NUCLEOTIDE SEQUENCE OF A BOVINE ARGinine TRANSFER RNA GENE

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

MASTER OF SCIENCE

By

Aleida C. Eubanks, B.S.

Denton, Texas

May, 1996
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A single plaque-pure lambda clone designated XBA84 that hybridized to a $^{32}$P-labeled bovine arginine tRNA was isolated from a bovine genomic library harbored in a lambda bacteriophage vector. A 2.3-kilobase segment of this clone was found to contain an arginine transfer RNA gene by Southern blot hybridization analysis and dideoxyribonucleotide DNA sequencing. This gene contains the characteristic RNA polymerase III split promoter sequence found in all eukaryotic tRNAs and a potential RNA polymerase III termination site, consisting of four consecutive thymine residues, in the 3'-flanking region. Several possible cis-acting promoter elements were found within the 5'-flanking region of the sequenced gene. The function of these elements, if any, is unknown.
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PEG/NaCl solution  20% (w/v) polyethylene glycol (MW 8,000)
                  2 M NaCl
                  Made up in SM buffer

TSE buffer        20 mM Tris-HCl (pH 7.5)
                  10 mM NaCl
                  0.1 mM Na₂EDTA

TE buffer         10 mM Tris-HCl (pH 7.5)
                  1 mM Na₂EDTA

YT medium         Per Liter
                  8 g Bactotryptone
                  5 g Yeast Extract
                  5 g NaCl

LB medium         (Luria-Bertani Medium)
                  Per Liter (pH 7.0)
                  10 g bacto-tryptone
                  5 g yeast extract
                  10 g NaCl

TSS solution      Per 100 ml
                  0.85 g Bactotryptone
                  0.43 g Yeast Extract
                  0.85 g NaCl
                  5.0 ml DMSO
                  10.0 g PEG (8000 MW) (10%)
                  50 mM MgCl₂ (pH 6.5)

NZY Agar           Per Liter (pH 7.5)
                  10 g NZ amine
                  15 g Difco agar
                  5 g NaCl
                  2 g MgSO₄·7H₂O

1X SSC            Sodium Standard Citrate
                  Per Liter (pH 7.0)
                  4.4 g Na₃Citrate 2H₂O
                  8.75 g NaCl
SM buffer  (salt magnesium buffer)  
Per Liter  
50.0 ml 1M Tris-HCl (pH 7.5)  
5.0 ml 2% (w/v) gelatin  
2.0 g MgSO₄·7 H₂O  
5.8 g NaCl

IPTG  Isopropylthiogalactoside  
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactoside  
EtBr  Ethidium Bromide  
kilobase pairs  
PEG  Polyethylene glycol  
DNA  Deoxyribonucleic Acid  
RNA  Ribonucleic Acid  
tRNA  Transfer Ribonucleic Acid  
bp  Base pair  
MW  Molecular Weight  
SDS  Sodium Dodecyl Sulfate  
ddNTP  Dideoxyribonucleotide Triphosphate  
dITP  Deoxyinosine Triphosphate  
dGTP  Deoxyguanosine Triphosphate  
rpm  Revolutions per minute  
XC  Xylene Cyanol FF  
nt  Nucleotide  
mRNA  Messenger Ribonucleic Acid
CHAPTER I

INTRODUCTION

Transfer RNA (tRNA) molecules are 73 to 93 nucleotides in length, with a molecular weight of about 25,000. They serve as adaptor molecules in the translation of mRNA into the amino acid sequence of a polypeptide. There is at least one, and usually more than one, specific tRNA molecule for each of the 20 different amino acids. Multiple tRNA molecules which bind to the same amino acid are designated as tRNA isoacceptors. During protein synthesis, aminoacyl-tRNA synthetases (TRSs) are responsible for esterifying the appropriate amino acid to its cognate tRNA species. There exists a different TRS for each amino acid, 20 in all (Normanly et al., 1986; Maclain and Foss, 1988).

All cytoplasmic tRNA molecules fold into a cloverleaf secondary structure (Rich and RajBhandary, 1976) and in so doing, they maximize hydrogen bonding with normal Watson-Crick base pairing (Holley et al., 1965), yielding double-stranded helical regions called stems and non-hydrogen-bonded regions called loops. There are four hydrogen-bonded stem regions in this secondary structure and four loop regions, including a variable loop which contains 4 to 21
nucleotides (Rich and Kim, 1978). This structure also consists of the D arm named for the presence of dihydrouridine, the TWc arm which contains pseudouridine, and the anticodon arm with the anticodon triplet. In addition, there is a 3'-terminal CCA trinucleotide located on the acceptor arm to which the amino acid is attached. As a general rule, there are seven base pairs in the acceptor arm, five in the TWc arm, five in the anticodon arm, and usually 3 (sometimes 4) in the D arm.

In addition to the cloverleaf secondary structure, X-ray diffraction studies have shown that tRNAs adopt a tertiary L-shaped structure, consisting of two double helices at a right angle to each other. In this structure, the anticodon loop is at one end of the L and the acceptor stem is at the other. This structure is stabilized by a large number of invariant and semi-invariant nucleotides which are identical or similar in sequence and position in all tRNAs and are involved in the large degree of base stacking and tertiary base-pairing found in the tertiary structure (Rich and RajBhandary, 1976). It is these secondary and tertiary structures which allow the tRNA molecule sufficient structural features that afford it the ability to interact with a diverse number of macromolecules involved in protein synthesis (Rich and Kim, 1978).
It is estimated that there are about 1300 tRNA genes (and pseudogenes) within the human and rat genomes (Hatlen and Attardi, 1971; Lasser-Weiss et al., 1981). This results in an average of 20 genes for each of the 60-90 different isoaccepting species of tRNA (Lin and Agris, 1980). All eukaryotic tRNA genes have many transcription-related structural similarities including: (1) tRNA structural genes do not encode the CCA trinucleotide found at the 3'-terminus of mature tRNAs (Sprinzl et al., 1991); (2) transcription of tRNA genes initiates at a purine residue located three to ten nucleotides upstream of the coding region in the sequence Py-Pu-Py (Ciliberto et al., 1983); (3) termination of transcription of tRNA genes occurs at four or more consecutive thymine nucleotides on the non-coding strand of DNA within the 3'-flanking region of the gene (Bogenhagen and Brown, 1981); and (4) all tRNA genes are transcribed by RNA polymerase III through an internal split promoter encompassing the A-block, corresponding to the D-loop region of the mature tRNA, and the B-block, corresponding to the T-loop region of the mature tRNA (Sharp et al., 1985; Ciliberto et al., 1983).

A variety of cis-acting structural motifs has also been characterized within the 5'- and 3'-flanking regions of a number of tRNA genes which can positively (or sometimes
negatively) regulate transcription of the gene (Goddard et al., 1983; Hipkinson and Clarkson, 1983; Schaack et al., 1984; Wilson et al., 1985; Gouilloud and Clarkson, 1986; Lofquist and Sharp, 1986; Morry and Harding, 1986; Young et al., 1986; Raymond and Johnson, 1987; Shortridge et al., 1989). The functional significance of these structures, including the decanucleotide (5'-CCCCGCCCCG-3') and hexanucleotide (5'-GGGCGG-3') GC boxes which can potentially bind the RNA polymerase II transcription factor Spl (Kodonaga et al., 1986), and CCAAT binding sites which can bind another RNA polymerase II transcription factor (Doran et al., 1987) are not well understood at this time. Transfer RNA genes may occur individually and be widely dispersed throughout the mammalian genome (Santos and Zasloff, 1981; Goddard et al., 1983; Shortridge et al.; 1992; Arnold et al., 1986; Van Tol and Beir, 1988). Alternatively, they can occur in gene clusters (Roy et al., 1982; Chang et al., 1986; Pirtle et al., 1986; Doran et al., 1987; Boyd et al., 1989; Craig et al., 1989; Shortridge et al., 1989; Morrison et al., 1991; Kacar et al., 1992). The clusters may be large, megaclusters, that may include tandemly repeated copies of one or more tRNA genes (Buckland, 1989), or they may include single copies of several separate tRNA genes in gene heteroclusters (e.g.,
Within the human genome, tRNA genes have been localized on several different chromosomes including: a selenocysteine tRNA gene on chromosome 19 (Mitchell et al., 1992); two methionine tRNA genes on chromosome 6 (Naylor et al., 1983); two glutamate tRNA genes on chromosome 1 (Boyd et al., 1989); a tRNA gene cluster encoding proline, threonine, and valine tRNAs on chromosome 5; a glycine tRNA$_{GO}$ gene and pseudogene on chromosome 16; and a glycine tRNA$_{GCC}$ gene on chromosome 1 (Shortridge et al., 1985; Pirtle et al., 1986; McBride et al., 1989; Shortridge et al., 1989). The localization of tRNA genes in other mammalian genomes has not yet progressed as far as in the human genome.

Retroposons in the form of SINEs (short interspersed nuclear elements) and LINEs (long interspersed nuclear elements) can be found within the mammalian genome (Weiner et al., 1986; Hutchison et al., 1989). In several studies, SINEs have been found in relatively close proximity to tRNA genes and tRNA pseudogenes (Rosen and Daniel, 1988; Doran et al. 1987; Thomann et al., 1989; Craig et al., 1989; Shortridge et al., 1989). Delta, sigma, and tau sequences as well as Ty elements have been found to be associated with tRNA genes in yeast (Sandmeyer et al., 1988; Humber et al.,
In humans, Alu-like elements have been found in the vicinity of tRNA genes (Pirtle et al., 1986; Chang et al., 1986; Shortridge et al., 1987), most often in the case of the human valine tRNA family, which is the most extensively characterized tRNA gene family to date (Arnold et al., 1986; Shortridge et al., 1989; Thomann et al., 1989). Additionally, a bovine tRNA gene cluster has been found having complete and partial copies of the BCS (bovine consensus sequence) SINE family occurring on both sides of two serine tRNA genes (Chee et al., 1991). The function, if any, of these elements is unknown.

The organization of tRNA genes within mammalian genomes and the expression of these genes is not entirely clear at this point. The location and characterization of tRNA genes within mammalian genomes should further progress in the physical mapping of the genomes of all mammalian species, since there are many essentially identical homologous genes within the genomes of all mammalian species (Womack and Moll, 1986). The characterization of mammalian tRNA genes should provide information about the organization and arrangement of tRNA genes, including the structures of the tRNA genes themselves and their 5'- and 3'-flanking regions which usually exhibit much variation.

The purpose of this study is to characterize the structure and organization of a tRNA gene from a mammalian
species other than human. A clone was isolated from a bovine genomic library harbored in a lambda vector which hybridized to a $^{32}$P-labeled bovine arginine tRNA. Dideoxyribonucleotide sequencing was carried out on a segment of this clone to determine the primary structure of the gene and its neighboring 5'- and 3'-flanking regions. The subclone was found to encompass an arginine tRNA$_{cys}$ gene with a characteristic internal split promoter and an RNA polymerase III termination site consisting of four thymine residues. The gene has no intervening sequences and does not encode the terminal CCA trinucleotide found at the 3'-end of mature tRNA molecules. Within the 5'-flanking region of the sequenced gene are several presumptive promoter elements for RNA polymerase II, including three hexanucleotide GC boxes and two regions that approximate CCAAT boxes. Whether these elements have any effect on the efficiency of transcription of this gene is currently under investigation in this laboratory.
CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

A bovine genomic library harbored in the Lambda Fix II vector and host cells, E. coli XL1-Blue MRAP2 and XL1-Blue MRA, were purchased from Stratagene. Restriction endonucleases were purchased from Promega and Gibco BRL Life Technologies. Host cells for the plasmid vector pUC18 (E. coli DH5α) and for M13 (E. coli DH5α F') and T4 DNA ligase were from Gibco BRL Life Technologies. Nitrocellulose used in library screening was purchased from Schleicher and Schuell. Agarose and Hybond nylon membranes were from FMC Corp. and United States Biochemical (USB), Amersham Life Sciences, respectively. Radioactive materials including [5'-32P]pCp and [35S] deoxyadenosine-5'-(α-thio)- triphosphate, were purchased from ICN/Biomedicals, Inc. and NEN Dupont, respectively. The Sequenase Version 2.0 DNA Sequencing Kit was purchased from USB, Amersham Life Sciences. Several sequencing primers were ordered from Genosys Biotechnology Inc., (The Woodlands, TX). Other chemicals, reagents and materials were purchased from Kodak,
Screening of a Bovine Genomic Library

A bovine genomic library harbored in the Lambda Fix II vector was screened using an arginine [3'-\(\text{\textsuperscript{32}}\text{P}\)]tRNA\textsubscript{cys} hybridization probe employing the plaque purification procedure of Benton and Davis (1977). Host bacteria (E. coli XL1-Blue MRA P2) were infected with recombinant lambda bacteriophage, incubated at 37°C for 15 minutes, mixed with top agarose, plated on plates containing NZY bottom agar and grown overnight at 37°C. After overnight incubation, the plates were cooled to 4°C and a nitrocellulose filter, saturated with 1 M NaCl, was gently pressed on each plate exhibiting sufficient titer and lysis. The phage adhering to these filters were subsequently denatured in alkali followed by neutralization and rinsing to remove excess agarose. These filters were then baked under vacuum at 80°C for two hours. The \(\text{\textsuperscript{32}}\text{P}\)-labeled arginine tRNA probe was hybridized to the single-stranded lambda DNAs on the nitrocellulose filters in 50% formamide solution (50% deionized formamide, 5X SSC, 1 mM Na\textsubscript{2}EDTA, pH 7.5, 0.1% SDS, E. coli carrier tRNA 2 \(\mu\)g/ml) at 43°C for 24 to 48 hours. The filters were rinsed in three successive 5X SSC washes.
followed by two washes in 50% formamide solution and a final wash in 5X SSC, until all non-specifically bound tRNA probe was removed. The dried filters were then exposed to Kodak X-OMAT film at -90°C with the aid of an intensifying screen. Plaques giving strong positive signals were replated for further plaque purification until all plaques on a single plate hybridized to the labeled probe, indicating plaque purity. The positive plaques were examined further for plaque purity by agarose gel electrophoresis of restriction endonuclease digests of the phage DNAs obtained through minilysate preparation as described below.

Minilysis Preparation of Lambda Phage DNA

Select recombinant phage which gave strong hybridization signals were mixed with an equal volume of SM buffer and LB with 5 mM CaCl$_2$ medium containing an overnight culture of *E. coli* XL1-MRA Blue. After shaking at 37°C for 15 minutes, allowing phage particles to adhere to host cells, the culture was transferred to 25 ml of LB medium containing 5 mM CaCl$_2$ and incubated overnight at 37°C with shaking. After incubation, 20 μl of chloroform were added to each lysate followed by centrifugation at 2,500 rpm for ten minutes to remove bacterial debris. DNase I and RNase A were added to the supernatant at a concentration of 1 μg/ml
followed by incubation at 37°C for 30 minutes. An equal volume of PEG/NaCl solution was added to each culture, and was incubated on ice for one hour. The precipitated phage particles were recovered by centrifugation at 13,200 x g for 20 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 400 μl of TSE Buffer. The phage suspension was extracted twice with equal volumes of phenol saturated in TE buffer and then with 800 μl of chloroform:isoamyl (24:1) to remove the protein coat. The phage was precipitated with two volumes of ethanol and re-dissolved in TE buffer. Analysis by agarose gel electrophoresis of restriction enzyme digests of the recombinant phage DNA was used to check purity. One clone was chosen for large scale phage preparation based on its purity upon ethidium bromide staining of the gel. It was designated as XBA84.

Large Scale Phage Preparation

A large scale phage preparation was performed based on modification of the procedures of Blattner et al. (1978) and Davis et al. (1986). A culture of host cells, E.coli XL1-MRA Blue, was inoculated in 50 ml of LB with 5 mM CaCl₂ and incubated overnight with shaking at 37°C. Approximately 8 ml of the overnight culture was mixed with an equal volume
of SM buffer and with about 2 ml of the phage stock solution. This mixture was incubated for 20 minutes at 37°C and was subsequently transferred to 400 ml of LB/CaCl$_2$ medium for overnight incubation. Following lysis, the cellular debris and chromosomal DNA were precipitated with the addition of NaCl to a final concentration of 1 M and the phage in the supernatant were precipitated with 0.7% polyethylene glycol. The phage were recovered by CsCl gradient ultracentrifugation in the Ti 75 rotor of a Beckman L5-65 ultracentrifuge at 120,000 x g at 4°C for 18 hours. After centrifugation, a sharp band, corresponding to the phage, was carefully removed and transferred to dialysis tubing for dialysis against 100 mM Tris-HCl (pH 8.0), 0.3 M NaCl for 24 hours at 4°C. The phage was further purified by phenol/chloroform extraction and recovered by ethanol precipitation. The concentration of the DNA was assayed by UV absorbance at 260 nm.
Subcloning into pUC18

Fragment Selection

To select appropriate restriction fragments for subcloning, single and double restriction enzyme digestions of the purified XBA84 DNA were performed. The digests were fractionated on 1.0 and 1.5% agarose gels, stained with ethidium bromide and photographed. The DNA was transferred from the agarose gels onto nylon membranes by alkaline blotting with 0.4 M NaOH (Reed and Mann, 1985). These membranes were prehybridized at 43°C in solution containing 50% formamide, 5X SSC, 0.1% SDS, E. coli carrier tRNA 2 μg/ml and 1 μg/ml 5S RNA. After prehybridization for two hours, hybridization of the arginine [3'-32P]tRNA was carried out in the same solution for 24 to 48 hours at 43°C. Unhybridized probe and non-specifically bound probe were subsequently removed by successive 30 minute washes in a solution containing 50% formamide, 5X SSC, 1 mM Na₂EDTA (pH 7.5), and 0.1% SDS at 43°C, twice, 5X SSC at 43°C, and 2X SSC at room temperature. Membranes were blotted and air-dried, followed by autoradiography. The relative mobilities of the hybridized fragments were measured on the autoradiograms and matched with the corresponding bands in the gel photographs.

A 2.3-kb AvaI fragment from the XBA84 clone, which
hybridized to the $^{32}$P-labeled arginine tRNA probe, was selected for isolation and subsequent cloning into the plasmid vector pUC18. The lambda phage DNA was digested to completion with Aval restriction enzyme, extracted with phenol, followed by diethyl ether. The DNA fragments were precipitated with 100% ethanol.

**Subcloning**

The Aval-derived fragments were subcloned into the multiple cloning site of the pUC18 plasmid vector. The linearized vector was dephosphorylated through treatment with bacterial alkaline phosphatase (BAP) in order to minimize the occurrence of self-ligation. The vector was treated with BAP for one hour at 37°C followed by extraction with phenol and diethyl ether and precipitation with 100% ethanol. The Aval fragments and linearized, dephosphorylated pUC18 DNA were mixed in a 1:5 ratio (vector:insert). T4 DNA ligase was added to ligate the 3'-hydroxyl termini of the pUC18 vector and the 5'-phosphate termini of the insert DNA. The reaction was incubated at 16°C overnight. The resulting recombinant plasmids were used for transformation of *E. coli* DH5α competent cells.

**Preparation and Transformation of E. coli DH5α Competent Cells**
For preparation of the competent cells, 50 μl of a frozen stock of *E. coli* DH5α cells was inoculated into 5 ml of YT medium, and the culture was grown overnight at 37°C at 250 rpm in a New Brunswick shaker/incubator. The overnight culture was diluted 1 to 100 with YT medium and placed at 37°C at 250 rpm for 2 to 2.5 hours. The OD$_{550}$ of the culture was monitored until it reached an absorbance of 0.45. At this point, the cells were incubated on ice for 20 minutes. The cells were collected by centrifugation for 10 minutes at 2200 rpm at 4°C. The pelleted cells were resuspended in 5 ml of TSS solution and were ready for transformation.

To transform the competent *E. coli* cells, approximately 10 ng of ligated pUC18 plasmid DNA was mixed with 200 μl of competent cells. This mixture was incubated on ice for 30 minutes followed by heat treatment at 42°C for two minutes. After heat treatment, 1 ml of YT medium was added to the cell suspension followed by incubation at 37°C for one hour at 250 rpm. Aliquots of the cell suspension (30 μl and 100 μl) were plated on LB/ampicillin plates containing 10 μl of 10 mM IPTG and 50 μl of 2% X-gal and incubated overnight at 37°C. After incubation, it was possible to distinguish white colonies containing recombinant plasmids from the colored colonies containing non-recombinants. The linearized and non-linearized pUC18 plasmid DNAs were plated as controls.
Isolation and Purification of Plasmid DNA

A small quantity (about 10 µg) of plasmid DNA was isolated from select colonies using a modification of the alkaline lysis procedure of Birnboin and Doly (1979) to determine if the colonies contained the desired recombinant plasmid. A single, isolated white colony was stabbed with a sterile toothpick and subsequently streaked on a "master plate" followed by inoculation of 5 ml of sterile LB medium containing 10 µl of ampicillin. The "master plate", which served as a library of the cloned DNA fragments, was incubated at 37°C overnight and then stored at 4°C. The inoculated cultures were incubated overnight at 37°C with shaking at 250 rpm. The cells were harvested by centrifugation and lysed by treatment with a solution of 50.0 mM glucose, 10.0 mM EDTA, and 25.0 mM Tris-HCl (pH 8.0). The chromosomal DNA of the bacterial cell was denatured by treatment with a solution of 0.2 M NaOH and 1% SDS at pH 12 - 12.5. This was followed by neutralization with 5 M potassium acetate (pH 4.8). The bacterial DNA was pelleted by centrifugation, leaving the plasmid DNA in the supernatant. The plasmid DNA was purified with phenol:chloroform (1:1) and precipitated with 100% ethanol. The recovered plasmid DNA was redissolved in 30 µl of TE buffer containing 25 µg heat-treated RNase A per ml. The
isolated plasmid DNA was digested with restriction enzymes and fractionated by agarose gel electrophoresis. Alkaline blotting and hybridization techniques, as previously described, were used to determine those subclones containing the appropriate 2.3 kb-AvaI fragment insert. One subclone, designated pBA84, was selected for preparation of larger quantities so that determination of the physical map could be achieved.

Physical Mapping of Recombinant Plasmid DNAs

Recombinant plasmid DNAs were digested with restriction enzymes having few or no sites in the pUC18 vector. Single and double digests were fractionated on 1% and 1.5% agarose gels. These gels were stained with ethidium bromide and photographed, allowing the relative mobilities and fragment sizes to be determined by comparison with molecular weight standards generated by restriction digests of bacteriophage lambda DNA that were also fractionated on the gels. These restriction fragments were transferred to nylon membranes using the alkaline blot technique of Reed and Mann (1985). Alkaline blotting and hybridization were carried out using the same procedure as described for the lambda phage subclone. After analysis of these data, the physical map of the pUC18 plasmid insert could be determined.
DNA Sequencing

Subcloning into M13 phage

Overlapping 0.8-kb XbaI and 1.0-kb PstI fragments which hybridized to the arginine [3'-32P]tRNA probe, were selected for subcloning into the bacteriophage M13mp18RF vector and incubated with T4 DNA ligase. The methods used were the same as those used in pUC18 plasmid subcloning.

Transformation of E. coli DH5αF' with Recombinant M13mp18RF DNAs

Approximately 30 ng of the recombinant M13mp18RF DNA was mixed with 100 μl of competent E. coli DH5αF' cells (Gibco BRL Life Technologies) followed by gentle mixing for five seconds. This mixture was incubated on ice for 30 minutes, followed by heat-shock treatment for 45 seconds in a 42°C water bath and incubation on ice for two minutes. The mixture was combined with 3 ml of YT top agar containing 50 μl of E.coli DH5αF' lawn cells, 50 μl 2% X-gal and 10 μl 100 mM IPTG, mixed well and poured and spread evenly on YT plates. The plates were incubated overnight at 37°C.

The plaques containing recombinant phage were clear and could easily be discerned from those containing nonrecombinant phage which were blue. Stock solutions of the selected recombinant plaques were made by inoculation of
1 ml of YT medium with a pulled plug of agar containing the plaque. These were stored at 4°C.

A small scale preparation of the single-stranded M13mp18 DNA was prepared by inoculation of 5 ml of YT medium with 100 µl of the appropriate stock solution and 100 µl of *E. coli* DH5αF'. After shaking at 37°C for six hours, the recombinant M13 phage containing single-stranded DNA could be isolated from the supernatant. This single-stranded DNA was purified by phenol/chloroform extraction and ethanol precipitation and subsequently used for C-test analysis and DNA sequencing.

**C-test**

The "C-test" (Sambrook et al., 1989) was used to determine the orientation of DNA fragments subcloned into M13 phage vectors. Equal amounts of supernatant from the single-stranded preparations of M13, containing an unknown orientation of the single-stranded insert, were lysed by 0.5% SDS and incubated at 65°C for five minutes. After incubation, 4 µl of 0.5 M NaCl was added and the reactions were incubated at 65°C for another hour. This mixture was fractionated on 1% agarose gels, stained with ethidium bromide and photographed. Those single-stranded inserts of opposite orientation hybridized to each other causing
retardation of band migration in the gel due to the formation of a dimer complex.

**DNA Sequence Determination**

Single-stranded bacteriophage M13 recombinants containing the 0.8-kb XbaI insert and the 1.0-kb PstI insert, in both orientations, were used for standard dideoxyribonucleotide chain-termination DNA sequence analysis (Sanger et al., 1980). The sequencing reactions were carried out using Sequenase 2.0 (US Biochemicals) and [α-³⁵S]dATP (New England Nuclear/Dupont).

Universal sequencing primers and unique sequencing primers were annealed to the template DNAs. The annealed primers were enzymatically extended, creating the newly synthesized chains which were terminated by incorporation of ddNTP.

To resolve the DNA fragments, the reactions were loaded and electrophoresed on 6% urea denaturing polyacrylamide gels. The samples were heat treated at 80°C for two minutes and cooled on ice for five minutes prior to loading on the gels which had been prewarmed to 50°C by a prerun at 1200 volts.

A combination of short (about 2 hours, XC dye at 30 cm) and long runs (about 6 hours, XC dye at 70 cm) were used to
obtain sequences close to the primer as well as sequence up to 400 nucleotides away. To clarify areas of sequence which contained compression artifacts, dGTP was substituted with dITP in the sequence extension reactions. At the end of the gel runs, the gels were soaked in a solution of 10% acetic acid and 12% methanol for 15 minutes and then dried in a Bio-Rad gel dryer at 60°C for one hour and 80°C for another hour. The dried gels were exposed to Kodak XAR-5 X-ray film for one to seven days.

Computer Analysis of the DNA Sequence

A search for restriction enzyme sites within the DNA sequence was done using the PCS DNA Sequence Analysis Package (Lagrimini et al., 1984). The presumptive arginine tRNA<sub>ccc</sub> gene was found by with the aid of a computer program specific for this purpose (Shortridge et al., 1986).
CHAPTER III

RESULTS

Isolation of a Lambda Phage Clone Encompassing a Bovine tRNA Gene

A bovine genomic library harbored in the Lambda Fix II vector was screened using an arginine \[3'\text{-}^{32}\text{P}]tRNA_{\text{cgc}} as a hybridization probe. A number of plaques which gave positive hybridization signals upon autoradiography were identified. One plaque which gave an intense positive signal was selected for plaque purification (Benton and Davis, 1977) and designated as \(\lambda\text{BA84}\). The purified phage was characterized by restriction endonuclease digestion, fractionated on agarose gels by electrophoresis, and subjected to alkaline blot hybridization. It was determined that the size of the bovine DNA insert within the vector was approximately 17.0 kb, based on analysis of restriction digests of the clone and on restriction information about the arms of the Lambda Fix II vector. Work on the \(\lambda\text{BA84}\) physical map is still in progress in our laboratory (Martin Roeder, unpublished results).
Construction of Physical Map of pBA84

Within this 17.0-kb insert, a 2.3-kb AvaI fragment was observed to hybridize to the arginine tRNA probe (Fig. 1). This fragment was subsequently isolated and subcloned into the AvaI site of the multiple cloning region of the pUC18 plasmid vector and the recombinant plasmid designated as pBA84. The physical map of pBA84 (Fig. 2) was constructed based on restriction endonuclease analysis and alkaline blot hybridization. The restriction enzymes used and the corresponding fragment sizes generated are summarized in Table 1.

DNA Sequence Analysis

Two overlapping fragments, a 1.0-kb PstI fragment and a 0.8-kb XbaI fragment, were subcloned (in both orientations) into the corresponding regions of the multiple cloning site of M13mp18 RF DNA for sequence analysis by the dideoxyribonucleotide chain termination method (Sanger et al., 1980). Both the XbaI and the PstI fragments hybridized strongly to the arginine tRNA probe (Fig. 3). These inserts were sequenced in the direction and to the extent shown in Figure 4 using both a universal sequencing primer and specific oligonucleotide primers. Sequencing of the PstI and XbaI fragments was done on both strands in order to
ensure the accuracy of the DNA sequences. The sequence of these overlapping fragments is 1,293 basepairs and is shown in Figure 5.

This resulting sequence was subjected to a computer search for tRNA genes (Shortridge et al., 1986), which revealed a potential arginine tRNA<sub>cys</sub> gene located at nt 386 to 459. It should be noted that the DNA sequence of the arginine tRNA gene is identical to that of the tRNA<sub>cys</sub> probe, allowing for modified nucleotides and uridines in the RNA. The area of sequence encompassing the potential gene is represented by the sequence gel in Figure 6. The cloverleaf secondary structure of the non-coding strand is depicted in Figure 7. Within the gene is a characteristic internal split promoter (Fig. 5). There are no intervening sequences within this gene, and it does not code for the CCA trinucleotide found at the 3'-end of a mature tRNA molecule. A potential RNA polymerase III termination site consisting of four consecutive T nucleotides is found in the 3'-flanking region of the gene at nt 472 to 475 (Fig. 5). It is interesting to note that some possible cis-acting structural motifs exist within the 5'-extragenic region. There are three potential hexanucleotide GC boxes located at nt 152 to 157, nt 234 to 240, and nt 328 to 333. Additionally, there are two areas of sequence which closely
resemble CCAAT boxes, but for a single nucleotide change in each, located at nt 270 to 276 and nt 287 to 292. The effect that these regions may have on the transcriptional efficiency of the arginine tRNA gene is being investigated in this laboratory (Martin Roeder and Irma Pirtle, unpublished results).
Table I. Hybridization of arginine [3'-32P] tRNA to alkaline transfers of restriction enzyme digests of pBA84.

<table>
<thead>
<tr>
<th>Primary digest</th>
<th>Secondary digest</th>
<th>Fragment sizes (kb)</th>
<th>Primary digest</th>
<th>Secondary digest</th>
<th>Fragment sizes (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ava I</td>
<td>--</td>
<td>2.30*</td>
<td>Pst I</td>
<td>--</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ava I</td>
<td>Pst I</td>
<td>2.60</td>
<td>1.00*</td>
<td>Pst I</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00*</td>
<td>0.60</td>
<td>Pst I</td>
<td>0.70*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
<td>Hind III</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>Ava I</td>
<td>Hind III</td>
<td>2.50</td>
<td>1.00*</td>
<td>Xba I</td>
<td>4.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.85</td>
<td></td>
<td>0.80*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xba I</td>
<td>Pst I</td>
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<tr>
<td>Hind III</td>
<td>--</td>
<td>3.20</td>
<td>1.00*</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1.00*</td>
<td></td>
<td></td>
<td>0.45</td>
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<td></td>
<td>0.80</td>
<td></td>
<td></td>
<td>0.30</td>
</tr>
</tbody>
</table>

The sizes of the DNA fragments (in kb) generated by the restriction enzymes AvaI, Hind III, PstI, XbaI, and combinations of these enzymes, are shown. The standards used for estimation of fragment sizes were derived from a Hind III digest of λ DNA and a Hind II digest of pBR322 DNA. The DNA fragments that hybridized to the tRNA probe are denoted by asterisks.
Figure 1. Hybridization of arginine [3'-32P]tRNA\textsubscript{Arg} to alkaline transfers of AvaI-digested λBA84. The left lane shows the EtBr-stained 1.5% agarose gel and the middle lane shows the corresponding autoradiogram. The size markers (in kb) were generated by digesting λ DNA with Hin dIII (shown in the right lane).
<table>
<thead>
<tr>
<th>Size in kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.72</td>
</tr>
<tr>
<td>9.46</td>
</tr>
<tr>
<td>6.67</td>
</tr>
<tr>
<td>4.26</td>
</tr>
<tr>
<td>2.25</td>
</tr>
<tr>
<td>1.96</td>
</tr>
</tbody>
</table>
Figure 2. Physical map of plasmid pBA84 DNA. The physical map of the 2.3-kb AvaI insert is depicted. Encompassed within this fragment is the presumptive arginine tRNA gene indicated by a black rectangle. The horizontal line represents bovine DNA and the hatched areas represent the pUC18 vector DNA. The polarity of the gene (from 5' to 3') on the non-coding strand is shown by the arrow.
Figure 3. Hybridization of arginine \([3'{}^{32}P]tRNA_{cys}\) to alkaline transfers of restriction digests of pBA84. Photographs of the EtBr-stained 1% agarose gel is shown in the left lanes and the corresponding autoradiograms are shown in the right lanes. Restriction enzymes used were PstI and XbaI. HindIII-digested \(\lambda\) DNA and HindIII-digested pBR322 were used as molecular weight standards (far left lane) as well as BstEII-digested \(\lambda\) DNA (far right lane).
<table>
<thead>
<tr>
<th>Size in kb</th>
<th>PstI</th>
<th>XbaI</th>
<th>Size in kb</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td>8.45</td>
</tr>
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<td>9.46</td>
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<tr>
<td>0.22</td>
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</tbody>
</table>
Figure 4. Sequencing strategy of the 1293-bp XbaI/PstI fragment. A 851-bp XbaI fragment and a 1022-bp PstI fragment were subcloned into M13mpl8 and sequenced in the direction and extent indicated by the arrows resulting in a 1293-bp XbaI/PstI fragment. The sawtooth lines represent the M13mpl8 vector. The solid boxes at the ends of some arrows indicate segments for which specifically designed oligonucleotide primers were used.
Figure 5. Nucleotide sequence of the 1293-bp fragment encompassing a bovine arginine tRNA\textsubscript{ccg} gene. The structural gene is contained within the rectangular box with an arrow showing its polarity from 5' to 3'. Indicated within the 5'-flanking region are three potential Sp1 binding sites occurring at nt 152 to 157, nt 235 to 240, and nt 328 to 333 and two presumptive CCAAT-like boxes at nt 271 to 276 and nt 287 to 292.
Arginine tRNA Gene ----->
Figure 6. DNA sequence gel exhibiting the nucleotide sequence of the arginine tRNA\textsubscript{ACC} gene.
Figure 7. Bovine arginine tRNA\textsubscript{GTS} gene drawn in the cloverleaf conformation. The numbers represent the nucleotide residue number. The dots indicate predicted Watson-Crick base pairs.
Bovine tRNA\textsubscript{Arg} Gene

\begin{align*}
\text{CGG} & \quad 15 \\
A & \quad A \\
T & \quad T \\
A & \quad T \\
\cdot & \quad \cdot \\
G & \quad G \\
T & \quad C \\
C & \quad C \\
G & \quad G \\
\cdot & \quad \cdot \\
G & \quad A \\
A & \quad A \\
A & \quad A \\
\bullet & \quad \bullet \\
G & \quad G \\
G & \quad T \\
C & \quad G \\
G & \quad T \\
T & \quad C \\
G & \quad T \\
C & \quad T \\
A & \quad A \\
T & \quad T \\
\end{align*}
CHAPTER IV

DISCUSSION

The tRNA gene family is a member of the middle repetitive class of DNA. There are about 1300 tRNA genes in the human haploid genome (Hatlen and Attardi, 1971) encoding 60-90 tRNA isoacceptors (Lin and Agris, 1980). To date, only a few mammalian tRNA genes have been isolated and characterized. The majority of these, approximately 40 to 50, are within the human genome (Sprinzl et al., 1991), the valine tRNA family being the most extensively characterized tRNA gene family (Arnold et al., 1986; Shortridge et al., 1989; Thomann et al., 1989). Thus far, only a single bovine tRNA gene cluster has been characterized (Chee et al., 1991). This heterogene cluster consists of two serine tRNAIGA genes, one serine tRNAUGA gene and a glutamine tRNAUGC gene.

In this study, an arginine tRNAArg gene was isolated and characterized for comparison with other mammalian tRNA genes. This arginine tRNA gene exhibits many of the similarities found in all other tRNA genes from mammals. The tRNAArg gene does not encode the CCA trinucleotide found at the 3'-end of mature tRNAs. The gene contains the
characteristic RNA polymerase III internal split promoter, and a putative transcription termination site (TTTT) is found within its 3'-flanking region. The DNA sequence of the gene harbored in the ABA84 clone was found to be identical to that of the tRNA_{CG} probe used during hybridization with the exception of uridine and modified nucleotides which occur in the probe. The sequence analysis revealed that the gene forms a typical cloverleaf structure and contains no intervening sequences.

Often there are cis-acting structural motifs found within the 5'- and 3'-flanking regions of tRNA genes. These regions have been observed to have either a positive or negative effect on the transcription efficiency of the corresponding tRNA genes (Goddard et al., 1983; Hipskind and Clarkson, 1983; Schaak et al., 1984; Wilson et al., 1985; Gouilloud and Clarkson, 1986; Young et al., 1986; Raymond and Johnson, 1987; Shortridge, et al., 1989). These structures include GC boxes which can bind the RNA polymerase transcription factor Spl (Kadonaga et al., 1986) and CCAAT-binding sites which can also bind RNA polymerase II transcription factors (Doran et al., 1987).

In one study (Shortridge et al., 1989), deletion constructs of a tRNA_{Thr}^UCA gene containing 272, 168, 33 and 2 bp of 5'-flanking region were compared for transcriptional
efficiency. It was found that the 272, 168 and 33 bp constructs had the same transcriptional efficiency, while that with 2 bp of the 5'-flanking DNA was noticeably reduced. This study implicates the upstream region, containing possible binding sites for RNA polymerase II transcription factors, as being crucial for high transcriptional efficiency. Three possible GC boxes and two possible CCAAT boxes are found within the 5'-flanking region of the presumptive tRNA\textsuperscript{Arg}_{CCG} gene sequenced in this study. The role, if any, that these potential binding sites play on the transcription of this gene is speculative. Further investigation, involving in vitro transcription experiments and deletion constructs, need to be done to test expression efficiency of the gene and any effect that the 5'-extragenic region may have on gene expression.

Finally, it will be necessary to localize this tRNA\textsuperscript{Arg}_{CCG} within the bovine genome. This would contribute further to understanding of the mammalian tRNA gene family structure, organization, and expression. Additionally, due to their sequence conservation and wide dispersal throughout the genome, it is possible that tRNA genes would make excellent candidates for the anchor (type I) loci necessary for the construction of a genome map. (Type I loci are defined as anchor loci necessary for the construction of a
genome map). These loci are generally evolutionarily conserved and exhibit only moderate levels of polymorphism (O'Brien, 1993). In contrast, type II anchor loci, such as microsatellite sequences, are highly polymorphic loci (Bishop, et al., 1994). Analysis of the bovine genome will assist in the understanding of the genetic control of economically important traits in cattle including those affecting disease susceptibility and disease resistance (Fries, 1993; Fries et al., 1989). With this understanding, it should be possible to manipulate these genetic loci within the breeding population (Fries et al., 1989), with the ultimate selection for a hardier more disease-resistant cattle population.
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