment from M13 mp8 (in pUC 8) and M13 mp9 (in pUC 9) which consist of a cluster of 9 single restriction enzyme sites in the lac Z gene. The pUC 8 and pUC 9 plasmids are 2717 bp long.

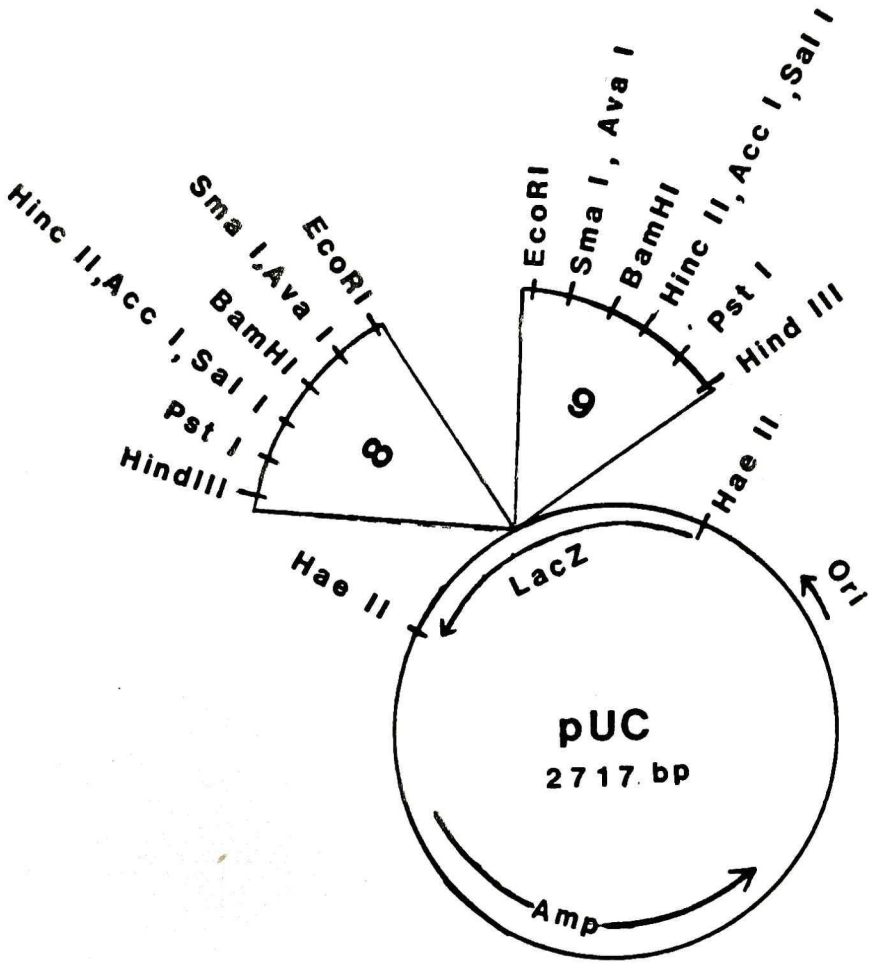
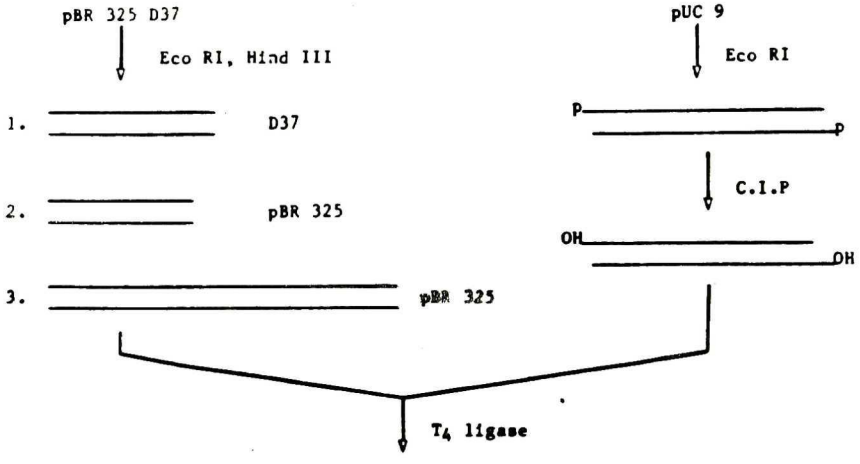

EcoRI
Sma I, Ava I
BamHI
Hinc II, Acc I, Sal I
Pst I
Hind III
Hae II
Ori
LacZ
Hae II
Hindlll
Pst I
BamHI
Sma I, Ava I
EcoRI
Hinc II, Acc I, Sal I
pUC
2717 bp
Amp

Figure 6

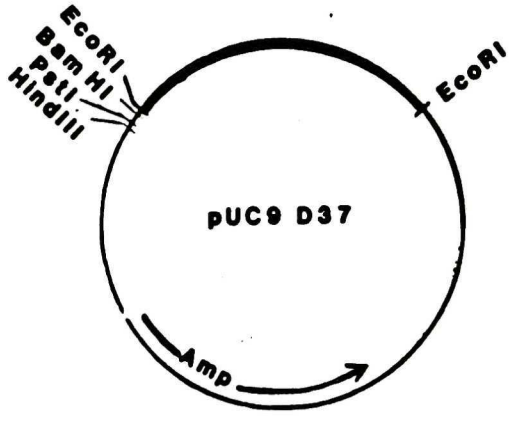
Strategy for subcloning D37 into pUC 9: pBR 325 D37: pBR 325 containing DNA fragment, D37, at its Eco RI site. C.I.P: Calf intestinal alkaline phosphase. pUC9 D37, pUC 9 plasmid containing DNA fragment identified as D37. The heavy line represents the DNA insert at the Eco RI site.



Possible ligations: fragment 1 and pUC 9 at Eco RI site to give pUC 9 D37
fragment 2 and fragment 2
fragment 3 and fragment 2
fragment 3 and fragment 3
fragment 1 and fragment 1
multiple ligations

↓ transform into JM-83 ($CaCl_2$ treated)

select for amp^R, lac^-. tet^S colonies



yield colonies that were resistant to both ampicillin and tetracyclin. Since there was a correlation between the size of the plasmids and the efficiency of transformation, DNA fragment 3 ligated to one another would probably be too large for efficient transformation.

Among 281 colonies obtained from plates containing ampicillin, X-gal and IPTG, 273 colonies were white and 8 were blue. When the white colonies were tested for resistance to both ampicillin and tetracycline, 207 colonies showed resistance only to ampicillin. This indicated that only 3% of the colonies analyzed were pUC9 that had self annealed and 76% of the colonies were pUC9 containing an insert. Twelve colonies (amp^R, lac^-,tet^S) were randomly selected and used in boiling lysate preparation. The DNAs were cut with EcoRI and analyzed on 1% agarose gel. Lanes c to n in Fig. 7 show that all the plasmids analyzed were pUC9 containing D37 insert.

Determination of the orientation of D37 in pUC9

Since D37 could be inserted in two orientations in the pUC9, it was also necessary to determine the orientation of the inserts. This was done by digesting pUC9 plasmids containing the inserts with Hae III, and analyzing the DNA fragments. Lanes b and c in Fig. 8a, show the two patterns of fragments of D37 identified as orientation I (D37 I) and orientation II (D37 II). Figure 8b shows the sizes of the Hae III DNA fragments of D37 I and II obtained after sequencing the complete D37 fragment later in the process. We wish to point out that the Hae III fragments containing D37 I and D37 II from the boiling minilysate analysis (Fig. 8a) were in agreement with those determined after the complete

Figure 7

In search for pUC9 D37 recombinant DNA: DNA was prepared using rapid boiling method as described under Materials and Methods. Approximately 1 ug of crude DNA was digested with Eco RI, separated on 1% agarose gel and stained with lug/ml ethidium bromide. Lane a, pUC 9; lane b and o, pBR 325 D37 cut with Eco RI; lane b to n, DNA sample 1 to 12 isolated from boiling minilysate preparations.

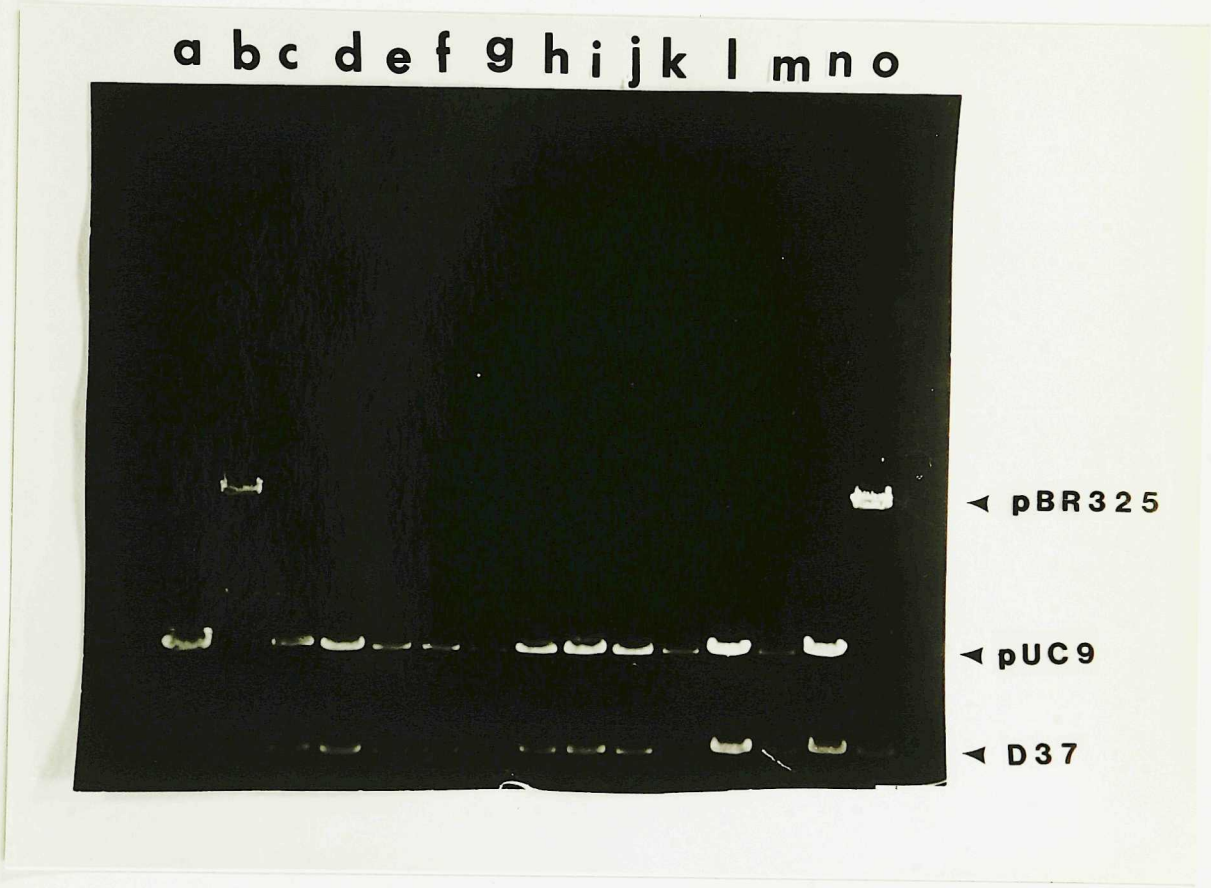
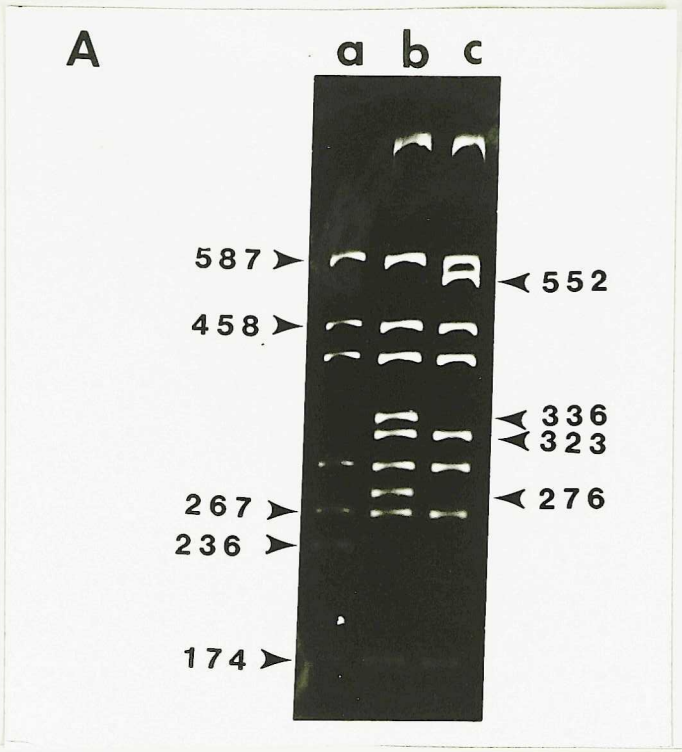
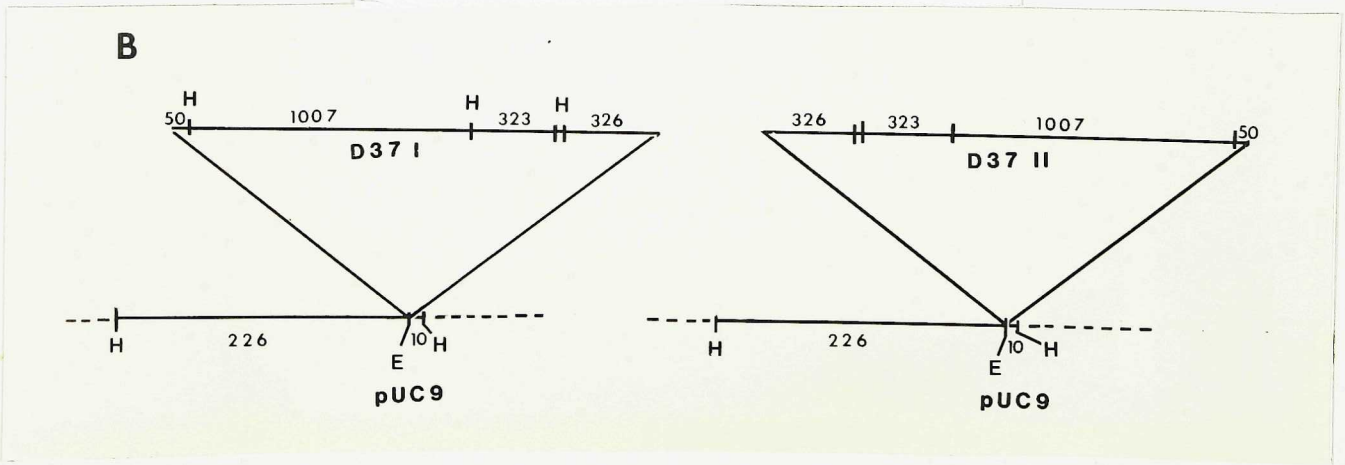

a b c d e f g h i j k l m n o
pBR325
pUC9
D37

Figure 8

Determination of the orientation of the insert: A) pUC9 D37 digested with restriction endonuclease Hae III and analyzed on 5% polyacrylamide gel in 1X E buffer. DNA bands were visualized by ethidium bromide. Lane a, pUC9 digested with Hae III; lane b, Hae III digestion pattern of pUC9 D37 dentified as orientation I (D37 I); lane c, Hae III digestion pattern of pUC9 D37 identified as orientation II (D37II). B) Diagrams showing D37 I and D37 II inserts in the Eco RI site of pUC 9. H, Hae III; E, Eco RI. The numbers correspond to the length of the DNA fragments determined from their DNA sequence.


A
a b c
587
458
267
236
174
552
336
323
276


B
H
50
1007
H
323
H
326
D37 I
H
226
E
10
H
pUC9
326
323
1007
50
D37 II
H
226
E
10
H
pUC9

sequence was known (Fig. 8b) Two DNA bands corresponding to the internal fragments, 1007 bp and 323 bp, of D37 can be seen in both orientations. The 236 bp Hae III fragment of pUC 9 containing the Eco RI site was split in two upon cloning D37 into this site. Therefore, two DNA bands corresponding to 336 bp and 276 bp in orientation I and one DNA band corresponding to 552 bp in orientation II can also be seen in Fig 8a. The 60 base-pair DNA fragment predicted from orientation II was too small to be detected on this gel.

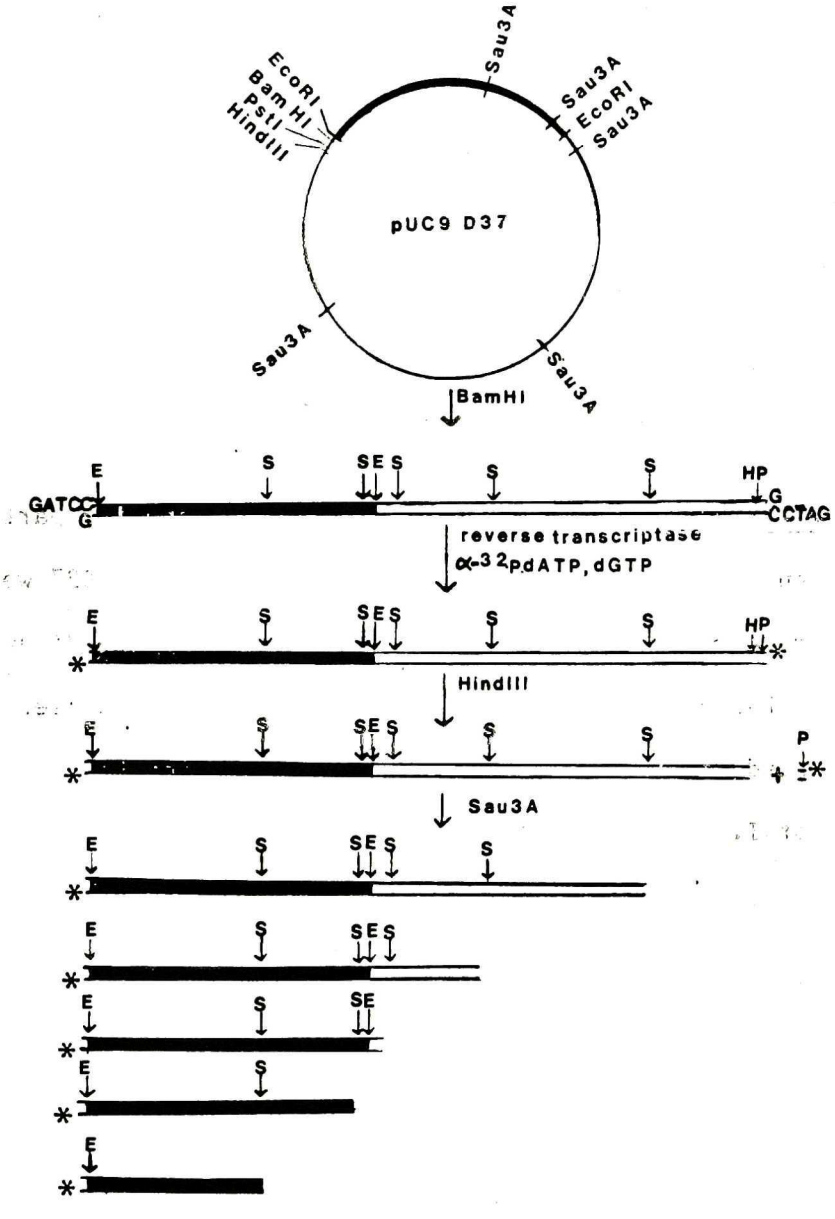
Restriction Enzymes Map of D37

D37 was further analyzed for restriction enzymes sites. Restriction enzyme sites were often very useful in the reorganization of a large DNA fragment after shotgun cloning and sequencing. They were also useful if one wished to selectively sequence a section of a DNA fragment. The restriction enzyme sites for Alu I (5'-AG'CT3'), Ava II (5'-G'G$\frac{A}{T}$CC-3'), Cfo I (5'-GCG'C-3'), Hae III (5'-GG'CC-3'), Hinf I (5'-G'ANTC-3'), Hpa II (5'-C'CGG-3'), Sau 3A (5'-'GATC-3'), and Taq I (5'-T'CGA-3') were determined. These restriction enzymes sites, with the exception of Taq I, are commonly found in DNA sequences. Taq I sites, however, are commonly found in tRNA genes in that Taq I is specific for sequence 5'-TCGA-3' which is often found in a highly conserved region of the ψ loop in many tRNA genes. However, some tRNA genes contain 5'-TCAA-3' in the ψ loop instead of the above sequence. Therefore, the number of Taq I sites may represent the number of tRNA genes in D37.

The strategy for restriction enzyme site mapping by partial digestion is shown in Fig. 9. The Sau 3A sites in the DNA fragment were

Figure 9

Strategy for restriction enzyme mapping: An example showing partial digestion with restriction enzyme, Sau 3A. The Sau 3A sites on pUC9 D37 were arbitrary assigned. Using a limited amount of restriction enzyme Sau 3A and removing aliquots of the DNA sample at different time intervals, a series of partially digested DNA fragments were collected. E, Eco RI; S, Sau 3A; H, Hind III; P, Pst I. * termini labeled with α -[^{32}P] ATP.


EcoRI
Bam HI
PstI
HindIII
Sau3A
Sau3A
EcoRI
Sau3A
pUC9 D37
Sau3A
Sau3A
BamHI
E
S
SE S
S
S
HP
G
GATCC
G
CCTAG
reverse transcriptase
α-32pdATP, dGTP
HindIII
P
Sau3A

arbitrary assigned for explanatory purpose. D37 was first linearized with Bam HI, then labeled with [^{32}P] dATP using reverse transcriptase. This was followed by Hind III digestion resulting in a large Bam HI/Hind III DNA fragment labeled at one end and containing most of the plasmid DNA as well as the complete insert. The small labeled Bam HI/Hind III DNA fragment (20 bp) could not be detected when the partial digested DNA was fractionated on a 2% agarose gel and therefore did not interfere with the analysis. The large Bam HI/Hind III fragment was then partially digested with each of the restriction enzyme as described under Materials and Methods. An example using Sau 3A partial digestion is shown in Fig 9. By using a limited amount of restriction enzyme Sau 3A and removing aliquots from the reaction mix at different times, a series of partially digested DNA fragments was obtained.

An autoradiogram of the partial digestion for D37 I, analyzed on 2% agarose gel is shown on Fig. 10. A similar autoradiogram was also obtained for D37 II (data not shown). The difference in size between the bands represents the distance between successive restriction enzyme sites. Since D37 was cloned into the Eco RI site of pUC 9, partial digestion with Eco RI should produce the largest DNA fragment containing the complete insert. Restriction enzyme sites within the D37 insert should result in DNA fragments which are smaller than the fragment produced by Eco RI digestion, while restriction enzyme sites in pUC 9 should result in DNA fragments that are larger than this fragment. This is also illustrated in Figure 9 or 10.

A restriction enzyme map of D37 I, deduced from the the partial digestions from both orientations, is shown in Fig 11a. In comparison, a restriction enzyme map of D37 I deduced from the complete DNA sequence of

Figure 10

Partial restriction enzymes digestion of pUC 9 D37I: The partial restriction enzyme digestion was carried out as described under Materials and Methods. Lane a and o, λDNA cut with Hind III; lane b and p, ϕX 174 DNA cut with Hpa II; lane c and q, ϕX 174 DNA cut with Hae III; lane d and r, pBR 322 DNA cut with Hinf I; lane f to n, pUC 9 D37I cut with Eco RI, Alu I, Ava II, Cfo I, Hae III, Hinf I, Hpa II, Sau 3A, and Taq I respectively. The sizes of DNA fragments are in base-pairs.

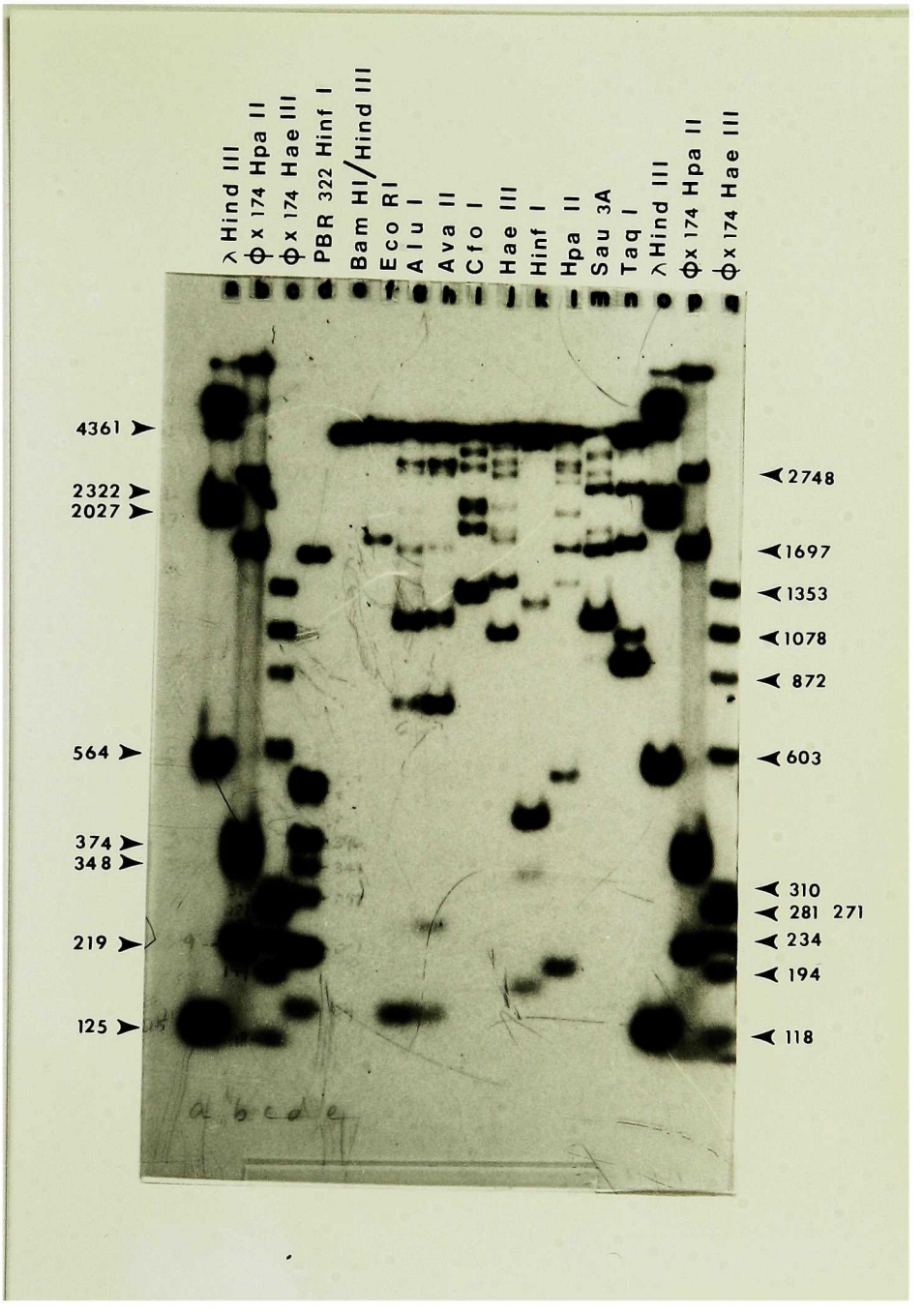
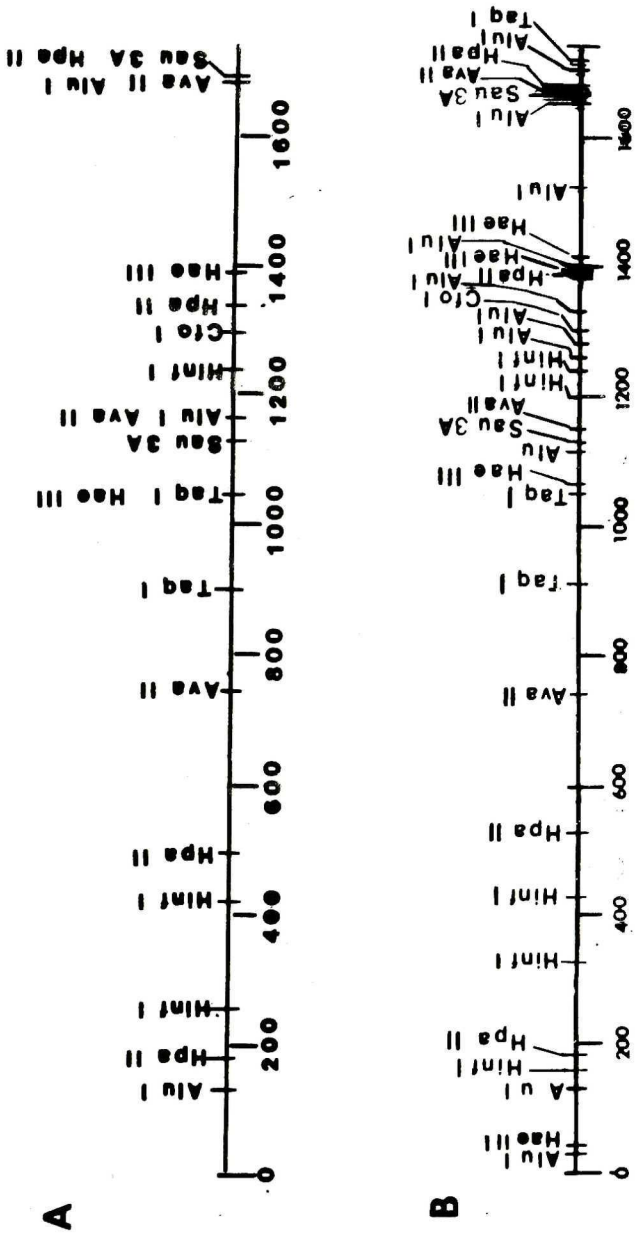

λ Hind III
φx 174 Hpa II
φx 174 Hae III
PBR 322 Hinf I
Bam HI/Hind III
Eco RI
Alu I
Ava II
Cfo I
Hae III
Hinf I
Hpa II
Sau 3A
Taq I
λ Hind III
φx 174 Hpa II
φx 174 Hae III
4361
2322
2027
564
374
348
219
125
2748
1697
1353
1078
872
603
310
281 271
234
194
118
a b c d e

Figure 11

a) Restriction enzyme map of D37 I determined by partial restriction enzyme digestion. Partial restriction enzyme digestion was carried out as described under Materials and Methods. The map for D37 I was determined from measuring the restriction enzyme fragments from partial digestions of both D37 I and D37 II.

b) Restriction enzyme map of D37 I determined from the complete DNA sequence of D37 (Fig. 20) deduced from dideoxysequencing.


A
Alu I
Hpa II
Hinf I
Hinf I
Hinf I
Ava II
Taq I
Taq I Hae III
Sau 3A
Alu I Ava II
Cfo I
Hpa II
Hae III
Ava II Alu I Sau 3A Hpa II
0
200
400
600
800
1000
1200
1400
1600
B
Alu I
Hae III
Alu I
Hpa II
Hinf I
Hinf I
Taq I
Ava II
Taq I
Hae III
Alu
Sau 3A
Ava II
Hinf I
Hinf I
Alu I
Alu I
Cfo I
Hpa II
Alu I
Hae III
Hae III
Alu I
Alu I
Sau 3A
Ava II
Hpa II
Alu I
Taq I
0
200
400
600
800
1000
1200
1400
1600

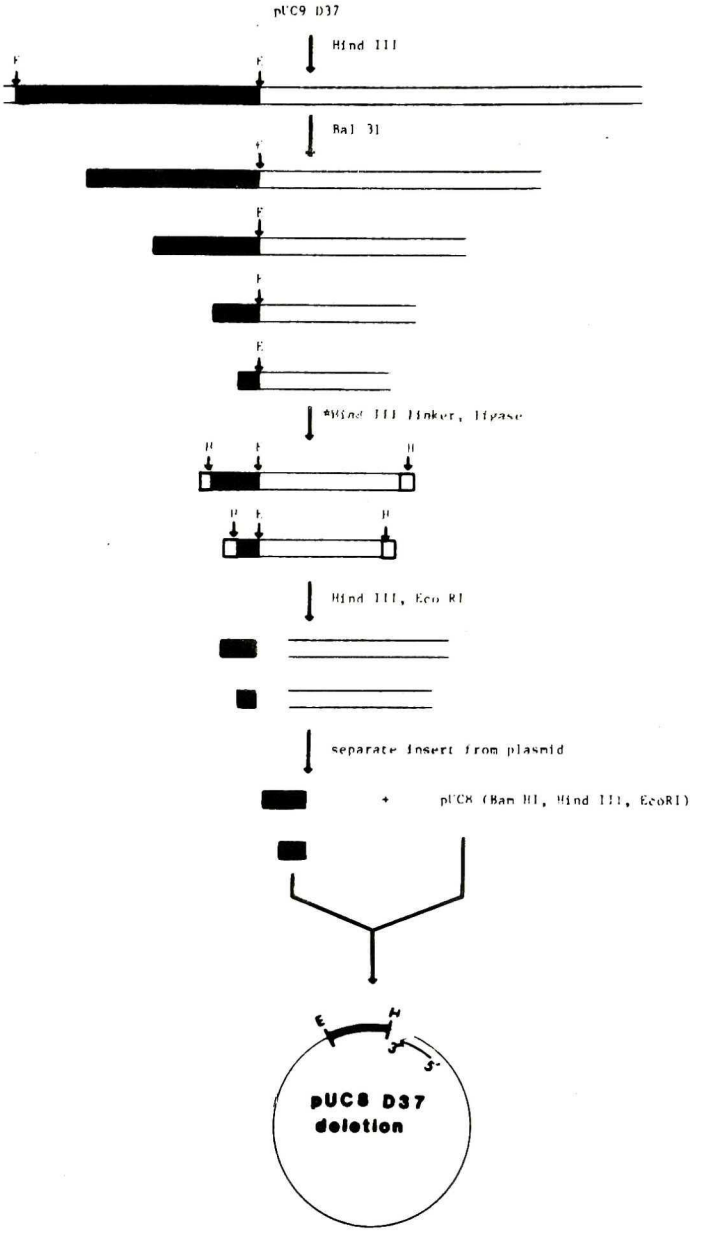
D37 fragment (Fig. 20) is shown in Fig. 10b. As one can observe, restriction enzyme digestion resulting in DNA fragments less than 100 bp could not be determined accurately by the partial digestion method. For example, restriction enzyme sites that were very close to the insertion sites, Alu I and Hae III at the 5' terminal and Alu I and Taq I at the 3' terminal of D37 were not detected. However, the number of sites and their approximate location in the DNA fragment could be determined when the restriction enzyme digested fragments were large. For example, the number of Cfo I, Sau 3A and Hpa II sites and their approximate positions could be determined accurately. The order of restriction enzyme sites in the DNA fragment could be determined if the sites were not very close to each other. For instance, the order of most restriction enzyme determined by partial digestion were in agreement with those determined by DNA sequencing. However, neither the order of Alu I, Ava II, Hpa II, and Sau 3A sites near the 3' end nor the order of some sites within the DNA fragment could be resolved because the fragments produced were very nearly the same size. Therefore, a restriction enzyme map of D37 I deduced from partial digestion with various enzymes was limited to resolving only the general order and the approximate positions of some but not all restriction sites. The exact order and position of each restriction enzyme site could only be determined by sequencing the the entire DNA fragment.

Construction of deletions in D37 I and D37 II

Our strategy was to sequence D37 in both directions by creating a set of deletions in each orientation followed by dideoxysequencing (Fig. 12). Nuclease Bal 31 was used to obtain such deletions. Bal 31 can degrade a

Figure 12

Strategy involved in creating a set of D37 deletions and recloning into EcoRI/Hind III site of pUC8. E, Eco RI; H, Hind III; *Hind III linker, kinase labeled Hind III linker.


pUC9 D37
Hind III
Bal 31
*Hind III linker, ligase
Hind III, Eco RI
separate insert from plasmid
pUC8 (Bam HI, Hind III, EcoRI)
pUC8 D37
deletion

linear double stranded DNA from both the 3' and the 5'termini in a progressive manner and at approximately the same rate. Figure 13 shows the Bal 31 digestion of pUC9 D37 after linearizing with Hind III. The rate of Bal 31 digestion was determined from agarose gel analysis as follows: pUC9 D37 was estimated to be approximately 4400 bp long and pUC9 was known to be 2717 bp long from its DNA sequence. Figure 13 shows that at 10 min approximately 1700 bp had been digested away by the nuclease. The rate of digestion was calculated to be approximately 170 bp/min and therefore D37 was probably completely deleted from pUC9 in approximately 20 minutes.

The deletions were ligated to kinased labeled Hind III linkers to create new Hind III sites for recloning the DNA into pUC8. D37 deletions were separated from their plasmid DNA by cutting with Eco RI and Hind III followed by electrophoresis on 1.2% low-melting agarose gel (Fig. 14). The DNA fragments were eluted from the gel and recloned into the Eco RI/Hind III site of pUC 8 (cut with Bam HI, Eco RI and Hind III) and transformed into E. coli DH-1 cells. pUC 8 was cut with Bam HI, Eco RI and Hind III to decreased the chances for the EcoRI/Bam HI and Bam HI/Hind III fragments to reintegrate between Eco RI/Hind III sites of pUC 8. The plasmid was also purified on 1% agarose gel and recovered by electroelution prior to use. pUC 8 containing various D37 I and II deletions are shown on Fig. 15 and and Fig. 16 respectively. These plasmids were used for DNA sequence analysis.

DNA Sequence Analysis

A region adjacent to the Hind III site of pUC 8 or Eco RI site of pUC 9 contains DNA sequences that are complementary to a 15-base primer (5'-AGTCACGACGTTGTA-3'). Dideoxysequencing requires a single-stranded

Figure 13

Bal 31 digestion of pUC9 D37: Progressive digestions of pUC9 D37 with Bal 31 were analyzed on 1% agarose gel and stained with ethidium bromide. Approximately 35 ug of purified pUC9 D37 was linearized by Hind III and digested with Bal 31 as described under Experimental Procedure.

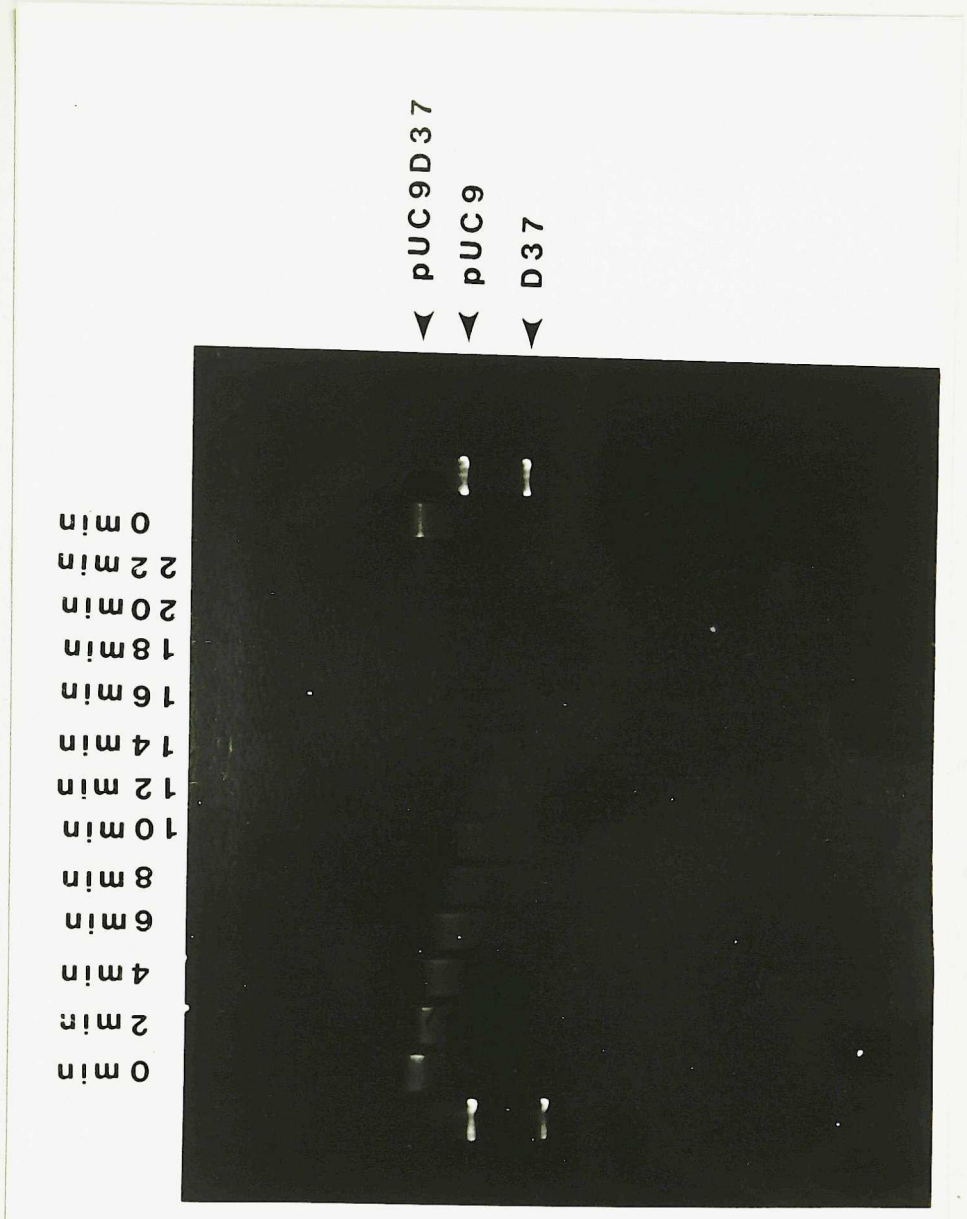

pUC9D37
pUC9
D37
0 min
2 min
4 min
6 min
8 min
10 min
12 min
14 min
16 min
18 min
20 min
22 min
0 min

Figure 14

Isolation of D37 deletions. Deleted D37 DNA was separated from plasmid DNA on 1.2% low-melting agarose gel. Lane A, pUC9 D37 digested with EcoRI; lane B,C and D, EcoRI/Hind III deletion fragments of pUC9 and D37; lane E, DNA fragments of ϕX 174 digested with Hae III.

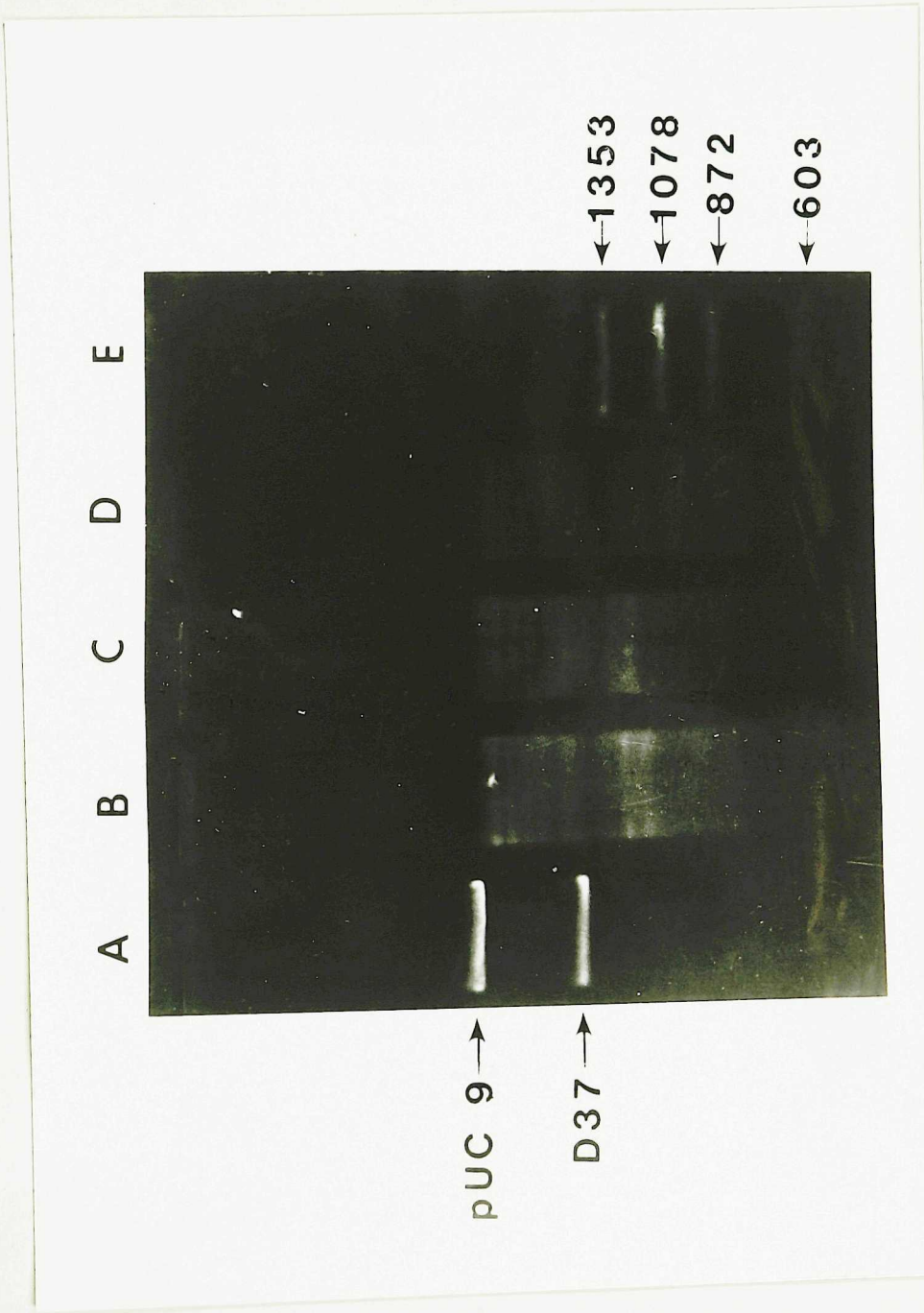

A
B
C
D
E
1353
1078
872
603
pUC 9
D37

Figure 15

Plasmids containing various D37 I deletions: The plasmids containing D37 I deletions, purified in CsCl gradients. were analyzed on 1.5% agarose gel and stained with ethidium bromide. Lane a and o, ϕX 174 cut with Hpa II; lane b and p, ϕX 174 cut with Hae III; lane c, pUC 9 D37I; lane d to n, pUC 8 containing D37 I deletions corresponding to those on lane b to 1 in Figure 19.

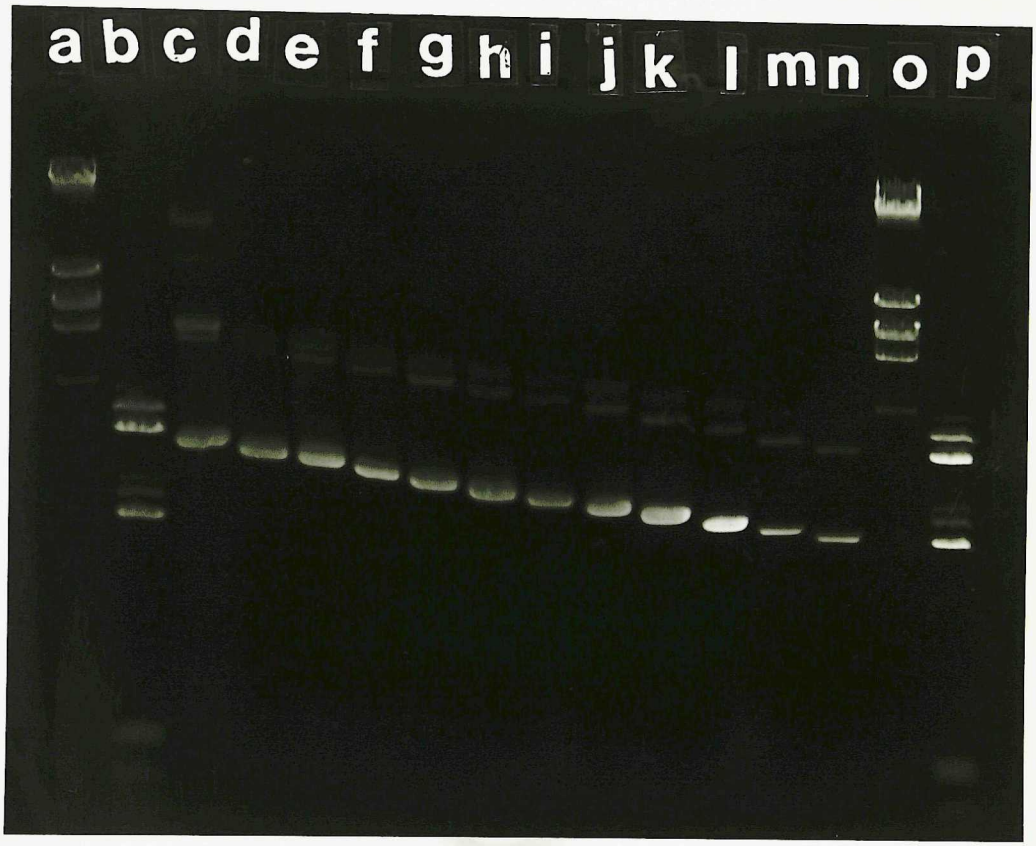
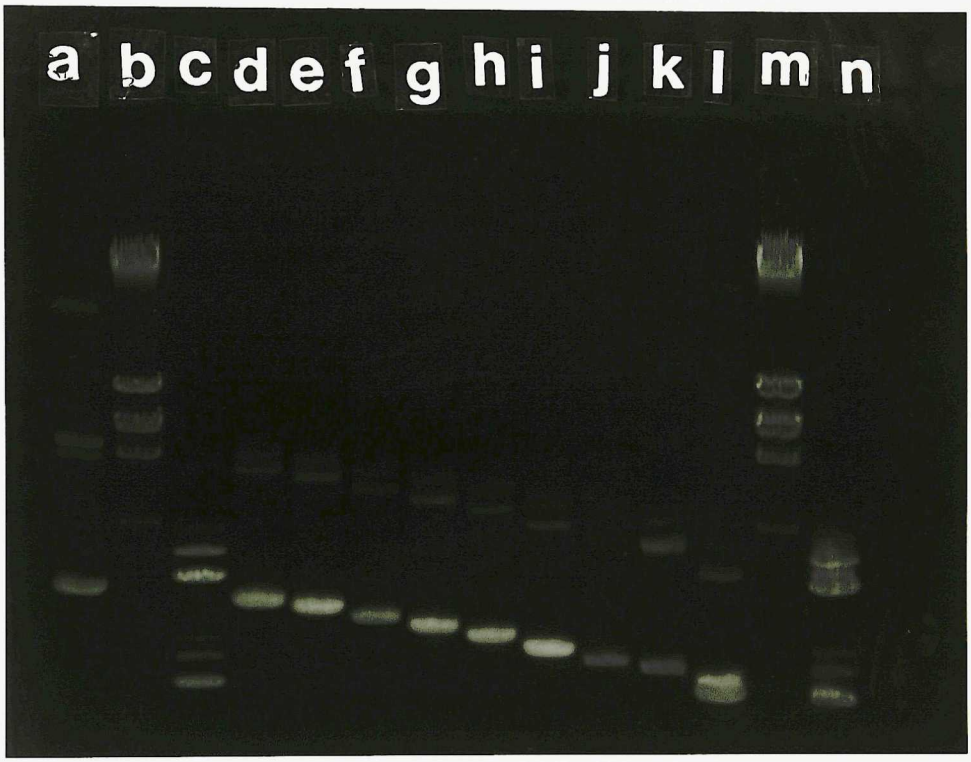

a b c d e f g h i j k l m n o p

Figure 16

Plasmids containing various D37 II deletions: The plasmids containing D37 II deletions, purified in CsCl gradient, were analyzed on 1.5% agarose gel and visualized with ethidium bromide. Lane a, pUC9 D37 II; lane b and m, ϕX 174 cut with Hpa II; lane c and n, ϕX 174 cut with Hae III; lane d to l, pUC 8 containing D37 II deletions corresponding to those on lane m to u in Figure 19.


a b c d e f g h i j k l m n

DNA as a template; therefore, it is necessary to create a single-stranded region in the plasmid if the technique is to be used. For inserts in pUC8, DNA was linearize with Eco RI, digested with Exo III and sequenced by dideoxy chain termination method. For inserts in pUC 9, the DNA was cut with Hind III followed by Exo III digestion and dideoxysequencing as described under Material and Methods. Exonuclease III removs 5' mononucleotides from a double-stranded DNA (3'->5') carrying a 3'-OH end. The strategy for sequencing DNA in pUC8 and pUC9 is shown in Fig. 17. α-[^{32}P] dATP was used to label the newly synthesized DNA fragment and dideoxynucleotides were used as chain terminators. The procedure for dideoxysequencing is described under Materials and Methods.

Due to the tendency of single stranded DNA which can form secondary structure, especially those contain tRNA genes, incorporation of nucleotides using DNA polymerase I ("Klenow fragment") at 30°C as suggested by Sanger et. al., was not very effective in sequencing these genes. We modified the temperature by incubating the reaction mix at 30°C and then shifting to 42°C as described under Materials and Methods. Figure 18 shows the effectiveness of this modification. One can see that when the entire sequencing reaction was carried out at 30°C, DNA polymerase I was not able to read through the secondary structures (indicated with arrows). However, when the temperature was shifted to 42°C after initial incubation at 30°C, the DNA polymerase I was able to read through these secondary structures.

Fig. 19 shows the sizes of D37 and some of the deletion mutants used for sequencing. The DNA inserts correspond to those contained in plasmid pUC8 shown in Fig. 15 and 16. The complete DNA sequence of D37,

Figure 17

Strategy for using pUC plasmids in dideoxysequencing: ++++, sequence complementary to 15-base primer; * radioactivity incorporated in the DNA

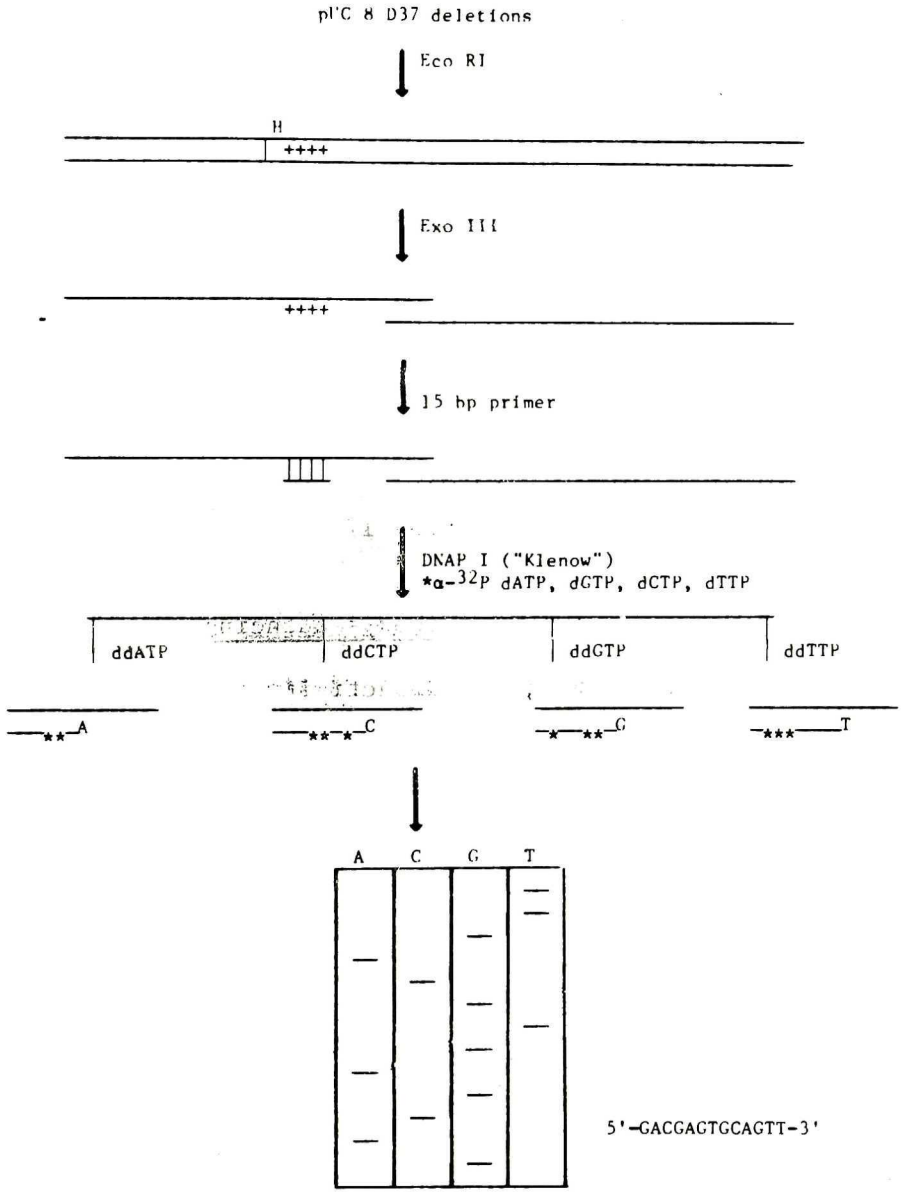

pUC 8 D37 deletions
Eco RI
H
++++
Exo III
++++
15 bp primer
DNAP I ("Klenow")
*α-32P dATP, dGTP, dCTP, dTTP
ddATP
ddCTP
ddGTP
ddTTP
A
C
G
T
5'-GACGAGTGCAGTT-3'

Figure 18

An autoradiogram comparing the sequence of DNA fragment, D6, obtained from dideoxysequencing reaction carried out at 30°C and 42°C: DNA sequencing using a modified Sanger dideoxysequencing method was described in detailed under Materials and Methods. Arrows show DNA regions that contained secondary structures. The DNA was analyzed by three loadings on an 8% polyacrylamide gel in 1.5 X TBE, 8 M urea, which was electrophoresed at 2000 V and exposed to XAR-5 film at -70°C.

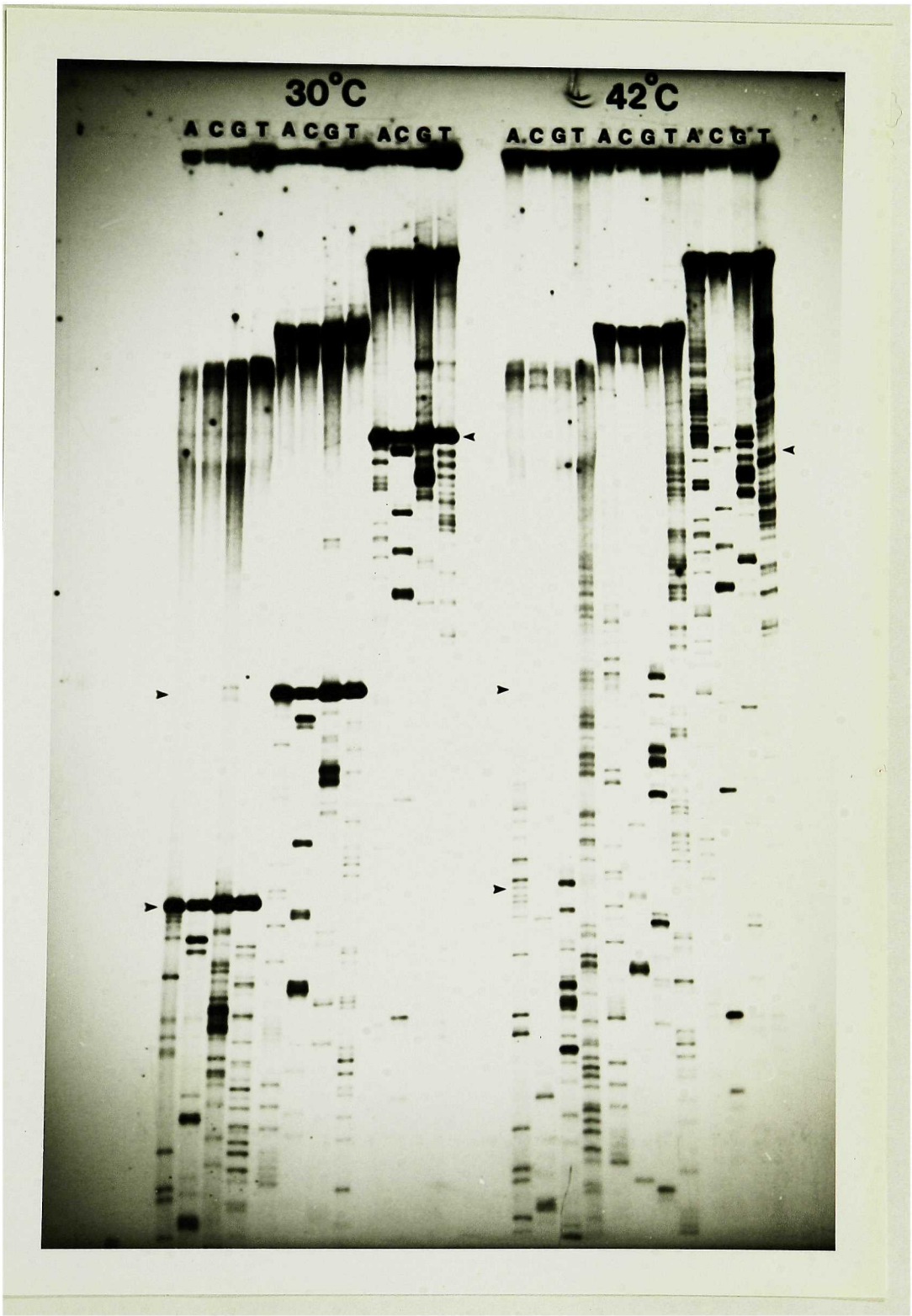
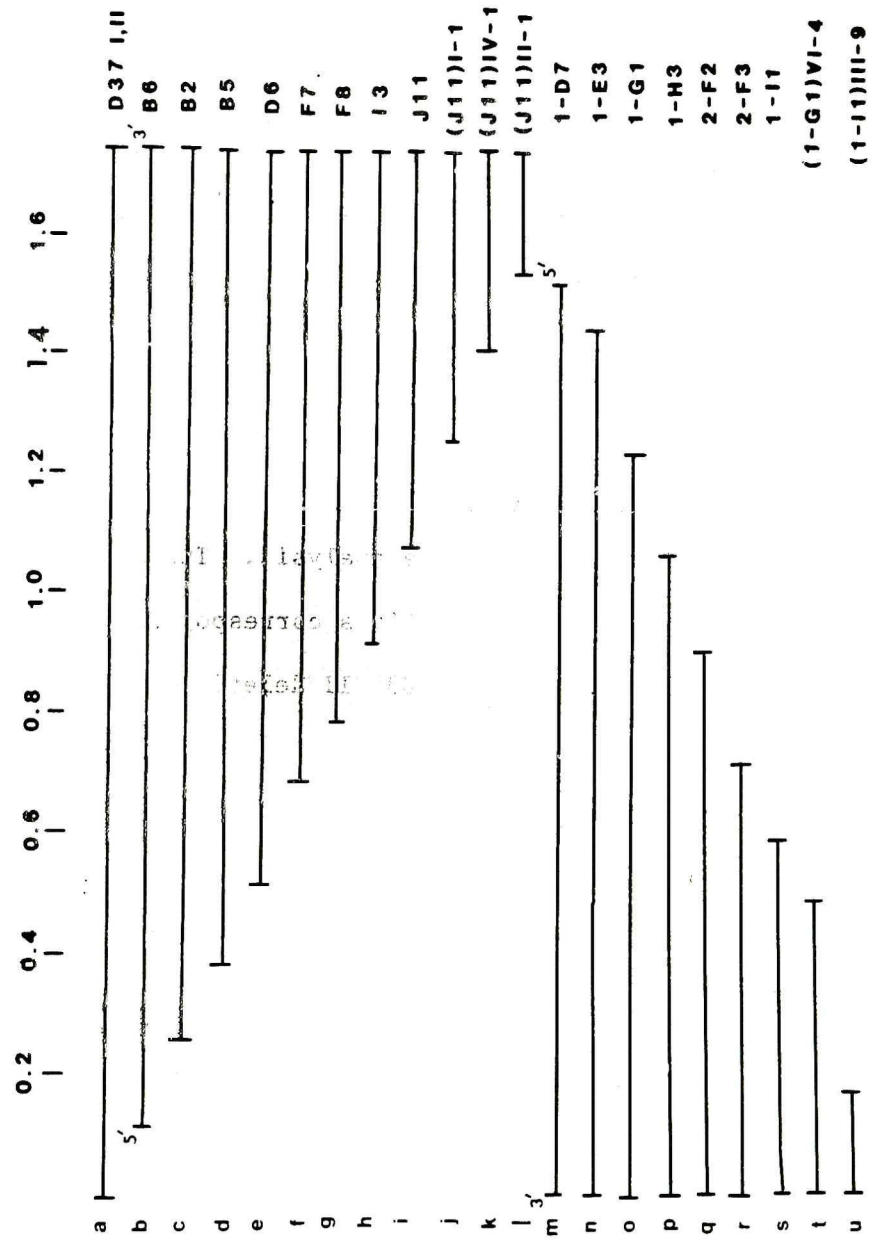

30°C
A C G T A C G T A C G T
42°C
A C G T A C G T A C G T

Figure 19

Line graph representing D37 and its deletions: The size of the DNA fragments was determined from DNA sequence analysis. The distance is shown in kilobases. Lane b to l, D37I deletions corresponding to those on lane d to n in Fig. 15; lane m to u, D37 II deletions corresponding to those on lane d to l in Figure 16.


0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
D37 I,II
B6
B2
B5
D6
F7
F8
I 3
J11
(J11)I-1
(J11)IV-1
(J11)III-1
1-D7
1-E3
1-G1
1-H3
2-F2
2-F3
1-I1
(1-G1)VI-4
(1-I1)III-9
5'
3'

1737 bp long, is shown in Fig. 20. A computer search for tRNA genes was done using known tRNA sequences in comparison to D37 DNA sequence. Only two regions in the entire D37 DNA sequence showed some homology to various known tRNA sequences. These two regions shown in Fig. 20, could be folded into tRNA "cloverleaf" structure using criterion established from other known tRNA genes. The anticodon (5'-GCA-3') deduced from these secondary structure suggested that the two tRNA genes code for cysteine tRNAs. These tRNA genes are transcribed in the same direction but are separated by a spacer DNA of 452 base pairs. They do not contain intervening sequences. No tRNA pseudogenes were detected in this DNA fragment by using the same secondary structure analysis.

The two tRNA genes on D37 were named $tRNA^{cys}_1$ and $tRNA^{cys}_2$ genes with respect to their order on this DNA fragment. Autoradiograms from dideoxysequencing showing a DNA fragment containing the mature $tRNA^{cys}_1$ coding sequence and its complementary sequences are shown in Fig. 21 and 22. Its proposed secondary structure is shown in Fig. 23. Likewise, the mature $tRNA^{cys}_2$ coding sequence and its complementary sequence are shown in Fig. 24 and 25; its proposed secondary structure shown in Fig 26. The two tRNA genes are 95.8% in homology resulting from only three base differences. These differences are indicated by the arrows in Fig. 23 and 26. Interestingly, little or no homology was observed in the DNA sequences immediately before or after the mature tRNA coding sequences.

The mature $tRNA^{cys}_1$ coding sequence was compared to other known tRNA gene sequences. It showed a 67% homology with $tRNA^{cys}$ gene of yeast, 65% homology with $tRNA^{phe}_{GAA}$ genes of X. laevis and D. melanogaster, 55% homology with $tRNA^{lys}_3$ gene of rabbit, and 30% homology with $tRNA^{pro}_{CGG}$ genes

Figure 20

Complete DNA sequence of D37 from both orientations: The complete D37 DNA sequence was deduced from sequencing the DNA fragments shown in Fig. 19. Vertical arrows show the Eco RI sites of the DNA fragment; highlighted regions show the DNA sequences of two mature tRNA genes; horizontal arrows show the polarity of the tRNA genes; the heavy bars show the anticodon of the tRNA genes.

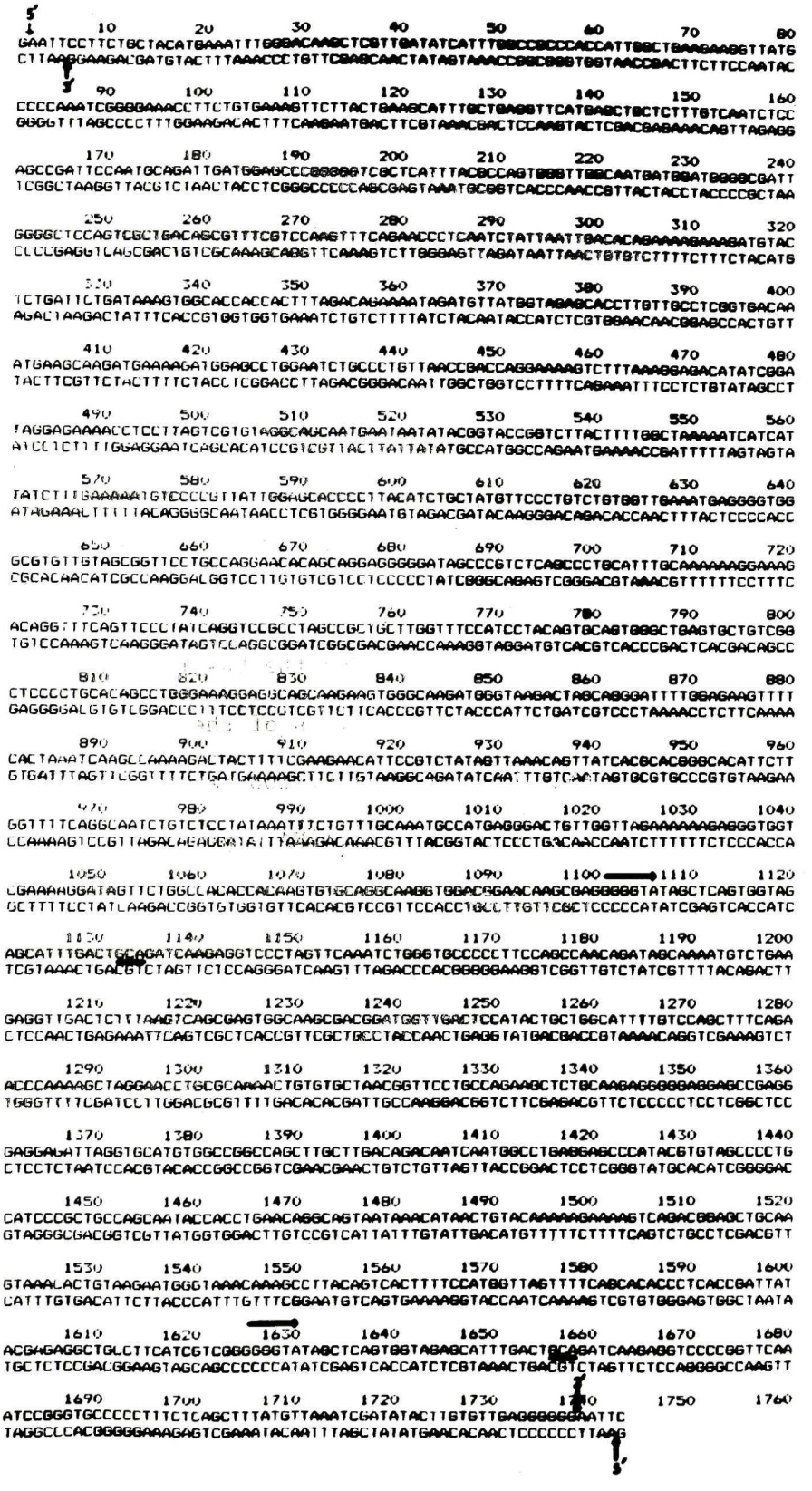


Figure 21

Autoradiogram of a dideoxysequencing gel showing a DNA fragment containing $tRNA^{cys}_1$ gene. The 5' end of this DNA sequence is at the bottom of the gel.


T C C C C G T G G T C T A A A C T T G A T C C C T G G A G A A C T A G A C G T C A G T T T A C G A G A T G G T G A C T C G A T A T G G G G G

Figure 22

Autoradiogram of a dideoxysequencing gel showing a DNA fragment containing the DNA sequence complementary to $tRNA^{cys}_{1}$. The bottom of the gel is the 5' end of this complementary sequence.

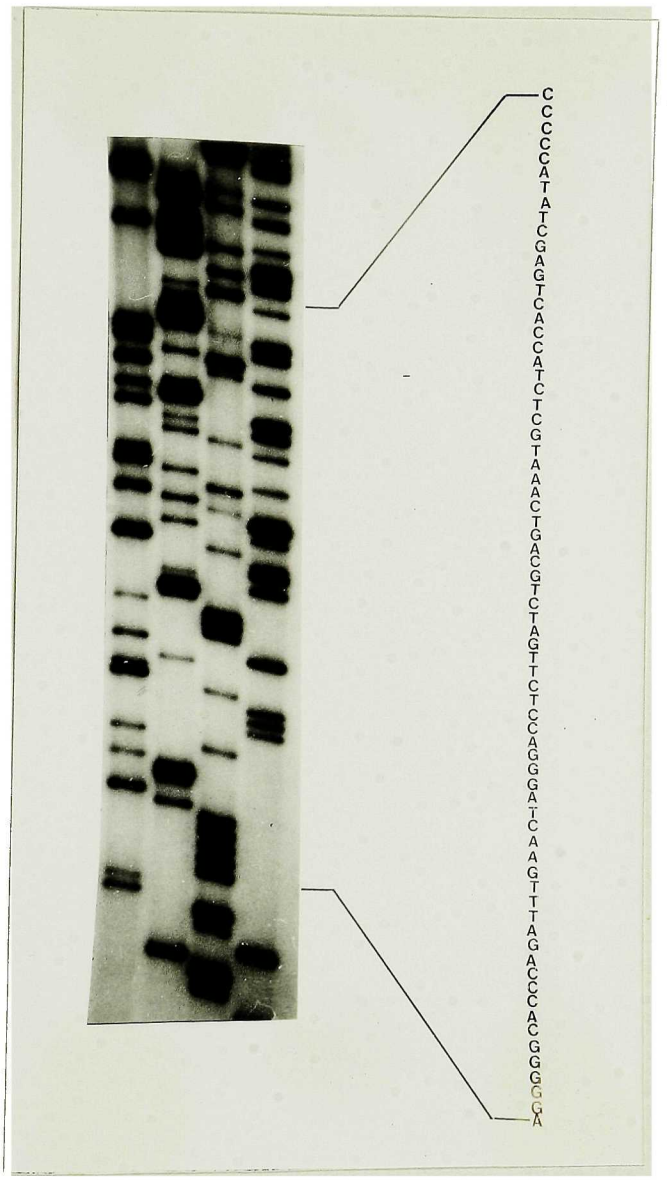

C
C
C
C
C
A
T
A
T
C
G
A
G
T
C
A
C
C
A
T
C
T
C
G
T
A
A
A
C
T
G
A
C
G
T
C
T
A
G
T
T
C
T
C
C
A
G
G
G
A
T
C
A
A
G
T
T
T
A
G
A
C
C
C
A
C
G
G
G
G
G
A

Figure 23

Secondary structure of $tRNA^{cys}_1$ gene deduced from its DNA sequence. The arrows indicate the nucleotides that are different in $tRNA^{cys}_1$ and $tRNA^{cys}_2$ (Fig. 26).

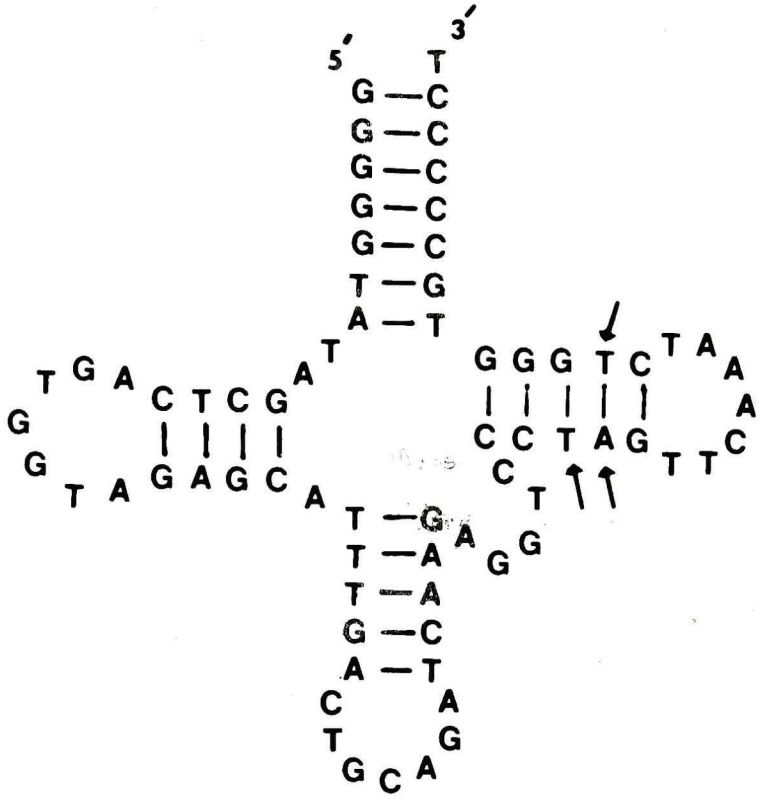

5′
3′
T
G — C
G — C
G — C
G — C
G — C
T — G
A — T
T
T G A
A
C T C G
G
G
T A
G A G C
A
G G G T C T A A
A
C C T A G T T C
C T
T — G
T — A
T — A
G — C
A — T
C
T
G C A
A
G
G

Figure 24

Autoradiogram of a dideoxysequencing gel showing a DNA fragment containing the DNA sequence of $tRNA^{cys}_2$. The 5' end of the sequence is at the bottom of the gel.


TCCCCCGTGGGCCTAAACTTGCCCCTGGAGAACTAGACGTCAGTTTACGAGATGGTGACTCGATATGGGG

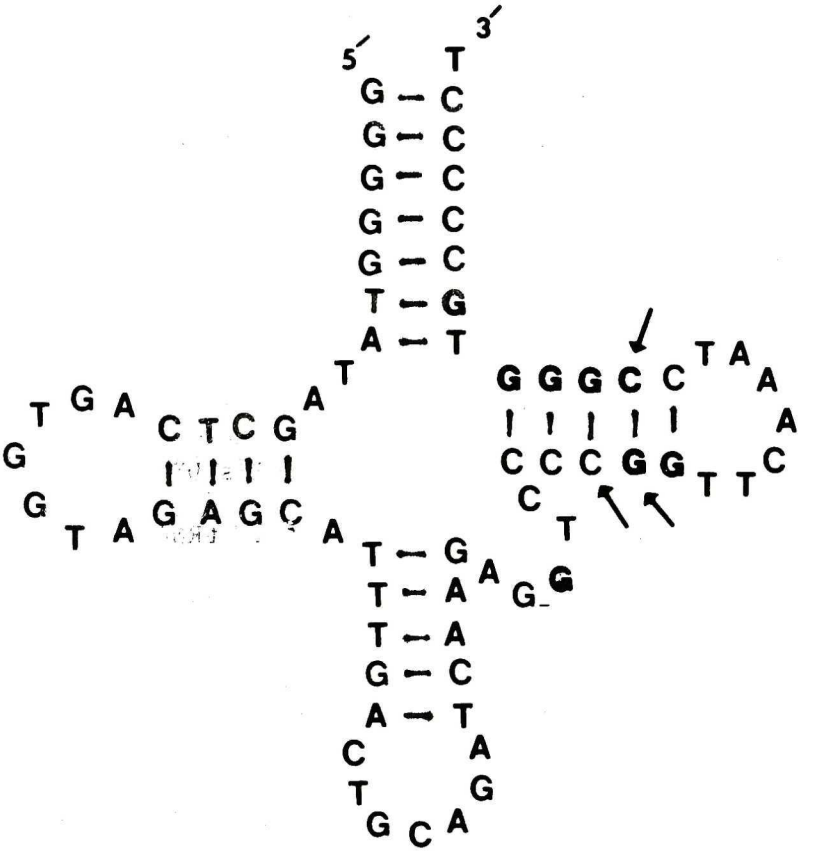
Figure 25

Autoradiogram of a dideoxysequencing gel showing a DNA fragment containing the DNA sequence complementary to $tRNA_2^{cys}$. The 5' end of this complementary sequence is at the bottom of the gel.


CCCCCATATCGAGTCACCATCTCGTAAACTGACGTCTAGTTCTCCAGGGGCCAAGTTTAGCCCCCACGGGGGA

Figure 26

Secondary structure of $tRNA^{cys}_2$ deduced from its DNA sequence. The arrows indicate the nucleotides that are different in $tRNA^{cys}_2$ and $tRNA^{cys}_1$ (Fig. 23).


5'
3'
T
G — C
G — C
G — C
G — C
G — C
T — G
A — T
T
A
T G A C T C G
G
G T A G A G C A
G G G C C T A A
A
C C C G G T T C
C T
T — G
T — A
T — A
G — C
A — T
C A
T G
G C A
A
G G

mouse and rat. It also showed 52% homology with $tRNA^{asp}_{GUU}$ and $tRNA^{lys}_{UUU}$ genes of B. subtilis. Most of these homologies are found in the D stem and loop regions and some in the ψ stem and loop regions. It has been known that some nucleotides in these regions appear to be promoter determinants and are highly conserved among different tRNA species.

Transcription of Cysteine tRNA Genes

These cysteine tRNA genes were tested for their ability to produce 4S RNA products when transcribed in vitro in Xenopus ovary S-100 cell extract. Figure 27 shows the transcription products obtained from the intact DNA and various deletions. Figure 28 shows a line diagram of the DNA fragments used in this transcription reactions. Lanes d to i in Fig. 27 show that 4S RNA products were observed when the DNA fragments contained either one or both of the tRNA genes; it was not observed in the DNA fragment where both genes were deleted (lane j). pXbs 1, a pBR 322 plasmid containing a 5S RNA gene, was used as a positive control; a blank, no exogenous DNA added, and pUC 8 were used as negative controls. From this experiment, we conclude that both of the cysteine tRNA genes in D37 are transcribed individually and that they are under separate control systems.

To Determine if D37 is a Tandem Repeated Unit of X. laevis

It has been known that some tRNA genes are contained on tandemly repeated DNA fragments in X. laevis (31). We wished to determine whether the D37 DNA fragment was also repeated tandemly in X. laevis. From the complete DNA sequence we obtained the restriction enzymes that have a

Figure 27

Transcription of tRNA genes: Autoradiogram of [^{32}-P]RNA products made by in vitro transcription in Xenopus S-100 extract. All reaction mixtures contained 0.5 ug of DNA template, 0.5 mM each of ATP, CTP, UTP, 0.025 mM GTP, 20 uCi [^{32}-P] GTP, 20 ul of Xenopus S-100 extract, 40 mM Tris-Cl(pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, and 10% glycerol in a final volume of 50 ul; incubated at 30°C for 2 hrs. The RNA products were separated on 8% polyacrylamide gel in 1.5% TBE buffer, 8 M urea, electrophoresed at constant voltage (2000 V) and exposed on XAR-5 film for 7 hrs at -70°C. Blank: no DNA added; pXbs 1, pBR 322 containing a DNA fragment of 5S RNA gene.

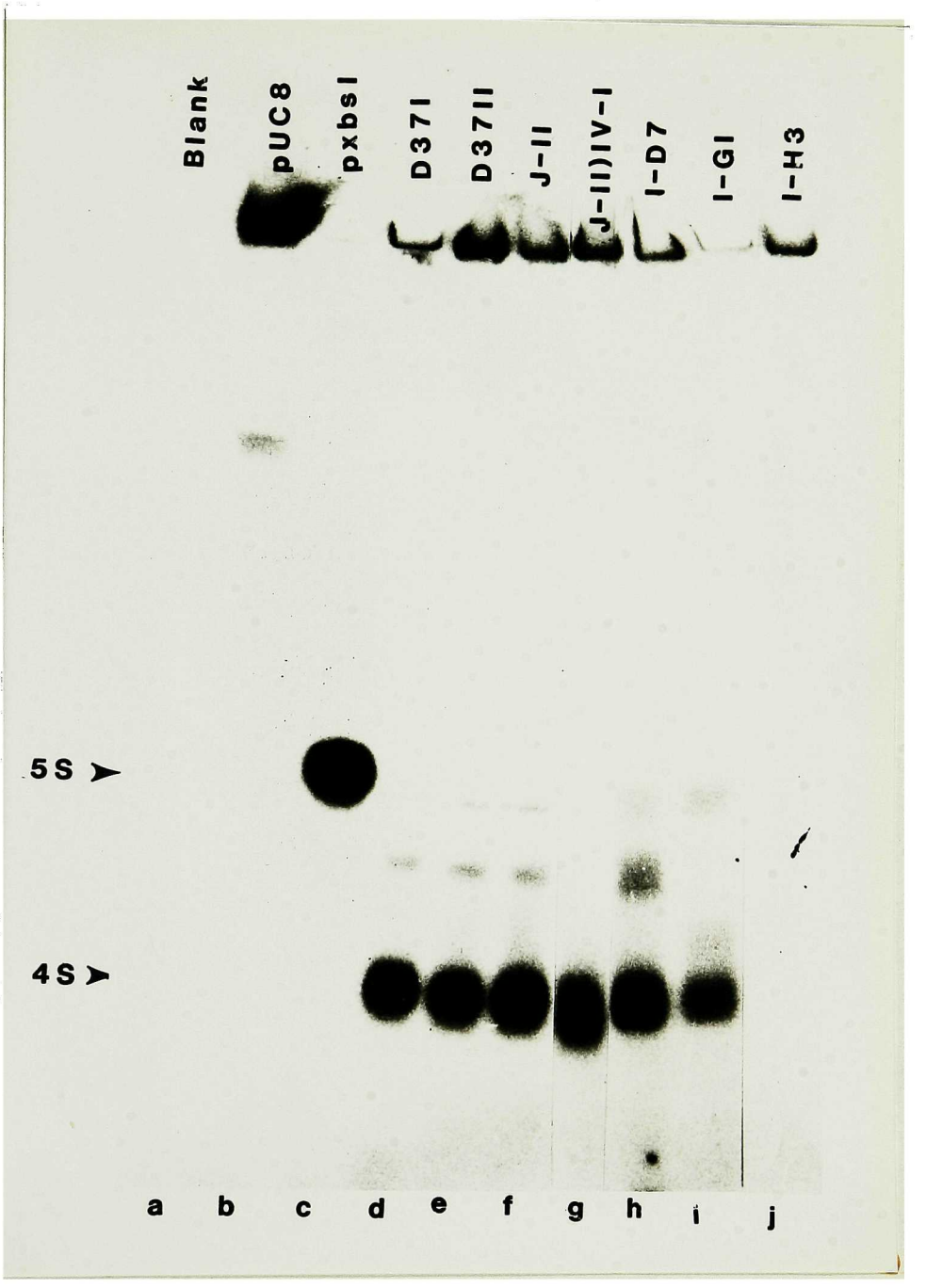
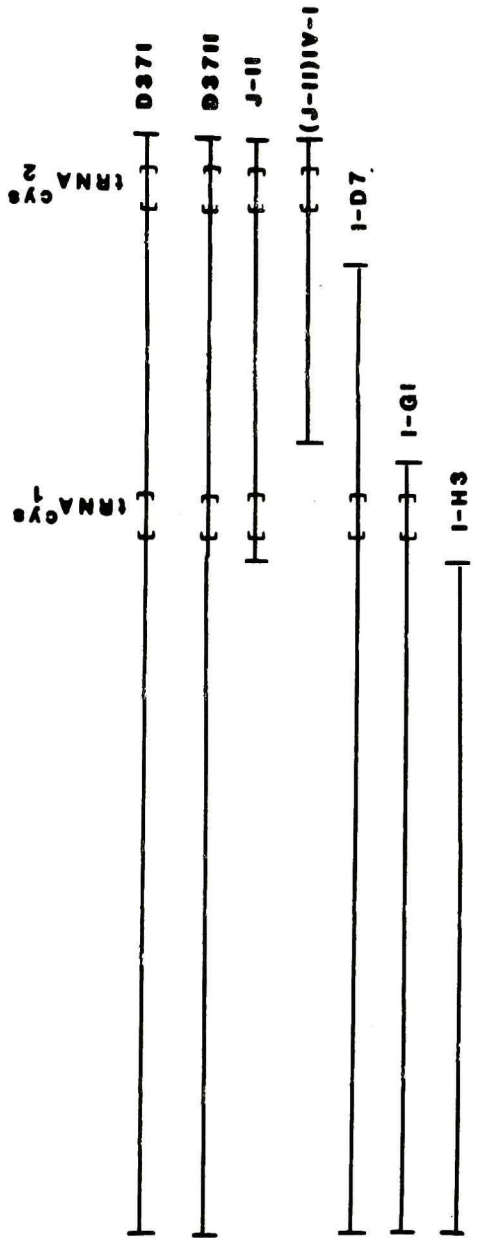

Blank
pUC8
pxbs I
D37 I
D37 II
J-II
J-II)IV-I
I-D7
I-GI
I-H3
5S
4S
a
b
c
d
e
f
g
h
i
j

Figure 28

DNA templates used in the transcription reactions shown in Fig. 23


tRNA$^{Cys}_{2}$
tRNA$^{Cys}_{1}$
D371
D371B
J-11
I-A(III-J)
I-D7
I-G1
I-H3

single site in D37. Among these were the restriction enzymes Kpn I, Bal I and Hpa I. Figure 29a shows a hypothetical model of a tandemly repeated DNA fragment in X. laevis with D37 as the tandemly repeated unit. Hypothetically, if D37 were tandemly repeated, X. laevis genomic DNA digested with Kpn I, Bal I or Hpa I should result in DNA fragments of the same length as those produced by Eco RI digestion (illustrated in Fig. 29a). An experiment was carried out by performing partial digestion of X. laevis genomic DNA with Eco RI or Kpn I or complete digestion with Hpa I or Bal I, followed by hybridization with labeled pUC9 D37. Southern transfer analysis, Fig. 30, showed that DNA fragments obtained from digestion with Kpn I, Bal I and Hpa I were the same size, but the DNA fragments obtained from Eco RI digestion were slightly smaller, by about 100 bp. This shows that D37 is contained in a tandemly repeated DNA fragment of approximately 1850 bp long. This tandemly repeated DNA fragment contains one restriction enzyme site for Kpn I, Bal I and Hpa I but at least two sites for Eco RI. Since the D37 fragment contains most of the tandem repeated unit, this repeat may contain only two or at the most three tRNA genes. Our proposed model for this tandemly repeated DNA of X. laevis is shown in Fig. 29b. This experiment shows that tRNA genes in a tandemly repeated DNA fragment of X. laevis may contain as few as two tRNA genes.

Figure 29

a) A hypothetical model of X. laevis DNA containing three D37 tandemly repeated units. b) A proposed model of X. laevis DNA containing three tandemly repeated units. E, Eco RI; H; Hpa I; K, Kpn I; B, Bal I; the dotted lines represent the distances between restriction enzyme sites.

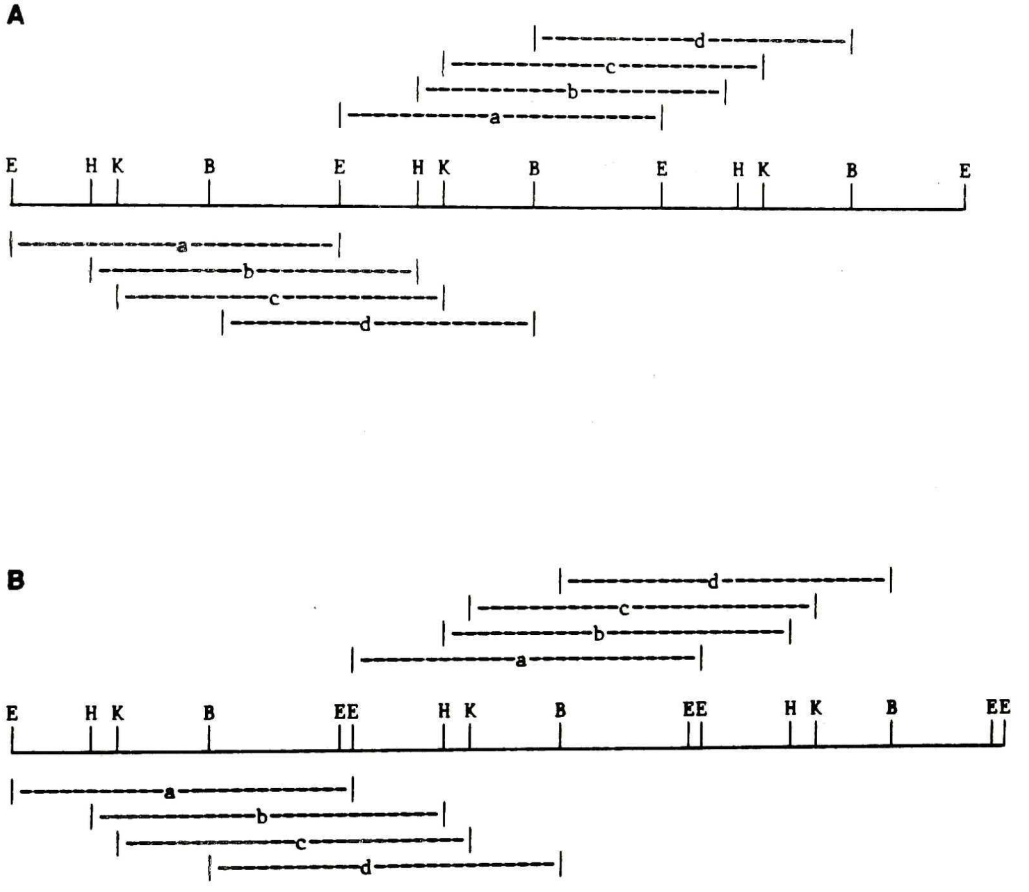
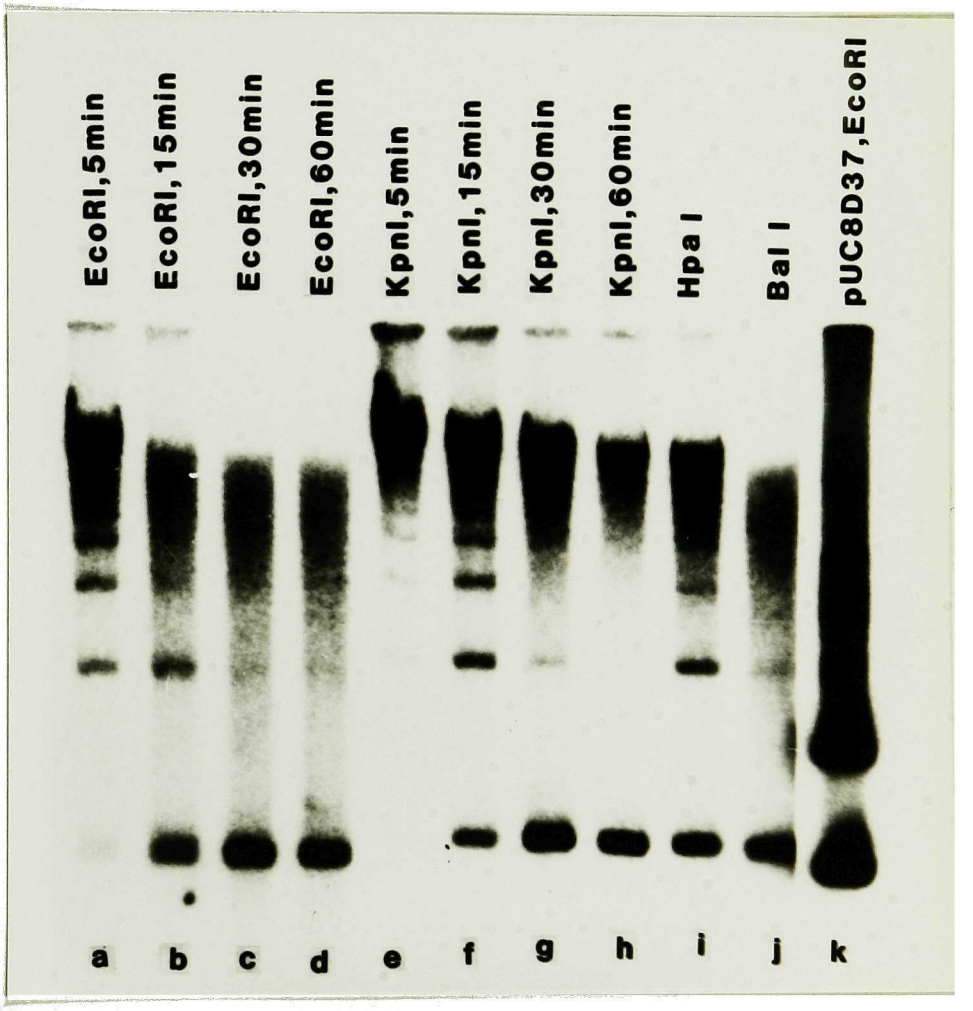

A
d
c
b
a
E H K B E H K B E H K B E
a
b
c
d
B
d
c
b
a
E H K B EE H K B EE H K B EE
a
b
c
d

Figure 30

An autoradiogram showing the Southern Transfer Analysis of X. laevis genomic DNA after digestion with various restriction enzymes and hybridization with labeled D37: Lane a to j; 5 ug of X. laevis was digested with the restriction enzymes shown above and transferred onto a nitrocellose filter after electrophoresis on agarose gel and hybridized with [^{32}P] labeled pUC9 D37 probe as described under Materials and Methods. pUC9 D37 cut with Eco RI was used to serve as a control in this reaction.


EcoRI,5min
EcoRI,15min
EcoRI,30min
EcoRI,60min
KpnI,5min
KpnI,15min
KpnI,30min
KpnI,60min
Hpa I
Bal I
pUC8D37,EcoRI
a
b
c
d
e
f
g
h
i
j
k

DISCUSSION

We are the first to report the characterization of a cysteine tRNA gene from Xenopus laevis. X. laevis DNA enriched for tRNA genes was digested with Eco RI and subcloned into pBR 325. One subclone, D37, containing a 1737 bp insert was further characterized. Both strands of the 1.74 kb insert were completely sequenced and found to encode two cysteine tRNAs. Assignment of these two tRNA genes was based upon the criterion derived from the secondary structure of known tRNAs.

All known tRNAs identified thus far share a unique two dimensional "cloverleaf" structure. A basic tRNA stucture must consist of four base-paired segments and three non-hydrogen bonded loops. These four base-paired segments are the amino-acid acceptor (AA) stem, the dihydrouridine (D) stem, the anticodon (AC) stem, and the TψCG (ψ)stem. The number of base pairs required in these stems are seven in the AA stem, three or four in the D stem, and five in the AC and ψ stem. The non-hydrogen bonded loops are the D loop, AC loop and the ψ loop extending from the D stem, the AC stem, and the ψ stem, respectively. The D loop contains six to eight nucleotides the AC and ψ loop each contain seven nucleotides.

The two regions from this 1.74 kb DNA fragment meet the criterion in that they can each form a two dimensional "cloverleaf" structure. The deduced structures contain exactly seven base pairs in the AA stem, four base pairs in the D stem, and five base pairs in the AC and ψ stems. Their D loops, AC loops, and ψ loops contain seven bases each. The anticodons 5'-GCA-3' deduced from the AC loops correspond with the sequence

expected for cysteine tRNAs. The two genes are named $tRNA^{cys}_1$ gene and the $tRNA^{cys}_2$ gene with respect to their order of transcription (5'->3') on the D37.

We have compared the organization of our tRNA genes on D37 to a 3.18 kb DNA fragment from X. laevis containing 8 tRNA genes. (14) This 3.18 kb DNA fragment is the only other DNA fragment from X. laevis reported containing tRNA genes. The tRNA genes on this 3.18 kb DNA fragment occur in a cluster and are separated by spacer DNAs of different lengths. Some of the tRNA genes are oriented in opposite polarities. The $tRNA^{tyr}$ gene is the only gene of this cluster contains an intervening sequence. (61) Our proposed cysteine tRNA genes are separated by a 452 base pair DNA spacer. The $tRNA^{cys}_1$ and $tRNA^{cys}_2$ genes are oriented in the same direction and do not contain intervening sequences.

Many DNA fragments are found which consist of only parts of a coding tRNA sequence. It has been suggested that these pseudogenes occur by a repeated insertion and excision of a transposible element within the intact tRNA genes. For example, a 1.5 kb DNA fragment of D. melanogaster contains four regions that display significant sequence homology with its $tRNA^{met}_i$. (78) In rat liver, a 3.2 kb DNA fragment contains $tRNA^{glu}$ and $tRNA^{gly}$ pseudogenes as well as the intact genes for $tRNA^{leu}_{CUG}$ and $tRNA^{asp}_{GAC}$. (53) However, no pseudogenes were found in our DNA fragment of X. laevis when analyzed by computer comparison with various known eukaryotic tRNA genes.

When compared with each other, these two proposed cysteine tRNA genes are 96% homologous resulting from 3 base substitutions. This is not uncommon in eukaryotic tRNA genes from the same or related species.

For instance, the coding sequence of $tRNA^{ser}_{UCG}$ and $tRNA^{ser}_{UCA}$ of S. cerevisiae are also 96% homologous resulting from three base changes. (6,64) These may have resulted from mutations leading to single base transitions. Some tRNA genes are identical among related species. For example, $tRNA^{Phe}$ of X. laevis contains the same primary sequence as $tRNA^{Phe}$ of rabbit, calf liver and human placenta (61) and the $tRNA^{lys}_{2}$ sequence in Drosophila and in mammals are identical. (88) This probably results from a simple duplication of an ancestral gene.

We have also compared the proposed cysteine tRNA genes to various known tRNA genes. A 67% homology is detected between the $tRNA^{cys}$ gene of yeast and $tRNA^{cys}_{1}$ gene of X. laevis. The cysteine tRNA gene of yeast, predicted from its RNA sequence, is the only other eukaryotic nuclear cysteine tRNA gene reported at the present time. Our tRNA genes showed approximately 65% homology with $tRNA^{phe}_{GAA}$ of X. laevis and D. melanogaster, 55% homology to $tRNA^{phe}_{GAA}$ of yeast, 45% homology with $tRNA^{gly}_{1}$ of B. mori, 36% homology with $tRNA^{lys}_{3}$ of rabbit, and 30% homology to $tRNA^{pro}_{CGG}$ of mouse and rat. They also show approximately 52% homology with the bacterial $tRNA^{asp}_{GUU}$ and $tRNA^{lys}_{UUU}$ of B. subtilis. Most of these homologies are in the D stem and loop regions and some in the ψ stem and loop regions. It has been known that some nucleotides in these regions appear to be promoter determinants and are highly conserved in both eukaryotic and prokaryotic tRNAs. Therefore, it is not surprising to find sequence homologies in these regions.

The tRNA genes in X. laevis are unique from tRNA genes of other species in that these genes are tandemly repeated. The 3.18 kb DNA fragment of X. laevis has been estimated to be tandemly repeated about 150 times at a single chromosomal locus. (15) Our results show that our subcloned 1.74 kb DNA

fragment is contained in a 1.85 kb tandem repeat. We have yet to sequence the remaining ≃ 100 bp. But, it is doubtful that this remaining 100 bp will contain a tRNA gene since a coding region of a tRNA gene is usually 72 to 73 bp long excluding 5' and 3' flanking regions and spacer DNA. However, we must not rule out the possibility that this tandem repeat may contain an additional tRNA gene with a very short spacer. Thus, this tandem repeat contains only two, at the most three, tRNA genes and probably only one tRNA species.

We have shown that these two proposed cysteine tRNA genes can be transcribed independently in Xenopus S-100 cell free extracts by the presence of 4S RNA products when each gene is presently separately. In the 3.18 kb DNA fragment of X. laevis, five out of the eight tRNA genes have been sequenced but only the $tRNA^{tyr}$ gene and one of the $tRNA_i^{met}$ have been reported to give transcriptional products. (45) We have not yet shown, however, that the 4S RNA products are indeed transcriptional products of these genes. This can be determined by RNA sequence analysis of the 4S RNA products using RNA fingerprinting methods. We can determine their RNA sequences and compare them with their DNA sequences to verify that the RNAs are indeed the transcriptional products from the tRNA genes on D37.

However, the above correlation does not prove that the transcriptional products of our two tRNA genes actually act as adaptors for cysteine incorporation in protein synthesis in vivo. We could prove that the transcriptional products of our two tRNA genes from this 1.74 kb DNA fragment of X. laevis indeed act as adaptors for cysteine incorporation in protein synthesis by conducting an experiment similar to that performed in 1962 demonstrating that the codon was recognized by the anticodon

rather than by the activated amino acid. (72) This experiment would be done as follows: labeled cysteine would be attached to isolated tRNA (4S RNA) using cysteine aminoacyl-tRNA synthetase. The attached unit would then be converted into alanine by reacting with Raney nickel, which removes the sulfur atom from the activated cysteine residue without affecting its linkage to the tRNA. Therefore, the aminoacyl-tRNA would have labeled alanine covalently linked to the tRNA instead of cysteine. This hybrid aminoacyl-tRNA would be isolated and used in a cell free protein synthesis system using templates that normally lead to the incorporation of cysteine. For example, hemoglobin mRNA containing codon for cysteine or a (UG) copolymer that leads to the incorporation of cysteine UGU could be used as templates. If radioactive alanine is incorporated in place of cysteine, we would have proven that our tRNA genes indeed code for cysteine tRNAs which act as adaptors for the amino acid cysteine in protein synthesis. Likewise, no radioactivity should be found using templates that lead to alanine but not cysteine incorporation.

Characterization of these two cysteine tRNA genes in our subclone has furthered our understanding of the organization of tRNA genes in X. laevis. Our results support the previous findings that X. laevis tRNA genes are organized in a cluster and are contained in tandemly repeated DNA fragments. Our immediate goal is to sequence the remaining, ≈100 bp, of this 1.85 kb tandem repeat. As outlined above, another goal would be to show that the 4S RNAs are indeed the transcriptional products of the cysteine tRNA genes and perhaps prove that they serve as adaptors for cysteine amino acids in protein synthesis. Previous studies show that the initiation site of RNA polymerase III on eukaryotic tRNA genes occur within 20 bases upstream of the mature tRNA coding sequence. The promoter control

regions are split into two essential elements composed of nucleotides 8-19 and 52-62, located within the D and the ψ stem and loop regions in the tRNA structure. It has been known that the 5'-flanking region of the tRNA genes plays an important role in transcription. For example, deletion of all but 14 bp of the 5'-flanking region of a B. mori $tRNA^{ala}$ gene abolished tRNA gene transcription in homologous cell free extract. (5) Also, a sequence located in the 5' flanking region of the $tRNA^{met}_{1B}$ gene of X. laevis was able to form Z-DNA. When a synthetic DNA fragment capable of forming Z-DNA was inserted preceeding the coding region, the expression of the gene was inhibited. (34) Transcription of tRNA genes involves other transcriptional factors, in addition to RNA polymerase III in cell free extracts. Most of these factors have not been identified and their exact involvement in transcription is not known. We have not observed any Z-DNA structure in the 5'-flanking regions in either of our tRNA genes. Future studies on this DNA fragment include the determination of the number of copies of cysteine tRNA genes in X. laevis, its site of RNA polymerase III initiation, the essential nucleotides in the promoter region, and the isolation of the transcriptional factors. We hope that these studies would give us to a better understanding of the control of eukaryotic tRNA genes transcription.

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