Investigation of Regulation of Transfer RNA Gene Expression in Mammalian Cells: Utilization of a Human Nonsense Suppressor Transfer RNA

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ABSTRACT

The regulation of eukaryotic transfer RNA gene expression serves largely to adapt the abundance of each isoacceptor tRNA species to the codon frequencies and amino acid usages particular to different cell types. tRNA genes are independently transcribed and, even within an isoacceptor family, can display considerable differences in transcriptional activity *in vitro* which cannot be ascribed to the highly conserved nature of their intragenic promoter elements. In this regard, it is well documented that expression of transfer RNA genes can be dramatically influenced in a wide variety of species by 5' flanking sequences. Unfortunately, it remains uncertain what role the majority of these modulatory elements play in their normal cellular context since most of these studies have utilized *in vitro* transcription systems whose ability to faithfully reproduce cellular conditions is questionable. Unfortunately, it is difficult to monitor the expression of an individual tRNA gene *in vivo* since the gene for any particular isoacceptor is reiterated in the eukaryotic genome. To overcome this, a nonsense suppressor transfer RNA gene has been utilized to study the regulation of tRNA gene expression in mammalian cells. Specifically, the functional expression of a human serine amber suppressor tRNA gene has been quantified *in vivo* by assaying its ability to suppress an amber nonsense mutation in the *Escherichia coli* chloramphenicol acetyltransferase gene following

cotransfection in mammalian cells.

Through utilization of the aforementioned *in vivo* assay the suppression activity of a series of upstream deletion and substitution mutants of the human serine tRNA gene was determined. Mutant genes in which the 18 nucleotides 5' proximal to the coding region were deleted, and replaced with heterologous sequences, were 2 to 5 fold more active *in vivo* in comparison to the wild type gene. The serine tRNA gene constructs were also transcribed *in vitro* using HeLa cell nuclear extracts. The strong correlation between the transcriptional activity *in vitro* and functional expression *in vivo* of the various mutants indicates that this negative element acts by modulating the transcriptional activity of the serine tRNA gene and suggests that this element plays a physiologically relevant role within the mammalian cell. Second template competition experiments demonstrate that the element reduces the ability of the serine tRNA gene to stably sequester limiting transcription components. The results from several insertion mutants, which effectively alter the orientation and upstream position of this negative element, suggest that it acts in a dominant negative manner *in vivo* and *in vitro*.

The precise mechanism by which extragenic flanking sequences modulate tRNA gene transcription is unknown and a direct comparison of identified modulatory regions has failed to reveal conserved sequence elements that could be ascribed to being positive or negative transcriptional modulators. One possibility is that specific regulatory factors bind these upstream sequences and attenuate tRNA gene transcription, but the isolation of any such activity has been difficult to demonstrate since the composition of higher

eukaryotic tRNA gene transcription complexes is unknown. As an alternative approach to examining this possibility, an oligonucleotide containing the recognition site for the *Escherichia coli lac* repressor was inserted at various positions in the 5' flanking region of the human serine tRNA gene and the consequences of binding *lac* repressor on *in vitro* transcription by RNA polymerase III was investigated. *lac* repressor prebound to operator sites centered at positions -9, -15, -35, and -37 upstream of the mature tRNA coding region completely inhibited transcription by interfering with the formation or stability of transcription complexes. *lac* repressor also inhibited transcription of serine tRNA gene derivates containing operator sites at -9 and -15 when added following transcription complex assembly or during ongoing synthesis, but had no effects on the other tRNA gene derivatives if added subsequent to complex assembly. *lac* repressor prebound at position -43 and -46 partially inhibited transcription and redirected initiation to sites farther downstream. These results show that the functional human RNA polymerase III transcription complex extends at least 35 nucleotides upstream of the tRNA gene coding region and that the sequences surrounding the transcription start site remain accessible to DNA-binding proteins throughout multiple rounds of transcription. Normal transcription was restored with the addition of the allosteric inducer IPTG demonstrating that these effects require the continued presence of bound repressor protein. In addition, *lac* repressor inhibited the functional expression of the human serine tRNA gene *in vivo* since inclusion of a plasmid expressing the *lac* repressor in the above cotransfection assay resulted in inhibition of suppression activity of *lac* operator-linked

genes. This effect was also alleviated with IPTG. These results demonstrate that a heterologous DNA binding protein bound to 5' flanking sequences can be used to regulate the expression of a mammalain tRNA gene *in vivo* and *in vitro*. Although artificial in its approach, this study shows that this is a potential mechanism by which naturally occcuring regulatory proteins may modulate the expression of cellular tRNA genes. In addition, these experiments revealed information regarding the upstream spatial arrangement and topological boundaries of functional mammalian tRNA gene transcription complexes.

LIST OF PUBLICATIONS

The studies presented in this thesis have also been reported in the following publications:

Tapping, R.I., Syroid, D.E., and Capone, J.C. (1994) Upstream Interactions of Functional Mammalian tRNA Gene Transcription Complexes Probed Using a Heterologous DNA Binding Protein. Journal of Biological Chemistry **269**, 21812-21819.

Tapping, R.I., Syroid, D.E., Bilan, P.T., and Capone, J.C. (1993) The 5'-Flanking Region Negatively Modulates the *In Vivo* Expression and *In Vitro* Transcription of a Human tRNA Gene. Nucleic Acids Research **21**, 4476-4482.

Syroid, D.E., Tapping, R.I., and Capone, J.C. (1992) Regulated Expression of a Mammalian Nonsense Suppressor tRNA Gene *In Vivo* and *In Vitro* Using the *lac* Operator/Repressor System. Molecular and Cellular Biology **12**, 4271-4278.

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A	adenosine
Ala	alanine
α	alpha
Am	amber stop codon/suppressor
Arg	arginine
Asn	asparagine
Asp	aspartic acid
β	beta
bp	base pair(s)
BCIP	5-bromo-4-chloro-3-indoyl phosphate
Brf	B-related factor
C	cytosine
°C	degrees celsius
CAT	chloramphenicol acetyltransferase
Ci	curie(s)
CoA	coenzyme A
CPM	counts per minute
Δ	delta

Da	dalton
dATP	deoxyadenosine triphosphate
DEAE	diethylaminoethyl
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
g	force of gravity
G	guanosine
gal	galactosidase
γ	gamma
Glu	glutamic acid
Gly	glycine
GTP	guanosine triphosphate
HBS	HEPES buffered saline
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His	Histidine
hrs	hours
Ile	isoleucine
IPTG	isopropylthio-β-D-galactoside

k	kilo
Kb	kilobase
kDa	kilodalton
L	litre
lac	lactose (operon)
Leu	leucine
LTR	long terminal repeat
Lys	lysine
mat.	mature
MEM	minimal essential media
Met	methionine
μ	micro
m	milli
M	molar
min	minutes
mol	moles
n	nano
N.E.	nuclear extract
NP-40	Nonidet P-40
NTP	nucleotide triphosphate
O.D.	optical density

oligo	oligonucleotide
ONPG	O-nitrophenyl-β-D-galactoside
%	percent
p	pico
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
pol	RNA polymerase
pre.	precursor
rep	*lac* repressor
RNA	ribonucleic acid
RPM	revolutions per minute
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
Ser	serine
Su	suppressor
SV	simian virus
T	thymidine
TAF	TATA-binding protein associated factor
τ	tau
TBE	Tris-borate EDTA
TEN	Tris-EDTA sodium chloride

TBP	TATA-binding protein
tDNA	transfer RNA gene
TE	tris-EDTA
TF	transcription factor
Thr	threonine
TLC	thin layer chomatography
trans.	transcription
Tris	Tris(hydroxymethyl)aminoethane
tRNA	transfer ribonucleic acid
Trp	tryptophan
Tyr	tyrosine
UV	ultra-violet
Val	valine
v/v	volume per total volume
WT	wild type
w/v	weight per total volume
YT	yeast extract, tryptone

INTRODUCTION

1.1 Overview

The main function of the transfer RNA (tRNA) is delivery of the correct amino acid to the growing polypeptide chain during translation. To fulfill this role, and maintain the fidelity of protein synthesis, tRNAs must interact with many components of the translation machinery including ribosomes, messenger RNAs and elongation factors, in addition to specific aminoacyl tRNA synthases. Therefore, in order for protein synthesis to proceed optimally, the transfer RNA must be synthesized by the cell in sufficient quantities in order to respond to the codon complement of the messenger RNAs being translated. This concept is central to this thesis. There are many biosynthetic processes involved in the production of the final functional aminoacylated tRNA and the first few sections of this introduction will describe these processes with an emphasis on transcription. This introduction will then describe the cellular adaptation of tRNA abundance in response to codon usage. The final sections of this introduction will discuss the control of tRNA abundance through the regulation of transcription of the tRNA gene itself.

1.2 Organization of Transfer RNA Genes

Transfer RNA genes are constituents of complex and dispersed multigene families (reviewed in Sharp *et al.*, 1985). In addition, the gene for any particular tRNA isoacceptor is reiterated in the genome. Transfer RNA genes are dispersed irregularly throughout the genome, either singly or as small clusters. In fact, even the genes for any particular isoacceptor are located at more that one chromosomal locus. For example, saturation hybridization analysis of *Saccharomyces cerevisiae* genomic DNA displayed approximately 360 tRNA genes, with an average frequency of 8 genes per isoacceptor, whose overall distribution in the genome appears to be random (Guthrie and Abelson, 1982).

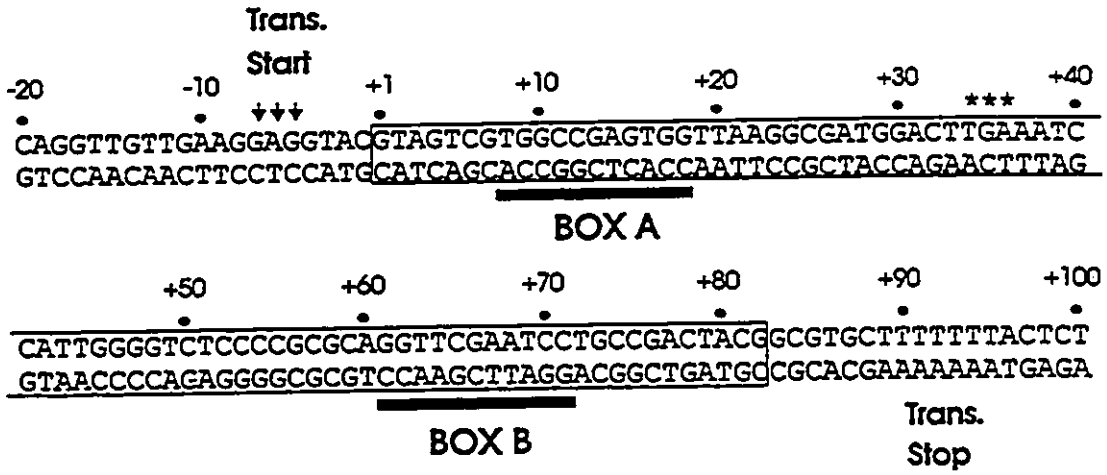
Over 1300 tRNA genes are contained within the human haploid genome representing about 60 different genes of 10 to 20 copies each (Hatlen and Attardi, 1971). The reiteration frequency and distribution of only a few human tRNA gene families has been determined. In the genome there are about 14 loci for $tRNA^{Tyr}$ (Van Tol and Beier, 1988) and at least 13 loci for the initiator $tRNA^{Met}$ (Santos and Zasloff, 1980), the $tRNA^{Glu}$ isoacceptors (Gonos and Goddard, 1990a), and two of the $tRNA^{Val}$ isoacceptors (Arnold *et al.*, 1986). At the other extreme, approximately 60 $tRNA^{Asn}$ genes are interspersed with other genes at only two loci on chromosome 1 (Buckland, 1989). In addition, there are several examples of heteroclusters of tRNA genes dispersed throughout the genome that differ in tRNA gene number and type (Pirtle *et al.*, 1993 and references therein). Thus, the organization and arrangement of tRNA genes in the

human genome is diverse and ranges from one tRNA gene megacluster, several tRNA gene heteroclusters and solitary tRNA gene family members widely dispersed on many different chromosomes.

1.3 Transfer RNA Gene Structure

In eukaryotes, transfer RNA genes are transcribed by RNA polymerase III (pol III) and are therefore, by definition, class III genes. The transcribed sequences of the tRNA gene are longer than the final mature sized transcript because the sites of transcription initiation and transcription termination lie upstream and downstream of the sequences encoding the mature tRNA, respectively. In addition, some eukaryotic tRNA genes contain introns. The extra nucleotides in the precursor transcript are subsequently removed by processing enzymes to yield the final functional mature tRNA (section 1.10). tRNAs are about 76 nucleotides in length which is suprisingly small given the large number of molecules with which they must interact. Thus, the nucleotide sequences within any tRNA are constrained by the interactions required for their function. A main determinant in tRNA recognition is their unique tertiary structure. Some of these concepts are illustrated in figure 1.3.1.

A surprising finding in the field of pol III transcription was that the promoter elements of many class III genes exist entirely within the coding sequences of the genes themselves, a discovery first made with the *Xenopus* 5S RNA gene (Sakonju *et al.*, 1980; Bogenhagen *et al.*, 1980). Shortly thereafter, the characterization of tRNA



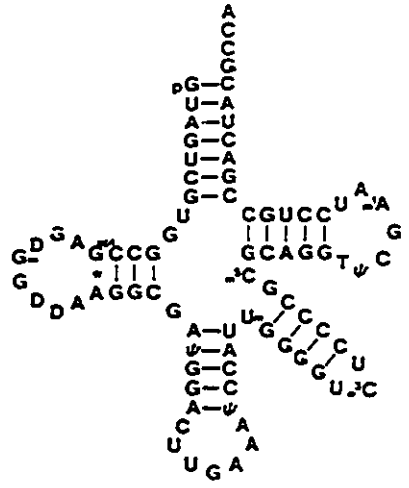


Figure 1.3.1 Diagram of a Human Serine tRNA Gene. The sequences shown are those of a human serine tRNA gene which is the focus of the studies described in this thesis. The top strand represents the nontemplate strand. Numbering of sequences is with respect to the first nucleotide present in the mature transcript which is taken as +1. The sequences encoding the mature transcript are outlined. The sequences of the box A and box B intragenic promoter elements are underlined in bold. The transcription initition sites are indicated by the arrows and transcription terminates within the stretch of seven consecutive T nucleotides of the nontemplate strand. The final mature tRNA transcript, with modified nucleosides, is shown under the gene in the typical cloverleaf form (adapted from Capone *et al.*, 1985). The sequences encoding the anticodon are indicated by the three astericks and were changed from TGA to CTA by site directed mutagenesis to generate the gene encoding the amber suppressor tRNA.

genes from a diversity of eukaryotes revealed an evolutionarily maintained split intragenic promoter made up of two conserved short sequence elements subsequently named box A and box B (Galli *et al.*, 1981; Hofstetter *et al.*, 1981; Sharp *et al.*, 1981). Other genes transcribed by pol III containing this promoter structure include the adenovirus VA gene and the human Alu1 gene family. The box A element constitutes nucleotide positions +8 to +19 with numbering starting at the 5' end of the mature tRNA. In all natural eukaryotic tRNA genes anywhere from 31 to 93 nucleotides can separate the box A element from the downstream 11 base pair box B element due to the presence of extra loops and, in some tRNA genes, introns (reviewed in Sharp *et al.*, 1985). It is important to note that because the promoter elements are intragencic, they also encode the sequences of conserved stem and loop structures required for tRNA function (Figure 1.3.1).

The box B element shows little variation among all tRNA genes (Sharp *et al.*, 1985) and a single point mutation is capable of resulting in a total absence of transcription (Newman *et al.*, 1983). This element has been demonstrated to be most critical for promoter strength and for the transcriptional commitment of the template (Schaack *et al.*, 1983; Sharp *et al.*, 1983). The box A element is more flexible with regard to nucleotide sequence and, although weaker in promoter strength, its position loosely selects the start site of transcription. This role for box A is best illustrated by the fact that if it is deleted, or placed too close to box B, another pseudo box A element will be selected resulting in a corresponding shift in the transcription initiation site (Baker *et al.*, 1987; Fabrizio *et al.*, 1987). The box A and box B promoter elements ultimately

act to recruit RNA polymerase III to the tRNA gene through the assembly of a transcription complex.

1.4 Transfer RNA Gene Transcription Complex Assembly

To establish a class III gene transcription complex several protein components, called transcription factors (TFs), are assembled in a sequential fashion on the template DNA. This was first demonstrated using separated mammalian pol III transcription components (Segall *et al.*, 1980; Lassar *et al.*, 1983; Fuhrman *et al.*, 1984). The first step in complex assembly is the binding of TFIIIC through recognition of the box A and box B promoter elements within the gene. Binding of TFIIIC is a prerequisite for the assembly TFIIIB whose addition greatly stabilizes the transcription complex. This stability has been demonstrated by the inherent ability of these complexes to resist a variety of challenges including large amounts of competitor DNA, high salt concentrations and extensive dilution. The resulting stable complex acts to recruit RNA polymerase III to the template for multiple rounds of transcription.

The transcription factor requirements and order of complex assembly is essentially conserved between yeast and higher eukaryotes. However, our understanding of the subunit composition, function, and spatial arrangement of these factors within higher eukaryotic pol III transcription complexes remains far less advanced than that in yeast. This is principally because, with the exception of yeast, the formation of functional pol III transcripition complexes using crude cell-free *in vitro* transcription

systems is extremely poor and does not improve upon separation and reconstitution of the individual components (addressed in Wolffe, 1991; Geiduschek and Kassavetis 1992). Yeast transcription components have also proven far more amiable to biochemical fractionation and reconstitution experiments. In addition, many yeast transcription components have been directly cloned through the advent of powerful yeast genetic techniques. Many of these advantages were predicted when the first cell free extract for pol III transcription was prepared from yeast cells (Klekamp and Weil, 1982).

1.5 Complex Assembly in Yeast

The binding of yeast TFIIIC relies solely on specific protein-DNA interactions between this factor and the box A and box B intragenic elements of the gene. These interactions can tolerate a wide variety of spacings between the box A and box B elements (Baker *et al.*, 1987) which has been attributed to the flexibility of TFIIIC itself (Camier *et al.*, 1990). The two DNA binding domains of TFIIIC from yeast, also called τ (*tau*), have been identified through limited proteolysis experiments (Marzouki *et al.*, 1986). When yeast TFIIIC is bound to a promoter and visualized by scanning electron microscopy it appears as a dumb-bell shaped molecule with two large DNA-binding domains separated by a thin flexible segment (Schultz *et al.*, 1989).

Biochemical characterization of TFIIIC has revealed that it is the largest and most complex transcription factor to date. The affinity-purified yeast factor is comprised of at least 5 polypeptides with masses of approximately 138, 131, 95, 65 and

55 kilodaltons (Gabrielson *et al.*, 1989; Parsons and Weil, 1990; Bartholomew *et al.*, 1990a, Conesa *et al.*, 1993). The 138 and 95 kDa subunits can be specifically crosslinked to tDNA by UV-irradiation (Gabrielson *et al.*, 1989) and photocrosslinking experiments have further mapped the 138-kDa subunit over the B block and the 95-kDa subunit over the A block (Bartholomew *et al.*, 1990a; 1991). These results indicate that the 95 and 138-kDa subunits are responsible for recognition of the A and B box elements, respectively. Precise positioning of the photoprobe in these studies has also determined that the 131-kDa subunit can be photocrosslinked to the region footprinted by TFIIIB making it a likely candidate for the recruitment of this factor. The position of the yeast TFIIIC subunits in an assembled tRNA gene transcription complex is schematically diagrammed in Figure 1.5.1.

Through yeast genetic techniques the three largest subunits of yeast TFIIIC have recently been cloned (Swanson *et al.*, 1991; Lefebvre *et al.*, 1992; Parsons and Weil, 1992; Marck *et al.*, 1993; Rameau *et al.*, 1994). The 131 kDa subunit contains a unique tetratricopeptide repeat (Marck *et al.*, 1993) and a mutation in this region affects the rate of recruitment of TFIIIB to the template further confirming the functional role of this subunit (Rameau *et al.*, 1994). Finally, all three of the cloned subunits of yeast TFIIIC appear to be phosphorylated *in vivo* (Conesa *et al.*, 1993).

The stablility of the yeast pol III transcription complex has been attributed to the DNA binding characteristics of TFIIIB itself. Footprinting *in vitro* of several yeast tRNA genes revealed protection of 40 base pairs of 5'-flanking DNA which was due to a protein that copurified with TFIIIB activity (Kassavetis *et al.*, 1989), and genomic

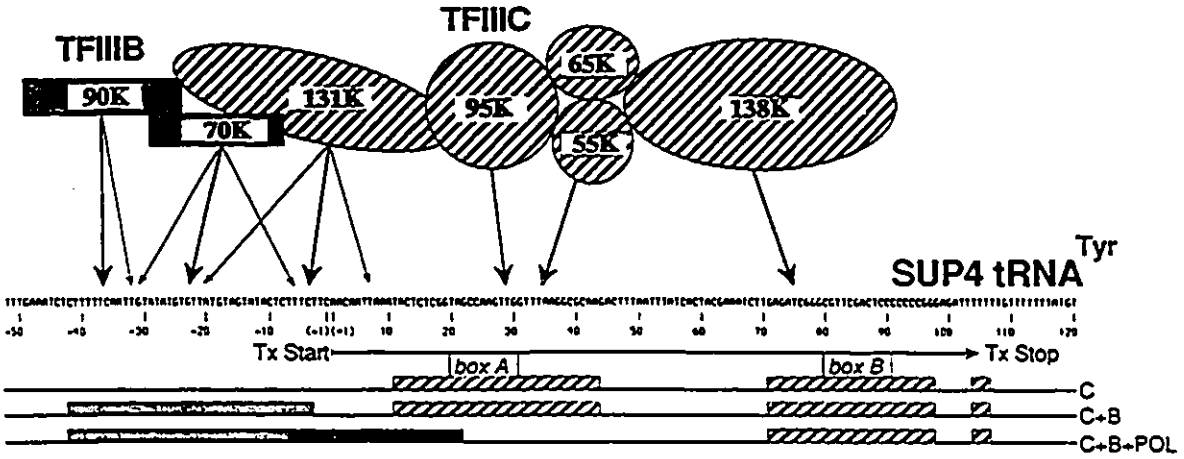


Figure 1.5.1 Schematic Diagram of the Assembled Yeast tRNA Gene Transcription Complex. The sequence shown is that of the nontemplate strand of a yeast $tRNA^{Tyr}$ gene. The position of the transcription start, transcription stop and box A and box B promoter elements are indicated. The rectangles beneath the gene indicate the regions of the gene which are footprinted at different stages of assembly of transcription complexes. A representation of the spatial arrangement of each of the individual polypeptide subunits of TFIIIC and TFIIIB are indicated above the sequence. Locations of DNA photoprobes that most efficiently crosslink individual subunits are indicated by thick arrows. Weaker photocrosslinking locations are indicated by thin arrows. Hatched, stippled and solid black shapes represent footprints or subunits of TFIIIC, TFIIIB and RNA polymerase III, respectively. (Adapted from Geiduschek and Kassavestis, 1992)

footprints of a yeast tRNA gene displayed this same protection (Huibregtse and Engelke, 1989). Using purified yeast components, it was subsequently demonstrated that TFIIIC can be selectively stripped, with heparin or high salt, from complexes assembled *in vitro*. In addition, the resulting TFIIIB-DNA complex was shown to be sufficent to recruit RNA polymerase III to the promoter for multiple rounds of transcription (Kassavetis *et al.*, 1990). This seminal study demonstrated that, in yeast, TFIIIB is the initiation factor proper, while TFIIIC is only required as an assembly factor. In fact, yeast TFIIIB is a remarkable factor which by itself displays no DNA binding ability, but, when recruited by TFIIIC binds DNA very tightly in a sequence independent manner.

Purification and characterization of yeast TFIIIB revealed two components of 90 and 70 kDa, both of which are required for heparin resistant DNA binding and the ability to recruit RNA polymerase III for transcription initiation (Kassavetis *et al.*, 1991; Bartholomew *et al.*, 1993). Photocrosslinking experiments, similar to those performed with TFIIIC, revealed that the 90 and 70 kDa polypeptides occupy the upstream region of the tRNA gene but on opposite faces of the DNA helix (Bartholomew *et al.*, 1991). These interactions are schematically diagrammed in Figure 1.5.1.

Genetic experiments in yeast demonstrated that TATA binding protein (TBP), the central RNA polymerase II transcription factor, is also essential for transcription from pol III promoters (Cormack and Struhl, 1992; Schultz *et al.*, 1992). In fact, a component of TFIIIB was cloned from yeast as a second-site suppressor of mutations in TBP (Buratowski and Zhou, 1992; Colbert and Hahn, 1992) and as a suppressor of a tRNA gene box A promoter mutation (Lopez-De-Leon *et al.*, 1992). The

gene product was named B-related factor (Brf) due to its amino acid sequence homology with the pol II-specific general factor TFIIB. Subsequent studies showed that Brf constitutes the properties of the 70 kDa component of TFIIIB and is recruited to the tRNA gene by the 131 kDa component of TFIIIC (Kassavetis *et al.*, 1992a).

Although TBP is required for TFIIIB to form a stable transcription complex (Huet and Sentenac, 1992), it is important to emphasize that the DNA binding properties of TBP are not important for its functional role as a subunit of TFIIIB (reviewed in Hernandez, 1993; Struhl, 1994). In fact, crosslinking data suggests that TBP acts by unmasking a cryptic DNA binding domain of Brf allowing it to interact upstream of the tRNA gene (Kassavetis *et al.*, 1992a). The subsequent addition of the 90 kDa subunit confers the full DNA binding and functional properties of yeast TFIIIB. Although these *in vitro* reconstitution experiments reveal interactions between the individual components, it is important to note that they may not reflect an actual assembly pathway *in vivo*. In fact, there is evidence that the subunits of yeast TFIIIB are already assembled *in vivo* since this factor does not readily separate into its components during purification (discussed in Kassavetis *et al.*, 1992a) and antibodies to TBP immunoprecipitate several polypeptides from yeast extracts, including those of 70 kDa and 90 kDa, that contain TFIIIB activity (Poon and Weil, 1993).

1.6 Complex Assembly in Mammalian Systems

As mentioned previously, the order of assembly and subsequent stability

of the pol III transcription complex has been conserved for all eukaryotes. In contrast to yeast, mammalian pol III transcription factors have proven difficult to purify and characterize even though their separated activities were first reported many years ago (Segall *et al.*, 1980). Human TFIIIC, purified from HeLa extracts, was initially shown to be made up of two functionally required components called IIIC1 and IIIC2 (Dean and Berk, 1987). It was discovered that TFIIIC2 binds to the promoter first, but can then recruit TFIIIB and TFIIIC1, in either order, to form the preinitiation complex (Dean and Berk, 1988). TFIIIC2 binds specifically to the box B promoter element, as determined by footprinting analysis, and the addition of TFIIIC1 was shown to extend this footprint over the box A element (Dean and Berk 1987; Yoshinaga *et al.*, 1987). Further characterization of TFIIIC2 activity, by sedimentation in sucrose gradients, indicated a molecular weight of approximately 400-500 kDa (Boulanger *et al.*, 1987). In agreement with this, highly purified IIIC2 activity contains 5 polypeptides with masses of 230, 110, 100, 80 and 60 kDa (Yoshinaga *et al.*, 1989). The largest of these polypeptides was identified as the box B binding subunit by UV crosslinking analysis (Boulanger *et al.*, 1987).

It is important to note that during this time other groups attempting to purify and characterize TFIIIC were obtaining quite different and contradictory results regarding its subunit composition (Cromlish and Roeder, 1989; Schneider *et al.*, 1989, 1990; discussed in Geiduschek and Kassavetis, 1992). For example, one group found that human TFIIIC activity consisted of a single polypeptide of 126 kDa which, by itself, bound the box B promoter element (Cromlish and Roeder, 1989). These contradictory

outcomes typify the difficulties in the the purification and characterization of the apparently complex mammalian pol III transcription components. The extensive purification of a more active form of TFIIIC (Hoeffler *et al.*, 1988) revealed a composition of five polypeptides of 220, 110, 102, 90 and 63 kDa, the largest of which has been cross-linked to the box B element (Kovelman and Roeder, 1992). These TFIIIC subunit sizes are in close agreement with those initially reported for the compostion of TFIIIC2 (Yoshinaga *et al.*, 1989). Furthermore, an additional activity required for pol III transcription was recently isolated by this group from a classical HeLa extract TFIIIB fraction (Chiang *et al.*, 1993). This activity, called TFIIIB1, appears to be functionally equivalent to the previously identified TFIIIC1 fraction (discussed in Lagna *et al.*, 1994). These recent observations appear to reconcile the existing data about the composition of human TFIIIC.

Although the mammalian pol III transcription components have proven difficult to clone, the sequence of the 220 kDa box B binding subunit of human and rat TFIIIC has recently been reported (L'Etoile *et al.*, 1994; Lagna *et al.*, 1994). The full length protein sequence obtained lacks convincing homology to any known structural motifs including any known DNA binding domains (Lagna *et al.*, 1994). Furthermore, the amino acid sequence showns surprisingly little homology with that of the 138 kDa box B binding subunit of yeast TFIIIC. This indicates that yeast and human TFIIIC may have diverged during evolution.

Mammalian tRNA gene transcription complexes assembled with HeLa extracts have been purified by ultracentrifugation in glycerol gradients and both TFIIIB

and TFIIIC in these complexes appear to be stable to high salt (Jahn *et al.*, 1987). Thus, in contrast to yeast, the stability of pol III transcription complexes assembled in HeLa cell extracts appears to be a collective property of both TFIIIC and TFIIIB. Unquestionably, protein-protein interactions are important for transcription complex stablility. In fact, cosedimenting complexes containing TFIIIC, TFIIIB and RNA polymerase III have been isolated from HeLa cell extracts in the absence of DNA (Wingender *et al.*, 1986). The only photocrosslinking study of the mammalian pol III complex was performed using photoaffinity labelled nascent RNA which, as expected, crosslinked the two largest subunits of RNA polymerase III, but provided little information about the position of transcription components on the gene (Bartholomew *et al.*, 1990b). Footprints of tRNA genes using purified mammalian TFIIIC show clear protection over the B box element (Fuhrman *et al.*, 1984; Carey *et al.*, 1986), however, similar footprints involving TFIIIC plus TFIIIB, RNA polymerase III, or both have not been published and are reported to not be reproducible (Carey *et al.*, 1986).

Until recently, the only study on the composition of mammalian TFIIIB was an activity that copurified with a single polypeptide of 60 kDa (Waldschmidt *et al.*, 1988). This situation changed rapidly with the finding that TBP, the central RNA polymerase II transcription factor, is required for RNA polymerase III mediated transcription of TATA-less class III genes containing internal promoter elements (reviewed in Sharp, 1992; White and Jackson, 1992a; Hernandez, 1993; Rigby, 1993). This was first demonstrated independently, and before the reports on the involvement of TBP in RNA polymerase III transcription in yeast (White *et al.*, 1992a; White *et al.*,

1992b). This seminal finding quickly provoked studies aimed at determining how the TATA-binding protein is involved in transcription of class III genes lacking a TATA-box (White and Jackson, 1992b; Simmen *et al.*, 1992; Lobo *et al.*, 1992; Taggart *et al.*, 1992). The collective discovery of this work was that TBP is in fact a component of TFIIIB.

In spite of this large volume of work the exact composition of mammalian TFIIIB is still unknown. One group discovered that pol III transcription requires a TBP complex that is made up of at least three TBP associated polypeptides (TAFs) of 150, 82 and 54 kDa (Lobo *et al.*, 1992). Others have found that classical pol III transcription involves a minimum of two TAFs, one tightly associated 172 kDa protein and another undefined loosely associated activity (Taggart *et al.*, 1992). More recently, the composition of TAFs has been determined by affinity purifying the TAF-TBP complexes from HeLa cells that express TBP as an epitope tagged fusion protein (Chiang *et al.*, 1993). Collectively, the simplest interpretation of these results is that TFIIIB is composed of TBP, a tightly associated 172 kDa TAF and three additional loosely associated TAFs of 96, 87 (82), and 60 (54) kDa. These studies are complicated by the fact that TBP is found in a large variety of complexes in extracts of mammalian cells (reviewed by Rigby, 1993). In fact, using a combination of conventional and immunoaffinity chromatography, it has been reported that the 172 kDa TAF does not appear to be a component of TFIIIB but is probably a component of the pol II general factor TFIID (Timmers *et al.*, 1992; Meyers and Sharp, 1993).

1.7 Additional Transcription Components

There is evidence for auxillary pol III transcription components from yeast, human and other eukaryotic sources. Recently, an additional transcription factor was identified from yeast nuclear extracts which is functionally distinct from TFIIIB and TFIIIC. This factor, denoted TFIIIE, is essential for single and multiple rounds of transcription from tRNA and 5S genes but does not play a role in the formation of stable preinitiation complexes (Dieci *et al.*, 1993). In addition, a transcription component which stimulates transcriptional activity by binding to the upstream region of 5S and tRNA genes has been isolated from HeLa extracts and shown to be distinct from all known pol III transcription factors (Oei and Pieler, 1990).

The silk gland of the silkworm *Bombyx mori* is a highly characterized pol III transcription system whose extensive fractionation has revealed an addition transcription component which is required for tRNA gene expression. This factor, called TFIIID, is essential for the formation of a full transcription complex which can occur with TFIIIB and TFIIID, or with TFIIIC and TFIIID as starting points (Ottenello *et al.*, 1987). Although a direct correspondence has not been demonstrated, TFIIID appears to be at least functionally homologous to the separable box B binding activity of human TFIIIC called TFIIIC2 (Dean and Berk, 1988). However, unlike human TFIIIC1 and TFIIIC2, reconstituted silkworm TFIIIC and TFIIID has an unusual footprint which extends far outside the coding region of the tRNA gene and into the 3' flanking sequences (Young *et al.*, 1991). A functional correlation between the silkworm, yeast

and human transcription factors has been proposed (Sprague, 1992).

1.8 Recruitment of RNA Polymerase III

RNA polymerases I, II and III are recruited to promoters through recognition of the assembled transcription factors, where they bind template DNA and nucleotide triphosphate substrates and catalyze the synthesis of RNA. All three eukaryotic RNA polymerases are large multisubunit enzymes composed of two very large subunits and several smaller polypeptides, some of which are common to all three enzymes (reviewed in Sentenac, 1985; 1992). Eukaryotic RNA polymerases have been most extensively characterized from *Saccharomyces cerevisiae*. RNA polymerase III appears to be the most complex of the three and consists of at least 15 peptide subunits of which a number have been cloned (reviewed in Sentenac *et al.*, 1992). The fact that the two largest subunits of both yeast pol II and yeast pol III display amino acid sequence homology to the the largest subunits of RNA polymerase from *Escherichia coli* underscores the evolutionary conservation of the enzyme (Allison *et al.*, 1985). Nevertheless, based on size and antigenic cross-reactivity, several subunits appear to be unique to pol III and are presumed to be involved in interactions which recruit the enzyme specifically to class III genes (reviewed in Gabrielson and Sentenac, 1991).

Recruitment of RNA polymerase III requires the prior assembly of a transcription complex on the gene to be transcribed. Yeast tRNA genes require both the 70 and 90 kDa subunits of TFIIIB assembled upstream for recruitment of pol III.

Footprinting and photocrosslinking analyses of yeast pol III complexes have shown that while 40 base pairs of DNA upstream of the gene is strongly protected, the transcription initiation site itself is largely unprotected until the addition of RNA polymerase III (Kassavetis *et al.*, 1989; 1990). Not suprisingly, the two largest subunits of RNA polymerase III dominated photocrosslinking studies of a yeast $tRNA^{Tyr}$ gene and at least nine different subunits of the enzyme could be crosslinked from photoprobes placed along the nontranscribed strand (Bartholomew *et al.*, 1993). In addition, a large number of pol III subunits were crosslinked from a single photoactive nucleotide (Bartholomew *et al.*, 1993; 1994). These results suggest that after the recruitment of RNA polymerase III the transcription complex comprises an ensemble of different structural states, rather than a single entity (Bartholomew *et al.*, 1994). These authors propose that these multiple states are generated by polymerase scanning for a transcription initiation site.

1.9 Transcription Initiation, Elongation and Termination.

Initiation of transcription of tRNA genes usually takes place at a purine residue within about 10 nucleotides upstream of the sequences encoding the mature tRNA. Once pol III is recruited to the template, DNA melting takes place at the transcription start site and the complex converts from a closed to an open state. This transition has been analysed on a $tRNA^{Tyr}$ gene using purified yeast pol III components and permanganate hyperreactivity assays (Kassavetis *et al.*, 1992b). This work has shown that this transition is an endothermic process in which the transcription bubble

extends 11 base pairs either side of the initiation site. After DNA melting there is evidence for two forms of post-initiation ternary complexes; those which are sensitive to polyanions such as heparin and have short RNA chains (less than 10 nucleotides), and those which are resistant to heparin and have longer RNA chains (Kassavetis *et al.*, 1992b). Unfortunately, it has not been possible to perform similar analyses with mammalian pol III complexes, however, after complex assembly a transition sensitive to 0.5 percent Sarkosyl has been revealed, the nature of which is unknown (Kovelman and Roeder, 1990). The addition of Sarkosyl or heparin after this transition has been reported to prevent reinitiation by mammalian RNA polymerase III thereby limiting transcription to a single round (Kovelman and Roeder, 1990). Any step leading to productive initiation, including the aforementioned transitions, can serve as control points through which RNA polymerase III transcription can be regulated.

Very little is known about elongation, termination and release of RNA polymerase III from the template DNA. Since RNA polymerases are enzymes, they all have the ability to process backwards yielding back the nucleoside substrates of RNA synthesis. In addition, prokaryotic RNA polymerase and eukaryotic RNA polymerase II have been shown to possess a second chain-retracting process that involves hydrolytic cleavage generating short chains of RNA (reviewed in Kassavetis and Geiduschek, 1993). Recently, it was demonstrated that RNA polymerase III also possesses this intrinsic activity (Whitehall *et al.*, 1994). An understanding of what controls these processes is important since transcription elongation is emerging as an important mechanism of control for eukaryotic gene expression (reviewed in Krumm *et al.*, 1993). In this regard,

our understanding of elongation by RNA polymerase III is less advanced, however, a thorough *in vitro* kinetic analyis of the rate of elongation by yeast pol III was recently undertaken which provided evidence that pol III can switch repeatedly between rapidly stepping and slowing stepping states during elongation of a single RNA molelcule (Matsuzaki *et al.*, 1994).

One of the major questions still outstanding in the pol III field is how the polymerase transcribes through an intragenically assembled transcription complex. It has been demonstrated that SP6 phage RNA polymerase transcribes through a complete *Xenopus* 5S RNA gene transcription complex with the structure of the complex retained, or at least restored, after passage (Wolffe *et al.*, 1986). Furthermore, yeast pol III initiated from a 3' overhanging end of linear DNA can transcribe through a $tRNA^{Tyr}$ gene bound by TFIIIC with only a very minor delay in elongation rate (Bardeleben *et al.*, 1994). Neither of these studies addresses the method by which RNA polymerase III naturally transcribes through a fully assembled class III gene transcription complex. Since yeast TFIIIB is the only factor required for multiple rounds of transcription initiation, and makes remarkably stable interactions outside of the transcribed gene, it can be envisioned that the intragenically bound TFIIIC is simply displaced by the transcribing polymerase and is no longer required or part of the yeast pol III elongation complex.

Termination is an important step in transcription since it involves arrest of elongation, release of the nascent RNA product and dissociation of the polymerase for another round of reinitiation. Termination of class III genes takes place at the first stretch of consecutive thymidine residues in the non-template strand (reviewed in

Geiduschek and Tocchini-Valentini, 1988). Genetic and biochemical studies indicate that RNA polymerase III itself is involved in recognizing and utilizing this termination signal (Cozzarelli *et al.*, 1983 and James and Hall, 1990). Another study has shown that recognition of the pol III termination signal can be experimentally uncoupled from polymerase release since the latter, but not the former, is dependent on RNA strand displacement during transcription elongation (Cambell and Setzer, 1992). In a groundbreaking study it was discovered that a 50-kDa phosphoprotein, known as the La antigen, is required for the synthesis of the terminal stretch of uridine residues within nascent pol III transcripts and for the efficient termination of transcription (Gottlieb and Steitz, 1989a; 1989b). A subsequent study, using immobilized templates, has revealed that the La protein increases both the utilization of the pol III termination signal and the efficiency of transcript release, thereby promoting efficent use of both template and mammalian RNA polymerase III (Maraia *et al.*, 1994).

1.10 Transfer RNA Processing, Modification and Aminoacylation.

Since tRNA genes are transcribed into molecules which are larger than the final product, the precursor tRNA transcript must be processed in order to yield the functional mature tRNA required in protein synthesis. These processing reactions include removal of the extra sequences from the 5' and 3' ends of the RNA, the addition of the trinucleotide CCA at the 3' end, and for some tRNAs the removal of introns. Furthermore, the nucleosides in tRNAs are extensively modified.

Despite extensive work there are major gaps in the understanding of tRNA gene processing pathways (Deutscher, 1984). Since the availability of tRNA processing mutants is largely confined to *Escherichia coli* much more is known about the details of prokaryotic tRNA maturation (reviewed in Deutscher, 1984; 1990). Most eukaryotic tRNA processing studies have been carried out *in vitro* using crude extracts, although some *in vivo* processing studies have introduced tRNA genes by injection into *Xenopus laevis* oocytes or by transfection into various cells. These studies have revealed that a single enzyme, called RNase P, is responsible for maturation of the 5' termini of transfer RNAs through site specific endonucleolytic cleavage of primary tRNA transcripts (reviewed in Pace and Smith, 1990 and Altman *et al.*, 1993). In addition, exoribonucleases acting at the 3' termini of tRNA precursors have been described in several eukaryotic systems, however, none have been purified or characterized (Deutscher, 1990). After trimming of the 3' end of the tRNA precursor, an enzyme called tRNA nucleotidyltransferase carries out the addition of the trinucleotide CCA which subsequently serves as the site of attachment for the amino acid to the tRNA and participates in the aminoacyl transfer reaction on the ribosome (reviewed in Deutscher, 1990). A primary determinant in tRNA processing appears to be the ability of the precursor to form a tRNA-like conformation (Koski and Clarkson, 1982; Nishikura *et al.*, 1982).

The nucleosides within the tRNA transcript are highly modified and about 75 different modifications have been identified in tRNAs all of which are derivatives of the normal nucleosides adenosine, guanosine, uridine and cytosine (Persson, 1993).

Modification of nucleosides surrounding the anticodon have been shown to influence translational efficiency, translational fidelity, reading frame maintenance and codon choice, while modified nucleosides outside this region are believed to stabilize tRNA conformation (reviewed in Bjork *et al.*, 1987). Thus, tRNA modification appears to have an important role in fine tuning the activity of the tRNA. The diversity of proteins with which tRNAs interact is at least one reason for their complex content of modified nucleosides.

Several enzymes involved in tRNA processing are present in *Xenopus* oocyte nuclei (De Robertis *et al.*, 1981; Solari and Deutscher, 1982). In addition, precursor tRNAs with 5'-leader and 3'-trailer sequences are not transported through the nuclear membrane and accumulate inside the oocyte nucleus (Melton *et al.*, 1980; Tobian *et al.*, 1985). These experiments indicate that tRNA processing takes place within the nucleus prior to transport to the cytoplasm. In this regard, a *Saccharomyces cerevisiae* gene involved in tRNA splicing has recently been identified as a component of the nuclear matrix (Shen *et al.*, 1993).

Once outside the nucleus, each isoacceptor tRNA is aminoacylated with the appropriate amino acid which is subsequently incorporated into the growing polypeptide chain. The specificity of tRNA identity depends on the each tRNAs productive interaction with the correct aminoacyl-tRNA synthetase, an action which is central to the maintenance of translational fidelity. In addition to being important for recognition by processing enzymes, the tertiary structure of the tRNA plays an active role in the recognition of the tRNA by aminoacyl-tRNA synthases (reviewed in Hou, 1993).

Discrimination between tRNAs is governed by an intricate set of rules because tRNAs are structurally quite similar. Recently, much progress has been made in identifying the major determinants of tRNA identity (reviewed in McClain, 1993; Saks *et al.*, 1994).

Since tRNAs are synthesized as precursors which undergo processing, base pair modifications and charging to yield the final functional aminoacylated tRNA then conceivably their abundance can be regulated at any step in this biosynthetic pathway. In this regard, there is an energetic advantage to the cell to control the level of functional aminoacyl tRNAs transcriptionally, at the beginning of their biosynthesis, rather than at a downstream processing, modification or aminoacylation step. Many earlier studies have shown that changes in chromatographic patterns of tRNA isoaccepting species are associated with differentiation, sporulation, phage and viral infection, hormonal stimulation, malignant degeneration, as well as different cell culture conditions (reviewed in Littauer and Inouye, 1973). It is important to note that such alterations in chromatographic patterns can occur through base pair modifications or through changes in the abundace of some tRNA species or in the levels of their cognate synthetases.

1.11 Transfer RNA Abundance and Codon Usage

Although almost all organisms have the same codon assignments for each amino acid, the preferred use of individual codons varies greatly among taxonomic groups (reviewed Andersson and Kurland, 1990). The nonrandom choices of synonomous codons in *Escherichia coli* and yeast genomes have been related to the

availability of tRNA molecules within the cell (Ikemura 1981a, 1981b, 1985; Ikemura and Ozeki, 1983). In this regard, a strong correlation between the preferential use of certain codons, found in genes which are highly expressed, and the abundance of the corresponding tRNA isoacceptor species has been well documented in *E. coli* grown under laboratory conditions (Ikemura, 1981a, 1981b, 1985; Ikemura and Ozeki, 1983). *E. coli* contains 79 tRNA genes in an arrangement where abundant tRNAs are generally expressed from three or four genes while minor tRNAs are often expressed from a single gene (Komine *et al.*, 1990). However, gene specific dose effects cannot completely account for the observed variations in abundance of different tRNA classes found within *E. coli* since there is often a 10 to 20 fold difference in abundance between major and minor tRNA (Emilsson *et al.*, 1993). In addition, it has been demonstrated that the major codons for leucine, glycine, proline and arginine increase in abundance as growth rate increases, while tRNAs cognate to the minor codons for these same amino acids decrease (Emilsson and Kurland, 1990; Emilsson *et al.*, 1993). This biased usage of both codons as well as their cognate tRNA species has been viewed as an arrangement to optimize the kinetic efficiency of translation in order to impove the growth efficiency of bacteria in rich media (discussed in Kurland, 1987, 1991; Andersson and Kurland, 1990). tRNA abundance is also regulated as part of the bacterial stringent response, in that bulk tRNA synthesis is curtailed following amino acid starvation (Ikemura and Dahlberg, 1973).

Significant correlations between synonymous codon frequencies and the distribution of the corresponding isoaccepting tRNA species have been found in many

different tissues from a variety of animals (compiled in Chavancy *et al.*, 1979; Hatfield *et al.*, 1979). Moreover, there are many instances where the distribution of isoaccepting tRNA species have been shown to adapt during cell differentiation in response to the synthesis of proteins with specific codon usages (reviewed in Garel, 1974). For example, during lens development epithelial cells differentiate into fiber cells resulting in the increased synthesis of phenylalanine rich proteins called lens crystallins (Delcour and Papaconstantinou, 1972). This differentiation event has been shown to coincide with a two fold increase in total $tRNA^{Phe}$ (Ortwerth *et al.*, 1975), which has been attributed to the appearance of a new $tRNA^{Phe}$ species (Lin *et al.*, 1980). The functional adaptation of tRNA population to codon usage has also been shown to take place during differentiation of mammalian erythrocytes to reticulocytes in preparation for the synthesis of hemoglobin (Smith, 1975; Hatfield *et al.*, 1982), and in specific tissues of chick embryos in preparation for the synthesis of collagen (Carpousis *et al.*, 1977).

The most striking example of an altered tRNA pool in response to biased codon usage exists within the silk gland of the silkworm, *Bombyx mori*. The posterior part of the mature silk gland of the larva produces massive quantities of fibroin, the principle protein component of the silk fiber, in preparation for spinning of the cocoon (Toshiro *et al.*, 1968). Fibroin is composed of 44 percent glycine, 29 percent alanine and 12 percent serine (Sprague, 1975) and a correlation between this composition and the total tRNA corresponding to these three isoacceptors has been demonstrated within the *B. mori* silk gland (Garel *et al.*, 1970; Chevallier and Garel, 1979). In addition, these isoacceptors have been shown to appear specifically during the period of silk gland

specialization (Garel *et al.*, 1971). Moreover, analysis of the mRNA sequence of fibroin (Suzuki and Brown, 1972) revealed a quantitative relationship between the frequency of synonomous codons and the distribution of the corresponding preponderant isoaccepting tRNA species for alanine, glycine and serine (Garel, 1976).

Thus, cells that synthesize predominantly crystallins, hemoglobin, collagen and silk fibroin have very different tRNA contents in which the isoacceptor levels reflect their requirements for the incorporation of certain amino acids in the corresponding proteins. This functional adaptation of tRNA population presumably allows the cell to obtain optimum efficiency during protein biosynthesis (discussed in Kurland, 1987, 1991). Indeed, variations in the rate of elongation of nascent proteins have been correlated with the concentrations of required amino acids (Varenne *et al.*, 1984).

1.12 Regulation of Transfer RNA Gene Expression

The diverse arrangement of tRNA genes in the eukaryotic genome (section 1.2) suggests that tRNA gene organization is not a method by which their expression is controlled. In addition, there is no experimental evidence to date to support the notion that tRNA gene organization plays any role in the regulation of eukaryotic tRNA gene expression. While there is no obvious reason for the high level of tRNA gene redundancy in the genome, it can be envisioned that it may be required in order to maintain high tRNA levels during peak periods of protein synthesis (Sharp *et al.*, 1985). The coding sequences between members of any isoacceptor tRNA gene family are, by

necessity, highly homologous and have been conserved throughout evolution. Conversely, the flanking sequences between these members are usually vastly divergent. For example, within the $tRNA^{Val}$ gene family, which is the most thoroughly studied human tRNA gene family, only two of the 13 cloned members shown any significant homology between their flanking sequences (Thomann *et al.*, 1989). In fact, it has been suggested that the evolutionary pressures of conserving a particular flanking sequence have probably arisen from mechanisms of transcriptional regulation (Sharp *et al.*, 1985). In this regard, human transfer RNA genes from the same isoacceptor family have been shown to exhibit vastly different transcriptional activities in extracts prepared from HeLa cell nuclei. These studies included three human $tRNA^{Lys}$ genes (Doran *et al.*, 1987), two human $tRNA^{Gly}$ genes (Doran *et al.*, 1988) and many members of the human $tRNA^{Val}$ gene family (Arnold *et al.*, 1986; Thomann *et al.*, 1989; Kacar *et al.*, 1992). Due to the highly conserved nature of the coding sequences the different individual transcriptional activities within these isoacceptor tRNA gene families is presumed to be a property of the unique extragenic flanking sequenes of each gene.

A large volume of research has directly demonstrated that flanking sequences outside of tRNA genes modulate their expression (reviewed in Sharp *et al.*, 1985; Geiduschek and Tocchini-Valentini, 1988; Geiduschek and Kassavetis, 1992). In the majority of these studies the *in vitro* transcriptional activity of tRNA genes with various deletions in their natural flanking sequences have been determined using a wide variety of eukaryotic *in vitro* transcription systems. For example, one of the human valine tRNA genes has been shown to possess a strong positive modulatory element

upstream of the gene when transcribed in extracts prepared from Hela cell nuclei (Arnold and Gross, 1987; Arnold *et al.*, 1988). Short regions upstream of a mouse $tRNA^{His}$ and a mouse $tRNA^{Asp}$ gene have been shown to modulate their activity when transcribed in HeLa extracts (Morry and Harding, 1986; Rooney and Harding, 1988). Similarily, a human $tRNA^{Met}$ and a human $tRNA^{Glu}$ both contain positive modulatory elements upstream (Wahab *et al.*, 1989; Gonos and Goddard, 1990b). Using yeast extracts, the transcriptional activity of various deletion mutants of a yeast $tRNA^{Leu}$ gene were compared which revealed a positive modulator of transcription between 15 and 2 base pairs upstream of the coding region (Raymond and Johnson, 1983; Johnson and Raymond, 1984). Upstream modulatory elements have also been discovered for several *Drosophilia* tRNA genes when transcribed in homologous cell free extracts including a $tRNA^{His}$ gene (Cooley *et al.*, 1984), two $tRNA^{Val}$ genes (Sajjadi *et al.*, 1987; Sajjadi and Spiegelman, 1987, 1989; MacKay, *et al.*, 1988), two $tRNA^{Arg}$ genes (Dingermann *et al.*, 1982; Schaack *et al.*, 1984; Schaack and Soll, 1985), and several $tRNA^{Asn}$ genes (Lofquist and Sharp, 1986; Lofquist *et al.*, 1988). Using *Xenopus* cell extracts short positive and negative modulatory elements have been identified upstream of a *Drosophilia* $tRNA^{Lys}$ gene (DeFranco *et al.*, 1981) and a *Xenopus* $tRNA^{Met}$ gene (Hipskind and Clarkson, 1983), respectively.

Extragenic flanking sequences of tRNA genes have been shown to confer developmentally regulated transcription. Four independent tyrosine tRNA genes, whose unprocessed transcripts are distinguishable from one another by their different 5' leader and intervening sequences, have been identified in *Xenopus*. Two of the $tRNA^{Tyr}$ genes

are specifically expressed in oocytes and two are expressed in somatic cells (Stutz *et al.*, 1989). The unique 5'-flanking sequences of a somatic type $tRNA^{Tyr}$ gene and an oocyte type $tRNA^{Tyr}$ gene have been found to be responsible for the observed six fold higher activity of the former gene in somatic S-100 extracts (Gouilloud and Clarkson, 1986). Phosphorylation of TFIIIC has been implicated in the selective inactivation of oocyte type $tRNA^{Tyr}$ gene expression during *Xenopus* development (Reynolds, 1993).

A direct correspondence between tRNA abundance and the modulation of tRNA gene expression by extragenic flanking sequences is best illustrated in the silk gland of *B. mori* where the levels of glycine, alanine, and serine isoacceptor tRNAs have been shown to increase in preparation for the synthesis of the silk protein fibroin (section 1.11). It was subsequently discovered that *B. mori* possesses two classes of alanine tRNAs which are identical in their anticodons, but differ by a single nucleoside in the anticodon stem allowing them to be electrophoretically separated (Sprague *et al.*, 1977). Surprisingly, one class of alanine tRNA is constitutive and found in all silkworm cell types, and the other is found only in the posterior part of the silk gland and contributes to the synthesis of fibroin (Sprague *et al.*, 1977). In silk gland extracts these two $tRNA^{Ala}$ gene classes display striking differences in their transcriptional behaviour *in vitro* which has been shown to be confered by the unique sequences found upstream of each gene (Young *et al.*, 1986). Subsequent studies have shown that AT rich upstream sequences have a strong positive effect on the transcription of one of these silk gland specific $tRNA^{Ala}$ genes in *B. mori* silk gland extracts (Sprague *et al.*, 1980; Palida *et al.*, 1993). Similarly, a different AT rich upstream element has been shown to positively

modulate the transcription of another silk gland specific $tRNA^{Ala}$ gene (Larson *et al.*, 1983). Thus, extragenic flanking sequences of individual alanine tRNA genes have been shown to restrict their expression specifically in the *B. mori* silk gland and appear to be responsible for the accumulation of this isoacceptor tRNA in preparation for silk fibroin production.

Much of our understanding of the sequences controlling tRNA gene transcription have come from *in vitro* studies using cell free transcription systems. Due to the inherent limitations of these systems to faithfully reproduce cellular conditions it remains uncertain what role the majority of these modulatory elements play in their normal cellular context. In fact, the *in vitro* transcriptional properties of tRNA genes usually only poorly correlate with their *in vivo* behaviour. For example, although a measurable difference in transcriptional activity is seen between the oocyte and somatic type $tRNA^{Tyr}$ genes in somatic cell extracts, both gene types are still transcriptionally active (Goullioud and Clarkson, 1986) even though oocyte type tyrosine tRNA precursors are totally absent in postembryonic somatic cells (Stutz *et al.*, 1989). Indeed, these researchers have questioned whether the oocyte-somatic switch of tyrosine tRNA expression can be reproduced in extracts. In the *B. mori* system, the ability to direct transcription *in vitro* from the silkgland specific $tRNA^{Ala}$ gene is extremely sensitive to variation between extract preparations. In fact, different silk gland extracts that are nearly equal in their transcriptional activity on constitutive $tRNA^{Ala}$ genes, vary widely in their activity on silkgland specific $tRNA^{Ala}$ genes (Young *et al.*, 1986).

1.13 Examining Transfer RNA Gene Expression *In Vivo*

The *in vivo* modulation of tRNA gene transcription is difficult to demonstrate due to the fact that there are several copies of each isoacceptor tRNA gene in the eukaryotic genome (section 1.2). To overcome this gene redundancy problem, primer extension (Dingermann and Nerke, 1987) or chromatographic separation (Francis and Rajbhandary, 1990) of heterologously expressed tRNA genes have been used to examine the expression of individual tRNA genes. Unfortunately, the biological significance of the expression of a heterologously expressed tRNA gene is questionable. For example, in the context of its own extragenic flanking sequences a human $tRNA^{Met}$ gene which is expressed in mammalian cells (Drabkin and Rajbhandary, 1985), is not expressed in yeast cells despite the presence of appropriate internal box A and box B consensus sequences (Francis and Rajbhandary, 1990). In addition, a large number of *in vitro* studies have shown that the effect of extragenic flanking sequences is dependent on the use of homologous transcription systems (Sprague *et al.*, 1980; Dingermann *et al.*, 1982; Raymond and Johnson, 1983; Schaack *et al.*, 1984; Cooley *et al.*, 1984). For example, in contrast to *B. mori* extracts, silkgland specific $tRNA^{Ala}$ genes do not require natural 5'-flanking sequences for correct transcription in *Xenopus* extracts (Sprague *et al.*, 1980).

Other researchers have used primer extension of synthetic tRNA genes to examine the transcription of individual tRNA genes and overcome the redundancy of genetic information for certain isoacceptor species (Marchalek and Dingermann, 1988;

Krieg *et al.*, 1991). Using this approach a tRNA gene was tagged by insertion of an intron-like sequence that could not be spliced out from the precursor transcript (Kreig *et al.*, 1991). Thus, quantitation of these precursor transcripts reflected the transcriptional activity of this gene without the complication of downstream processing. The disadvantage to this approach is that since the transcription elements are intragenic, changes to the coding sequences of the tRNA alters the promoter structure of the gene itself. Primer extension coupled with sequence analysis has also been utilized to compare the *in vivo* expression of the various members of the human valine tRNA gene family based on the knowledge of each genes unique immediate upstream sequences giving rise to identifiable precursor tRNAs (Schmutzler and Gross, 1990). This method suffers from the fact that tRNA transcripts are highly processed and the majority of tRNAs in the cell are found in the mature form (Dingermann and Nerke, 1987; Schmutzler and Gross, 1990). Furthermore, this method requires that the immediate upstream sequences of a majority of constituents of a single isoacceptor tRNA gene family be known and differ considerably between individual members.

1.14 Utilization of Suppressor Transfer RNAs

Termination of polypeptide chain synthesis is encoded by the three nonsense codons UAG (amber), UAA (ochre), and UGA (opal). These codons are recognized and decoded by protein release factors in the cell. Transfer RNAs capable of suppressing these termination codons and allowing continued polypeptide elongation were initially

isolated from *E. coli* (reviewed in Smith, 1979), yeast (reviewed in Sherman, 1982) and *Caenorhabditis elegans* (reviewed in Hodgkin *et al.*, 1987) through mutagenesis and genetic screening. Suppressor tRNA activity is easily quantified *in vivo* by determining the efficiency of translational readthrough of premature nonsense codons. In fact, suppressor tRNAs have been used to study many aspects of aminoacyl tRNA biosynthesis and tRNA function in translation (reviewed in Hatfield *et al.*, 1990). Indeed, the efficiency of suppression is the result of a competition for the nonsense codon between the release factor and all the aminoacyl tRNAs (complexed with elongation factor and guanosine triphosphate) among which one is acting as a suppressor (discussed in Valle and Morch, 1988; Nierhaus, 1993). Since suppression is the result of a competition event the efficiency of nonsense suppression reflects the amount of suppressor tRNA in the cell. Therefore, nonsense suppressor tRNA genes afford a facile biological assay system by which to examine the expression of individual tRNA genes *in vivo* and overcome the problem of reiterated isoacceptor tRNA genes in the genome.

In yeast, suppressor tRNAs have been used to examine the effect of extragenic flanking sequences on tRNA gene expression. For example, the expression of extragenic deletion mutants of a *Saccharomyces* ochre suppressor $tRNA^{Tyr}$ gene revealed that the 5'flanking sequence of this gene is a strong positive modulator of its expression as measured by the growth of yeast strains dependent on the ochre suppression phenotype (Shaw and Olson, 1984). Conversely, the 3'-flanking sequences of this ochre-suppressing $tRNA^{Tyr}$ gene, up to the stretch of thymidine residues, were found to have no effect on its expression (Allison and Hall, 1985). Similarly, measurement of

suppression dependent growth of yeast strains transformed with various 5'-flanking deletion constructs of an amber suppressor $tRNA^{Leu}$ gene, revealed a positive modulatory element upstream of this gene (Cambell-Raymond *et al.*, 1985).

The worm, *Caenorhabditis elegans*, is the only multicellular organism in which nonsense suppressors have been recovered through classical genetic techniques (reviewed in Hodgkin *et al.*, 1987). Utilizing this approach, eight individual amber suppressor $tRNA^{Trp}$ genes have been isolated in *C. elegans* (Kondo *et al.*, 1988; 1990). Through cross-suppression tests each individual $tRNA^{Trp}$ gene has been shown to be expressed at different relative levels in individual tissues and in a developmental stage-specific manner, both of which has been attributed to the unique extragenic flanking sequences possessed by each gene (Kondo *et al.*, 1988, 1990).

As yet, there are no definitive studies that clearly establish the molecular basis for the regulation of biosynthesis of a specific tRNA isoacceptor species in higher eukaryotic systems analogous to the hallmark example of regulated $tRNA^{Ala}$ gene expression in the silk gland of *B. mori*. Nonsense suppressor tRNAs which are active in higher eukaryotes have been constructed from cloned tRNA genes by alteration of the sequences encoding the anticodon using recombinant DNA techniques (reviewed in Hatfield *et al.*, 1985, 1990). In addition, a number of suppressor tRNAs have been constructed and shown to possess activity in mammalian cells (Temple *et al.*, 1982; Laski *et al.*, 1982, 1984; Summers *et al.*, 1983, Young *et al.*, 1983; Capone *et al.*, 1985, 1986). One of these suppressor tRNAs has been utilized to examine the regulation of tRNA gene expression in mammalian cells (Capone, 1988).

1.15 Mechanisms of Transfer RNA Gene Regulation.

The aforementioned studies of the *B. mori* $tRNA^{Ala}$ genes, *Xenopus* $tRNA^{Tyr}$ genes, and *C. elegans* $tRNA^{Trp}$ genes demonstrate that the 5'-flanking region of tRNA genes can confer tissue specific and developmentally regulated expression. In almost all the *in vitro* and *in vivo* studies cited above the extragenic region responsible for transcriptional modulation of each tRNA gene lies within 50 base pairs upstream of the initiation site. With the exception of the general sequence TNNCT as a positive modulator of some *Drosophilia* tRNA genes (Sajjadi and Spiegelman, 1987), direct comparison of the modulatory regions identified in these studies has failed to reveal conserved sequence elements that could be ascribed to being positive or negative transcriptional modulators. Furthermore, tRNA genes show no sequence similarities in their flanking sequences even within the same isoacceptor family from the same species (section 1.12). The above facts suggest that if sequence specific regulatory factors operate at these extragenic sequences they are extremely diverse, being gene and genus specific, and therefore not strongly conserved. In this regard, the binding of a protein to the immediate upstream region of a *B. mori* $tRNA^{Gly}$ gene has been detected, however, the functional significance of this is not clear since the activity was not isolated (Taneja *et al.*, 1992; Fournier *et al.*, 1993). In fact, in only one reported case has a tRNA gene regulatory activity been isolated and shown to be distinct from the general RNA polymerase III transcription machinery (Oei and Pieler, 1990). This activity, isolated from *Xenopus* oocyte extracts, binds the 5'-flanking region of a *Xenopus* $tRNA^{Met}$ gene

resulting in the stimulation of its transcription.

During the course of this work many additional hypotheses have been put forward to explain the diversity of 5'-flanking sequences which modulate tRNA gene transcription. Two early studies speculated that the cause for the observed transcriptional modulation was the conformation of the DNA, which included a possible Z-DNA conformation (Hipskind and Clarkson, 1983) and a potential tRNA-like structure (Goddard *et al.*, 1983), found within identified upstream elements. The modulatory regions of three $tRNA^{Asn}$ were suggested to affect an initiation event, such as open complex formation, based on the finding that they redirect the transcriptional start site and inactive tRNA gene deletion mutants appear to be deficient in transcription initiation (Lofquist *et al.*, 1988). Similarily, the *Drosophilia* TNNTC element was proposed to increase the rate of transcription initiation (Sajjadi and Spiegelman, 1989). The proposal that certain 5'-flanking sequences affect the rate of formation or final stability of the transcription complex is supported by several second template experiments which reveal a correlation between the transcriptional activity of tRNA deletion constructs and their ability to stably sequester transcription components (Sharp *et al.*, 1983, Schaack *et al.*, 1983, 1984; Cooley *et al.*, 1984; Morry and Harding, 1986; Arnold *et al.*, 1987, 1988; Rooney and Harding, 1988; Wahab *et al.*, 1989). Thus, 5'-flanking sequences may modulate transcription by acting through the basal transcription machinery. This hypothesis is strengthened by the finding that yeast TFIIIB displays extended interactions with sequences upstream of tRNA genes (Kassavetis *et al.*, 1989, 1990). It is worth noting here, that the interactions of both yeast TFIIIB and yeast TFIIIC with a tRNA

gene have been shown to bend the DNA (Leveillard *et al.*, 1991), and this in itself is an action which displays a certain degree of sequence discrimination (reviewed in Travers, 1991). Recently, competition assays using separated silkworm components has shown that the primary discriminator between transcription of constitutive and silk gland specific $tRNA^{Ala}$ genes is competition for the TFIIIB and RNA polymerase III fractions (Sullivan *et al.*, 1994).

1.16 The Thesis Project

The overall aim of the research project described in this thesis was to investigate the regulation of mammalian transfer RNA gene expression. Specifically, this research focused on examining the role of the sequences upstream of a human serine tRNA gene, shown in Figure 1.3.1, in transcriptional modulation and in basic RNA polymerase III mediated transcription. To accomplish this, and ascribe physiological significance to these studies, experiments were performed both *in vivo* and *in vitro*. The results obtained are discussed throughout this thesis in the context of our current knowledge of tRNA gene expression in order to provide a comprehensive understanding of the field as it exists at this time.

Since monitoring the expression of a specific tRNA gene *in vivo* is hindered by the large number of endogenous isoacceptor tRNA genes, the *in vivo* studies described here relied on the use of a human serine tRNA gene which had been converted to an amber suppressor (Capone 1985). The phenotypic expression of this gene is readily

quantified *in vivo* by assaying its ability to suppress an amber nonsense mutation in the *E. coli* chloramphenicol acetyltransferase gene following cotransfection in mammalian cells (Capone, 1986). An initial study, using this *in vivo* suppression assay, revealed that sequences within the first 66 base pairs immediately upstream modulate the expression of this tRNA gene *in vivo* (Capone, 1988). The initial focus of the research described here was to further delineate the modulatory region of this gene and to elucidate the mechanism for its action. Towards this end a series of upstream deletion and insertion mutants of the human $tRNA^{Ser}$ gene were constructed. The phenotypic expression of these constructs was assessed *in vivo* using the above suppression assay, which was improved to attain statistically relevant results. This work is described in Chapter 3 of this thesis.

The suppression assay does not distinguish transcriptional activity from post-transcriptional processing or modification events. Therefore, the deletion and insertion constructs of the human $tRNA^{Ser}$ gene were analyzed using an *in vitro* transcription assay in order to further understand the functional role of this region. These analyses relied on quantitative *in vitro* transcription and second template competition assays which utilized HeLa cell nuclear extracts as a source of RNA polymerase III transcription components. A comparison between the results obtained from these experiments and those obtained from the *in vivo* suppression assay are described in Chapter 4 of this thesis.

Although the promoter elements necessary for transcription are found within the tRNA gene itself, the majority of tRNA transcriptional modulatory sequences are

found extragenically, within 50 base pairs upstream of the tRNA coding region. Whether these elements act directly through the basal pol III transcription machinery or through specific regulatory binding proteins is largely unknown. To address this it was ascertained whether a well characterized heterologous DNA binding protein bound within the upstream region could modulate the transcriptional activity of a tRNA gene. Specifically, an oligonucleotide containing the recognition site for the *E. coli lac* repressor was inserted at various positions upstream of the serine tRNA gene and the consequences of binding the *lac* repressor on transcription *in vitro* and expression *in vivo* was investigated. This work, described in Chapter 5 of this thesis, also revealed spatial and functional characterisics of the human pol III transcription complex within the upstream region of the serine tRNA gene.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

acetyl Coenzyme A	Pharmacia
acrylamide	Gibco/BRL
actinomycin D	Sigma Chemical Co.
agarose	Gibco/BRL
ampicillin	Boehringer Mannheim
bacto-agar	Difco
bacto-tryptone	Difco
Biorad protein dye reagent	Biorad
5-bromo-4-chloro-3-indoyl phosphate (BCIP)	Gibco/BRL
calf serum	Gibco/BRL
calf thymus DNA	Pharmacia
cesium chloride	Terochem Labs
chloramphenicol	Sigma Chemical Co.
deoxynucleotide triphosphates	Pharmacia
dideoxynucleotide triphosphates	Pharmacia
DEAE dextran	Sigma Chemical Co.
dimethyl-sulphoxide (DMSO)	BDH Chemicals
DNA ladder (1 Kb)	Gibco/BRL
dithiothreitol (DTT)	Boehringer Mannheim
Econofluor	Dupont/NEN
ethidium bromide	Sigma Chemical Co.
ethylene diaminetetraacetic acid (EDTA)	BDH Chemicals
fluorescent TLC plates (Kieselgel 60 F_{254})	EM Science
formamide	BDH Chemicals
glutamine	Gibco/BRL
heparin	Sigma Chemical Co.
isopropylthio-β-D-galactoside (IPTG)	Gibco/BRL

lac repressor protein (*E. coli*)	Stratagene
low melting point agarose	Gibco/BRL
MEM non-essential amino acids	Gibco/BRL
MEM vitamen solution	Gibco/BRL
N,N'-methylene bisacrylamide	Biorad
O-nitrophenyl-β-D-galactoside (ONPG)	Sigma Chemical Co.
Nonidet P-40 (NP-40)	United States Biochemical Corp.
nitrocellulose (.45 μm pore size)	Schleicher and Schuell Inc.
nucleotide triphosphates	Pharmacia
Nusieve agarose	FMC Bioproducts
phenol	Gibco/BRL
phenylmethylsulfonyl fluoride (PMSF)	United States Biochemical Corp.
Qiagen columns	Qiagen Inc.
rubidium chloride	Sigma Chemical Co.
salmon sperm DNA	Sigma Chemical Co.
sephadex G-50	Pharmacia
silica gel 150A plates	Whatman
spinbind units	FMC Corp.
transfer RNA (from *E. coli*)	Pharmacia
Triton X-100	Sigma Chemical Co.
urea	Gibco/BRL
X-ray film (X-omat AR)	Eastman Kodak Co.
yeast extract	Difco

2.1.2 Radiochemicals

[^{3}H]acetyl coenzyme A (10 Ci/mmol)	ICN Radiochemicals
[^{14}C]chloramphenicol (60 mCi/mmol)	Dupont/NEN
[α-^{32}P]dATP (3000 Ci/mmol)	ICN Radiochemicals
[γ-^{32}P]dATP (3000 Ci/mmol)	Dupont/NEN
[α-^{32}P]GTP (400 Ci/mmol)	Amersham and ICN Radiochemicals

2.1.3 Enzymes

Bal 31 exonuclease	International Biotechnologies Inc.
calf intestinal phosphatase	Gibco/BRL, New England Biolabs
chloramphenicol acetyltransferase (*E. coli*)	Pharmacia
DNA ligase (T4)	New England Biolabs, Pharmacia
DNA polymerase (modified T7, sequenase)	United States Biochemical Corp.
DNA polymerase I, Klenow fragment (*E. coli*)	Gibco/BRL, pharmacia
lysozyme	Sigma Chemical Co.
polynucleotide kinase (T4)	New England Biolabs
restriction endonucleases	Boehringer Mannheim, Gibco BRL, NewEngland Biolabs, Pharmacia
reverse transcriptase (avian myeloblastosis virus)	Life Sciences Inc.
ribonuclease I "A" (bovine pancreas)	Pharmacia
S1 nuclease	Gibco/BRL
trypsin	Gibco/BRL

2.1.4 Oligonucleotides

All oligonucleotides were synthesized and purified at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. They are listed in Table 2.1.1.

2.1.5 Cloning Vectors

pUCtS Su^+(am) is a human serine tRNA gene with the normal serine TGA anticodon changed to an amber suppressor CTA anticodon (Capone *et al.*, 1985). The vector pRSVcat contains the *E. coli* chloramphenicol acetyltransferase (CAT) gene under

OLIGO	SEQUENCE (5′ to 3′)	PURPOSE
AB49	GTCTAGAC	*Xba*1 linker for *Bal*31 deletions
AB586	GGATTTCCTCTACCCGAG	Sequencing primer for tRNA gene
AB2343	CCTTAACCACTCGGC	Oligo for primer extension of tRNA transcripts
AB1442 AB1443	CTAGGTTGTTGAAGGAGGTAC CTAGGTACCTCCTTCAACAAC	Negative element of tRNA gene with *Xba*1 sticky ends
AB882	ATTGTGAGCGCTCACAAT	Short *lac* operator site
AB883	CTAGATTGTGAGCGCTCACAAT	Short *lac* operator site with *Xba*1 sticky ends
AB1465	TGTGGAATTGTGAGCGCTCAGAATTCCACA	Extended *lac* operator site

Table 2.1.1 List of Oligonucleotides.

the control of the Rous sarcoma virus long terminal repeat (Gorman *et al.*, 1982). pRSVcat(am27) is a derivative of pRSVcat that contains a suppressible nonsense TAG codon in place of the serine TCA codon at amino acid position 27 of the CAT gene (Capone *et al.*, 1986). The vector pRSVβgal was obtained from Dr. R. Rosenberger (National Institute for Medical Research, London, England) and contains the *E. coli* β-galactosidase gene under control of the Rous sarcoma virus long terminal repeat. pBRVA was obtained from Dr. R. Bhat (Northwestern University Medical School, Chicago, Illinois) and contains the adenovirus type 5 VA1 RNA gene cloned into pBR322 (Bhat *et al.*, 1983). pALU was obtained from the lab of Dr. J. Smiley (McMaster University, Hamilton, Ontario) and contains a single human *Alu* element cloned into pUC18. pRSVIns was obtained from Dr. N. Davidson (California Institute of Technology, Pasadena, California) and expresses the *E. coli lac* repressor modified by the addition of a nuclear targeting signal, from simian virus 40 large T antigen, to the carboxyl terminus (Hu and Davidson, 1991).

2.1.6 *E. coli* Strains

All cloning, except for the contruction of the *lac* operator containing plamids, was performed in *E. coli* strain HB101, genotype: F⁻Δ(mcrC-mrr) leu supE44 ara14 galK2 *lac*Y1 proA2 rpsL20(Str^r) xyl-5 mtl-1 recA13. Cloning of plasmids containing the *lac* operator site was performed in *E. coli* strain DH5α, genotype: F'/endA1 hsdR17 (r_k^- m_k^+) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)U169 (ϕ80dlacΔ(lacZ)M15).

2.1.7 Cell Lines

BSC-40, Vero, 293, HeLa, and HeLa S3 cell lines were obtained from the American Type-Culture Collection, Rockville, Maryland.

2.2 Enzymatic Manipulation of DNA

2.2.1 Restriction Digestion

Plasmid DNA was digested using the suppliers restriction enzyme buffer and incubation conditions. Often Pharmacia's one-for-all-plus buffer (a potassium acetate based buffer) was used for double digests. Typically at least 5 units of enzyme was used for every 1 μg of plasmid DNA digested. Unless specified otherwise, restriction digests were carried out in Eppendorf tubes and incubated for 1 hour at 37°C.

2.2.2 *Bal* 31 Digestion

Bal 31 digestions were carried out on the plasmid ptS-66 which was previously linearized by digesting with either *Hin*D3 or *Sna*B1. A typical *Bal* 31 digestion contained 50 μg of linearized plasmid and 20 Units of *Bal* 31 exonuclease in a 200μl final volume under buffer conditions specified by the supplier (International Biotechnologies Inc.). The reaction was incubated at room temperature and, at one

minute intervals, 20 μl aliquots were added to ice cold Eppendorf tubes containing 10 μl of 150mM EDTA to terminate the reaction. Each 30 μl sample was extracted with an equal volume of Tris (pH 8.0) saturated phenol chloroform (1:1), then extracted twice with an equal volume of chloroform and ethanol precipitated (Maniatis *et al.*, 1982). The extent of *Bal* 31 digestion was checked by analysing a small portion of each sample by agarose gel electrophoresis.

2.2.3 Phosphorylation and Dephosphorylation

DNA fragments or synthetic oligonucleotides were phosphorylated with T4 polynucleotide kinase under conditions specified by the supplier. Dephosphorylation of DNA fragments was performed with calf intestinal phosphatase under conditions specified by the supplier. Dephosphorylation reactions were then heated at 70°C and extracted with phenol/chloroform followed by ethanol precipitation in order to ensure inactivation of the enzyme.

2.2.4 Filling in of 3' Recessed Ends

The Klenow fragment of *E. coli* DNA polymerase I was used to fill in 3' recessed ends of DNA fragments and synthetic oligonucleotides. The enzyme was used at 1 unit per μg DNA in a variety of buffers supplemented with 33 μM of each deoxynucleotide triphosphate.

2.2.5 Ligation

To ligate DNA fragments less than 1 μg of vector and a molor excess of insert were incubated overnight at 16°C in the presence of 10 units of T4 DNA ligase under buffer conditions recommended by the supplier. Blunt-end ligations were performed with at least 50 units of T4 DNA ligase.

2.3 Cloning Techniques

2.3.1 Isolation and Recovery of DNA Fragments

After restriction endonuclease digestion samples were made up in agarose gel loading buffer (0.04% bromophenol blue, 0.04% xylene cyanol, 6.7% (w/v) sucrose) and analyzed by electrophoresis in 0.8% agarose gels cast in TBE buffer (89 mM Tris-borate 89 mM boric acid, 2.5 mM EDTA) containing 0.5 μg/mL ethidium bromide. The gel was run in the same buffer at about 15 Volts/cm using a Hoefer submarine gel apparatus and DNA fragments were visualized under short wavelength ultraviolet light.

To facilitate cloning, a desired DNA fragment was cut out of low melting point agarose gel and placed in an Eppendorf tube. To this gel slice was added 200 μl of 0.2M Tris (pH 8.0) and 1 μl of *E. coli* tRNA (10 mg/ml) as a carrier. The tube was heated at 65°C until the gel slice melted, typically about 10 minutes. To the melted slice was added an equal volume of phenol which was saturated with 0.2 M Tris (pH 8.0).

The sample was vortexed and then spun in a microfuge at top speed for 2 minutes. The upper aqueous phase was collected and extracted with an equal volume of Tris (pH 8.0) saturated phenol/chloroform and then extracted with chloroform. The DNA in the aqueous phase was precipitated by the addition of one-tenth the volume of 3M Sodium Acetate (pH 5.2) and one volume of isopropanol followed by storage at -20°C. The DNA was recovered by spinning the sample for 15 minutes at top speed in a microfuge. The pellet was rinsed with ethanol and resuspended in TE buffer (10mM Tris (pH 8.0), 1mM EDTA).

Alternatively, DNA fragments were recovered from agarose gels by adding three volumes of binding solution (7.2 M sodium iodide, 0.1 M sodium phosphate, pH 6.0) to the agarose gel slice and heating the sample at 60°C until the slice dissolved. The sample was then applied to a spinbind unit (silica glass matrix column, FMC Corp.), which was fitted into an eppendorf tube, and recovered under the suppliers guidelines through washes with sodium iodide and ethanol solutions followed by elution with TE.

2.3.2 Growth and Maintenance of *E. coli*

Liquid cultures of *E. coli* were grown overnight at 37°C with agitation in 2YT media (15% (w/v) bactotryptone, 10% (w/v) yeast extract, 5% (w/v) sodium chloride). Cultures of 50 mL or less were inoculated directly from frozen stock or from an isolated plate colony. Larger cultures were inoculated with a saturated culture grown the day before. *E. coli* colonies were obtained either by streaking frozen stock or by

spreading liquid cultures on plates containing 2YT, 1.5% bactoagar and incubating overnight at 37°C. When the bacteria were under selection the media and plates contained 50 μg/ml ampicillin. Frozen stocks of *E. coli* were obtained by taking 1 ml of a saturated culture, adding 70 μl of dimethlysulphoxide, flash freezing in liquid nitrogen and storing at -80°C.

2.3.3 Preparing Transformation Competent *E. coli*

A 100 ml culture of *E. coli* was grown to an $O.D._{600}$ of 0.4 to 0.5 and then placed on ice for ten minutes. The culture was pelleted and resuspended in 20 ml of RF1 buffer (100 mM rubidium chloride, 50 mM manganese chloride, 30 mM potassium acetate, 10 mM calcium chloride, 15% glycerol adjusted to pH 5.8 with acetic acid) and incubated on ice for one hour. After pelleting the *E. coli* were resuspended in 8 ml of RF2 buffer (10 mM MOPS, 10 mM rubidium chloride, 75 mM calcium chloride, 15% glycerol adjusted to pH 6.8 with sodium hydroxide). The cells were placed into Eppendorf tubes as 200 μl aliquots, flash frozen in liquid nitrogen and stored at -80°C.

2.3.4 Transformation of *E. coli*

Approximately 0.1 μg of ligation or plasmid DNA was added to a 200 μl aliquot of competent *E. coli*. The cells were incubated on ice for 40 minutes and then heat shocked for two minutes at 42°C. 1 ml of 2YT was added to the tube and the cells

were incubated for approximately one hour at 37°C. Varying quantities of the cells (25 to 200 μl aliquots) were spread on plates containing 2YT, 1.5% bacto-agar and 50 μg/ml ampicillin. Plates were incubated overnight at 37°C.

2.3.5 Screening *E. coli* Transformants

Colonies of *E. coli* transformants were picked with a sterile toothpick from agar plates and used to inoculate 3 ml of 2YT containing 50 μg/ml ampicillin. The bacteria were grown overnight at 37°C with shaking after which half of the culture was transferred to an Eppendorf tube while the other half was stored at 4°C. Plasmid DNA was isolated by the rapid boiling method as described (Maniatis *et al.*, 1982). Briefly, the Eppendorf tubes were spun in a microfuge for two minutes and to each cell pellet was added 350 μl of lysis buffer (10 mM Tris (pH 8.0), 8% (w/v) sucrose, 0.5% Triton X-100, 50 mM EDTA). After adding 30 μl of lysozyme (10 mg/ml in 10 mM Tris, pH 8.0) the tube was vigorously vortexed and placed in a boiling water bath for 40 seconds. The tube was then spun in a microfuge for 10 minutes and the resulting pellet was removed with a toothpick. To the supernatant was added 200 μl 7.5 M ammonium acetate and 700 μl isopropanol. The tube was incubated for 15 minutes at -20°C and spun in a microfuge for 15 minutes at 4°C. The DNA pellet was rinsed with 70% ethanol, dried, and resuspended in 50 μl of TE containing 10 ug/ml DNAse free ribonuclease I "A" (bovine pancreas; Pharmacia). This DNA was subjected to restriction endonuclease analysis and agarose gel electrophoresis to test for positive clones which,

when identified, could be grown directly from the cultures saved at 4°C for large scale plasmid purification.

2.4 Preparation, Sequencing and Quantitation of Plasmid DNA

2.4.1 Large Scale Preparation of Plasmid DNA

Plasmids containing a *lac* operator site within the upstream region of the human $tRNA^{ser}$ gene were prepared and purified on Qiagen P-500 columns (Qiagen Inc.) using the supplier's guidelines. Plasmids containing the upstream deletion, collapse or insertion constructs of the human $tRNA^{ser}$ gene were prepared by the alkaline lysis technique (Maniatis *et al.*, 1982) and purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients as described below.

Overnight 500 ml cultures of *E. coli* were pelleted in a Sorvall GSA rotor at 7,000 RPM for 10 minutes. The bacterial pellet was resuspended in 40 ml of 25 mM Tris (pH 8.0), 50 mM glucose, 10 mM EDTA and to this suspension was added 80 ml of 0.2 M sodium hydroxide, 1% sodium dodecyl sulphate. The solution was mixed and left at room temperature for 10 minutes after which 40 ml of 5 M potassium acetate (prepared at 3 M potassium and 5 M acetate, pH 4.8) was added. The solution was mixed gently, left at room temperature for 10 minutes and centrifuged in a Sorvall GSA rotor at 7,000 RPM for 10 minutes. The supernatant was decanted through four layers of cheeze cloth into a 250 ml centrifuge bottle which was subsequently filled with

isopropanol and centrifuged at 7,000 RPM for 10 minutes to pellet the nucleic acids. The pellet was washed with absolute ethanol and resuspended in 7.5 ml of TE buffer. To the sample was added 8.8 grams of cesium chloride, which was dissolved, and 0.8 ml of 10 mg/ml ethidium bromide. The solution was left on ice for 15 minutes and centrifuged at 7,000 *g* for 30 minutes. The supernatant was transferred into two 5ml Beckman ultracentrifuge tubes which were then sealed. The tubes were centrifuged in a Beckman VTi65 rotor at 55,000 RPM at 15°C for at least 16 hours. The band of plasmid DNA was visualized with long wavelength ultraviolet light and recovered by side puncture with a 3 ml syringe fitted with an 18 gauge needle. The ethidium bromide was removed from the sample by repeated extractions with cesium chloride/water saturated butanol and the final aqueous phase was dialysed overnight against several volumes of 10 mM Tris (pH 8.0), 10 mM sodium chloride, 1mM EDTA. The DNA within the dialysis bag was ethanol precipitated and suspended in TE.

2.4.2 Sequencing of Plasmid DNA

Plasmid DNA was sequenced either directly from crude minipreparations of DNA or from large scale plasmid preparations. All sequencing was carried out using the Sequenase enzyme (United States Biochemical Corp.) under the suppliers guidelines using synthetic oligonucleotides as primers.

2.4.3 Quantitation of Plasmid DNA

The $O.D._{260}$ to $O.D._{280}$ ratio was between 1.8 and 2.0 for all the large scale plasmid preparations which signifies relatively pure plasmid DNA (Maniatis *et. al.*, 1982). However, despite this purity, the $O.D._{260}$ was found to be a poor indicator of the quantity of DNA in each plasmid preparation.

Fluorometry was used to quantify all large scale plasmid preparations subsequently used in transient transfection or *in vitro* transcription analyses. 2 ml of fluorometry buffer (20 mM potassium phosphate, 0.5 mM EDTA, 0.5 ug/ml ethidium bromide, pH adjusted to 11.8 with potassium hydroxide) in a 5 ml borosilicate tube was used to zero the fluorometer. To this was added 5 μl of F standard (0.1 μg/μl calf thymus DNA) and the fluorometer was adjusted to a setting of 150. Plasmid DNA was diluted to obtain a reading between 50 and 150. DNA concentrations were calculated based on the observation that covalently closed circular plasmid DNA intercalates 72% as much ethidium bromide as an equivalent amount of linear DNA (Morgan *et al.*, 1979).

The purity and quantity of each large scale plasmid preparation was confirmed by agarose gel electrophoresis. Based on the fluorometry calculations, 0.5 and 1 μg of several plasmids were ran together on a 0.8% agarose gel and the intensity of the bands were visually compared under ultraviolet light. The agarose gels were also checked for significant amounts of relaxed or nicked plasmid DNA in the large scale preparations.

2.5 Transfection of Mammalian Cells

2.5.1 Maintaining Mammalian Cells in Culture

BSC 40, Vero, 293, and Hela cells were maintained at 37°C and 5% carbon dioxide on 10 centimeter dishes. Cells were grown in Dulbecco's modified essential medium (supplied by the Cancer Research Group, McMaster University) supplemented with 10% calf serum, 1% glutamine, 1% penicillin and 1% streptomycin. Cells were split at a ratio of 1 to 10 upon reaching confluency by rinsing with phosphate buffered saline (PBS), treating them with trypsin and diluting them into fresh medium.

2.5.2 Calcium Phosphate Transfections

Initially, transfections were carried out using the calcium phosphate coprecipitation technique (Graham and Van der Eb, 1973). Cells were split the previous day in 6 centimeter dishes and grown overnight to approximately 80% confluency. In a sterile Eppendorf tube the desired plasmid DNA was mixed with salmon testes DNA to make the total DNA content 20 μg. To this was added 31 μl of 2M calcium chloride and water so that the final volume was 0.25 ml. This mixture was then added dropwise, over a 30 second time span, to a sterile 5 ml plastic tube containing 0.25 ml of HBS (50 mM HEPES, 0.28 M sodium chloride and 1.5 mM sodium phosphate, adjusted to pH

7.12 with sodium hydroxide) while continuously vortexing. After allowing the mixture to sit at room temperature for 20 minutes it was gently mixed and added dropwise to a 6 centimeter plate of cells containing 3 ml of fresh medium. The plates were then incubated at 37°C, 5% carbon dioxide, for 6 hours and the medium was then aspirated off. The cells were shocked for two minutes through the addition of 2 ml of serum free medium containing 10% dimethylsulfoxide. After shocking, the cells were quickly washed three times with PBS and incubated in 5 ml of complete medium at 37°C, 5% carbon dioxide, until being harvested 48 hours later.

2.5.3 DEAE Dextran Transfections

All large scale transfections were carried out using the DEAE dextran transfection method (Ausubel *et al.*, 1988) on BSC-40 cells. Cells were split the previous day in 6 centimeter dishes and grown overnight to almost 100% confluency. In a sterile 5 ml plastic tube the desired plasmid DNA was mixed with salmon testes DNA to make the final DNA content 20 μg. To this was added 2 ml of serum free medium containing 0.1 mM HEPES (pH 7.3) and 0.2 mg/ml DEAE dextran. After washing twice with PBS the DEAE solution was placed on the cells and the plates were incubated at 37°C, 5% carbon dioxide, for 4 hours. The cells were then shocked by replacing the DEAE solution with 2 ml of serum free medium containing 10% dimethylsulfoxide. After incubating for 2 minutes at room temperature the solution was aspirated and the cells were quickly washed twice with PBS. The cells were incubated

at 37°C, 5% carbon dioxide, in 5 ml of complete medium until being harvested 48 hours later.

2.5.4 Harvesting Mammalian Cells After Transfection

Mammalian cells were harvested by first aspirating the medium and washing the cells three times with PBS. After the final wash was aspirated, 1 ml of ice cold TEN buffer (40 mM Tris (pH 7.4), 0.15 M sodium chloride, 1mM EDTA) was added to each plate of cells after which the plates were kept on ice. The cells were scraped off the plates with a rubber policeman and transferred into Eppendorf tubes kept on ice. After pelleting in a microfuge for 2 minutes at 4°C the cells were resuspended in 100 μl of ice cold CAT buffer (250 mM Tris (pH 7.8), 1mM phenylmethylsulphonyl fluoride (PMSF), 0.5% NP-40) by vortexing the Eppendorf tubes for at least 10 minutes in a 4°C room. The tubes were sonicated for a 10 second period three times and spun in a microfuge for 5 minutes at 4°C. The supernatant containing the cell extract was transferred to a fresh Eppendorf tube and immediately assayed for chloramphenicol acetyltransferase and β-galactosidase activity. The extracts could be stored for many months at -20°C without loss of chloramphenicol acetyltransferase activity.

2.6 Chloramphenicol Acetyltransferase Assays

2.6.1 CAT Activity Assayed using Thin Layer Chromotography

Initially CAT activity was determined using an assay that separated the various unacetylated and acetylated forms of chloramphenicol by thin layer chromotography (Gorman *et al.*, 1982). To an Eppendorf tube was added 10 μl of cell extract, 1 μl [^{14}C]chloramphenicol, 70 μl of 1 M Tris (pH 7.8), 25 μl 5 mM acetyl Coenzyme A and water to a final volume of 150 μl. The reaction was mixed and incubated for 1 hour at 37°C and terminated by the addition of 1 ml of ethyl acetate. The tube was vortexed and microfuged for 2 minutes at room temperature. The upper ethyl acetate phase was transferred to a new tube and dried down in a Speed Vac. The dried pellet was resuspended in 25 μl ethyl acetate and spotted on a prescored silica gel 150A thin layer chromotography plate (Whatman). The plate was placed into a thin layer chromotography chamber containing 95% chloroform and 5% methanol. When the solvent reached the top of the plate it was removed, air dried, marked with radioactive ink and exposed to film overnight at room temperature. Using the film as a template, the radioactive silica spots were scraped into vials and radioactivity was quantified by scintillation counting.

2.6.2 CAT Activity Assayed by Direct Diffusion

Most CAT assays were performed using a method that utilizes the diffusion of acetylchloramphenicol, from the aqueous reaction, directly into a water-immiscible liquid scintillation counting cocktail which can be quantified by counting over selected time intervals (Neumann *et al.*, 1987). First, 60 μl of extract was heated at 70°C for 5 minutes followed by a 10 minute spin at 4°C. This heating step is necessary to get rid of acetyl Coenzyme A consuming cellular activity (Sleigh, 1986) and to inhibit the acetylchloramphenicol deacylating activity found in a number of cell extracts (Crabb and Dixon, 1987). 50 μl of the resulting supernantant was placed in a 7 ml scintillation vial and 200 μl of reaction mixture (63 mM Tris (pH 7.8), 1.25 mM chloramphenicol containing 0.1 μCi [^{3}H]acetyl Coenzyme A (10 Ci/mmole)) was added immediately followed by 5 ml of Econofluor (Dupont/NEN). The samples were immediately counted for 150 minutes over 15 minute intervals and the slope of counts per minute versus reaction time was used to calculate the CAT activity.

2.6.3 CAT Assay Normalization

Initially CAT activity was normalized to the protein concentration determined in each mammalian cell transfection extract using the Bradford method of protein determination (Bradford, 1976). In a 1 ml disposable cuvette 2 μl of cell extract was made up to 0.8 ml with distilled water and mixed with 0.2 ml of protein dye reagent

(Biorad). The $O.D._{595}$ of each sample was measured and the protein concentration was calculated from a standard curve obtained by performing the assay with 4, 8, 12, 16, 20 and 24 μg of bovine serum albumin.

Most CAT assay data was normalized to transfection efficiency by including the plasmid pRSVβgal as an internal transfection control and measuring β-galactosidase activity in each mammalian cell extract. β-galactosidase assays were performed as previously described (Sato *et al.*, 1986). In a 1 ml disposable cuvette was added 40 μl of cell extract and 400 μl of reaction buffer (100 mM sodium phosphate (pH 7.5), 10 mM potassium chloride, 1 mM magnesium sulfate and 50 mM 2-mercaptoethanol). The cuvettes were then warmed at 37°C for several minutes and 150 μl of O-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 100 mM sodium phosphate (pH 7.5)) was added. The reactions were incubated at 37°C for 2 hours and were terminated by the addition of 200 μl of 1 M sodium carbonate. The $O.D._{420}$ was measured directly after adding 50 μl of isopropyl alcohol to clear turbidity caused by the presence of NP-40.

2.7 *In Vitro* Transcription Assays

2.7.1 Growth of HeLa Cells in Suspension

HeLa S3 cells were grown from frozen stock on 10 centimeter plates at 37°C and 5% carbon dioxide in Dulbecco's modified essential medium containing 10% calf serum, 1% glutamine, 1% penicillin and 1% streptomycin. Upon obtaining 10 confluent

plates, the cells were trypsinized and put into suspension in Joklic's medium containing 5% fetal calf serum, 2% glutamine, 1X MEM vitamens, 1X MEM non-essential amino acids, 1% penicillin and 1% streptomycin. Suspension cells were grown at 37°C in spinner flasks and maintained at cell densities between 2 x 10^5 and 8 x 10^5 cells/ml.

2.7.2 Preparation of HeLa Extracts

HeLa extracts were prepared as described in Dignam *et al.*, 1983. HeLa S3 cells were split to a cell density of 2.5 x 10^5 cells/ml and incubated overnight at 37°C. The next day at least 3 litres of cells between densities of 4 x 10^5 and 6 x 10^5 cells/ml were pelleted by centrifuging at 1,500 *g* for 10 minutes at room temperature. The cells were resuspended in 50 ml of PBS and pelleted as before. After resuspending in 5 pelleted cell volumes of ice cold buffer A (10 mM HEPES (pH 7.9), 10 mM potassium chloride, 1.5 mM magnesium chloride and 0.5 mM dithiothreitol (DTT)) the cells were left on ice for 10 minutes to allow them to swell. After centrifuging at 1,500 *g* for 10 minutes at 4°C the cells were resuspended with 2 pelleted cell volumes of ice cold buffer A. The cells were lysed to isolate the nuclei by dounce homogenization using 15 to 20 strokes of a B pestle. Extent of cell lysis was checked microscopically and the nuclei were pelleted by centrifuging at 1,500 *g* for 10 minutes at 4°C. After carefully removing the supernatant with a pipet, the remaining nuclei were centrifuged at 25,000 *g* for 20 minutes at 4°C to remove residual cytoplasmic material. The pelleted nuclei were resuspended in ice cold buffer C (20 mM HEPES (pH 7.9), 0.42 M sodium chloride,

25% (v/v) glycerol, 1.5 mM magnesium chloride, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) using 3 mLs of buffer C per 10^9 harvested cells. The nuclei were dounce homogenized with 10 strokes of a B pestle and the resulting extract was gently stirred for 30 minutes at 4°C using a magnetic stir bar. The extract was centrifuged at 25,000 *g* for 30 minutes at 4°C and the resulting supernatant was dialysed for 5 hours at 4°C against 50 volumes of buffer D (20 mM HEPES (pH 7.9), 0.1 M potassium chloride, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The dialysed extract was centrifuged at 25,000 *g* for 20 minutes at 4°C and the supernatant was aliquoted in 200 μl volumes, flash frozen in liquid nitrogen, and stored at -80°C. The protein concentration of the extract was checked using the Bradford method (Bradford, 1976) and was typically between 7 and 10 mg/ml of protein.

2.7.3 Transcription Reactions

Transcription reactions were optimized for the amount of HeLa cell nuclear extract, magnesium concentration, template concentration, and total amount of DNA in preliminary experiments. Usually half the volume of a standard 20 μl transcription reaction was nuclear extract making the protein concentration approximately 4 or 5 mg/ml and the reaction buffer conditions 10 mM HEPES (pH 7.9), 10% (v/v) glycerol, 50 mM potasium chloride, 0.1 mM EDTA, 0.25 mM PMSF and 0.25 mM DTT. The optimal magnesium concentation was usually about 2.5 mM in the form of magnesium chloride and creatine phosphate was added to a final concentration of 8 mM. The

nucleotide concentrations were 0.4 mM for ATP, CTP, UTP and 0.04 mM cold GTP with 2μCi [α-^{32}P]GTP (400 Ci/mmole) in each reaction. Reactions were incubated at 30°C for 90 minutes and terminated by the addition of 200 μl of 300 mM sodium acetate (pH 5.0) containing 0.5% sodium dodecylsulphate. After adding 200 μl of water saturated phenol, the samples were vigorously vortexed and then centrifuged in a microfuge for 2 minutes. Exactly 180 μl from the upper aqueous layer of each sample was precipitated in 400 μl of ice cold ethanol. The samples were stored at -20°C for 20 minutes and centrifuged at 4°C in a microfuge. The supernantant was removed and the pellet briefly air dried before adding 10 μl of formamide load buffer (50 mM Tris-borate (pH 8.3), 80% (v/v) formamide, 1 mM EDTA, 0.1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue). Samples were vigorously vortexed to ensure complete recovery before being analysed on 10% acrylamide/7 M urea gels. Products were visualized by autoradiography and quantified by either densitometry or phosphoimager analysis.

2.7.4 Template Competition Experiments

Template competition experiments were performed as described previously (Fowlkes and Shenk, 1980; Wormington *et al.*, 1981; Sharp *et al.*, 1983). Initially, the optimal amount of total DNA for the extract was determined by performing transcription reactions with increasing amounts of the wild type tRNA gene. The amount of DNA that resulted in a maximal level of transcription was considered the optimal amount of total

DNA for the transcription reaction. This amount of total DNA was kept constant, using pBR322 as a filler, in transcription reactions which contained increasing amounts of tRNA gene or VA gene template. The amount of template DNA that resulted in a just maximal level of transcription was considered to be saturating. Typically, approximately 1 μg of total DNA was found to be optimal and between 0.1 and 0.4 μg of template DNA, depending on template being tested, was found to be saturating in a 20 μl transcription reaction.

2.7.5 Transcription with Exogenous Components

The stock solution of *lac* repressor (Stratagene) was 1 mg/ml in 20 mM Tris (pH 7.4), 500 mM sodium chloride, 10 mM magnesium chloride, 5 mM β-mercaptoethanol, 0.1 mM EDTA and 50% (v/v) glycerol. *lac* repressor was diluted in the same buffer and transcription reactions contained μg/ml of this protein added in a 1 or 2 μl volume. Control transcription reactions conducted in the absence of *lac* repressor contained an equivalent volume of this buffer. Derepression experiments were performed in the presence of 50 mM IPTG (Gibco/BRL) which was diluted in water. Single round transcription reactions contained 400 μg/ml of heparin (Sigma) which was also diluted in water. IPTG and heparin were added to reactions in a 1 or 2 μl volume and control reactions contained an equivalent volume of water.

2.8 Transcription Initiation Site Determination

2.8.1 Preparation of End Labelled Primer

Approximately 50 μg (10 nmoles) of a 15 nucleotide primer 5'-CCTTAACCACTCGGC-3', complementary to nucleotides +10 to +24 with respect to the mature tRNA, was gel purified on a 20% polyacrylamide/7 M urea gel. The oligonucleotide band was visualized by placing the gel on a fluorescent TLC plate (Kieselgel 60 F_{254}; EM Science) under long wavelenth ultraviolet light. A slice corresponding to the oligonucleotide band was cut out of the gel and eluted in an eppendorf tube as described (Sambrook *et al.*, 1989) by adding 400 μl of gel elution solution (0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% SDS) and incubating overnight at 37°C. The gel was pelleted by centrifugation in a microfuge and to the supernatant was added 40 μl of 3 M sodium acetate (pH 5.2) and 1.1 ml of ice cold ethanol. After storing for 20 minutes at -20°C the precipitated oligonucleotide was pelleted by centrifuging for 15 minutes at 4°C. The pellet was air dried and resupended in 40 μl of TE. 4 μl of the purified oligonucleotide was end-labelled for 1 hour at 37°C with 20 units of bacteriophage T4 polynucleotide kinase (NEB) in a 50 μl reaction containing 5 μl of [γ-^{32}P]ATP (3000 Ci/mmole). After adding 50 μl of TE the reaction was placed in a 1 ml syringe containing 0.8 ml of packed Sephadex G-50 and spun in a clinical centrifuge to separate the oligonucleotide from unincorporated [γ-^{32}P]ATP (Maniatis *et al.*, 1982). The end-labelled primer in the eluent was stored at -20°C and

1 μl was 1 x 10^6 cpm by Cerencov counting.

2.8.2 Primer Extension Analysis

Primer extension analyses were performed by a previously described method (Jones *et al.*, 1985) using a 15 nucleotide end-labelled primer 5'-CCTTAACCACTCGGC-3' complementary to nucleotides +10 to +24 with respect to the mature tRNA. Unlabelled transcription products were obtained by performing *in vitro* transcription reactions with 0.4 mM of each nucleotide triphosphate (NTP). After extraction and precipitation each RNA pellet was resuspended in 8 μl of 10 mM Tris (pH 7.9), 1 mM EDTA containing 2 x 10^5 cpm of end-labelled primer. After the addition of 2 μl of 10 mM Tris (pH 7.9), 1 mM EDTA, 1.25 mM potasium chloride the sample was incubated at 50°C for 60 minutes to allow the primer to hybridize to the tRNA transcripts. Samples were cooled to room temperature and diluted with 25 μl of primer extension reaction buffer (20 mM Tris (pH 8.7), 10 mM magnesium chloride, 0.33 mM of each dNTP, 5 mM DTT, 10 mg/ml actinomycin D) containing 10 units of avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.). Reactions were incubated at 42°C for 60 minutes, precipitated with 300 μl of ethanol and analysed on 20% polyacrylamide-7M urea gels followed by autoradiography. Primer extension products were identified by running a dideoxy sequencing ladder obtained by using 1 x 10^6 cpm of the end-labelled primer and 2 μg of the wild type tRNA gene as a template.

2.8.3 Preparation of S1 Mapping Probes

The S1 probe used to map transcripts derived from wild type and p*Lac*OtS-46 templates was a 140 base pair fragment obtained by digesting 150 μg of ptS-66 with *Pst*1 and *Bst*B1. The *Pst*1 site is at nucleotide position -74 of the desired strand and the *Bst*B1 site is within the mature tRNA at nucleotide position +66. The S1 probe used to map transcripts derived from p*Lac*OtS-43, p*Lac*OtS-52 and pMCtS was a 115 base pair DNA fragment obtained by digesting 150 μg of pMCtS with *Sac*1, at nucleotide position -49, and *Bst*B1. The digests were phenol/chloroform extracted and ethanol precipitated. The DNA fragments were directly end-labelled for 1 hour at 37°C with 20 units of T4 polynucleotide kinase (NEB) in a 50 μl reaction containing 5 μl of [γ-^{32}P]ATP (3000 Ci/mmole). After adding 50 ul of formamide load buffer the reactions were ran on 12% acrylamide/7M urea strand separation gels and the desired single stranded probes were visualized by a 5 minute exposure to film. Using the film as a template the probe DNA was cut out of the gel and the gel slices were incubated overnight at 37°C in 400 μl of gel elution solution (0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% (w/v) SDS). The gel was pelleted by centrifugation in a mircofuge and to the supernantant was added 40 μl of 3 M sodium acetate (pH 5.2) and 1.1 ml of ice cold ethanol. After pelleting in a microfuge the probes were relabelled for 1 hour at 37°C using 20 units of T4 polynucleotide kinase (NEB) in a 50 μl reaction containing 5 μl of [γ-^{32}P]ATP (3000 Ci/mmole). After adding 50 μl of TE the reaction was placed in a 1 ml syringe containing 0.8 ml of packed Sephadex G-50 and spun in a clinical centrifuge to separate

the end-labelled probe from unincorporated [γ-^{32}P]ATP (Maniatis, 1982). The eluents were stored at -20°C and 1 μl was approximately 2 x 10^5 cpm for both probes as determined by Cerencov counting.

2.8.4 S1 Mapping Analysis

S1 mapping experiments were performed following an established protocol (Sambrook *et al.*, 1989) using unlabelled transcription products obtained by performing *in vitro* transcription reactions with 0.4 mM of each nucleotide triphosphate. Each RNA pellet was resuspended in 30 μl of hybridization buffer (40 mM pipirazine-N,N'bis[2-ethanesulfonic acid] (pH 4.6), 400 mM sodium choride, 1 mM EDTA and 80% deionized formamide) containing 2 x 10^5 cpm of the appropriate DNA probe. The reaction was heated for 10 minutes at 90°C and quickly transferred to 60°C for 16 hours. To each sample was added 300 μl of ice cold S1 nuclease buffer (50 mM sodium acetate (pH 4.6), 150 mM sodium chloride and 50 mM zinc sulphate) containing 230 units of S1 nuclease (Gibco/BRL). The reactions were incubated at 23°C for 1 hour and, after chilling on ice, were terminated by the addition of 80 μl of S1 nuclease stop mixture (4 M ammonium acetate, 50 mM EDTA containing 50 μg/ml *E. coli* tRNA as a carrier). The products were precipitated by the addition of 1 ml of ice cold ethanol and pelleted in a microfuge for 15 minutes at 4°C. Pellets were washed with 70% ethanol and resuspended in 10 μl of formamide load buffer by vigorous vortexing. The S1 digestion products were analysed on 8% polyacrylamide/7M urea gels and sized by comparison to dideoxy sequencing reactions of the wild type plasmid run in parallel.

THE IN VIVO EXPRESSION OF A HUMAN SERINE tRNA GENE

In eukaryotes the genes encoding a particular isoacceptor tRNA are present in multiple copies which are located at more than one chromosomal locus (section 1.2). This redundancy makes it difficult to demonstrate the *in vivo* activity of a specific gene. To overcome this, researchers have attempted to selectively determine the *in vivo* expression of a single tRNA gene largely through the use of primer extension analysis of tRNA genes heterologous to the organism (Dingermann and Nerke, 1987) and tRNA genes which are naturally or artificially variant (Marschalek and Dingermann, 1988; Schmutzler and Gross, 1990; Krieg *et al.*, 1991). Unfortunately, these methods have a number of inherent limitations (section 1.13).

An alternative approach has been the utilization of nonsense suppressor tRNA genes, which afford a facile phenotypic assay for their functional expression *in vivo* through the direct analysis of translational readthrough of termination codons (section 1.14). Through utilization of yeast suppressor tRNA genes it has been demonstrated that extragenic flanking sequences of certain tRNA genes are important in the regulation of their expression *in vivo* (Shaw and Olson, 1984; Allison and Hall, 1985; Campbell-Raymond *et al.*, 1985). Suppressors shown to be active in mammalian cells have been constructed using site-directed mutagenesis of cloned tRNA genes (Hudziak *et al.*, 1982; Temple *et al.*, 1982; Laski *et al.*, 1982, 1984; Summers *et al.*, 1983; Capone, *et al.*,

1985). Using this approach, amber, ochre and opal suppressors were derived from a human serine tRNA gene (Capone *et al.*, 1985). Functional expression of these human suppressor $tRNA^{Ser}$ genes has been detected and quantified *in vivo* in mammalian cells by measuring suppression of an *E. coli* CAT gene containing the corresponding nonsense mutation (Capone *et al.*, 1986). The functional expression of a series of extragenic deletion mutants of this human amber suppressor $tRNA^{Ser}$ gene were quantified in mammalian cells through the utilization of this co-transfection assay (Capone, 1988). These results, and those reported here, represent the only experiments that have measured the modulatory effect of extragenic sequences on tRNA gene expression *in vivo* using a suppression assay in mammalian cells. The results from the initial study revealed that the first 66 base pairs immediately upstream of this human $tRNA^{Ser}$ gene modulates the expression of this gene in mammalian cells *in vivo* (Capone, 1988). The purpose of the work described here was to further delineate the modulatory region of this gene and to elucidate the mechanism for its action.

3.1 Construction of Upstream Deletion Mutants of the Amber Suppressor $tRNA^{Ser}$ Gene

The first step toward delineating this modulatory region was to construct a series of linker scanning mutations across this 66 base pair upstream region of the human serine tRNA gene. Therefore, two sets of *Bal* 31 deletion mutants, each containing *Xba*I linkers at the junction of their deletion, were created. One set of deletions was created from base pair -83 and the other set deleted sequences from base pair -1. It was assumed

that there would be a heterogenous population of deletions in each set so that if enough mutants were sequenced the position of the *Xba*1 linker in one set would perfectly match the position of the *Xba*1 linker in the other set. In this way the natural sequences upstream and downstream of the linker would be restored, by ligation of two matched deletion mutants at their unique *Xba*1 sites, thereby creating a series of linker scanning mutations across this region. Unfortunately, upon sequencing it was discovered that the *Bal* 31 exonuclease paused at certain sequences within the upstream region resulting in several deletion mutants with identical *Xba*1 linker positions and stretches of the upstream region with a total absence of *Xba*1 linkers. The constructs obtained were therefore treated as strict deletions with completely different replacement sequences.

ptS-32X, ptS-4X, ptS-1XA and ptS-1XB are four mutants from one set of deletions which were derived from ptS-66 by bidirectional *Bal* 31 exonuclease digestion from a unique *Hin*D3 site (present at nucleotide position -83 with respect to the mature tRNA) followed by blunt end ligation of an *Xba*1 linker 5'-GTCTAGAC-3' to the ends, *Xba*1 digestion and recircularization. These four constructs are missing the natural 5' flanking sequences of the human $tRNA^{Ser}$ gene up to nucleotides -32, -4, -1 and -1, respectively, and are shown in Figure 3.1.1. The deletion mutants ptS-66, ptS-18 and ptS-1 are missing the natural 5' flanking sequences of the human $tRNA^{Ser}$ gene up to and including nucleotide -66, -18 and -1, respectively. These contructs were derived directly from the use of unique restriction sites in pUCtS Su^{+}(am) and have been described previously (Capone, 1988). Since they are different constructs, ptS-1, ptS-1XA and ptS-

PLASMID:

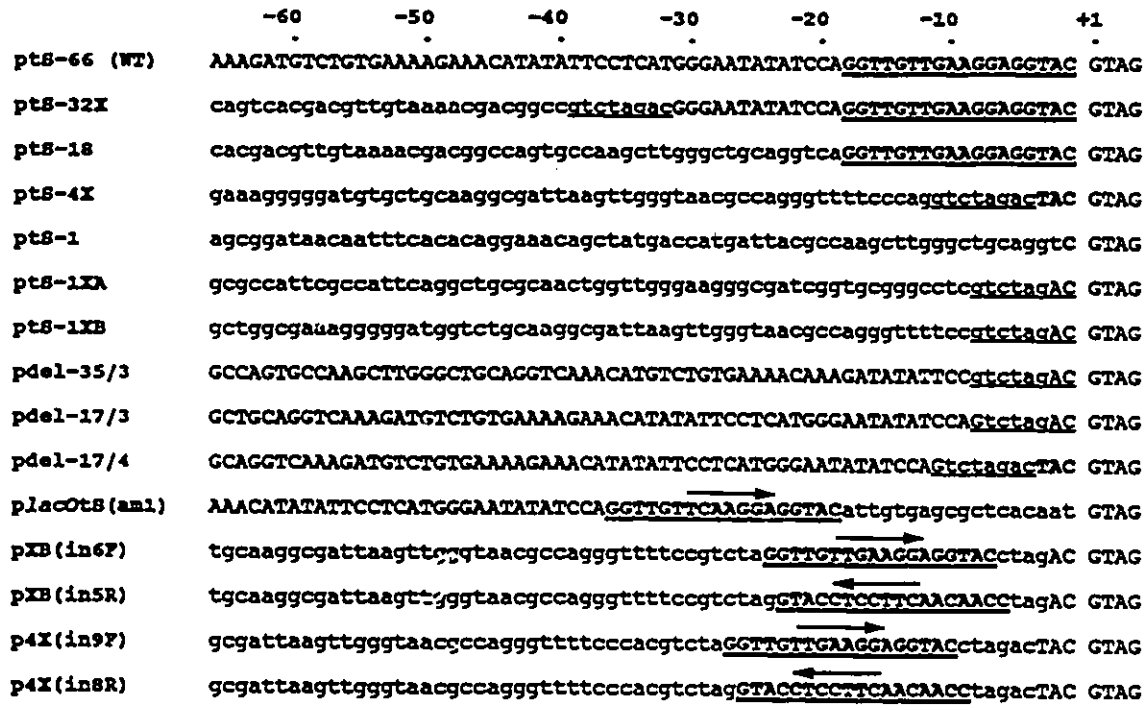


Figure 3.1.1 5'-Flanking Sequences of Deletion and Insertion Constructs of the Serine tRNA Gene. The first line shows the sequence of the upstream region of the wild type gene. Numbering is with respect to the first nucleotide present in the mature transcript which is taken as +1. Upper case letters denote sequences corresponding to the natural 5'-flanking region while lower case letters are sequences derived from the pUC vector. Sequences corresponding to the *Xba*1 linker are underlined. The sequence shown in bold and double underlined represents the -1 to -18 element, with arrows indicating its orientation with respect to the wild type gene.

1XB differ from each other in the vector sequences that replace the natural 5' flanking sequences of the gene.

The second set of *Bal* 31 deletion mutants were constructed from ptS-66 using the unique *Sna*B1 site at nucleotide position -1. These mutants were subsequently used to generate a number of human $tRNA^{Ser}$ gene constructs containing internal deletions in the region immediately upstream and are shown in Figure 3.1.1. In the construct pDel-35/3, sequences upstream to nucleotide -36 have been removed, but the sequences encoding the mature tRNA remained intact. Therefore, the natural upstream sequences from -35 to -1 are missing in pDel-35/3, and have been replaced with the *Xba*1 linker which restores the first two nucleotides of the 5' flanking sequence. Bidirectional *Bal* 31 digestion gave rise to pDel-17/+9 where the natural sequences from -17 to +9, within the mature tRNA, have been replaced with the *Xba*1 linker. The small fragment of an *Xba*1 and *Aat*2 digest of pDel-17/+9 was ligated to the large fragment of a similarily digested ptS-1XA giving rise to pDel-17/3. Thus, the natural 5' flanking sequences between nucleotides -17 and -1 of this latter clone have been replaced with the *Xba*1 linker (which restores nucleotides -1 and -2). Similarily, pDel -17/4 was constructed through ligation of the small fragment of an *Xba*1 and *Aat*2 digest of pDel-17/+9 with the large fragment of a similarily digested ptS-4X.

3.2 Initial Suppression Assay Results

The first set of deletion mutants constructed were tested in various

mammalian cell types using the *in vivo* suppression assay (Capone, 1988) and the calcium phosphate method of transfection (Graham and van der Eb, 1973). The cotransfections were performed using 5 μg of pRSVcat(am) and 3 μg of the suppressor tRNA construct. Each construct was tested once in any experiment, therefore, each replicate was performed on a different day. The CAT assays were performed using the direct diffusion method and all CAT activities were corrected for total protein in the extract. These corrected CAT activities were then directly compared to pUCtS Su^+(am), the amber suppressor $tRNA^{Ser}$ gene with wild type extragenic sequences, by normalizing their values to those obtained for this construct. The four cell types utilized were Vero, BSC-40, 293 and HeLa, and the first constructs tested were pUCtS Su^+(am) (wild type), ptS-66, ptS-18, ptS-1 and ptS-1XA. The mean suppression results are shown in Table 3.2.1 and indicate that deletion up to base pair -66 has little effect on suppression activity in that ptS-66 has similar suppression levels as the wild type gene in all the cell types tested. This finding is in agreement with the previous assay results which used Vero cells (Capone, 1988). The mean suppression results for ptS-1 and ptS-1XA, which delete the entire upstream region, indicate a small increase in suppression activity. However, the results proved difficult to reproduce, as demonstrated by the high standard deviations shown in Table 3.2.1, and the differences between the means proved to be statisitically insignificant by one tailed t test.

These results were concerning and the assay was tested for linearity by cotransfecting Vero cells with varying amounts (0.5, 1, 2, 4, 6, and 10 μg) of pUCtS Su^+(am) and 5 μg of pRSVcat(am). Each transfection condition was performed in

Construct	Vero		BSC-40		HeLa		293	
pUCtS Su^+	**1.00**	N/A	**1.00**	N/A	**1.00**	N/A	**1.00**	N/A
ptS-66	**0.85**	0.51	**0.49**	0.15	**0.88**	0.28	**0.96**	0.42
ptS-18	**0.82**	0.63	**1.57**	0.67	**0.78**	0.26	**2.12**	1.29
ptS-1	**1.85**	0.67	**1.38**	0.37	**1.96**	2.86	**1.87**	2.63
ptS-1XA	**2.03**	1.05	**1.89**	1.52	**3.04**	2.69	**4.64**	3.52

Table 3.2.1 Relative Nonsense Suppression Efficiency of Some Serine tRNA Gene 5'- Flanking Deletion Constructs in Various Cell Lines. The bold numbers on the left side of each column represent the mean suppression level from three separate transfection experiments performed with 3μg of the suppressor tRNA construct and 5μg pRSVcat(am) using the calcium phosphate transfection method. The number to the right of each mean is the standard deviation of that mean. All values shown are relative to the suppression activity of the wild type gene (pUCtS Su^+ (am)) which was normalized to 1.

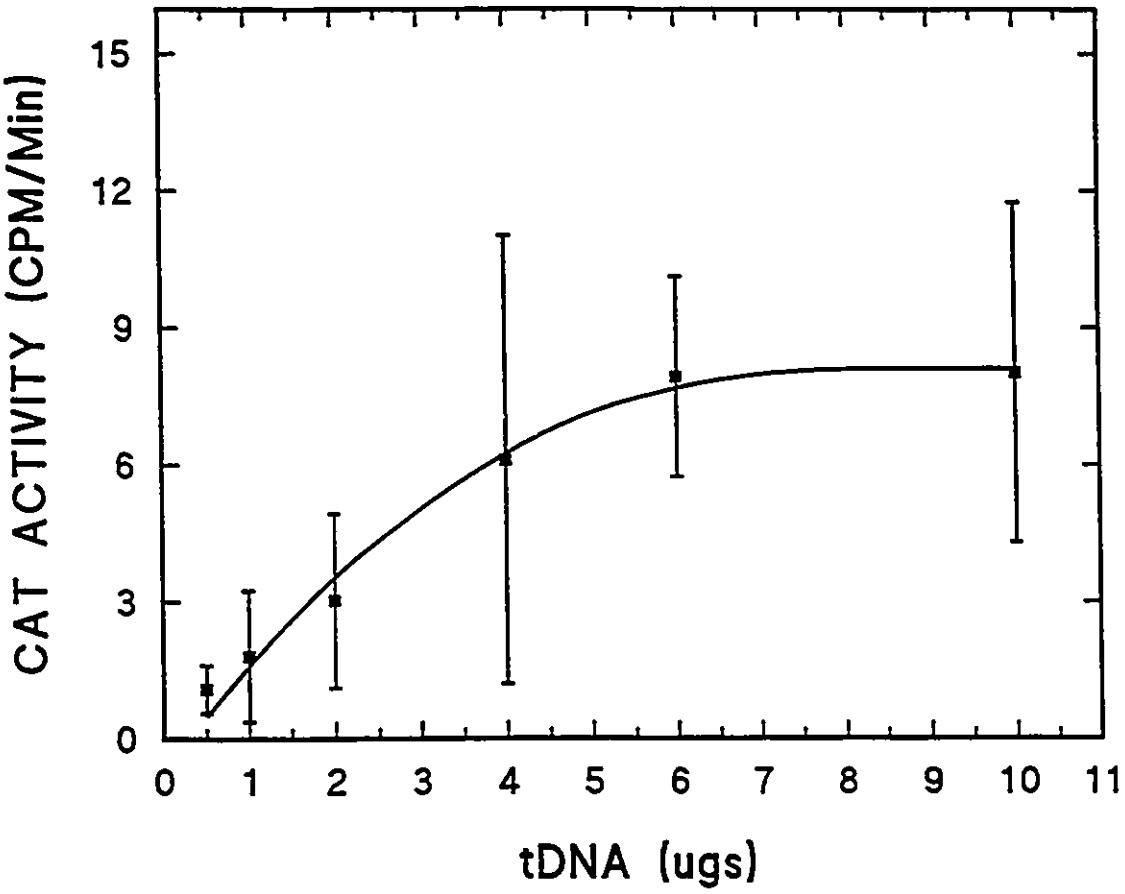


Figure 3.2.1 Suppression as a Function of Increasing Amounts of Amber Suppressor tRNA Gene Using the Calcium Phosphate Method of Transfection. Vero cells were cotransfected with 5 μg of pRSVcat(am27) and increasing amounts of pUCtS Su^+(am) as indicated on the bottom axis. Transfections were performed in triplicate using the calcium phosphate method of transfection and CAT activity was quantitated using the direct scintillation counting method.

triplicate. The results shown in Figure 3.2.1 demonstrate that the assay is linear up to approximately 5 μg of wild type suppressor tRNA and begins to saturate after this point. The results also illustrate a substantial standard deviation for each mean. In fact, the coefficient of variation, which is the standard deviation of the mean expressed as a percentage of that mean, ranges from 28 to 80 percent in Figure 3.2.1. In addition, the differences in the levels of suppression obtained using 1 μg of pUCtS Su^+(am) versus that obtained using 4 μg of pUCtS Su^+(am) are statistically insignificant. This indicates that the assay exhibits a high level of variability even between transfections performed on the same cell type on the same day.

3.3 Obtaining a Suppression Assay with Lower Variability

In an effort to find an assay with lower variability the DEAE-dextran transfection method (Lopata *et al.*, 1984) was tested as an alternative to the calcium phosphate precipitation method since the former method has a premade transfection mix and does not rely on separate and possibly variable precipitation reactions. Since DEAE dextran transfection is especially effective on BSC-40 cells they were chosen for further study. As in previous experiments, all CAT activities were normalized to the activity of the wild type construct. The accumulated data of numerous CAT assays is shown in Table 3.3.1. These results are in agreement with the earlier findings and suggest that the 5'-flanking region can be deleted up to base pair -66 with no effect on suppression while deletion to base pair -1 results in a 3 to 5 fold increase in suppression levels.

Furthermore, the results of Table 3.3.1 display less variation than the previous assays, even between transfection experiments performed on different days, as demonstrated by the lower standard deviations and reduced coefficient of variations which range from 27 to 53 percent.

The improvement using the DEAE-dextran method of transfection suggests that the largest variable in the assay is transfection efficiency. Therefore, to further improve the assay we tested the use of a plasmid as an internal transfection control which could be added to all the transfections and easily assayed in the resulting cell extracts as a measure of transfection efficiency. In preliminary experiments up to 0.5 μg of the plasmid pRSV-β-gal gave measurable β-galactosidase activity without having any competitive effect on CAT activity when cotransfecting 5 μg of pRSVcat. Therefore, 0.5 μg of pRSV-β-gal was tested in a suppression assay using 5 μg of pRSVcat(am) and 5 different amounts of pUCtS Su^+(am). Each transfection condition had 4 replicates. When plotted as amount of suppressor tDNA versus CAT activity a linear relationship similar to that of Figure 3.2.1 was obtained except with lower coefficient of variations ranging from 20 to 51 percent for the five amounts of pUCtS Su^+(am). Surprisingly, correcting these CAT activities for the protein concentration in each extract did not improve this observed variation. However, correcting these CAT activities for β-galactosidase activity substantially reduced the standard deviations for every amount of pUCtS Su^+(am) plotted. In fact, the coefficient of variations were reduced and are in the range of 6 to 29 percent in the final plot shown in Figure 3.3.1.

In conclusion, these results confirm the finding that differences in transfection

Construct	Number of Determinations	Mean Suppression	Standard Deviation
pUCtS Su^+ (am)	20	1.00	N/A
ptS-66	19	1.31	0.55
ptS-18	16	1.70	0.90
ptS-1	20	3.32	0.90
ptS-1XA	9	4.27	1.48
ptS-1XB	8	4.65	1.38

Table 3.3.1 Relative Nonsense Suppression Efficiency of some Serine tRNA Gene 5'-Flanking Deletion Constructs in BSC-40 Cells. All results are from transfection experiments performed using 3 μg of each construct and 5 μg of pRSVcat(am) and the DEAE-dextran method of transfection. The values shown are relative to the activity of the wild type gene (pUCtS Su^+ (am)) which was normalized to 1.

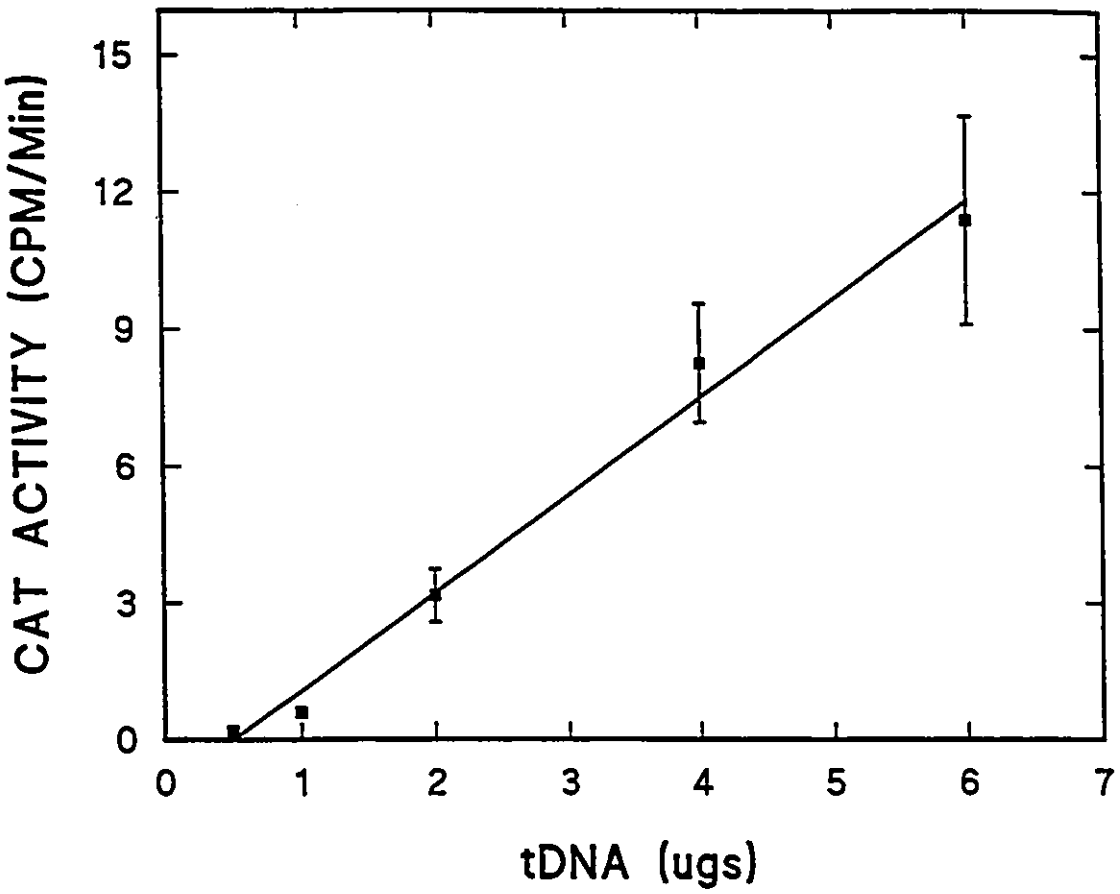


Figure 3.3.1 Suppression as a Function of Increasing Amounts of Amber Suppresor tRNA Gene Using the DEAE-Dextran Method of Transfection and an Internal Transfection Control. BSC-40 cells were cotransfected with 5 μg of pRSVcat(am27) and increasing amounts of pUCtS Su^+(am) as indicated on the bottom axis. Transfections were performed in triplicate using the DEAE-dextran method of transfection and CAT activity was quantitated using the direct scintillation counting method. Every transfection included 0.5 μg of pRSV-β-gal as an internal control and all CAT activities were subsequently normalized for β-galactosidase activity.

efficiency account for the observed variation in the suppression assay. The use of a measureable internal transfection control, such as a plasmid expressing the β-galactosidase gene, in all the transfections greatly reduces this variation. At this time a study which directly examined variation in transient gene expression assays was published (Hollen and Yoshimura, 1989). In this work it was discovered that the coefficient of variation observed in multiple transfections is reduced from approximately 40 percent down to 20 percent through the use of an internal transfection control. This reduction is similar to the improved variation observed using an internal transfection control in the indirect suppression assay. In addition, in order for the actual suppression levels to be meaningful the experminents must be performed within the linear range of the assay. Since many of our constructs exhibit suppression activities greater than that of the wild type tRNA gene, there was concern that they may saturate the assay. Thus, in order to ensure linearity, cotransfections with increasing amounts of each construct were performed.

3.4 Final Suppression Assay Results from Deletion Constructs of the $tRNA^{Ser}$ Gene

The suppression assay in its final form involved the use of 5 different amounts of each construct to be tested (0.25, 0.5, 1, 2, and 4 μg) transfected into BSC-40 cells using the DEAE-dextran transfection method. Every transfection included 5 μg of pRSVcat(am) and 0.5 μg of pRSV-β-gal, the latter included as an internal transfection control. Each transfection condition had 4 replicates and β-galactosidase activity was

measured and used to correct the CAT activity in every extract. The slope of a plot of the amount of tranfected tDNA verus the corrected CAT activity is a measure of the suppression activity of each construct. As before, the suppression activity for each construct was measured in relation to the activity of the wild type gene by normalizing all the values to that of wild type. Therefore, to ensure consistency, a similar determination of the activity of the wild type gene was performed in every experiment.

The final suppression results obtained using the above assay are shown graphically in Figure 3.4.1 and are summarized in Table 3.4.1. The inset of Figure 3.4.1 shows representative plots used to calculate these activities. All plots were linear with coefficient of correlations greater than 0.9 indicating that all assays were performed within the linear range. The values in Table 3.4.1 clearly show that deletion of sequences up to base pair -18 has no effect on the functional expression of the human serine tRNA gene in that those constructs with these sequences deleted, including ptS-66, ptS-32X and ptS-18, all exhibit levels of suppression which are similar to that of the wild type gene. In contrast, constructs ptS-4X, ptS-1, ptS-1XA and ptS-1XB all display a 2.5 to 5 fold increase in suppression levels over the wild type gene. This increase is statistically significant as determined by the one tailed t-test, at the 95 percent confidence interval, demonstrating that deletion of all the 5' flanking sequences results in higher functional tRNA expression levels. Since the three -1 deletion constructs all exhibit higher suppression activities than wild type, yet differ in the substituting vector sequences which replace the natural 5' flanking region, strongly indicates that this increase in expression is not related to the fortuitous placement of stimulatory vector sequences

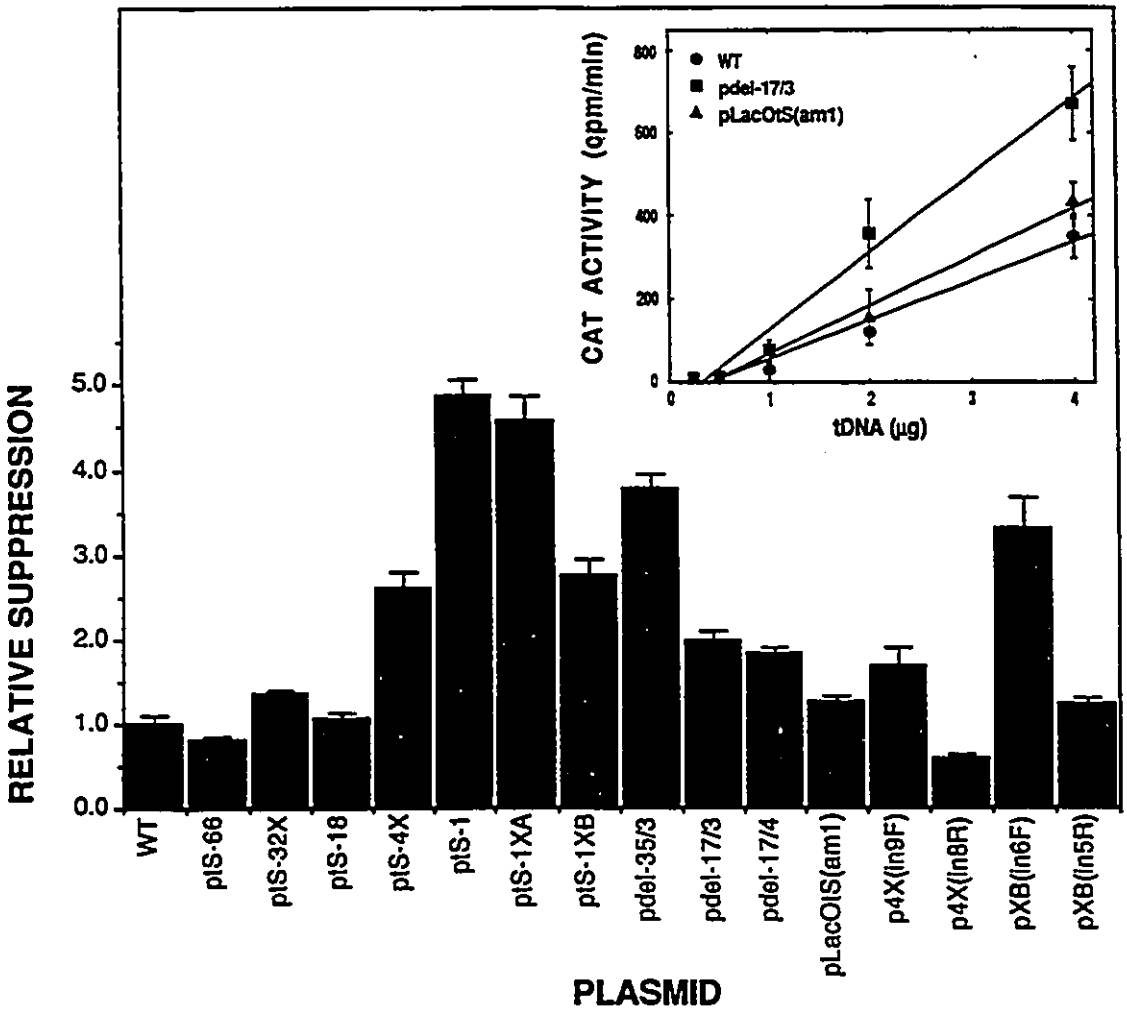


Figure 3.4.1 Final Suppression Results of Serine tRNA Gene Constructs with Altered 5'-Flanking Sequences. BSC-40 cells were cotransfected with pRSVcat(am27), a serine tRNA gene construct and pRSV-β-gal as an internal transfection control. CAT activity was determined and normalized for β-galactosidase activity for each plate of cells transfected. Each tRNA gene construct was transfected in quadruplicate at each of 5 different DNA concentrations (0.25, 0.5, 1, 2, and 4 μgs). The suppression activity represents the slope of the CAT activity versus the amount of transfected DNA as determined by linear regression analysis. The values shown are relative to the wild type gene which was normalized to 1. This data is also numerically summarized in Table 3.4.1. The inset shows representative data for pUCtS Su^+(am), pdel-17/3, and p*Lac*OtS(am).

Construct	Coefficient of Correlation of Slope	Suppression Activity	Standard Deviation
pUCtS Su^+ (am)	0.918 to 0.982	1.00	0.06 to 0.23
ptS-66	0.980	0.80	0.04
ptS-32X	0.984	1.34*	0.06
ptS-18	0.968	1.05	0.08
ptS-4X	0.957	2.58*	0.20
ptS-1	0.983	4.85*	0.22
ptS-1XA	0.962	4.57*	0.30
ptS-1XB	0.954	2.73*	0.20
pDel-35/3	0.985	3.77*	0.17
pDel-17/3	0.975	1.98*	0.12
pDel-17/4	0.976	1.81*	0.09
pLacOtS(am1)	0.970	1.25	0.07
pXBin6F	0.917	3.28*	0.38
pXBin5R	0.969	1.22	0.08
pXBin9F	0.934	1.68*	0.21
pXBin8R	0.958	0.58	0.05

Table 3.4.1 Summary of Final *In Vivo* Suppression Results for $tRNA^{Ser}$ Gene Constructs With Altered 5'-Flanking Sequences. The above data is shown graphically in Figure 3.4.1 and was obtained as described in the legend of that figure. The data is a summary of 8 separate experiments one of which is shown in the inset of Figure 3.4.1. Since the wild type gene was included in each of these experiments the range of values obtained for the correlation and standard deviation of these slopes are shown. The asterisks indicate values that are statistically higher than wild type in each experiment as determined by one tailed t-tests performed at the 95% confidence interval.

upstream.

Taken together, the results suggest that the natural sequences immediately upstream reduce the functional expression of the human $tRNA^{Ser}$ gene. To further confirm this, three internal deletion mutants, missing the natural sequences -35 to -3, -17 to -4 and -17 to -3, were constructed and are named pDel-35/3, pDel-17/4 and pDel-17/3, respectively. The *in vivo* suppression assay results of these deletion mutants are shown graphically in Figure 3.4.1 and numerically in Table 3.4.1. All three constructs exhibit an approximate 2 to 4 fold increase in suppression activity over the wild type gene and each increase statistically significant as determined by one tailed t-tests performed at the 95 percent confidence interval. These results further indicate that the natural sequences between base pairs -18 and -4, while not essential for biological activity, negatively modulate the expression of the $tRNA^{Ser}$ gene *in vivo*.

3.5 Construction and Suppression of $tRNA^{Ser}$ Gene Insertion Mutants

The possible role of position and orientation dependence for the modulatory effect of this element was explored by reintroducing, in both orientations, the negative element back into ptS-4X and ptS-1XB. To construct these insertion mutants two complementary oligonucleotides (5'-ctaGGTTGTTGAAGGAGGTAC-3' and 5'-ctaGGTACCTCCTTCAACAAC-3') were synthesized which together comprise the 18 base pair region immediately upstream of the human $tRNA^{Ser}$ gene. The oligonucleotides were constructed with *Xba*1 sticky ends to facilitate cloning. Insertion mutants pXBin6F

and pXBin5R were created by inserting this synthetic double stranded region into the *Xba*1 site of ptS-1XB in both the forward and reverse orientation, respectively. Similarily, p4Xin9F and p4Xin8R were created by insertion of the synthetic region into the *Xba*1 site of ptS-4X in both the forward and reverse orientation, respectively. The sequences of the insertion constructs are shown in Figure 3.1.1 and the suppression results are shown graphically in Figure 3.4.1 and numerically in Table 3.4.1. In general, the higher levels of suppression activity observed with the parental plasmids ptS-4X and ptS-1XB are negated by the reinsertion of this element, irrespective of orientation, resulting in constructs which often exhibit suppression levels similar to that of the wild type gene. This suggests that the modulatory effect on functional expression exerted by this element on the serine tRNA gene is independent of the element's position and orientation. One exception to this is pXBin6F, whose suppression activity remains elevated at a level similar to that of the parental plasmid ptS-1XB. An explanation for this is offered in section 4.1 of this thesis. Another construct tested in the suppression assay was p*Lac*OtS(am1). This construct is described in Chapter 5 of this thesis and contains an 18 base pair insertion of heterologous DNA at position -1 which effectively displaces the 5' flanking region of the tRNA gene 18 nucleotides farther upstream. p*Lac*OtS(am1) exhibits suppression activity statistically indistinguishable from the wild type gene, further supporting the finding that this element reduces the functional expression levels of the serine tRNA in a position independent manner.

3.6 Summary of *In Vivo* Suppression Data

The results of an earlier study revealed that the first 66 base pairs immediately upstream of a human $tRNA^{Ser}$ gene modulates the expression of this gene in mammalian cells *in vivo* (Capone, 1988). To further delineate the sequences responsible for this modulation *Bal*31 deletion mutants were constructed and tested in the *in vivo* suppression assay. The initial results using the calcium phosphate method of transfection resulted in statistically insignificant results and demonstrated the assays high inherent variability. This variability was reduced by using the DEAE dextran method of transfection, including an internal transfection control, and by performing transfection replicates on the same day, and therefore, on the same set of cells. The assay in its final form allowed the measurement of the functional expression of a series of extragenic deletion and insertion mutants of this gene so that the differences in suppression levels reported between the various $tRNA^{Ser}$ gene constructs are statistically significant at the 95% confidence interval.

The final assay results indicate that the suppression strength of the amber suppressor $tRNA^{Ser}$ gene is unaffected by the deletion of its natural 5'-flanking sequences up to base pair -18, since constructs deleting these sequences (such as ptS-66, ptS-32X and ptS-18) all exhibit wild type levels of suppression. Conversely, four other $tRNA^{Ser}$ gene constructs with upstream sequence deletions beyond base pair -18 (such as ptS-4X, ptS-1, ptS-1XA and ptS-1XB) all display a 2.5 to 5 fold increase in suppression levels over that of the wild type gene. The increase in suppression strength observed with three

collapse mutants, (pDel-35/3, pDel-17/4 and pDel-17/3) which remove the sequences immediately upstream, confirm the importance of the first 18 nucleotides of the upstream region. In addition three out of four derivatives (including pXBin5R, p4Xin9F and p4Xin8R) which reinsert this 18 base pair element back into the upstream region negate the increased suppression phenotype of the deletion constructs from which they were derived. These later results, and the results from p*Lac*OtS(am1), suggest that this 18 base pair element is moveable and can function in either orientation.

Taken together, the above suppression assay results indicate that the natural upstream region between base pairs -1 and -18 exerts a dominant negative effect on the functional expression of this human $tRNA^{Ser}$ gene *in vivo*. The modulatory effect of flanking sequences on tRNA gene transcription is believed to be a general method by which tRNA levels are regulated in the cell, and 5'-flanking sequences have been clearly shown to confer tissue specific and developmentally regulated tRNA gene expression in *B. mori*, *Xenopus*, and *C. elegans* (section 1.12 and 1.14). In addition, tRNA populations have been shown to adapt to new codon usage requirements, and significant correlations between the distribution of individual isoaccepting tRNA species and their corresponding codon frequencies have been found in many different tissues in a variety of eukaryotes (section 1.11). In this regard, a 2.5 to 5 fold effect, like that observed with this human $tRNA^{Ser}$ gene, on the abundance of a single isoacceptor species would be quite substantial. Unfortunately, it is unknown how the expression of this individual serine tRNA gene contributes to the cellular population of this particular isoacceptor and until every member of the $tRNA^{Ser}$ gene family is identified and analyzed

transcriptionally, as is currently being attempted with the human $tRNA^{Val}$ family (Thoman *et al.*, 1989; Kacar *et al.*, 1992), this issue will remain unresolved. Nevertheless, it is interesting that the serine tRNA gene possesses an element which negatively controls its expression in that this gene has the biological capacity to be upregulated, perhaps, tissue specifically or during a certain stage in human development.

Since the *in vivo* assay measures final functional tRNA it is impossible to comment of the mechanism of action by which this element appears to exert its dominant negative effect. Since biosynthesis of the final aminoacylated tRNA involves a number of steps (section 1.10), the differences in functional tRNA expression observed with the various 5' flanking tRNA gene constructs could be the consequence of a number of post-transcription modification and maturation events such as 5' and 3' end maturation, CAA trinucleotide addition, or specific base pair modifications. In order to address this, and determine the mechanism by which this modulatory element exerts its effect, *in vitro* transcription experiments were performed and are described in the next chapter of this thesis.

TRANSCRIPTION OF SERINE tRNA GENE DERIVATIVES *IN VITRO*

The experiments described below were conducted based on the results from the *in vivo* suppression assay, provided in the previous chapter, which show that the 5'-flanking region between base pairs -1 and -18 negatively modulates the functional expression of a human serine tRNA gene. Unfortunately, the suppression assay does not distinguish transcriptional activity from post-transcriptional RNA stability or modification events that are also involved in the biosynthesis of final functional mature tRNA. Thus, to further our understanding of the role of this region the transcriptional activity of the various 5'-flanking deletion and insertion constructs of the human $tRNA^{Ser}$ gene were analysed using an *in vitro* transcription assay. To ascertain if the differences observed using the *in vivo* suppression assay are related to the *in vitro* transcriptional efficiency of each mutant tRNA gene, these two sets of data were directly compared. In addition, *in vitro* transcription competition experiments were performed in order to address the mechanism underlying this negative modulatory effect.

4.1 *In Vitro* Transcriptional Activity of Upstream Deletion and Insertion Contructs of the Human $tRNA^{Ser}$ Gene

The *in vitro* transcription assay used plasmid DNA as a template in extracts

prepared from HeLa cell nuclei (Dignam *et al.*, 1983). Transcription was measured by incorporation of radiolabelled GTP into the RNA transcripts analysed by fully denaturing polyacrylamide gel electrophoresis and autoradiography. The system was optimized for amount of extract, total DNA, template DNA and magnesium. The wild type $tRNA^{Ser}$ gene and all of the constructs were transcribed in triplicate and the resulting transcripts were quantified by densitometric analysis of the autoradiograms. All results were normalized to the activity of the wild type gene.

The products from a typical transcription reaction are shown in Figure 4.1.1. The transcription reactions generally contain very few background products. In addition, the HeLa cell nuclear extracts contain active tRNA processing enzymes and the gels resolve unprocessed precursor transcripts from the final mature form. The quantitative *in vitro* transcription results for all of the $tRNA^{Ser}$ gene constructs are summarized in Table 4.1.1. All of the constructs deleting sequences up to base pair -18 exhibited levels of *in vitro* transcriptional activity which were comparable to the wild type gene (Figure 4.1.1, lanes a-d). In constrast, constructs which delete sequences beyond base pair -18, with the exception of ptS-4X, displayed 3 to 7 fold higher levels of transcriptional activity than the wild type gene (lanes f-k). These levels are statistically higher than wild type as determined by performing one-tailed t-tests at the 95 percent confidence interval. The p*Lac*OtS(am1) construct, in which the entire 5' flanking region is 18 base pairs farther upstream, retained wild type levels of *in vitro* transcriptional efficiency (lane l). As shown in Figure 4.1.2 and Table 4.1.1 constructs pXBin6F, pXBin5R, p4Xin9F and p4Xin8R, created by reintroduction of the 18 base pair element into ptS-1XB and ptS-4X,

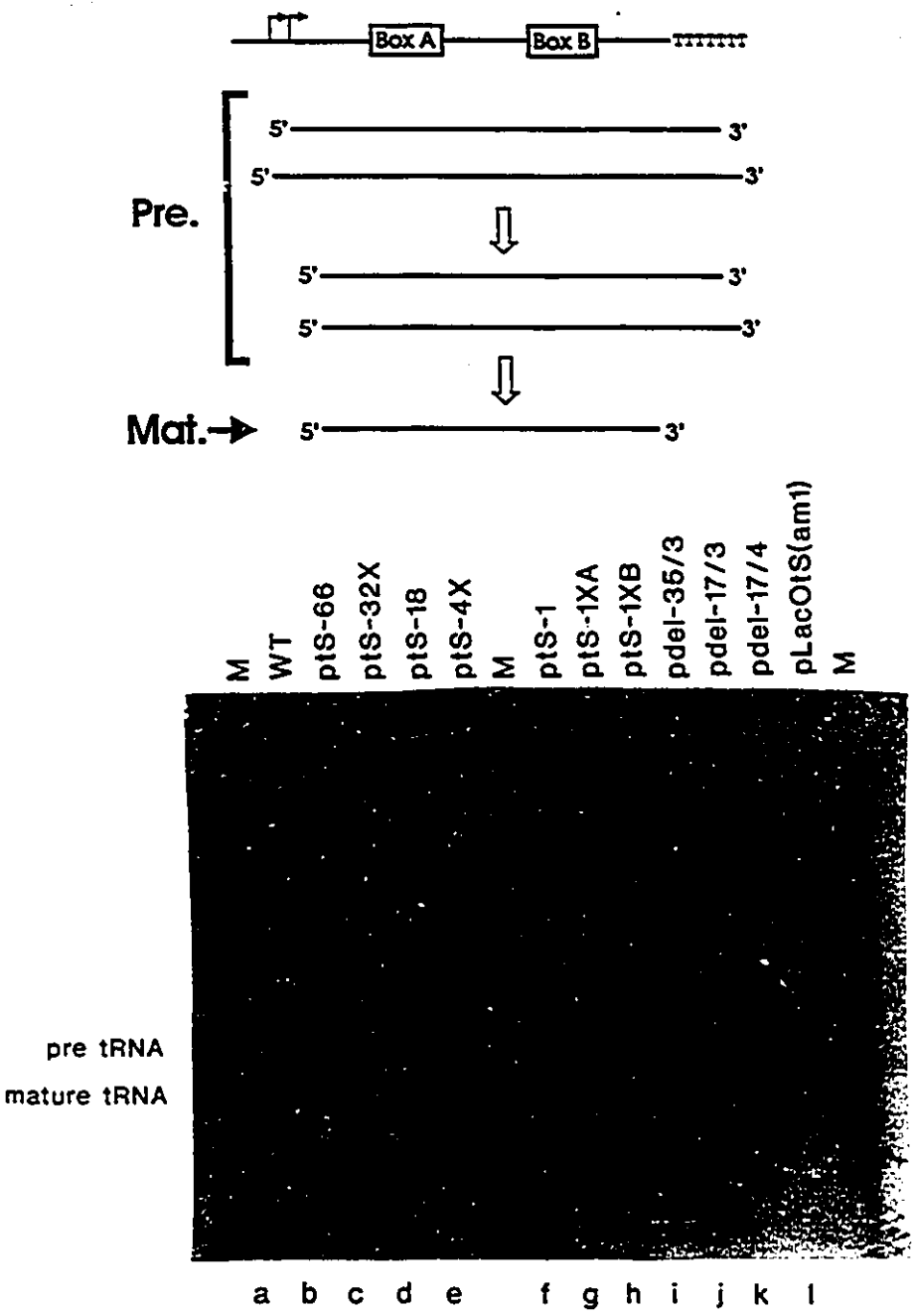


Figure 4.1.1 ***In Vitro*** **Transcription of Serine tRNA Gene Constructs with Altered 5'-Flanking Sequences.** The top of the figure is a schematic representation of a tRNA gene where transcription usually initiates from more that one site and terminates at multiple places within the stretch of thymidine residues. This results in a heterogeneous population of precursor tRNA transcripts (pre.) which are subsequently processed at the 5' and 3' ends (indicated by open arrows) to yield the mature sized transcript (mat.). The positions of the these precursor and mature sized tRNA transcripts are indicated on the gel. Exactly 0.1 μg of each tRNA gene construct, as indicated at the top of the gel, was transcribed in HeLa cell nuclear extract and analyzed by gel electrophoresis. M represents size markers derived from *Hpa2* digested and Klenow labelled pBR322.

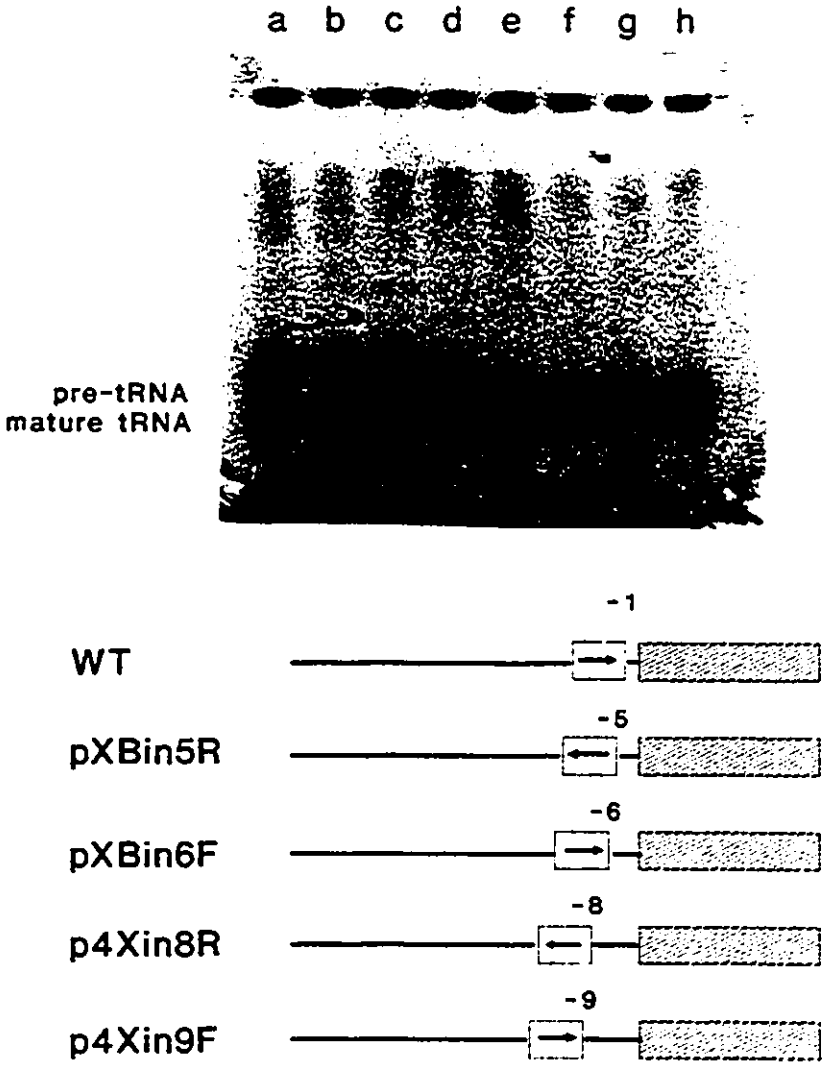


Figure 4.1.2 ***In Vitro*** **Transcription of Serine tRNA Gene 5'-Flanking Insertion Constructs.** Exactly 0.1 μg of each tRNA gene insertion construct was transcribed in HeLa nuclear extract and analyzed by gel electrophoresis. The positions of the precursor and mature sized transcripts are indicated. Lanes a and b, wild type gene; lane c, ptS-1XB; lane d, ptS-4X; lane e, p4Xin9F; lane f, p4Xin8R; lane g, pXBin6F; lane h, p4Xin5R. The insertion constructs are schematically digrammed at the bottom of the figure. The open boxes with the arrows indicate the relative position and orientation of the 18 nucleotide long proximal element for the various insertion mutants. The hatched boxes represent the sequences encoding the mature tRNA.

Construct	Transcriptional Activity	Standard Deviation
pUCtS Su^+ (am)	1.00	0.26
ptS-66	1.04	0.25
ptS-32X	1.20	0.19
ptS-18	1.70	0.81
ptS-4X	1.42	0.40
ptS-1	4.58*	0.37
ptS-1XA	3.55*	0.72
ptS-1XB	6.73*	2.10
pDel-35/3	7.08*	1.50
pDel-17/3	3.96*	0.90
pDel-17/4	3.02*	0.55
p*Lac*OtS(am1)	0.64	0.30
pXBin6F	0.68	0.13
pXBin5R	0.24	0.05
p4Xin9F	1.24	0.13
p4Xin8R	0.23	0.08

Table 4.1.1 ***In Vitro*** **Transcriptional Activity of Serine tRNA Gene Constructs with Altered 5'-Flanking Sequences.** Transcription reactions were performed in HeLa extracts using 0.1 μg of template DNA. Transcripts were analysed by gel electrophoresis and autoradiography. Quantitation was carried out by densitometric scanning and normalized against the activity of the wild type gene which was taken as 1. The values represent the average and standard deviation obtained from three separate transcription reactions. The asterisks indicate values that are statistically higher than wild type as determined by one tailed t-tests at the 95% confidence interval.

resulted in wild type or below wild type levels of transcription. These results indicate that the 5' proximal 18 base pair region of this human $tRNA^{Ser}$ can exert a dominant negative effect on transcription *in vitro*, and can do so even if placed at different positions and orientations with respect to the coding region.

4.2 Comparison of *In Vivo* Suppression and *In Vitro* Transcription Results.

A large number of *in vitro* studies have demonstrated that extragenic sequences can modulate the transcription of eukaryotic tRNA genes (reviewed in Geiduschek and Tocchini-Valentini, 1988; Geiduschek and Kassavetis, 1992). However, due to the inability of these systems to faithfully reproduce the conditions found within the cell it remains uncertain whether these studies constitute true biological events (section 1.12). In only a few cases has the effect of these sequences on *in vivo* expression been examined (sections 1.13 and 1.14). Some studies in yeast, have utilized nonsense suppressor tRNA genes which provide a simple phenotypic assay that overcomes the large number of endogenous isoacceptor tRNA genes and allows an assessment of the functional expression of an individual tRNA gene *in vivo*. Only one study, examining a yeast $tRNA^{Leu}$ gene, has quantitatively compared the *in vivo* expression and *in vitro* transcription levels of a eukaryotic tRNA gene (Cambell-Raymond *et al.*, 1985). The nonsense suppression assay described in this thesis, coupled with *in vitro* analyses, provides a quantitative strategy to examine the relationship between transcriptional activity *in vitro* and functional expression *in vivo* of mammalian

tRNA genes. Thus, the work described here provides an opportunity to address various aspects of mammalian tRNA gene regulation *in vivo* in a manner which has been used successfully in lower eukaryotic systems.

A direct comparison of the accumulated results, shown in Table 4.2.1, reveals that the increased functional expression observed with the various $tRNA^{Ser}$ constructs *in vivo*, correlates well with their increased transcriptional activity *in vitro*. The coefficient of correlation between both data sets is 0.64 and this linear correlation is statistically nonrandom at the 95% confidence interval. However, this correlation was not a strict one. For example, the construct with the highest suppression level was not the one with the highest transcriptional activity. Furthermore, the *in vitro* transcriptional efficiencies of ptS-4X and pXBin6F were similar to that of the wild type gene even though these constructs displayed significantly higher levels of suppression.

This correlation exists despite the fact that the *in vivo* suppression assay does not distinguish transcriptional activity from post-transcriptional modification or processing events. This is especially significant since the human $tRNA^{Ser}$ gene initiates transcription at purine nucleotides -5, -6 and -7, with respect to the tRNA coding region (see Section 5.6), and many of our constructs are altered through the start site up to the mature tRNA. Therefore, some of the discrepancies between the *in vivo* and the *in vitro* measurements may be due to differences in RNA processing or RNA stability of slightly different primary transcripts. None of our constructs appeared to be aberrantly processed, as judged using high resolution sequencing gels, and with only two exceptions they all appear to be processed with comparable efficiency. One of these exceptions, the

Construct	Suppression Activity	Transcription Activity	Competitive Strength
pUCtS Su^+ Su	1.00	1.00	1.00
ptS-66	0.80	1.04	1.51
ptS-32X	1.34*	1.20	1.81
ptS-18	1.05	1.70	0.69
ptS-4X	2.58*	1.42	1.05
ptS-1	4.85*	4.58*	2.86*
ptS-1XA	4.57*	3.55*	2.26*
ptS-1XB	2.73*	6.73*	2.01*
pDel-35/3	3.77*	7.08*	3.77*
pDel-17/3	1.98*	3.96*	2.57*
pDel-17/4	1.81*	3.02*	1.64
pLacOtS(am1)	1.25	0.64	1.43
pXBin6F	3.28*	0.68	3.92*
pXBin5R	1.22	0.24	3.14*
p4Xin9F	1.68*	1.24	3.04*
p4Xin8R	0.58	0.23	1.03

Table 4.2.1 Summary of the *In Vivo* Suppression, *In Vitro* Transcription and Competition Data of $tRNA^{Ser}$ Constructs with Altered 5'-Flanking Sequences. The values for suppression activity, transcripitional activity, and competitive strength are taken from Tables 3.4.1, 4.1.1, and 4.3.1, respectively. The asterisks indicate that these values are higher than the wild type as determined by one tailed t-test at the 95% confidence interval.

pXBin6F construct, appeared to be almost completely processed *in vitro* and the ratio of mature tRNA to primary transcript was consistently much greater than that observed for any other template (Figure 4.1.2, lane g). This may explain the observed 3 fold higher suppression activity of this construct *in vivo* even though the overall *in vitro* transcriptional activity was similar to that of the wild type gene. Furthermore, if this explanation is accepted and the data from this construct is ignored, then the coefficient of correlation for the remaining data set increases from 0.64 to 0.72 and the confidence interval over which this linear correlation is nonrandom increases from 95 to 99 percent.

In conclusion, the functional expression *in vivo* and transcriptional efficiency *in vitro* of a human $tRNA^{Ser}$ gene appear to be both negatively modulated by the proximal 5'-flanking 18 nucleotides. Deletion of this upstream element, and replacement with heterologous sequences, results in a 2 to 5 fold increase in suppression activity and a 2 to 7 fold increase in transcriptional efficiency in comparison to the wild type gene. The results obtained from the insertion mutants indicate that this modulatory element may act in a dominant negative manner *in vivo* and *in vitro*. The strong correlation between the *in vivo* and *in vitro* results suggests that the proximal 5'-flanking sequence negatively modulates transcription of the human $tRNA^{Ser}$ *in vivo*. Thus, in this instance, the *in vitro* results appear to accurately reflect *bona fide* cellular processes.

4.3 Determining the Mechanism by which the Negative Modulatory Element Exerts its Transcriptional Effect.

Since the overall data strongly suggests that the 18 base pair region negatively modulates transcription of this $tRNA^{Ser}$ gene, we were interested in determining the mechanism underlying this effect. Most 5' modulatory elements identified thus far exert a positive effect on expression, however, negative acting elements have been identified for a *Drosophilia* $tRNA^{Lys}$ (Defranco *et al.*, 1981), a *Drosophilia* $tRNA^{Arg}$ (Dingermann *et al.*, 1982) and a *Xenopus* $tRNA^{Met}$ gene (Hipskind and Clarkson, 1983). There is no obvious similarity in the sequence of these negative modulatory elements with the proximal region of the human $tRNA^{Ser}$ gene. This observation is in accord with the lack of sequence homology amoung previously identified tRNA gene transcriptional modulatory elements as discussed in section 1.15 (reviewed in Geiduschek and Tocchini-Valentini, 1988; Geiduschek and Kassavetis, 1992). In fact, extragenic flanking sequences of tRNA genes in general display no significant sequence homology even between tRNA genes within the same isoacceptor family. Some studies have speculated that the basis for 5'-flanking sequence modulation of tRNA gene transcription is the conformation of the DNA (Hipskind and Clarkson, 1983) or potential for secondary structure (Goddard *et al.*, 1983), however, there is nothing noteworthy in this regard within the region immediately upstream of the human $tRNA^{Ser}$ gene.

Many other hypotheses have been put forward to explain the diversity of extragenic flanking sequences that modulate tRNA gene transcription (section 1.15). One

theory is that highly specific, nonconserved, regulatory factors bind to these modulatory elements and attenuate transcription. However, in only one case has the existence of such a factor been unequivocally proven (Oei and Pieler, 1990). To address whether a specific regulatory factor binds to the negative element of this human $tRNA^{Ser}$ gene we tested the ability of a double stranded oligonucleotide encoding the 18 base pair region to compete for such a factor in an *in vitro* transcription assay. Since this oligonucleotide, as well as a variety of other control oligonucleotides, were found to nonspecifically reduce transcription from the serine tRNA gene this approach was deemed unsuitable for this analyis. Thus, the 18 base pair region was cloned into pUC19 and varying amounts of this construct were then added as a competitor to transcription reactions containing the wild type $tRNA^{Ser}$ gene. Similarily, equivalent amounts of the pUC19 parental plasmid were added to control reactions. Both plasmids behaved identically as competitors and caused slightly reduced levels of tRNA gene transcription (data not shown). To confirm these results the experiment was performed under constant DNA conditions while altering the ratio of the construct DNA to the parental pUC19 vector DNA. All of these reactions exhibited indistinguishable levels of tRNA gene transcription (data not shown) suggesting that this 18 base pair region of the $tRNA^{Ser}$ gene modulates transcription in the context of the other RNA polymerase III promoter elements farther downstream.

The natural position of this negative element is consistent with the finding that extragenic sequences responsible for tRNA gene transcriptional modulation are usually found within 50 base pairs upstream of the initiation site. The position of this negative element, immediately upstream of the coding region of the gene, suggests that

it may still act through the general pol III transcription machinery by inhibiting some other aspect of transcription such as DNA melting, open complex formation or transcription initiation (section 1.9). Attenuation of transcription initiation has been a suggested mechanism of action for some other tRNA gene modulatory elements (Lofquist, 1988; Sajjadi and Spiegelman, 1989).

In previous studies, extragenic sequences of tRNA genes have been shown to influence the assembly or stability of transcription complexes (Sharp *et al.*, 1983; Schaak *et al.*, 1983, 1984; Cooley *et al.*, 1984; Morry and Harding, 1986; Arnold and Gross, 1987; Arnold *et al.*, 1988; Rooney and Harding, 1988; Wahab *et al.*, 1989). The fact that yeast TFIIIB, the pivotal pol III transcription component for this organism, displays extended interactions upstream of tRNA genes (Kassavetis *et al.*, 1989, 1990) also suggests that 5'-flanking sequences may modulate transcription by acting through the basal transcription machinery. The 5'-flanking sequences of $tRNA^{Ala}$ genes in *B. mori* are the primary discriminators for their constitutive or silkgland specific transcription (see Section 1.12), and recently, this differential transcription has been ascribed to differences in the ability of these genes to compete for TFIIIB and subsequently, RNA polymerase III (Sullivan *et al.*, 1994).

To determine if the 18 base pair region influences the interaction of the $tRNA^{Ser}$ gene with transcription components we performed template competition assays on each of our constructs. The template competition assay assesses the ability of a gene to stably assemble RNA polymerase III transcription complexes by measuring the reduction in transcription of a simultaneously incubated second reference template

(Fowlkes and Shenk, 1980; Wormington, 1981). The adenovirus VA1 gene was utilized as the second template due to the similarity of this gene to tRNA genes in both promoter structure and transcription factor requirements (Segall *et al.*, 1980), and because the resulting transcripts are easily discerned from those arising from the $tRNA^{Ser}$ gene. These assays were performed using an established procedure (Sharp *et al.*, 1983) as described in section 2.7.4.

A representative competition experiment is shown in Figure 4.3.1 and the results obtained with all the tRNA gene variants are summarized in Table 4.3.1. The results from the 10 deletion mutants indicate that those constructs with higher *in vitro* transcriptional activity also exhibit a small, but statistically significant, 2 to 3 fold increase in competitive strength. In fact, the linear coefficient of correlation between transcriptional activity and competitive strength for the 10 deletion mutants in Table 4.2.1 is 0.77, and nonrandom at the 99% confidence interval. Furthermore, with the exception of ptS-4X, this increase in competitive strength correlates with the removal of the immediate 5'-flanking 18 base pairs of the $tRNA^{Ser}$ gene. These results indicate that the 18 base pair region indeed influences the affinity of the tRNA gene for limiting transcription factors resulting in inhibited transcriptional activity *in vitro*. However, when the *in vitro* transcription and competition data from all of the constructs, including the 5 insertion mutants, are compared in this fashion this correlation becomes statistically random and the coefficient of correlation drops to 0.31. The results obtained from pXBin6F, pXBin5R and p4Xin9F are largely responsible for this poor correlation in that these constructs all display wild type or below wild type levels of transcription yet exhibit

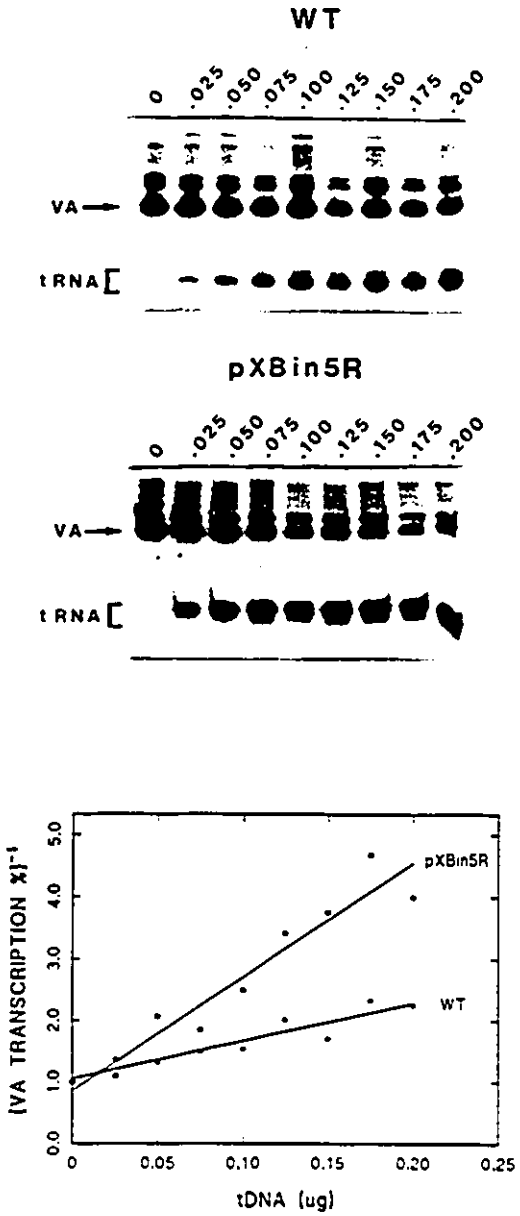


Figure 4.3.1 Representative Second Template Competition Experiment. A constant amount of pBRVA (0.4 μg) was incubated in an *in vitro* transcription reaction in the absence of competitor DNA or in the presence of vary amounts (shown in μgs) of the wild type gene or the pXBin5R construct, as indicated at the top of each gel. Total DNA was normalized to 1 μg in each reaction with pBR322. The amount of VA1 transcription was quantitated by phosphoimager analysis and expressed as a percentage of the amount of VA1 made in the absence of competitor tDNA. The reciprocal of this value was plotted against the amount of competitor DNA, as shown at the bottom of the figure, and the competitve strength was determined from the slope of the line by linear regression analyes. Similar analyses were performed for each tRNA gene construct and the results are summarized in Table 4.3.1.

Construct	Coefficient of Correlation	Competitive Strength	Standard Deviation
pUCtS Su^+ (am)	0.908	1.00	0.50
ptS-66	0.941	1.51	0.58
ptS-32X	0.982	1.81	0.38
ptS-18	0.899	0.69	0.36
ptS-4X	0.903	1.05	0.54
ptS-1	0.917	2.86*	1.32
ptS-1XA	0.902	2.26*	1.16
ptS-1XB	0.964	2.01*	0.57
pDel-35/3	0.956	3.77*	1.24
pDel-17/3	0.928	2.57*	1.08
pDel-17/4	0.886	1.64	0.91
pLacOtS(am1)	0.826	1.43	1.05
pXBin6F	0.954	3.92*	1.33
pXBin5R	0.960	3.14*	0.97
p4Xin9F	0.987	3.04*	0.54
p4Xin8R	0.886	1.03	0.58

Table 4.3.1 Competitive Ability of Serine tRNA Gene Constructs with Altered 5'-Flanking Sequences. The competitive strength of each construct was determined as described in Section 2.7.4 and in the legend of Figure 4.3.1. All the values shown have been normalized to the competitive strength of the wild type gene which is taken as 1. The coefficient of correlation indicates the linearity of each plot and the standard deviation of the slope is also shown. An asterisk indicates that this value is statistically higher than the wild type gene as determined by a one tailed t-test at the 95% confidence interval.

a 3 to 4 fold increase in competitive strength (Table 4.2.1). In this regard, it is possible that another transcription process, other than stable complex formation, which inhibits the transcriptional activity of the $tRNA^{Ser}$ gene may be effected as a result of the altering the position of the negative element with respect to the other promoter elements farther downstream.

4.4 Summary

This chapter has demonstrated that the human serine tRNA gene possesses a negative modulatory element within the 5'-flanking sequences immediately upstream of the gene in that tRNA gene constructs which delete this element result in increased *in vitro* transcriptional activity. Since the ability of *in vitro* transcription systems to faithfully reproduce cellular conditions is questionable, this finding requires supporting data from *in vivo* experiments to ascribe physiological relevence to this finding. The *in vivo* suppression strength of each of the $tRNA^{Ser}$ gene constructs strongly correlate with their *in vitro* transcriptional activity indicating that this negative element indeed modulates the transcriptional activity of this gene within the mammalian cell. In addition, tRNA gene constructs which delete this element display an enhanced ability to stably sequester limiting transcription components indicating that this is at least one mechanism by which this element exerts its dominant negative effect. The binding of a specific regulatory factor to the negative element might be capable of sterically interfering with stable complex assembly. This aspect of tRNA gene transcriptional regulation is addressed in the next chapter of this thesis.

REGULATION OF *IN VITRO* TRANSCRIPTION AND *IN VIVO* EXPRESSION OF A HUMAN SERINE tRNA GENE

A comparison of extragenic regions that have been shown to modulate tRNA gene transcription has failed to reveal any sequence homology between positive or negative transcriptional modulators despite the large number of these elements that have been identified (section 1.12). In addition, the flanking sequences of tRNA genes show no sequence conservation, even between members of the same isoacceptor family from the same species. The vast majority of transcriptional modulatory sequences are found within 50 base pairs upstream of the initiation site (reviewed in Geiduschek and Tocchini-Valentini, 1988; Geiduschek and Kassavetis, 1992).

The ability of such a diversity of sequences to modulate tRNA gene transcription could reflect the existence of gene specific factors which may bind these elements and attenuate the interaction of transcription components with the upstream region. Even though only one reported case, from *Xenopus*, has demonstrated the isolation of such a factor (Oei and Pieler, 1990), this is not a strong indication that gene specific modulatory factors do not exist, especially since the exact composition of the basal pol III transcription machinery is not completely known even in the well characterized yeast system. In this regard, factors required for basal pol III transcription in yeast are still being isolated (Dieci *et al.*, 1993). Moreover, our understanding of the

composition and spatial arrangement of the various constituents of mammalian pol III transcription complexes remains less advanced than that in yeast (addressed in section 1.4). This is principally due to difficulties in purifying mammalian pol III transcription factors and the poor efficiency of formation of functional pol III transcription complexes using crude mammalian extracts (Kovelman and Roeder, 1990), or following reconstitution with purified components (addressed in Geiduschek and Kassavetis, 1992).

Given the above limitations in isolating regulatory components of the mammalian pol III transcription machinery, this chapter describes experiments in which a reverse approach to studing the modulation of tRNA gene transcription by 5'-flanking sequences was taken. The focus of this approach was to ascertain whether a sequence specific DNA binding protein bound within the upstream region of a mammalian tRNA gene could potentially modulate its transcriptional activity. The experiments described in the previous chapters of this thesis demonstrate that the proximal 5'-flanking region of a human serine tRNA gene acts as a negative modulator of its functional expression *in vivo* and transcriptional activity *in vitro*. Specifically, this chapter describes experiments in which an oligonucleotide containing the recognition site for the *Escherichia coli lac* repressor was inserted at various positions in the 5'-flanking region of the human serine tRNA gene, and the consequences of binding *lac* repressor on *in vitro* transcription by RNA polymerase III were investigated. In addition to providing insights into potential mechanisms of transcriptional modulation of tRNA genes, these studies should also reveal information about the upstream spatial arrangement and topological boundaries of functional mammalian pol III transcription complexes.

5.1 *lac* Repressor Inhibits the Transcription of a tRNA Gene Containing a 5'-Proximal *lac* Operator Site.

The *Escherichia coli lac* operator/repressor system was chosen as a probe of RNA polymerase III transcription complexes for many reasons, most of which are based on the binding characterisics of the repressor to the operator site (reviewed in Barkly and Bourgeois, 1980). First, binding of *lac* repressor to the *lac* operator site is highly specific. Second, *lac* repressor has an extreemely high affinity for the operator site, with a dissociation constant of 10^{-13} M. Finally, repression can be relieved by the allosteric inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG) which greatly lowers the affinity of the repressor for the operator site. In addition to these advantages, the prokaryotic *lac* operator/repressor system has been shown to function in a variety of mammalian cells (Brown *et al.*, 1987; Hu and Davidson, 1987; Figge *et al.*, 1988; reviewed in Gossen *et al.*, 1993).

A *lac* operator site was initally positioned immediately upstream of the coding region of the human amber suppressor $tRNA^{Ser}$ gene to create p*Lac*OtS(am1), also known as p*Lac*OtS-9 (Figure 5.2.1). This plasmid was constructed from pUCtS-Su^{+}(am) by inserting the double stranded oligonucleotide 5'-ATTGTGAGCGCTCACAAT-3', which is a short *lac* operator sequence (Brown *et al.*, 1987), into the unique *Sna*B1 site at position -1, immediately upstream of the coding region of the tRNA gene, by blunt-end ligation. This construct was tested for *in vitro* transcriptional activity in HeLa cell nuclear extracts where the effect of adding purified *lac* repressor protein and the

allosteric inducer IPTG was determined. The results in Figure 5.1.1 show that the transcriptional activity of p*Lac*OtS(am1) is close to that of the parental wild type gene (compare lanes a and e), and the quantitative results in the last chapter demonstrate that this *lac* operator insertion has no measureable transcriptional effect on this tRNA gene. Preincubation of the p*Lac*OtS(am1) with purified *lac* repressor, prior to the addition of nuclear extract and nucleotide triphosphates, resulted in a complete inhibition of transcription (lane g). This *lac* repressor dependent inhibition of transcription is largely relieved through the addition of IPTG to the reactions (lane h). Control reactions containing IPTG alone show that this molecule has no effect on the transcriptional activity of either template (lanes b and f). *lac* repressor has no effect on transcription directed by the wild type gene (lane c) even when added in 10-fold higher concentrations. These results show that prebound *lac* repressor selectively and completely inhibits the transcription of a tRNA gene containing a cognate operator site immediately upstream of the coding region and that this repression can be largely relieved with the allosteric inducer IPTG.

Transcription complexes assembled on tRNA genes are stable, even during multiple rounds of transcription, and are refractory to a variety of challenges including high salt and excess second template DNA (section 1.4). This has been attributed to the ability of TFIIIB and TFIIIC to form a highly stable preinitiation complex. In the experiments described above *lac* repressor was preincubated with the p*Lac*OtS(am1) plasmid and allowed to bind to its target site before the addition of nuclear extract. To determine if *lac* repressor can still inhibit transcription after the assembly of a stable

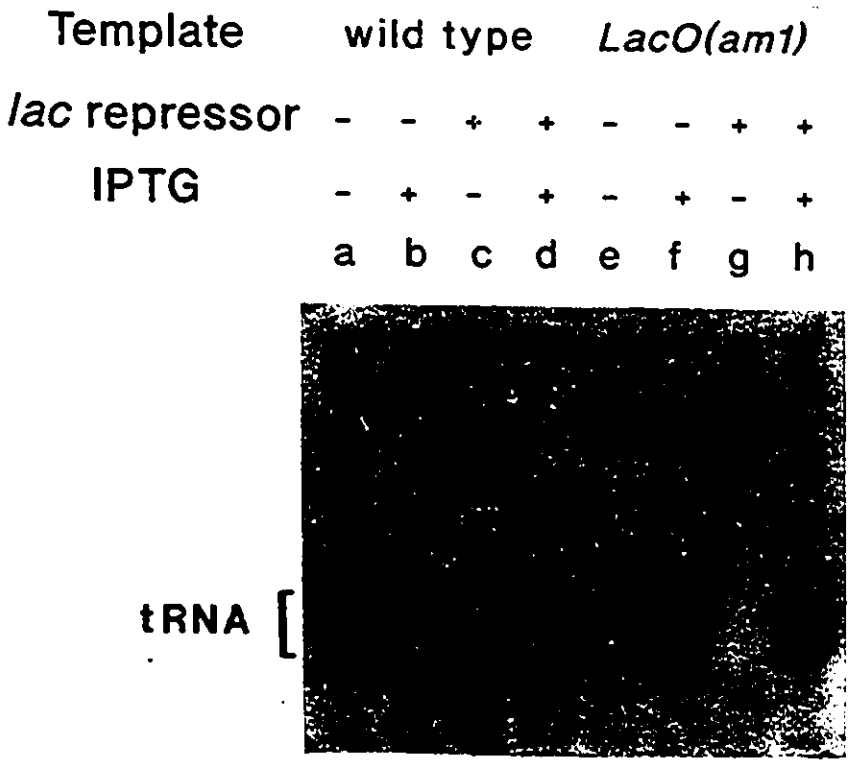


Figure 5.1.1 Inhibition of tRNA Gene Transcription by *lac* Repressor. pUCtS-Su$^+$ (lanes a to d) and p*Lac*OtS(am1) were transcribed *in vitro* using HeLa cell nuclear extracts. Where indicated, the templates were preincubated with 50 ng of purified *lac* repressor (Stratagene) in the presence or absence of 60 mM IPTG for 10 minutes prior to addition of nuclear extract and nucleotide triphosphates. Transcription reactions were incubated for two hours and analyzed by gel electrophoresis.

transcription complex, order of addition experiments were conducted by Dan Syroid and are shown schematically in Figure 5.1.2. As already demonstrated above, when the the p*Lac*OtS(am1) template was preincubated with *lac* repressor prior to the addition of extract and nucleotide triphosphates, transcription was prevented (lane d). Previous second template committment experiments, using HeLa nuclear extracts, have determined that this tRNA gene assembles stable transcription complexes in approximately five minutes (see also Figure 5.5.2). When p*Lac*OtS(am1) was preincubated with both *lac* repressor and nuclear extract for ten minutes prior to the addition of nucleotide triphosphates, transcription was completely inhibited (lane f). This result shows that *lac* repressor is capable of actively competing with the assembly of pol III transcription factors on this template. Moreover, transcription was still abolished even after p*Lac*OtS(am1) was preincubated with only nuclear extract for ten minutes, prior to the addition of *lac* repressor and nucleotide triphosphates. This latter result demonstrates that *lac* repressor is capable of accessing an operator site immediately upstream of a tRNA gene and abolishing transcription, even after this gene has assembled a stable transcription complex. This effect is specific for this template since *lac* repressor had no effect on transcription of the wild type gene under any of the above conditions (lanes c,e and g).

A kinetic analysis of transcription was carried out in order to ascertain whether *lac* repressor can prevent multiple rounds of transcription from actively transcribing complexes. Thus, the effect of adding repressor at various times during 90 minute transcription reactions was ascertained. The results, shown in Figure 5.1.3,

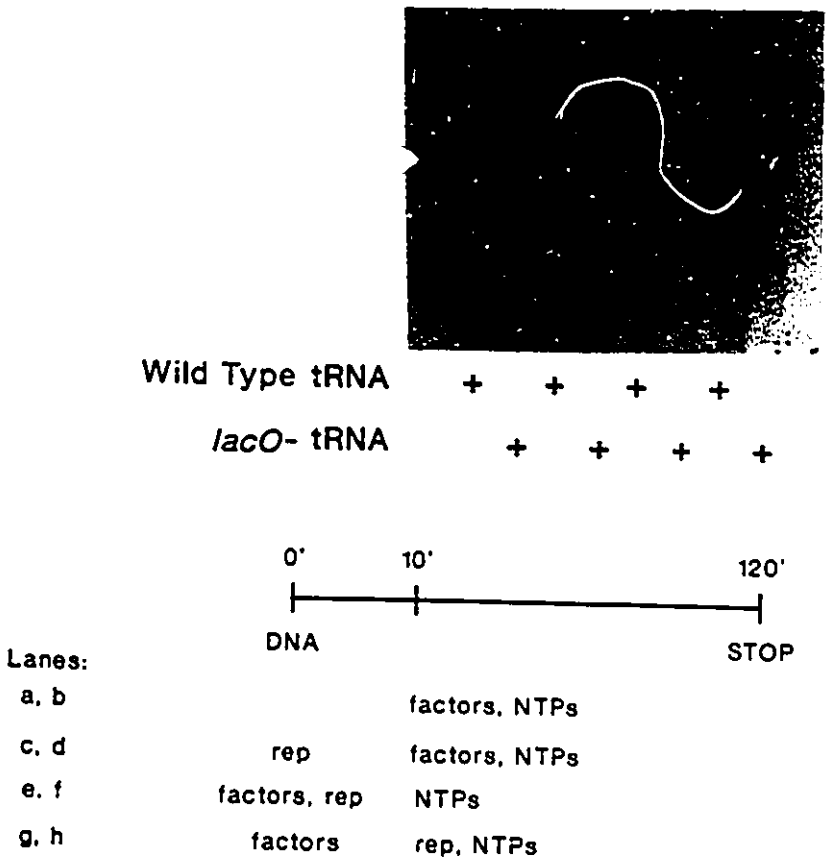


Figure 5.1.2 ***lac* Repressor Prevents Transcription of a tRNA Gene already Assembled into a Preinitiation Complex.** Order of addition experiments were performed as schematically diagrammed at the bottom of the figure. Lanes a and b are control transcription reactions using 0.1 μg of pUCtS-Su$^+$(am) (wild type-tRNA) and p*Lac*OtS(am1) (*lac*O-tRNA), respectively. In lanes c and d, these templates (DNA) were preincubated with 50 ng of *lac* repressor (rep) prior to the sequential addition of nuclear extract (factors) and nucleotide triphosphates (NTPs). In lanes e and f, templates were preincubated with nuclear extract and *lac* repressor for ten minutes prior to the addition of nucleotide triphosphates. In lanes g and h, templates were preincubated with nuclear extract for ten minutes prior to the addition of *lac* repressor and nucleotide triphosphates. Reaction mixtures were incubated for two hours following the addition of nucleotide triphosphates. This experiment was performed by Dan Syroid.

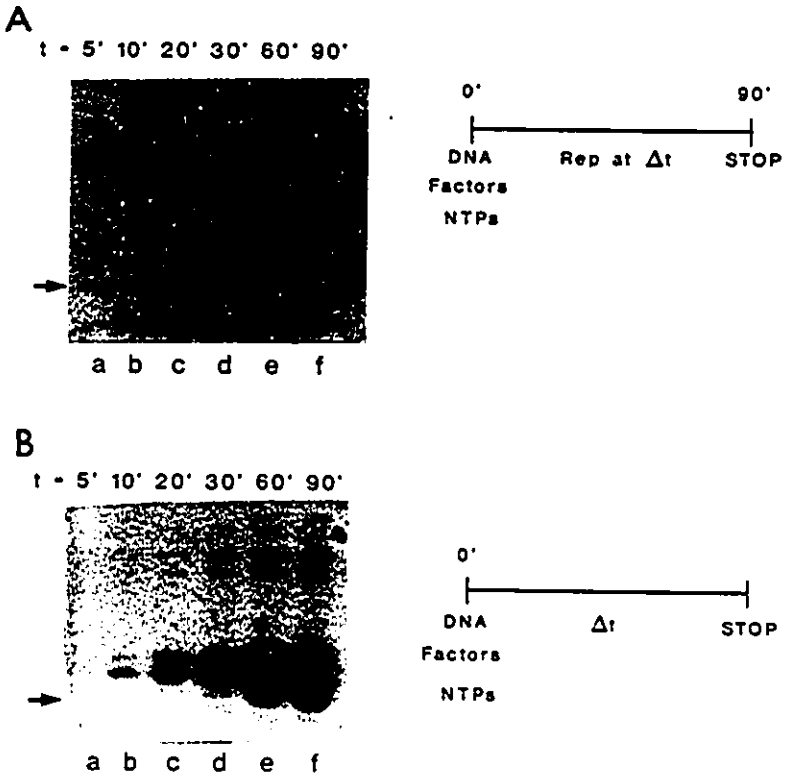


Figure 5.1.3 Prevention of Reinitation of Transcription by *lac* Repressor. Transcription reactions were carried out as schematically diagrammed on the right. In panel A, 0.2 μg of p*Lac*OtS(am1) (DNA) was incubated with nuclear extract (Factors) in the presence of nucleotide triphosphates (NTPs). *lac* repressor (Rep) was added at various times afterward (Δt) as indicated. Reaction mixtures were incubated for 90 minutes following the addition of nucleotide triphosphates. In panel B, 0.2 μg of p*Lac*OtS(am1) was incubated with nuclear extract and nucleotide triphosphates, and transcription was terminated, at the times indicated, by the addition of 10 volumes of 0.3 M sodium acetate (pH 5.0) containing 0.5% SDS. The arrows correspond to the position of the mature tRNA transcripts.

indicated that the amount of transcripts synthesized from p*Lac*OtS(am1) were proportional to the time interval that elapsed prior to *lac* repressor addition (panel A). In addition, the amount of product made coincided with control reactions carried out in parallel in the absence of *lac* repressor, but which were prematurely terminated at the corresponding times (compare panels A and B). These findings demonstrate that *lac* repressor is able to prevent multiple rounds of transcription from actively transcribing complexes, presumably by inhibiting the reinitiation of transcription.

5.2 Construction of Serine tRNA Gene Derivatives Containing *lac* Operator Sites at Varying Positions Upstream

The demonstrated ability of *lac* repressor to inhibit transcription of a tRNA gene containing a cognate operator site immediately upstream of the coding region prompted the construction of a series of tRNA gene derivatives containing a *lac* operator site at varying distances upstream of the tRNA gene. These constructs, made by Dan Syroid, are diagrammed in Figure 5.2.1. The majority of these constructs were made thorough use of the double stranded oligonucleotides 5'-ATTGTGAGCGCTCACAAT-3' and 5'-TGTGGAATTGTGAGCGCTCACAATTCCACA-3', which correspond to a short *lac* operator sequence (Brown *et al.*, 1987) and an extended *lac* operator sequence (Sadler *et al.*, 1983), respectively. p*Lac*OtS-9 is described in section 5.1. p*Lac*OtS-15 contains the extended *lac* operator sequence blunt-end ligated into the *Sna*B1 site at position -1 of the tRNA gene. The numbering of these and other constructs corresponds

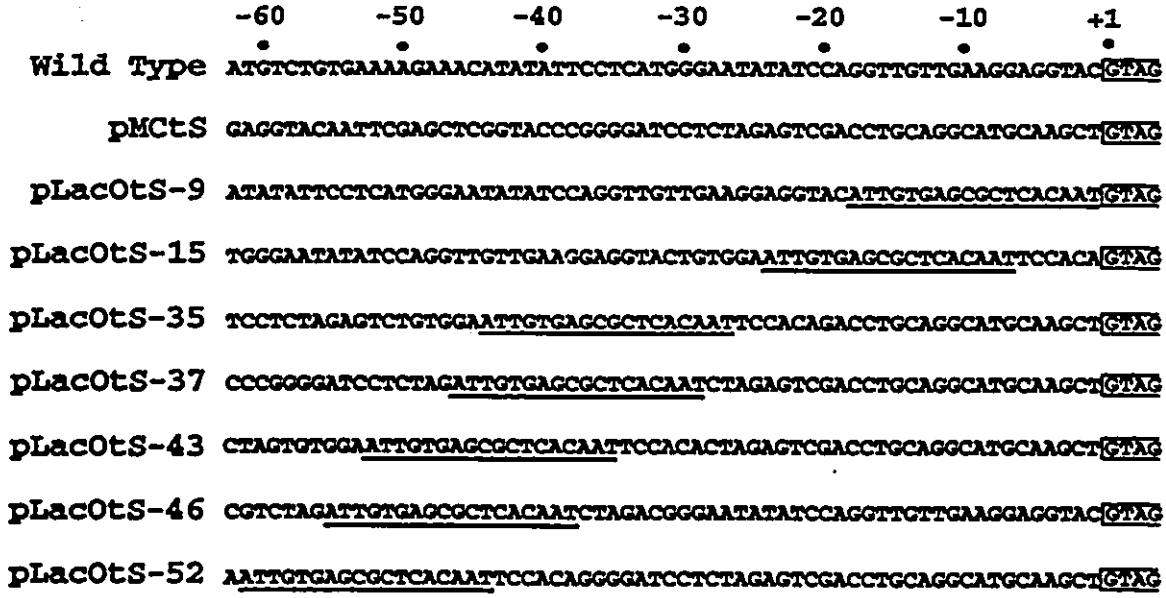


Figure 5.2.1. Sequence of *lac* Operator-Containing tRNA Genes. The open box represents the sequences encoding the mature tRNA and the underlined sequences correspond to the *lac* operator site. The numbering of the plasmids corresponds to the position of the central nucleotide of the *lac* operator site with respect to the first nucleotide of the coding region of the mature tRNA. Wild type is the parental plasmid of p*Lac*OtS-9, -15, and -46, while pMCtS is the parental plasmid of p*Lac*OtS-35, -37, -43 and -52.

to the upstream position of the central nucleotide of the *lac* operator site with repect to the first nucleotide of the coding region of the mature tRNA. Four other unique operator containing clones were derived from the construct pMCtS, which contains the pUC19 multiple cloning site immediately upstream of the human serine tRNA gene. pMCtS was constructed by ligation of this Klenow treated *Hin*D3 to *Eco*R1 multiple cloning site fragment into the unique *Sna*B1 site at nucleotide position -1 of the $tRNA^{Ser}$ gene. p*Lac*OtS-37 and p*Lac*OtS-43 were constructed by inserting the above double stranded oligonucleotides, corresponding to the short or extended *lac* operator sequences respectively, into the unique *Xba*1 site of pMCtS after first treating the *Xba* overhangs with Klenow polymerase. p*lac*OtS-35 and p*lac*OtS-52 were constructed by blunt-end ligation of the longer *lac* operator oligonucleotide into the unique *Hinc*2 and *Sma*1 sites of pMCtS, respectively. p*Lac*OtS-46 was derived from ptS-32X, a derivative of pUCtS-Su^+(am) described in Section 3.1, in which sequences upstream from position -32 were deleted and replaced with an *Xba*1 linker 5'-GTCTAGAC-3'. p*Lac*OtS-46 was constructed by inserting a double stranded oligonucleotide corresponding to the short *lac* operator sequence, except with a four-base extension to facilitate direct ligation, into the unique *Xba*1 site of ptS-32X.

5.3 Effect of *lac* Repressor on *In Vitro* Transcription of Serine tRNA Gene Derivatives.

In vitro transcription products from tRNA gene derivatives, containing *lac*

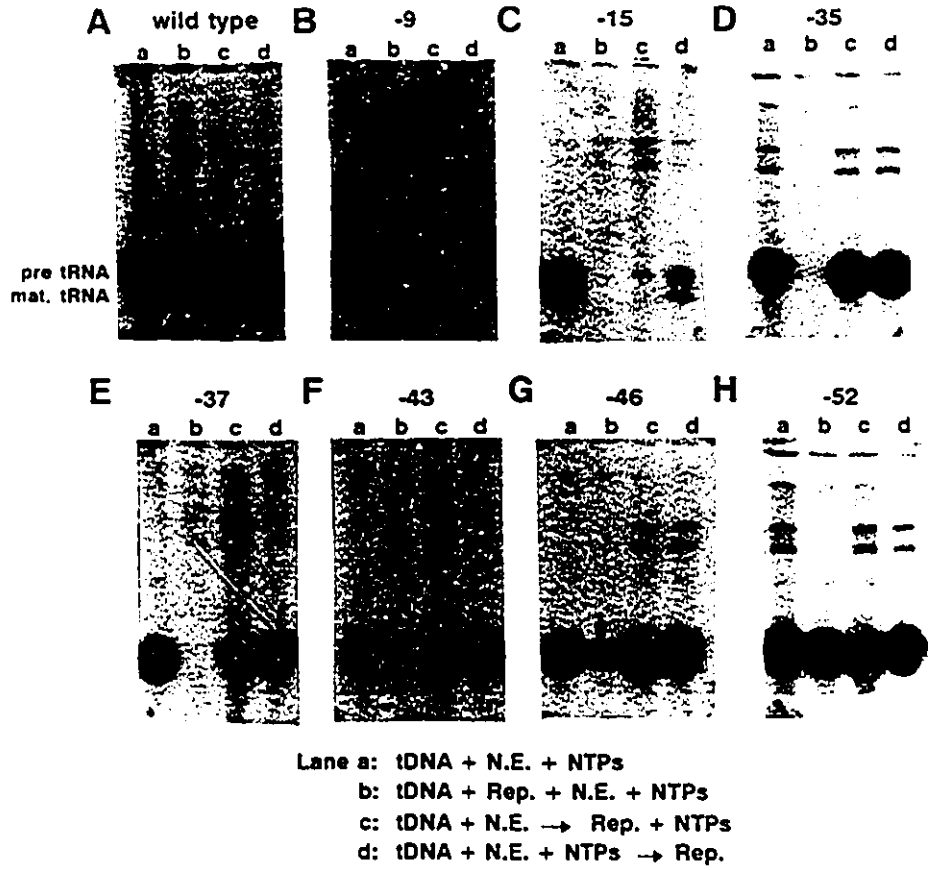


Figure 5.3.1 ***lac*** **Repressor Conditionally Inhibits tRNA Gene Transcription.** The various serine tRNA gene derivatives, as indicated at the top of each panel, were transcribed *in vitro* under the following conditions: lane a, standard incubation carried out for 60 minutes; lane b, same as lane a except repressor protein (Rep) was added to the tRNA gene just prior to adding nuclear extract (N.E.) and nucleotide triphosphates (NTPs); lane c, the tRNA gene was preincubated with extract for 20 minutes after which repressor protein and NTPs were added; lane d, same as lane a except repressor protein was added 20 minutes following the addition of nuclear extract and NTPs. The total time of incubation upon addition of NTPs was 60 minutes in all cases. The position of the precursor tRNA transcripts and the mature tRNA are indicated. The arrows in lane b of panels F and G highlight the synthesis of shortened transcripts generated in the presence of prebound repressor.

operator sites at varying positions in the 5'-flanking region, are shown in Figure 5.3.1. The results in the last chapter demonstrated that although the natural sequences immediately upstream of the human $tRNA^{Ser}$ gene coding region can have a modulatory effect, they are not essential for the transcription of this gene or for the processing of the tRNA transcripts. In this regard, all of the tRNA gene derivatives are transcriptionally active *in vitro*. In addition, the resulting precursor transcripts appear to be undergoing normal processing and yielding mature sized tRNA in these *in vitro* reactions (Panels A to H, compare lane a).

In section 5.1, a thorough *in vitro* transcription analysis of p*Lac*OtS-9 ascertained the ability of *lac* repressor to inhibit transcription prior to complex assembly, after complex assembly, or during ongoing transcription. In a similar manner, the ability of *lac* repressor to inhibit transcription under these three conditions was determined for each of the various tRNA gene derivatives (Figure 5.3.1). *lac* repressor completely inhibited transcription of p*Lac*OtS-15 whether pre-bound to the template or added following a 20 minute pre-incubation of the tDNA with nuclear extract (panel C, lanes b and c). Repressor protein also inhibited ongoing transcription from this template as indicated by the reduction in final transcripts synthesized in a reaction where *lac* repressor was added 20 minutes following transcription initiation (panel C, compare lanes a and d). Identical results were obtained using p*Lac*OtS-9 as a template (panel B, compare lane a with b, c and d) confirming the findings described in section 5.1. Thus, transcription is abolished for tRNA genes containing a *lac* operator site centred at base pairs -9 or -15 whether *lac* repressor is added before or after complex assembly, or to

ongoing transcription reactions.

The *lac* operator oligonucleotide contributes the transcription start site for p*Lac*OtS-9 and p*Lac*OtS-15, which initiate from base pairs -3 and -4 as determined by primer extension analysis (section 5.6). The demonstration that repressor can still access its binding site in these tDNA derivatives following the assembly of functional transcription complexes suggests that the region surrounding the transcription start site is exposed during multiple rounds of reinitiation. These findings are also in agreement with available footprinting and photocrosslinking analyses of yeast pol III complexes which show that while 40 base pairs of DNA upstream of the transcription initiation site is strongly protected by TFIIIB, the transcription initiation site itself is largely unprotected until the addition of RNA polymerase III (Kassavetis *et al.*, 1989, 1990).

Prebound *lac* repressor also abolished transcription of p*Lac*OtS-35 and p*Lac*OtS-37, however, in these cases transcription was not affected when *lac* repressor was added after complex formation or to ongoing reactions (Figure 5.3.1D and E, compare lanes a with b, c, and d). These findings suggest that *lac* repressor cannot access target sites located at these positions once the transcription complex is assembled. It remains possible, however, that *lac* repressor may still bind to these sites after complex formation but have no effect on transcription.

Prebound repressor protein significantly diminished, but did not abolish, transcription of p*Lac*OtS-43 and p*Lac*OtS-46 which contain operator sequences centred at -43 and-46, respectively (Figure 5.3.1F and G, compare lanes a and b). Repressor had no effect if added subsequent to complex formation or to ongoing transcription

reactions (Figure 5.3.1F and G, lanes c and d). The residual transcriptional activity observed in the presence of prebound repressor was greater for p*Lac*OtS-46 than for p*Lac*OtS-43 and corresponded to approximately 50% and 10% of transcription levels obtained in the absence of repressor protein, respectively. Interestingly, in both cases the residual transcriptional activity observed was associated with shortened primary transcripts (see section 5.6).

Thus, *lac* repressor inhibits transcription of tRNA gene derivatives containing operator sites centered at nucleotide positions -35, -37, -43 and -46 only when repressor is prebound to the tDNA or added at the same time as nuclear extract. This suggests that the assembled transcription complex occludes the binding of *lac* repressor binding to these sites. Repressor had no effect on transcription of p*Lac*OtS-52, which contains the operator centered upstream at nucleotide -52, or on the wild type gene (which does not contain an operator site), under any of the conditions tested (Figure 5.3.1A and H, respectively). Therefore, the boundary at which *lac* repressor has no observable effect on pol III transcription of this human tRNA gene lies at operator sites centered between 46 and 52 nucleotides upstream from the mature tRNA. Since the bound *lac* repressor tetramer is 80 Å long (Ohshima *et al.*, 1975; Zingsheim *et al.*, 1977) and footprints 25 base pairs of DNA (Schmitz and Galas, 1979), this places the edge of the functional human pol III transcription complex at approximately 35 to 40 nucleotides upstream of the tRNA gene coding region. This is consistent with the region upstream that is bound by TFIIIB in assembled yeast tRNA gene transcription complexes as revealed by footprinting and photocrosslinking analyses (Kassavetis *et al.*, 1989, 1990; Bartholomew

et al., 1991; section 1.5).

5.4 Effect of *lac* Repressor on the Assembly of the Preinitiation Complex.

The implication from the above findings is that *lac* repressor perturbs, in a position dependent manner, the formation or stability of transcription complexes through steric hinderance. Thus, template competition experiments were carried out as described in Section 2.7.4, using the adenovirus VA gene as the reference template, to determine whether *lac* repressor affects the interaction of limiting transcription factors with the various tDNA derivatives.

Saturating levels of the VA gene and each of the various tDNA derivatives were incubated prior to the addition of extract and nucleotide triphosphates. As expected, the amount of VA RNA synthesized in the presence of the wild type tDNA template was significantly reduced, compared to the VA gene incubated on its own, due to mutual competition for at least one limiting transcription factor (Figure 5.4.1A, compare lanes c and d). Similarly, there was a marked reduction in VA gene transcription in direct competition reactions carried out with all the tDNA derivatives in the absence of *lac* repressor (Figure 5.4.1B to H, compare lanes c and d). When repressor was included with the *lac* operator containing tDNA derivatives and the VA gene prior to nuclear extract and nucleotide addition, VA gene transcription was restored in each case to levels that were observed for the VA gene alone (Figure 5.4.1B to H, compare lanes c and e for each case). *lac* repressor had no effect on the competitive

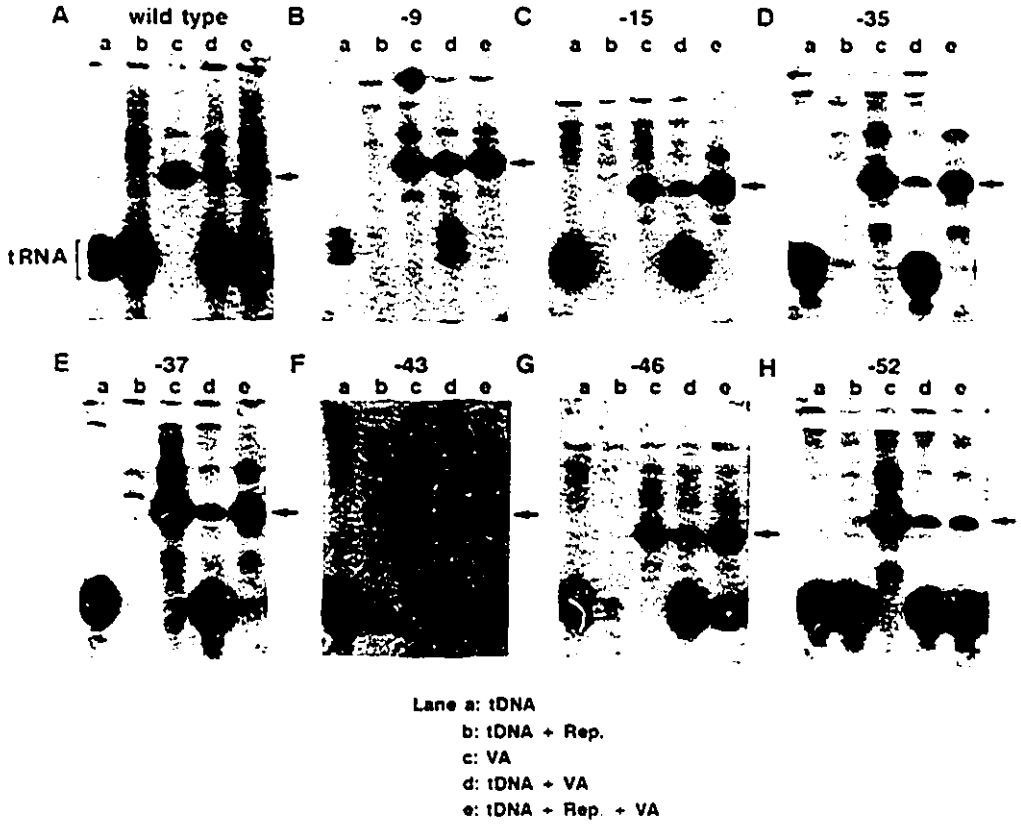


Figure 5.4.1. Template-Bound *lac* Repressor Reduces the Competitive Ability of the tRNA Gene Derivatives. Saturating amounts of the various tRNA gene (tDNA) and VA gene (VA) templates were pre-incubated under the conditions shown at the bottom of the figure prior to the addition of nuclear extract and nucleotide triphosphates: lane a, tRNA gene alone; lane b, tRNA gene and repressor protein (Rep); lane c, VA gene alone; lane d, tRNA gene and VA gene; lane e, tRNA gene, repressor protein and VA gene. Reactions were incubated for 60 minutes following the addition of nuclear extract and nucleotide triphosphates. All components were added within 1 minute of each other. The VA transcripts are indicated by the arrows. The tRNA gene derivatives are indicated above each gel.

abilities of the wild type gene or of p*Lac*OtS-52 (Figure 5.4.1A and H, lanes d and e, respectively). These results indicate that the interaction of *lac* repressor with the 5'-flanking region of the $tRNA^{Ser}$ gene, up to nucleotide position -46, prevents or hinders the stable assembly of at least one limiting factor(s) on the tRNA gene that is required for maximal levels of VA gene transcription. Furthermore, this effect is only observed with those tRNA gene derivatives in which prebound *lac* repressor severely reduces or abolishes transcription. The boundary upstream of the tRNA gene at which *lac* repressor has no observable effect on competitive strength coincides with the boundary at which *lac* repressor has no affect on pol III transcription.

5.5 Instability of Complexes Assembled on a Serine tRNA Gene Derivative Containing Prebound *lac* Repressor

As shown above, prebound *lac* repressor inhibited transcription of p*Lac*OtS-43 and p*Lac*OtS-46 by approximately 90% and 50%, respectively, following a 1 hour incubation. A kinetic analyses of transcription of p*Lac*OtS-46 in the presence of prebound *lac* repressor protein showed that the overall rate of transcription was reduced by approximately 50% over the course of the incubation, both prior to and after allowing sufficient time for formation of the preinitiation complex (Figure 5.5.1A and B, respectively). This suggests that the observed reduced rate of transcription is not directly related to a reduced rate of stable complex assembly. Template commitment experiments support this conclusion. In these experiments p*Lac*OtS-46 was preincubated with nuclear

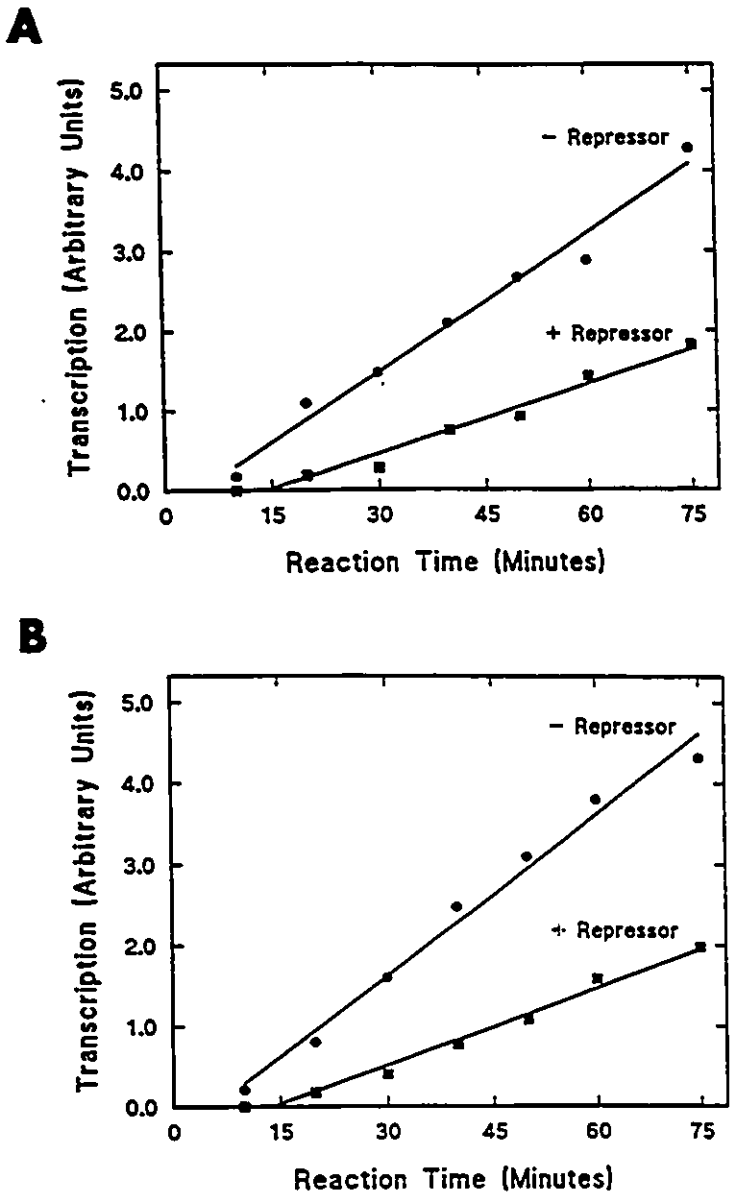


Figure 5.5.1. *lac* Repressor Reduces the Rate of Transcription of p*Lac*OtS-46. p*Lac*OtS-46 was transcribed in the absence or presence of prebound *lac* repressor and the reactions were terminated at various times following the addition of nucleotide triphosphates and analysed by gel electrophoresis. Transcription levels were quantitated by densitometric scanning of the autoradiograph and plotted as arbritrary units versus the time (in minutes) of incubation following the addition of nucleotide triphosphates. In panel A, tDNA, *lac* repressor, nuclear extract, and nucleotide triphosphates were added sequentially within 1 minute of each other. In panel B, pt*SLac*O-46 was preincubated with nuclear extract, in the presence or absence of repressor protein, for 30 minutes prior to the addition of nucleotide triphosphates.

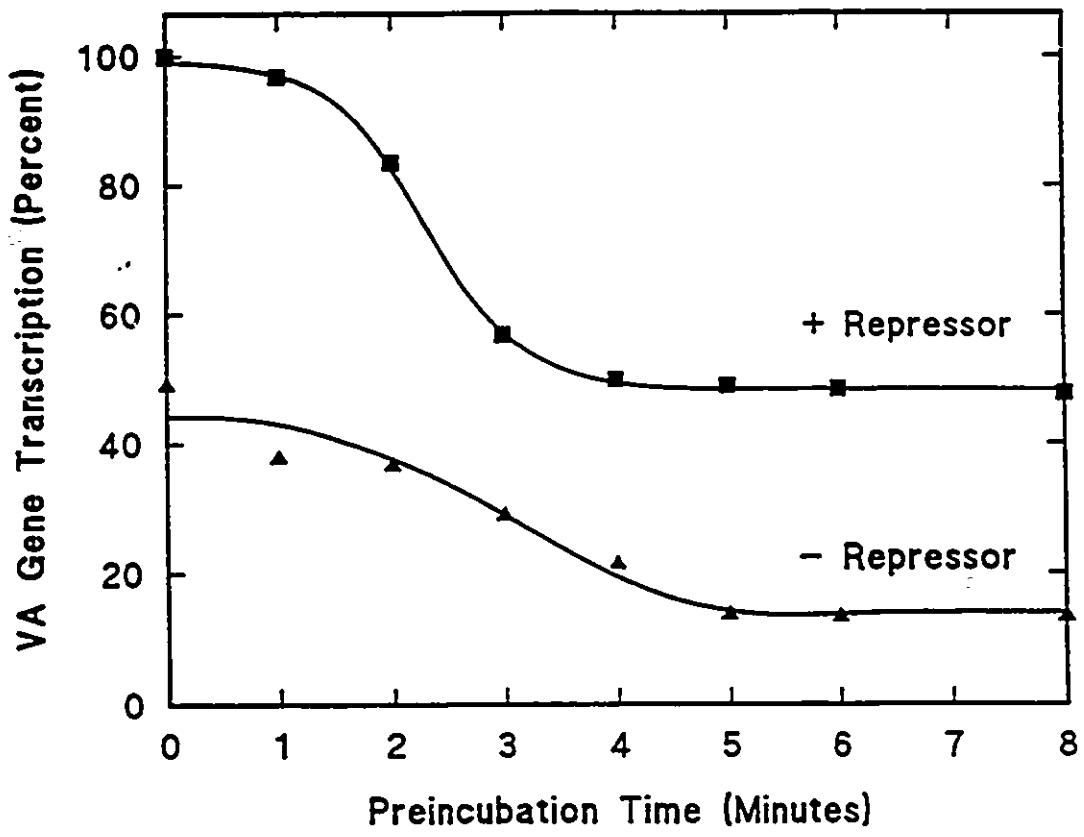


Figure 5.5.2. Prebound Repressor does not Affect the Rate of Formation of Transcription Complexes on p*Lac*OtS-46. Template commitment experiments were carried out as described in Section 2.7.4. A saturating amount of p*Lac*OtS-46 was preincubated for varying times, shown on the horizontal axis, with nuclear extract in the presence or absence of *lac* repressor. Reactions were supplemented with saturating amounts of pBRVA and nucleotide triphosphates and the incubation was continued for 60 minutes. Reactions were analysed by gel electrophoresis followed by autoradiography. The level of VA RNA synthesized was quantitated by densitometric scanning analysis and plotted as a percentage of VA RNA synthesized in a separate 60 minute reaction carried out in the absence of p*Lac*OtS-46.

extract in the presence or absence of *lac* repressor for various times prior to the addition of the VA gene and nucleotide triphosphates. The incubation was continued for 60 minutes and the levels of VA gene transcription were quantified. The results, shown in Figure 5.5.2, indicate that repressor protein does not appreciably affect the rate at which the preinitation complex is assembled on the tRNA gene, since maximal levels of transcription inhibition of the challenge VA template occurred within 5 minutes whether in the presence or absence of prebound *lac* repressor. The final extent of VA transcription was reduced by only 50% in the presence of repressor compared with approximately 90% in the absence of repressor, suggesting that either the number of stable transcription complexes is reduced or that the transcription complexes are themselves less stable in the presence of repressor.

To explore this further, experiments were performed with p*Lac*OtS-46 in the presence of heparin. Heparin has been shown to prevent reinitiation by yeast RNA polymerase III (Kassavetis *et al.*, 1990) and has been reported to limit transcription to a single round in HeLa extracts (Kovelman and Roeder, 1990). In the experiment shown in Figure 5.5.3, p*Lac*OtS-46 was preincubated with extract in the absence or presence of repressor protein for 30 minutes to allow formation of the preinitiation complex. Heparin and nucleotides were then added to the reactions which were terminated at varying times thereafter. In the absence of repressor a very low level of transcription was observed which peaked within 2 minutes following heparin and nucleotide addition indicating that, as expected, heparin prevents transcription reinitiation (compare lanes a-c with k-m). The same low level of transcription was observed when repressor was added

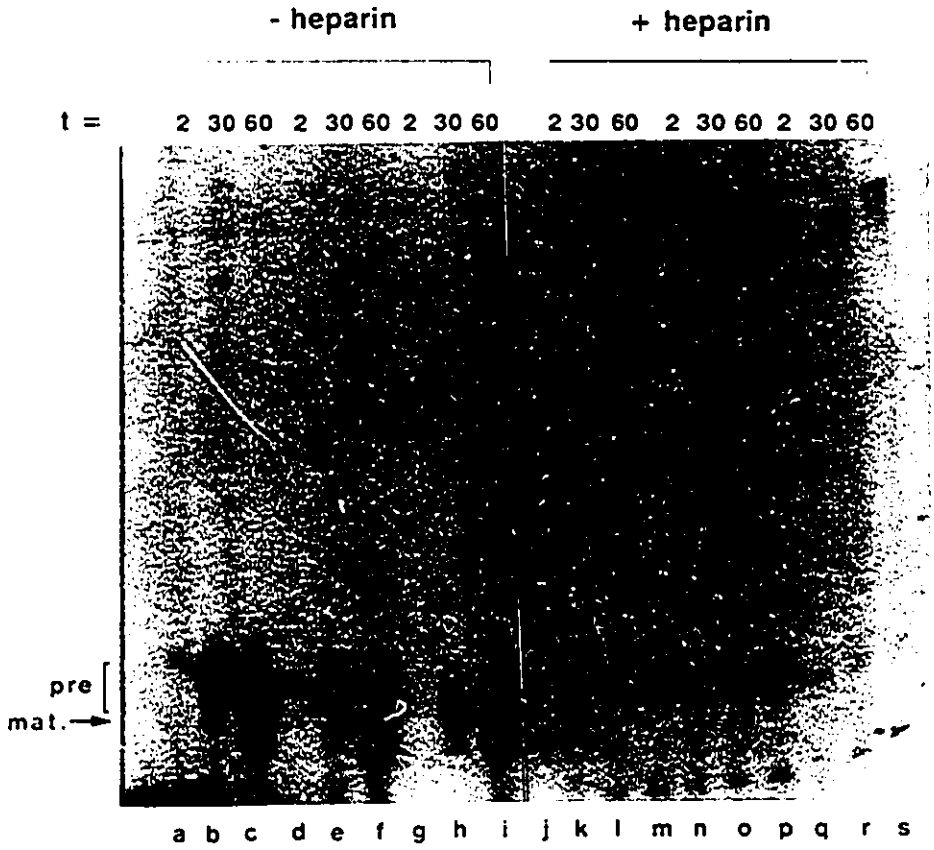


Figure 5.5.3. Transcription Complexes Formed on p*Lac*OtS-46 in the Presence of Prebound *lac* Repressor are Sensitive to Heparin. In lanes a-c, p*Lac*OtS-46 was preincubated with nuclear extract for 30 minutes prior to the addition of nucleotide triphosphates. In lanes d-f, *lac* repressor was added along with the nucleotide triphosphates, while in lanes g-i, *lac* repressor was present in the preincubation. Reactions were incubated for 2, 30, and 60 minutes following the addition of nucleotide triphosphates as indicated at the top of the figure, and analyzed by gel electrophoresis. Lanes k-s are identical to lanes a-i, respectively, except that heparin was added 3 minutes prior to nucleotide triphosphate addition. Lane j is a negative control which is identical to lane c except that heparin was added just prior to nuclear extract in the preincubation thereby preventing transcription. The figure is a composite of the same gel in which lanes a-i were exposed for 1 hour and lanes j-s were exposed for 48 hours in order to visualize the low levels of transcription products synthesized in the presence of heparin.

after the preincubation along with heparin and nucleotides (lanes n-p). This was expected since repressor has been shown to have no effect on the transcription of this template if added subsequent to the assembly of the transcription complex. Since inclusion of repressor in the preincubation reduced transcription of p*Lac*OtS-46 by only 50% in the absence of heparin (lanes g-i), one would expect to see a proportionate level of transcription in the presence of heparin if this diminished level of transcription was due to a reduced number of active transcription complexes. However, when repressor was included in the preincubation, transcription was not observed following heparin addition, even after longer incubation times or extended exposure of the gel (lanes q-s). These results demonstrate that transcription complexes assembled on p*Lac*OtS-46 in the presence of bound *lac* repressor are more sensitive to heparin.

The residual transcription observed with p*Lac*OtS-46 and p*Lac*OtS-43 in the presence of prebound *lac* repressor demonstrates that functional transcription complexes can still assemble to some extent on these templates. The rate of complex assembly with repressor bound at position -46 is not altered, however, the final transcription complex is compromised in second template assays and is completely sensitive to heparin. The heparin experiments also show that the residual transcription observed with these templates does not arise from a small subpopulation of normally assembled complexes. Together, these findings suggest that prebound *lac* repressor centred upstream at position -46 qualitatively alters the assembled transcription complex, rendering it less stable.

5.6 Repositioning of the Transcription Initiation Site by *lac* Repressor

As described above, shortened transcripts were generated from both p*Lac*OtS-46 and p*Lac*OtS-43 in the presence of prebound repressor protein. One explanation for this result is that the spatial constraints imposed by the bound repressor protein alters the position of the transcription complex, thereby forcing transcription to initiate at sites farther downstream. To investigate this, the 5' ends of the longest precursor tRNA transcripts generated in the presence and absence of prebound repressor were mapped through both primer extension and S1 mapping analyses.

The primer extension reactions display many bands, shown in Figure 5.6.1, most probably due to pausing of the reverse transcriptase while traversing through tRNA secondary structure (Dingermann and Nerke, 1987). The primer presumably hybridized to endogenous tRNAs and possibly other pol III transcripts present in the Hela extract since primer extension of transcription reactions performed in the absence of plasmid DNA (lanes a and l) also gave rise to the variable appearance of specific products. Nevertheless, as shown in Figure 5.6.1, intense bands arising from transcription products of our tRNA constructs are easily discernable above this background. The longest products generated from the wild type gene map to purine nucleotides at positions -5, -6 and -7, relative to the 5' end of the mature tRNA. This pattern of initiation was not altered when transcription was carried out in the presence of *lac* repressor (compare lanes b and c). Transcription of pLacOtS-46, whose upstream sequences are identical to wild type up to the operator site, also initiate from these same purine nucleotides except that there is stronger initiation events at nucleotide -7 and minor initiation events at nucleotide

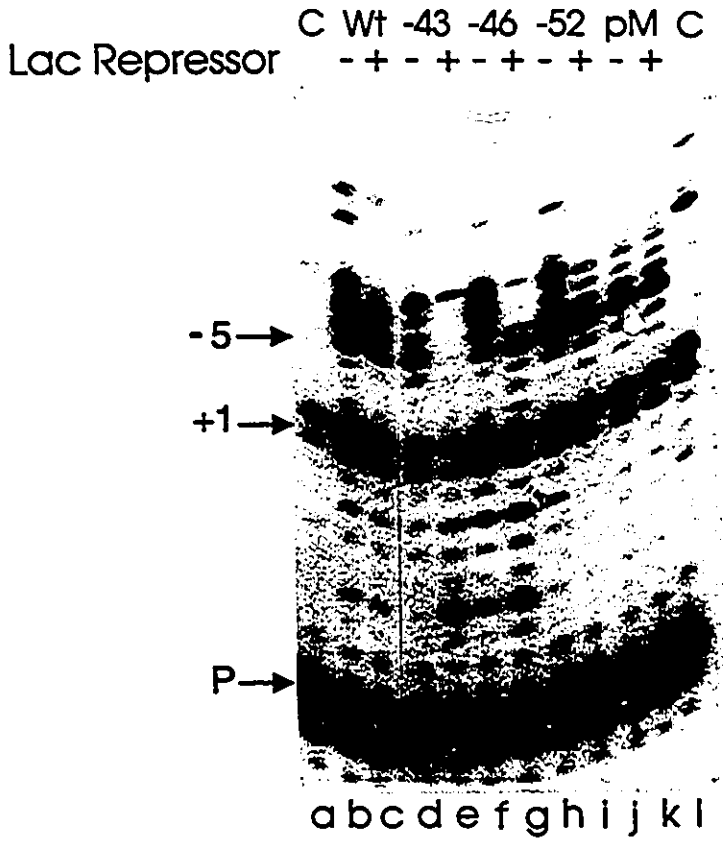


Figure 5.6.1. Prebound *lac* Repressor Alters the Site of Transcription Initiation of p*Lac*OtS-43 and p*Lac*OtS-46. Cold 90 minute *in vitro* transcription reactions were performed on various tDNA derivatives, in the presence and absence of *lac* repressor, as indicated at the top of the figure. The products of these reactions were subjected to primer extension analysis using a 5' end labelled oligonucleotide primer complementary to nucleotides +10 to +24 of the mature tRNA. The sizes of the extension products were determined by comparison to dideoxy sequencing reactions of the wild type tRNA gene using the same primer (not shown). Lanes a and l are control lanes where *in vitro* transcription reactions carried out in the absence of tDNA were used. The 5' ends of the primer extension products are indicated with respect to the 5' end of the mature tRNA, taken as +1. pM is the parental plasmid pMCtS. The position of the free primer oligonucleotide (P) is also indicated.

-8 (lane f). However, in the presence of prebound *lac* repressor transcription initiation occurs predominantly from position -5, to a much lesser extent from position -6 and is not detected from nucleotide positions farther upstream. Interestingly, a band corresponding to transcription initation from within the mature tRNA, at nucleotide position +6, displayed a slight repressor dependent intensification. Transcripts of p*Lac*OtS-43 and p*Lac*OtS-52 generated in the absence of repressor initiate mostly at purine nucleotide -5 with weak initation detected at positions -6 to -8 (lanes d,h and j). The pattern of initiation observed with p*Lac*OtS-52 and the parental plasmid pMCtS was not altered by *lac* repressor (compare lanes h-k). In contrast, transcription initation from specific sites upstream of p*Lac*OtS-43 were not detected in the presence of prebound repressor (compare lanes e and l). Instead, an extension product that maps to position +6 was greatly intensified (compare lanes d and e) suggesting that initiation is redirected to this downstream site within the mature tRNA coding region.

To support the primer extension results obtained with p*Lac*OtS-46 and p*Lac*OtS-43, S1 nuclease mapping experiments, shown in Figure 5.6.2, were carried out. Two single stranded DNA probes; one extending from position -74 to +66 (specific for wild type and p*Lac*OtS-46) and another extending from position -49 to +66 (specific for p*Lac*OtS-43, p*Lac*OtS-52, and the parental plasmid pMCtS) were used. The probes (shown undigested in lanes b and p) did not give rise to any S1 protected bands from control *in vitro* transcription reactions carried out in the absence of plasmid DNA (lanes d and o). A control lane of *in vitro* transcribed p*Lac*OtS-9, whose sequences upstream of the mature tRNA are different than wild type and therefore not complementary to the probe, showed a protected band of 66 nucleotides demarcating the 5' end of the mature

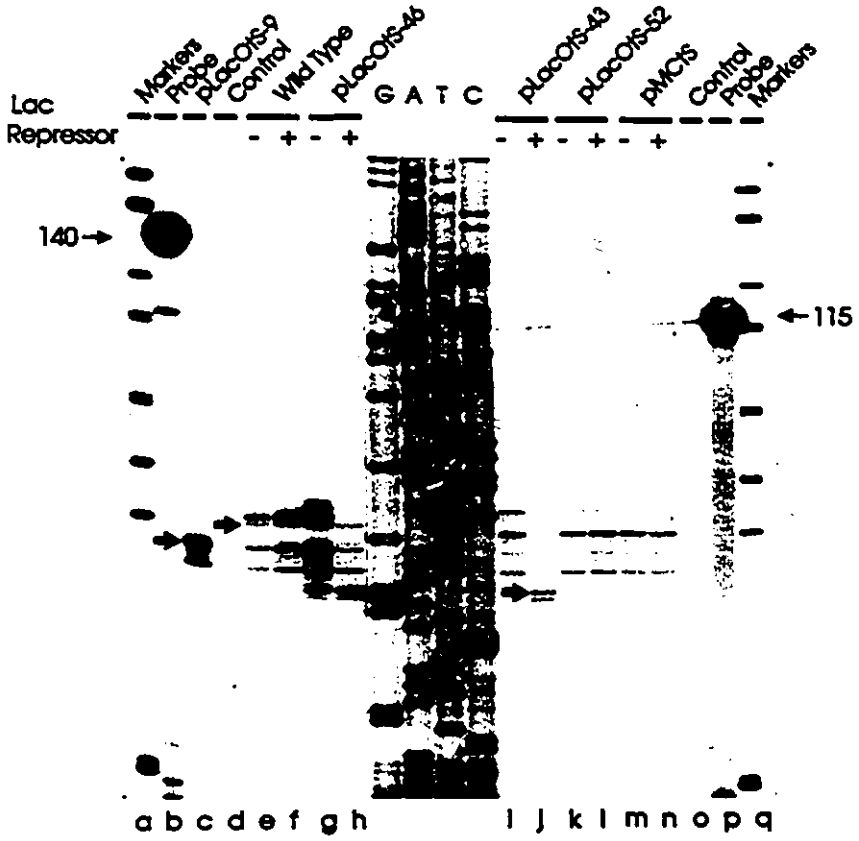


Figure 5.6.2. S1 Nuclease Protection Analysis of Transcription Products from p*Lac*OtS-43 and p*Lac*OtS-46. Unlabelled products from *in vitro* transcription reactions, as indicated at the top of the figure, were subjected to S1 nuclease mapping analysis. A 140 base pair probe (used in lanes c to h) and a 115 base pair probe (used in lanes i to o) are shown undigested in lanes b and p, respectively. Lanes d and o are control lanes where *in vitro* transcription reactions carried out in the absence of tDNA were used. The arrow for lane c indicates a 66 nucleotide protected band which demarcates the 5' end of the mature tRNA (since p*Lac*OtS-9 differs from the 140 base pair probe in sequences immediately upstream of the mature tRNA coding region). The arrows for lanes e and j indicate 71 and 61 nucleotide protected bands, and correspond to transcription initiation at -5 and +6, respectively. Lanes a and q are labelled markers derived from *Hpa*II digested pBR322. A sequencing ladder from the wild type tRNA gene is shown in the center lanes.

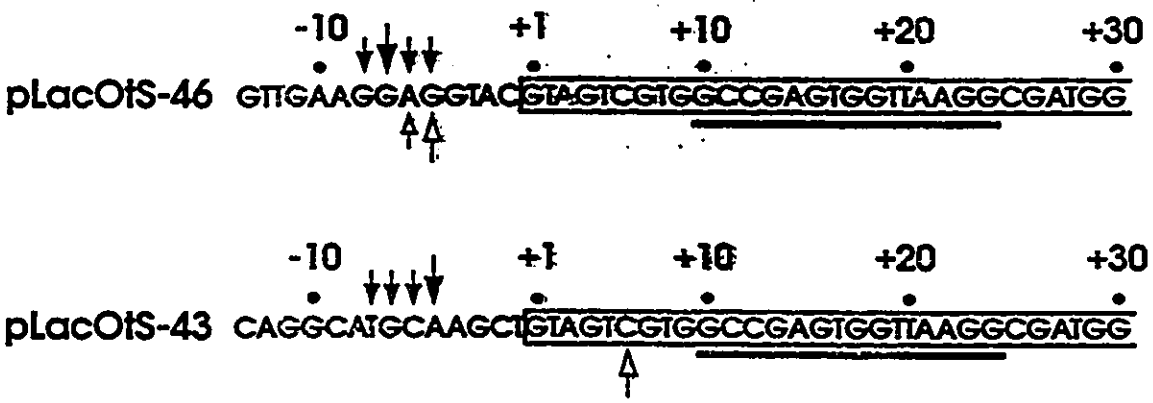


Figure 5.6.3. Summary of Primer Extension and S1 Mapping Results. The transcription initiation sites for p*Lac*OtS-46 and p*Lac*OtS-43 observed in the absence and presence of *lac* repressor are diagrammed below. The sequences of the nontemplate strand of both constructs is shown. The beginning of the mature tRNA starts at nucleotide position +1 and is indicated by the boxed sequences. The transcription initiation sites observed in the absence of *lac* repressor are indicated by the downward pointing arrows while those observed in the presence of *lac* repressor are indicated by the upward pointing arrows. Smaller arrows indicate minor initation sites. The oligonucleotide used in primer extension analysis was complementary to nucleotides +10 to +24 which is the underlined sequence.

tRNA (lane e, arrow). Transcripts derived from the wild type gene, obtained in both the presence and absence of *lac* repressor, protected bands of 71, 72 and 73 nucleotides (lanes e and f) corresponding to initiation events at nucleotide positions -5, -6 and -7, respectively. The same protected fragments were observed with p*Lac*OtS-46 transcripts obtained in the absence of repressor, however, in the presence of repressor a single protected species of 71 nucleotides was generated (lanes g and h). Transcripts derived from p*Lac*OtS-43, p*Lac*OtS-52 and pMCtS generated a strong protected band of 70 nucleotides and weaker protected products of 71-74 nucleotides (lanes i,k and m) corresponding to a major initation site at -4 and minor initiation sites farther upstream. Prebound *lac* repressor had no effect on initation site selection for p*Lac*OtS-52 or pMCtS (compare lanes k-n) but completely eliminated the protected bands seen for p*Lac*OtS-43 (compare lanes i and j). Instead, novel protected bands of 60 and 61 nucleotides were observed with p*Lac*OtS-43 (lane j, arrow), corresponding to initiation within the mature tRNA coding region at positions +6 and +7. Overall, the S1 mapping results are in agreement with the primer extension analysis, and confirm that *lac* repressor redirects transcription initation of p*Lac*OtS-46 and p*Lac*OtS-43 to downstream sites. These collective results are summarized diagramatically in Figure 5.6.3.

To determine if the altered sites of transcription initiation observed with p*Lac*OtS-46 persist after releasing repressor from the template, reactions were carried out in the presence of the allosteric inducer IPTG under various conditions. The results of these experiments are shown in Figure 5.6.4. As expected, when both repressor and IPTG were present in the preincubation the level and pattern of transcription was the same as that obtained without repressor (compare lanes a and c), whereas when repressor

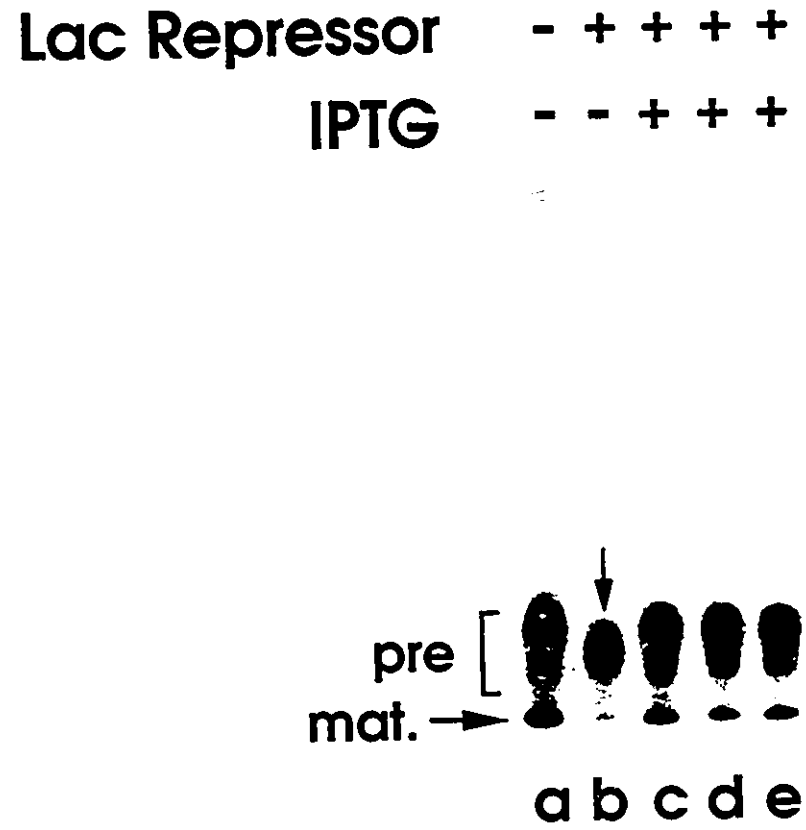


Figure 5.6.4. Redirected Transcription Initiation of p*Lac*OtS-46 is Not Sustained Following Removal of *lac* Repressor. pt*SLac*OtS-46 was preincubated for 30 minutes with nuclear extract after which nucleotide triphosphates were added and the reaction continued for another 60 minutes. Lane a, control reaction in the absence of *lac* repressor. Lane b, *lac* repressor was present in the preincubation and was added 1 minute prior to nuclear extract. Lanes c, d and e are identical to lane b except IPTG was included in the preincubation (lane c), added 5 min prior to nucleotide triphophate addition (lane d), or added 5 min after nucleotide triphosphate addition (lane e).

alone was present in the preincubation, transcription was reduced and associated with shortened primary transcripts (lane b). Addition of IPTG immediately following the preincubation period, or 5 minutes following nucleotide addition, restored wild type levels of transcription and the synthesis of shortened transcripts was not observed (compare lanes a with d and e). These results demonstrate that both transcription inhibition and redirected initiation are dependent on the continued presence of bound repressor. Thus, the preinitiation complex formed on p*Lac*OtS-46 in the presence of repressor protein is an adaptable state which can apparently readjust to restore normal transcription once the constraints imposed by bound repressor are alleviated.

In summary, *lac* repressor centred at positions -46 and -43 forces transcription to initiate at sites progressively farther downstream. When repressor is bound at position -43 the major site of initiation maps to position +6 which is 10 nucleotides downstream from the normal start site and within 2 nucleotides upstream of the consensus A box internal control region recognized by TFIIIC. These findings are consistent with those of a recent study showing that the yeast GCN4 protein bound upstream of the transcription start site of a *Saccharomyces cerevisiae* tRNA gene repositions the interaction of yeast TFIIIB with upstream DNA, resulting in both diminished transcription and redirected transcription initiation to sites farther downstream (Leveillard *et al.*, 1993). Thus, there is an inherent flexibility in pol III transcription complexes and start site selection in both yeast and mammalian systems. In the GCN4 study, the synthesis of downstream initiated transcripts persisted following removal of the GCN4 protein from the complexes, likely because yeast TFIIIB remains strongly bound to upstream DNA once it is assembled into a functional transcription complex. In contrast,

our findings demonstrate that both normal transcription levels and initiation sites are restored following release of the *lac* repressor protein from p*Lac*OtS-46. This demonstrates that active mammalian transcription complexes assembled on templates constrained by *lac* repressor can rearrange following removal of the block, perhaps reflecting a less avid interaction of transcription factors with the 5' flanking region of the gene in comparision to TFIIIB in the yeast pol III complex. In this regard, the stability of pol III transcription complexes assembled in HeLa extracts has been suggested to be a collective property of both TFIIIB and TFIIIC (Jahn *et al.*, 1987).

5.7 Effect of *lac* Repressor on Functional Expression of Serine tRNA Gene Derivatives *In Vivo*.

All of the *lac* operator sites were inserted upstream of a human serine tRNA gene which had previously been converted to an amber suppressor (Capone *et al.*, 1985). Therefore, the functional expression of these $tRNA^{Ser}$ gene derivatives is easily determined *in vivo* following cotransfection with pRSVcat(am27), an expression plasmid in which the chloramphenicol acetyltransferase gene contains a suppressible UAG nonsense codon (Capone *et al.*, 1986, see chapter 3). The effect of *lac* repressor on the functional expression of the various tRNA gene derivatives was determined by Dan Syroid who included pRSVIns, a plasmid expressing the *lac* repressor protein (Hu and Davidson, 1991), in the *in vivo* suppression assay. As shown in Figure 5.7.1, inclusion of pRSVIns in the transfections resulted in a substantial inhibition in the suppression level of p*Lac*OtS-9, p*Lac*OtS-15, and p*Lac*OtS-46, but had no apparent effect on the

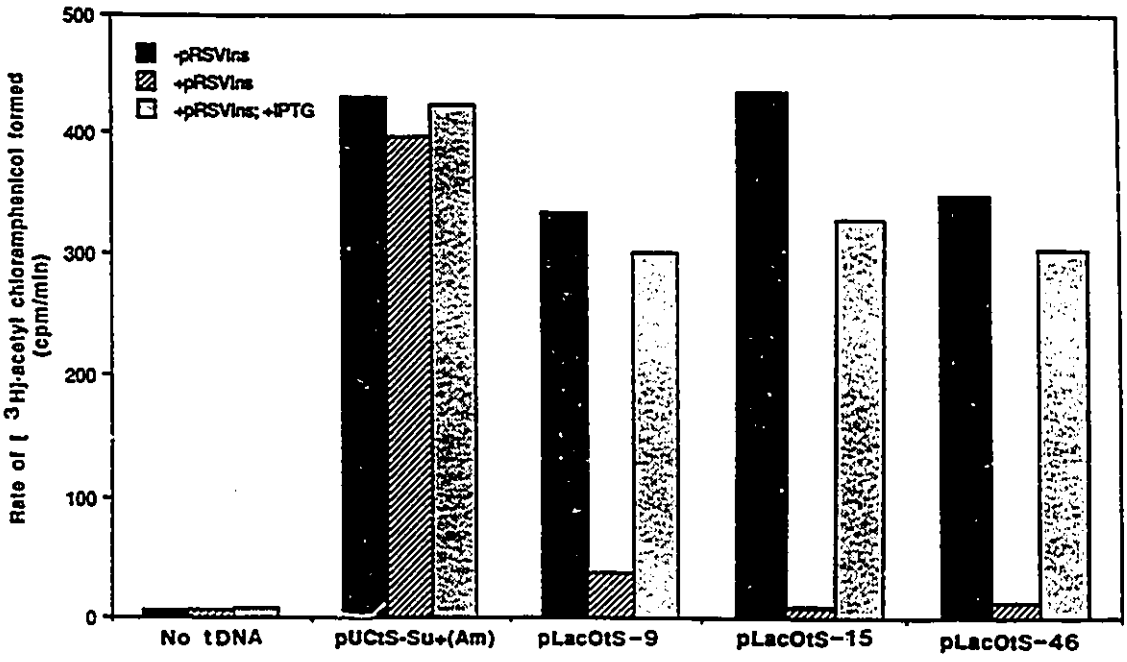


Figure 5.7.1 Inhibition of the Phenotypic Expression of Serine tRNA Gene Derivatives *In Vivo* by *lac* repressor. BSC-40 cells were cotransfected with 5 μg of pRSVcat(am27) either alone (no tDNA), or with 1μg of pUCtS-Su$^+$(am), 1μg of p*Lac*OtS-9, 0.25 μg of p*Lac*OtS-15 or 0.25μg of p*Lac*OtS-46, as indicated. In order to examine repression, cotransfections included nothing (black bars) or 1 μg of pRSVIns (hatched bars). To examine derepression, cotransfections included 1μg of pRSVIns and cells were incubated in the presence 20mM IPTG for the course of the transfection (stippled bars). CAT activity was determined 48 hours later using the scintillation counting method. The results are averages from at least two independent transfections. These experiments were performed by Dan Syroid.

suppression level of the parent plasmid pUCtS-Su$^+$(am). Furthermore, the inclusion of 20mM IPTG in the cotransfection reversed the repressor-dependent inhibition. These results demonstrate that *lac* repressor is capable of inhibiting the functional expression of tRNA gene derivatives containing operator sites centered upstream at nucleotide positions -9, -15 and -46. Since the previous *in vitro* studies demonstrate that *lac* repressor inhibits transcription through steric effects on the pol III transcription complex, this is presumably the mechanism by which repressor inhibits the phenotypic expression of these tRNA gene derivatives *in vivo*.

The above findings demonstrate that a heterologous DNA binding protein bound within the 5'-flanking region can modulate the functional expression of a tRNA gene in mammalian cells. While this work was in progress, the expression of suppressor tRNA genes in both *Saccharomyces cerevisiae* and *Dictyostelium discoideum* was demonstrated to be inhibited at the level of transcription using the bacterial tetracycline operator/repressor system by inserting the operator site immediately upstream of the coding region of the genes (Dingermann *et al.*, 1992a, 1992b). Thus, it appears that tRNA gene expression can be effectively modulated by heterologous DNA binding proteins bound upstream in at least three diverse eukaryotes.

5.8 Summary

The demonstration of stable upstream pol III transcription complexes has been difficult to establish by conventional approaches that directly examine protein/DNA interactions because of the poor efficiency of formation of functional transcription complexes using crude extracts or reconstituted components. The studies described in

this chapter have shown that a heterologous DNA binding protein can be used to probe the characteristics of functional pol III transcription complexes. The results demonstrate for the first time that the functional human pol III transcription complex extends at least 35 to 40 nucleotides upstream from the tRNA coding region and that the sequences surrounding the transcription initiation site remain accessible to DNA binding proteins throughout multiple rounds of transcription.

It is well established that tRNA gene transcription is modulated by nonconserved sequence elements which are generally found within 50 base pairs upstream of the coding region (section 1.12). In addition, chapters 3 and 4 of this thesis describe the identification of a negative transcripitonal modulatory elcment upstream a human $tRNA^{Ser}$ gene. While the mechanisms by which 5'-flanking sequences effect transcription are largely unknown, one theory is that gene specific DNA binding proteins bind these nonconserved elements and attenuate the assembly or function of the transcription complex. This chapter has shown that positioning of a heterologous DNA binding protein upstream of a human tRNA gene conditionally inhibits its transcriptional activity *in vitro* and its functional expression *in vivo*. This inhibition is principally due to steric effects on mammalian complex formation and stability, and transcription is progressively diminished as *lac* repressor is positioned closer to the coding region of the gene. These findings demonstrate that sequence specific DNA binding proteins positioned upstream of the tRNA gene coding region can modulate their transcriptional activity. Although artifical in its approach, this study shows that this is a potential mechanism by which naturally occuring regulatory proteins may modulate RNA polymerase III mediated transcription of cellular tRNA genes.

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