Nonsense Suppressor tRNAs in the Study of Class II and Class III Gene Expression and Regulation.

by

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ABSTRACT

The ability of suppressor (su^+) tRNAs to abrogate the effects of otherwise-lethal nonsense mutations forms the basis of a genetic screen for the identification of essential genes. The availability of numerous nonsense su^+ tRNA strains of *E. coli* and yeast led to the rapid development of prokaryotic and yeast genetics. The recent availability of mammalian nonsense su^+ tRNA genes has afforded not only the opportunity of applying similar strategies towards the advancement of mammalian viral and cellular genetics, but also the opportunity to examine the regulation of transcription by RNA polymerase III (polIII) *in vivo*. This thesis comprises two parts. The initial aim of this work involved the development and use of mammalian systems of nonsense mutation suppression to examine the mechanisms of eukaryotic class II gene expression and regulation. Herpes simplex virus type-1 (HSV-1) was chosen as a model system since HSV-1 genes are expressed in a regulated cascade. A natural progression of this work involved the study of the molecular mechanisms of mammalian polIII transcription *in vivo* and *in vitro*.

Initial work involved the identification and propagation of conditional-lethal nonsense mutants of HSV-1 using a mammalian cell line which can be induced to express high levels of a human amber su^+ $tRNA^{Ser}$ gene as the permissive host in a simple host-range screening system. Several HSV-1 mutants have been isolated from virus stocks mutagenized with nitrous acid and have been partially characterized. Such mutants exhibit

a number of interesting temperature- and/or host-range-dependent phenotypes including variations in growth, cell-to-cell spread, and plaque morphology. Mutant A7'(A9-2) is particularly interesting since this isolate is defective in an essential function required for HSV-1 late gene expression. Isolate A7'(A9-2) is a potential nonsense mutant since a growth enhancement of 100 fold is exhibited under conditions of suppression.

To enhance the utility of mammalian systems of nonsense mutation suppression, a novel and more generally applicable means of efficiently regulating the expression of a su^+ tRNA gene was developed. Stringent control of su^+ tRNA gene expression is required since constitutive high levels of nonsense suppression activity is deleterious to mammalian cells. The *Escherichia coli* (*E. coli*) *lac* operator/repressor system has been adapted to confer inducibility upon the human su^+ $tRNA^{Ser}$ gene both *in vivo* and *in vitro*. *Lac* repressor protein, bound to its cognate *lac* operator site appropriately positioned upstream of the su^+ tRNA gene, stringently inhibited tRNA gene expression *in vivo*, as determined by quantitating suppression of an indicator gene nonsense mutation following transfection of mammalian cells. However, su^+ tRNA gene expression was quantitatively restored with the allosteric inducer isopropylthio-β-D-galactoside (IPTG). Similarly, *lac* repressor effected the complete inhibition of *lac* operator-linked tRNA gene transcription *in vitro* in HeLa cell nuclear extracts by precluding the assembly of an active polIII transcription complex, and this inhibition was reversible with IPTG. The results demonstrate that a DNA binding protein positioned upstream of a eukaryotic tRNA gene may impart transcriptional regulation upon tRNA gene expression, a novel and particularly intriguing finding in view of the intragenic nature of tRNA gene promoters.

The ability to conveniently and reversibly manipulate su^+ tRNA gene expression may foster rapid progress in the development of mammalian nonsense su^+ tRNA genetics, perhaps through the establishment of additional mammalian cell lines which conditionally express a variety of nonsense su^+ tRNA genes.

The second aspect of this thesis involved an examination of the molecular mechanisms that govern transcription by polIII which, in mammalian systems, has remained largely refractory to study through conventional approaches. A novel approach was adopted and involved use of the *lac* repressor protein as a reagent to probe various stages of polIII transcription to define the promoter disposition and functional properties of mammalian polIII transcription complexes. The effect on tRNA gene transcription, of varying the position of *lac* repressor protein upstream and downstream of the tRNA gene, was examined *in vitro* in HeLa cell nuclear extracts. *Lac* repressor differentially and reversibly interfered with different stages of transcription complex assembly and productive initiation. Such analyses have identified distinct functional and structural properties of mammalian polIII transcription complexes, and have provided a view of the spatial arrangement of mammalian polIII preinitiation complexes. The mammalian polIII transcription complex extends at least 35 nucleotides (nts) upstream, and to within 10 nts downstream of the tRNA gene. Moreover, sequences directly upstream of the coding region remain accessible to DNA binding proteins throughout multiple rounds of transcription. The results illustrate a number of potential mechanisms whereby DNA binding factors may modulate transcription initiation by polIII.

The *lac* operator/repressor system has also afforded a novel means of examining transcription elongation and termination by mammalian polIII. *Lac* repressor protein, appropriately positioned downstream of the tRNA gene coding region, reversibly blocked elongation by polIII *in vitro* in HeLa cell nuclear extracts, thereby resulting in either the formation of paused polIII ternary transcription complexes, a subset of which maintained the ability to undergo direct transcriptional readthrough of the *lac* repressor obstacle, or in premature transcription termination. The formation of paused polIII ternary complexes effectively mediated the inhibition of tRNA gene transcriptional activity, which was restored upon removal of the repressor-mediated block with IPTG. The results demonstrate that DNA binding proteins can modulate elongation and termination by polIII *in vitro*, and suggest that conditional factor-mediated blocks to elongation by polIII may function *in vivo* to attenuate transcription of class III genes. The ability to selectively arrest elongation by polIII at defined positions within the tRNA gene transcription unit has permitted the identification of discrete functional properties of paused mammalian polIII ternary complexes and may afford a novel strategy for the generation of homogeneous populations of stalled polIII ternary complexes, in which to study their biochemical properties.

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In memory of my mother,

Lucille

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am	amber
ATP	adenosine triphosphate
bp	base pair(s)
CAT	chloramphenicol acetyltransferase
Ci	Curie(s)
cpe	cytopathic effect
cpm	counts per minute
CTP	cytidine triphosphate
Da	dalton
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
βgal	β-galactosidase
gC	glycoprotein C
gD	glycoprotein D
gI	glycoprotein I
GTP	guanosine triphosphate

HBSS	hepes-buffered saline solution
HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HSV-1	herpes simplex virus type-1
IPTG	isopropylthio-β-D-galactoside
lac	lactose (operon)
*lac*O	*lac* operator
μ	micro
M	molar
MCS	multiple cloning site
min	minute(s)
moi	multiplicity of infection
mol	mole(s)
M_r	relative molecular mass
mRNA	messenger RNA
NP40	Nonidet P-40
nt	nucleotide(s)
NTP	nucleoside triphosphate(s)
oc	ochre
op	opal
ORF	open reading frame
PBS	phosphate buffered saline
pfu	plaque forming unit(s)

PMSF	phenylmethylsulphonylfluoride
pol	RNA polymerase
pvi	post virus infection
RF	release factor
RNA	ribonucleic acid
RNase A	ribonuclease A
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
snRNA	small nuclear RNA
SSC	sodium chloride-sodium citrate buffer
su^+	suppressor
SV40	simian virus 40
syn	syncytial
TAF	TBP-associated factor(s)
TBP	TATA element binding protein
TE	10 mM Tris-HCl (pH 7.8), 1 mM EDTA
TF	transcription factor
TK	thymidine kinase
Tris	Tris(hydroxymethyl)aminoethane
tRNA	transfer ribonucleic acid
ts	temperature-sensitive
UTP	uridine triphosphate
X-gal	5-bromo-4-chloro-3-indolyl-β-D galactoside

1 INTRODUCTION

1.1 Nonsense Codons, Suppressor tRNAs and Translation Termination

The information for directing the termination of polypeptide chain synthesis is encoded within 3 translation termination or nonsense codons: UAG (amber (am)), UAA (ochre (oc)), and UGA (opal (op)). Protein release factors (RF) specifically recognize and decode these termination codons. In prokaryotes, there are 3 release factors (RF1, 2, and 3). RF1 is specific for UAG and UAA termination codons while RF2 recognizes UGA and UAA codons. RF3 lacks codon specificity but recognizes GDP and GTP and is thought to stimulate the binding and release of RF1 and RF2 from the ribosome. In eukaryotes, a single release factor, which possesses GTPase activity (Konecki *et al*, 1977) and is specific for all three termination codons, is sufficient to effect translation termination. Release factor and GTP binding to the ribosome is thought to stimulate the hydrolysis of ribosomal-bound peptidyl-transfer RNA (tRNA) by the ribosomal peptidyltransferase, thereby releasing the nascent polypeptide chain; GTP hydrolysis ensues facilitating dissociation of the release factor and deacylated tRNA from the ribosome, which then is released from the messenger RNA (mRNA) (Caskey, 1980).

Open reading frame (ORF)-internal nonsense codons cause premature translation termination generating truncated, often non-functional polypeptides and hence, may be lethal. Nonsense codons can also modulate steady-state levels of mRNA by influencing

mRNA processing (Urlaub *et al*, 1989; Naeger *et al*, 1992), transport (Humphries *et al*, 1984; Baserga and Benz, 1988) and stability (Peltz *et al*, 1990, 1993; Barker and Beemon, 1991) and hence maintain ancillary functions aside from acting solely as signals marking translation termination. Nonsense codons may be bypassed through exon-splicing (Bond *et al*, 1990; Dietz *et al*, 1993), ribosomal hopping or frameshifting, or through suppressor (su^+) tRNA-mediated translational readthrough (Valle and Morche, 1988; Weiss, 1991). These mechanisms determine nonsense codon usage and thus can play integral roles in the regulation of gene expression.

Nonsense codons can also function normally in support of polypeptide chain elongation. In mitochondria and *Mycoplasma*, UGA codons normally encode tryptophan (Engelberg-Kulka and Schoulaker-Schwarz, 1988), while in ciliates, UAG and UAA codons specify glutamine (Hanyu *et al*, 1986; Cohen *et al*, 1990). Moreover, cessation of translation at some nonsense codons is not absolute; rather termination codons may be rendered leaky and read as sense codons through the insertion, at varying efficiencies, of an amino acid by a nonsense su^+ tRNA.

Nonsense su^+ tRNAs in *Escherichia coli* (*E. coli*) (Celis and Smith, 1979; Eggertsson and Soll, 1988) and yeast (Sherman, 1982) were initially isolated from mutagenized cells and as such, are termed genetic su^+ tRNAs. The suppressor phenotype most often originates from a mutation within the anticodon region of a normal aminoacyl-tRNA gene. Interestingly, the suppressor phenotype conferred upon an *E. coli* $tRNA^{Trp}$ gene is due to a base substitution in the dihydrouracil stem (Hirsh, 1971). While genetic su^+ tRNAs have subsequently been isolated from *Caenorhabditis elegans* (Wills *et al*,

1983), such approaches have not facilitated the isolation of su$^+$ tRNA genes from higher eukaryotes, perhaps signifying the deleterious nature of nonsense suppression activity to higher organisms. However, with the advent of recombinant DNA technology, such nonsense su$^+$ tRNAs have been constructed from cloned tRNA genes through alteration of the anticodon, and have been shown to possess suppression activity in *Drosophila* (Laski *et al*, 1989), plant cells (Szweykowska-Kulinska and Beier, 1991; Franklin *et al*, 1992), and in mammalian cells (Capone *et al*, 1985,1986; Laski *et al*, 1982,1984; Temple *et al*, 1982; Young *et al*, 1983).

Naturally occurring eukaryotic su$^+$ tRNAs have recently been identified (Hatfield *et al*, 1990). The first class corresponds to normal major cytoplasmic aminoacyl-tRNAs which, in addition to maintaining their normal specificity and function in polypeptide chain synthesis, can occasionally misread a termination codon. Readthrough by these tRNAs is inefficient since termination codon recognition is mediated through unconventional non-Watson-Crick base pairing (Valle and Morch, 1986). Amber su$^+$ tRNAs^{Tyr}(GψA) have been isolated from *Drosophila* (Bienz and Kubli, 1981), and wheat (Beier *et al*, 1984b). As well, two novel amber su$^+$ tRNAs^{Leu} (CAA,CAG) have been isolated from calf liver (Valle *et al*, 1987). Translational readthrough mediated by this class of su$^+$ tRNAs is thought to play a critical role in the regulation of viral gene expression. Generation of the Moloney murine leukemia virus encoded protease is dependent upon the suppression of an amber termination codon separating the *gag* and *pol* ORFs (Yoshinaka *et al*, 1985) and is due to misreading by a minor amber su$^+$ tRNA^{Gln}(UmUG), recently identified in mouse liver (Kuchino *et al*, 1987). Similarly,

generation of the 183 kd protein of tobacco mosaic virus is dependent upon suppression of an amber termination codon at the end of the 126 kd protein gene ORF (Pelham, 1978) and is due to misreading by two su^+ $tRNAs^{Tyr}(G\psi A)$ found in tobacco plants (Beier *et al*, 1984a). It is not known whether functional suppression mediated by these su^+ tRNAs plays a role in the regulation of cellular genes. Ochre su^+ tRNAs have recently been identified in yeast (Boone *et al*, 1992) and in plants (Zerfass and Beier, 1992).

The second class of naturally occurring eukaryotic su^+ tRNAs maintains specialized cellular functions. These minor tRNA species possess anticodons which are directly complementary to the nonsense codon. Recognition of defined nonsense codons serves to direct the co-translational incorporation of modified amino acids. Opal su^+ $tRNAs^{Ser}$ have been isolated from diverse organisms and are converted to selenocysteyl-tRNAs, through a phosphoseryl-tRNA intermediate, prior to direct incorporation into selenium-containing polypeptides at specific non-terminator UGA codons (Engelberg-Kulka and Schoulaker-Schwarz, 1988; Stadtman, 1990; Hill *et al*, 1991).

Hence, su^+ tRNAs have natural physiological roles. The regulation of translation termination, through su^+ tRNA-mediated recognition of specific nonsense codons, represents not only a mechanism for the regulation of gene expression, but also a novel strategy for modulating protein function (Morch *et al*, 1987; Valle and Morche, 1988).

1.2 Nonsense Codon Recognition and Efficiency of Translational Readthrough

Adopting suppression of translation termination as a regulatory strategy necessitates that termination codon readthrough be highly regimented and that recognition

of nonsense codons be highly selective. The factors which influence suppression efficiency or which specify nonsense codons as substrates for su^+ tRNA-mediated translational readthrough are poorly understood.

The efficiency of suppression at a defined nonsense codon is thought to reflect a competition between two mutually exclusive processes; recognition of the nonsense codon by the su^+ tRNA, promoting translational readthrough, and recognition by the release factor, promoting termination (Beaudet and Caskey, 1970). An alteration of the availability or activity of the release factor or su^+ tRNA will thereby influence the efficiency of translational readthrough (Engelberg-Kulka and Schoulaker-Schwarz, 1988; Pedersen and Curran, 1991). Indeed, the relative suppression strength of a su^+ tRNA at a defined nonsense codon is directed by structural characteristics of the tRNA, including the extent of base modification and the nature of the anticodon loop, which influence the interaction between the su^+ tRNA and the mRNA (Eggertsson and Soll, 1988; Kleina *et al*, 1990). A reduction in the activity of RF1 or RF2 in *E. coli* results in an increase in UAG (Ryden and Isaksson, 1984; Ryden *et al*, 1986) or UGA suppression (Kawakami *et al*, 1988), respectively. As well, enhanced suppression activity in [psi^+] strains of yeast is thought to be due to defective or reduced levels of release factor (Tuite *et al*, 1987). Extrinsic factors can also influence the competitive abilities of the release factor or su^+ tRNA. Alterations to ribosome structure can differentially alter both release factor and su^+ tRNA recognition of a nonsense codon and thereby influence the efficiency of translational readthrough (Eggertsson and Soll, 1988; Prescott and Kornau, 1992).

The mRNA sequence surrounding the nonsense codon or codon context is a

primary determinant in establishing whether a nonsense codon serves as a site for translational termination or readthrough. Studies of the influence of mRNA codon context on nonsense suppression have been carried out almost exclusively in *E. coli* and suggest that the efficiency of translational readthrough by su^+ tRNAs is modulated by sequences downstream of the nonsense codon; for UAG nonsense codons, readthrough is more efficient when the base directly 3' of the nonsense codon is a purine (Bossi and Roth, 1980; Bossi, 1983; Miller and Albertini, 1983) according to the rank order: UAGG $\geq$ UAGA > UAGC > UAGU (Stormo *et al*, 1986). Furthermore, the efficiency of UGA suppression is subject to influence by nucleotides both upstream (Buckingham *et al*, 1990) and downstream (Kopelowitz *et al*, 1992) of the UGA nonsense codon. Recent work suggests that mRNA codon context also functions in eukaryotic systems to influence the efficiency of nonsense suppression, and apparently does so in a manner which is distinct from that observed in *E. coli* (Phillips-Jones *et al*, 1993).

A preferred codon context may also favour translation termination. Sequence compilations of natural prokaryotic termination codons have revealed a strong bias towards uridine use following tight stops (Kohli and Grosjean, 1981; Brown *et al*, 1990), whereas the presence of adenosine directly 3' to UGA codons is associated with leaky stops (Engelberg-Kulka, 1981). Moreover, release factor recognition of nonsense codons in *E. coli* (Martin *et al*, 1988) and termination efficiency in yeast (Miller and Hinnebusch, 1989) are subject to context effects.

The nature of such mRNA context effects in regulating the efficiency of translational readthrough is poorly understood. A mRNA codon context favourable for

translational readthrough may reflect an enhanced su^+ tRNA affinity for the nonsense codon. Extended anticodon loop/mRNA interactions of 4 to 5 base pairs (bps) or stabilizing tRNA-tRNA interactions between the peptidyl-tRNA at the ribosomal P site and the aminoacylated su^+ tRNA at the ribosomal A site may account for enhanced nonsense codon recognition by a su^+ tRNA (Bossi, 1983; Miller and Albertini, 1983). Interestingly, a number of leaky eukaryotic termination codons are flanked by CAA codons specifying glutamyl-tRNAs; stabilizing tRNA-tRNA interactions may account for this inherent leakiness (Valle and Morche, 1988). Stabilization of the codon/anticodon complex may also be due to base stacking interactions with downstream nucleotides (Stormo *et al*, 1986). The corollary, in which mRNA codon context favours release factor interaction with a nonsense codon, may underly stringent termination signals. Enhanced release factor recognition of termination codons may arise through contacts with the nucleotide directly downstream of the nonsense codon, thereby increasing the rate of codon selection and hence, termination (Pedersen and Curran, 1991).

Natural termination codons are apparently refractory to use as sites for su^+ tRNA-mediated translational readthrough. Injection of *Xenopus laevis* oocytes with yeast amber, ochre, and opal su^+ tRNAs results in only a slightly altered 2-dimensional gel electrophoresis polypeptide profile (Bienz *et al*, 1981). A yeast opal su^+ tRNA which can only partially suppress the UGA terminator of a β-globin mRNA (Kohli *et al*, 1979), can suppress with high efficiency, five UGA nonsense codons on a mitochondrial mRNA (De Ronde *et al*, 1980), *in vitro*. Moreover, mammalian cells can apparently tolerate low levels of su^+ tRNA activity (Hudziak *et al*, 1982; however, see section 3.2). The

frequency of tandem or close, in-frame, second termination codons in eukaryotic mRNAs is low and hence alone cannot account for the high fidelity of polypeptide chain termination (Kohli and Grosjean, 1981). Rather, signals for termination may comprise two domains, thereby favouring release factor recognition; a termination codon may serve as the primary signal directing release factor interaction, while distal, variable sequences may act as secondary signals which modulate the efficiency of polypeptide chain release (Bossi, 1983).

The ability of naturally occurring su^+ tRNAs to specifically recognize certain nonsense codons may also be governed by the surrounding mRNA sequence (Weiss, 1991). Translational readthrough *in vivo* of the leaky tobacco mosaic virus UAG termination codon is dependent upon downstream sequence elements (Skuzeski *et al*, 1991), while readthrough of the Moloney murine leukemia virus UAG codon is stimulated through the formation of a downstream pseudoknot (Wills *et al*, 1991). An extensive stem-loop structure is required for selenocysteyl-$tRNA^{Sec}$ recognition of a non-terminator UGA codon in *E. coli* formate dehydrogenase mRNA (Zinoni *et al*, 1990), while incorporation of selenocysteine at a specific mammalian UGA codon is dependent upon a 3'-untranslated region (Berry *et al*, 1991). Interestingly, structural features of the bacterial selenocysteyl-$tRNA^{Sec}$ apparently contribute to the specificity of UGA nonsense codon selection (Li and Yarus, 1992).

In summary, su^+ tRNAs can selectively recognize nonsense codons which act as sites of protein modification or gene regulation. A primary determinant in establishing the consequence of a nonsense codon on polypeptide synthesis and thus, the efficiency

of translational readthrough, is the mRNA sequence context. Moreover, while the signals remain poorly defined, an active mechanism apparently serves to protect natural termination signals from translational readthrough. Implicitly, random nonsense codons or mutations are likely to undergo preferential suppression over natural termination sites.

1.3 Nonsense Su^+ tRNAs in the Examination of Biological Function

Su^+ tRNA activity is easily quantitated both *in vivo* and *in vitro* by determining the efficiency of translational readthrough of premature nonsense codons. Hence, the mechanisms by which components of the translational apparatus ensure translational fidelity are readily amenable to investigation, both in prokaryotic and in eukaryotic systems. Su^+ tRNAs have advanced our understanding of how mRNA codon context (Bossi and Roth, 1980; Bossi, 1983; Miller and Albertini, 1983; Phillips-Jones *et al*, 1993) and anticodon loop nucleotide composition (Valle *et al*, 1987; Kleina *et al*, 1990) influence anticodon/codon recognition; of the importance of tRNA base modification in anticodon/codon interaction (Zerfass and Beier, 1992; Valle and Morch, 1988); and of ribosome structure and function (Prescott and Kornau, 1992). Extrinsic factors which influence translational fidelity, such as the specificity of aminoacyl-tRNA synthetases for cognate tRNAs, have been readily investigated using su^+ tRNAs (McClain *et al*, 1990; Normanly *et al*, 1990; Lee and RajBhandary, 1991).

Su^+ tRNAs have afforded a powerful approach to conducting protein structure/function analyses (Miller *et al*, 1979; Glass *et al*, 1982; Kleina and Miller, 1990) and maintain the potential to play an integral role in the engineering of proteins

with defined properties (Miller *et al*, 1989; Miller, 1991). Indeed, su^+ tRNAs have facilitated the incorporation of unnatural amino acids into polypeptide chains (Noren *et al*, 1989, 1990). Moreover, su^+ tRNA-mediated nonsense suppression has been adopted as a means to conditionally express protein-coding genes in *E. coli* (Cohen *et al*, 1990). A human su^+ tRNA gene which may be conditionally expressed may serve as a generally applicable regulatory switch to confer regulated gene expression, at the level of translation, in eukaryotic systems (Syroid *et al*, 1992).

Su^+ tRNAs have provided the opportunity of investigating tRNA gene expression. In eukaryotes, following the expression of individual tRNA genes *in vivo* is problematic owing to the existence of multiple, identical genes for most tRNA isoacceptors (Sharp *et al*, 1986). Su^+ tRNA-mediated nonsense suppression, however, is a phenotype which is readily distinguishable from that of other tRNAs and has been exploited as a means to define the differential regulatory patterns of individual members of tRNA gene families (Kondo *et al*, 1988, 1990). Moreover, studies aimed directly towards understanding the mechanisms of tRNA gene regulation (Tapping *et al*, 1993) and tRNA processing (Kirsebom *et al*, 1988) have been carried out *in vivo* through quantitation of nonsense suppression activity.

Su^+ tRNAs have mediated the identification and characterization of nonsense mutations in prokaryotic, viral, and cellular genes (Gesteland and Wills, 1979). In humans, nonsense mutations have been implicated in the manifestation of numerous disease states. Su^+ tRNA technology has afforded not only a means by which nonsense mutations which underly genetic lesions may be identified, but also a potential strategy

for the control or alleviation of such conditions (Temple *et al*, 1982; Ho *et al*, 1986).

Hence, the utility and scope in application of nonsense su$^+$ tRNAs is considerable. However, su$^+$ tRNAs have perhaps been most invaluable in their application towards genetic analyses in *E. coli* and yeast. The ability of su$^+$ tRNAs to abrogate the effects of otherwise-lethal nonsense mutations by allowing the generation of a full length polypeptide, forms the basis of a genetic screen for the identification of essential genes.

1.4 The Project

The availability of numerous nonsense su$^+$ tRNA strains of *E. coli* and yeast led to the rapid development of prokaryotic and yeast genetics (Celis and Smith, 1979; Sherman, 1982). The recent availability of mammalian nonsense su$^+$ tRNA genes has afforded the opportunity of applying similar strategies towards the advancement of mammalian viral and cellular genetics. Moreover, that su$^+$ tRNA-mediated nonsense suppression activity is readily amenable to quantitation in mammalian cells (Capone *et al*, 1986), has provided a means to examine the regulation of transcription by RNA polymerase III (polIII) *in vivo*.

The initial aim of this research involved the further development and use of mammalian systems of nonsense mutation suppression for the genetic analysis of mammalian viruses. Specifically, the underlying objective of this work was to examine the mechanisms which underly eukaryotic class II gene expression and regulation. As such, herpes simplex virus type-1 (HSV-1) was chosen as a model system since HSV-1 genes are expressed in a regulated cascade. Furthermore, HSV-1 encodes numerous

ORFs that have largely been unmarked in previous genetic analyses and moreover, HSV-1 undergoes a complex replication cycle. Hence, HSV-1 presents a stringent evaluation of the feasibility of adopting currently available mammalian systems of nonsense mutation suppression in the genetic analysis of large animal viruses.

A related aspect of this work involved the enhancement of the utility of mammalian systems of nonsense mutation suppression through the development of novel systems for the regulation of su^+ tRNA genes in mammalian cells.

Lastly, a natural progression of this work involved the utilization of such a conditional system of su^+ tRNA gene expression to examine the molecular mechanisms of transcription by mammalian polIII both *in vivo* and *in vitro*.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

The following is a list of specialized reagents and the companies from which they were purchased.

acycloguanosine	Sigma Chemical Company
calf serum, fetal bovine serum, fetal bovine serum (dialyzed)	Gibco/BRL
creatine phosphate	Boehringer Mannheim
crystal violet	BDH Chemicals LTD.
dimethylformamide	BDH Chemicals LTD.
E. coli lac repressor	Stratagene
geneticin (G418)	Gibco/BRL
L-glutamine (100X; 200 mM)	Gibco/BRL
heparin	Sigma Chemical Company
hydroxylamine	Fluka Chemical Corp.
immune serum globulin (human)	Miles Canada Inc.
IPTG (isopropylthio-β-D-galactoside)	Gibco/BRL
MEM non-essential amino acid solution (10 mM; 100X)	Gibco/BRL

MEM vitamin solution (100X)	Gibco/BRL
minimal essential media (methionine deficient)	Flow Laboratories Inc.
molecular weight standards	
(i) 1 kbp DNA ladder	Gibco/BRL
(ii) lambda DNA (*EcoR*1/*Hind*III restriction) (*Hind*III restriction)	Gibco/BRL
(iii) ^{14}C-labeled protein standards (high range molecular weight 14,300-200,000 Da)	Gibco/BRL
nucleoside triphosphates (ATP, GTP, UTP, CTP)	Pharmacia Inc.
penicillin (5000U/ml)-streptomycin (5000 μg/ml) cocktail	Gibco/BRL
phenylmethylsulphonylfluoride (PMSF)	Sigma Chemical Company
protein A sepharose	Pharmacia Inc.
rabbit anti-HSV-1	Dakopatts (Denmark)
salmon testes DNA	Sigma Chemical Company
sephadex G-50	Pharmacia Inc.
sodium nitrite	Fluka Chemical Corp.
spermine-HCl	Sigma Chemical Company
trypsin-EDTA	Gibco/BRL
urea	Gibco/BRL
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Gibco/BRL

2.1.2 Radiochemicals

^{14}C-chloramphenicol mCi/mmol; 0.1 μCi/μl)	Dupont/NEN Canada
^{3}H-acetyl-coA (3.7 Ci/mmol; 0.25 μCi/μl)	Amersham Canada Ltd.
[α-^{32}P]dATP (3,000 Ci/mmol; 10 μCi/μl)	Amersham Canada Ltd.
[α-^{32}P]GTP (650 Ci/mmol; 10 μCi/μl)	ICN Biomedicals Inc.
L-[^{35}S]-methionine (1151 Ci/mmol; 10μCi/μl)	Amersham Canada Ltd.

2.1.3 Cloning Vectors and Host Bacterial Strains

Subcloning was carried out using the vectors pUC18 (Yanisch-Perron *et al*, 1985) and pUC118 (Vieira and Messing, 1987) as indicated. Host *E. coli* strains HB101 and DH5α used for cloning were purchased from Gibco/BRL.

2.1.4 HSV-1 Marker Transfer Vectors

Plasmids pSS17L and pSS17B were provided by Dr. D.C. Johnson, Department of Pathology, McMaster University. These plasmids contain a copy of the herpes simplex virus type-1 (HSV-1) KOS *Bam*H1 J fragment altered through the introduction of a polylinker and a unique *Bgl*II site, respectively, within the HSV-1 glycoprotein I gene (gI). Plasmid pTKSB was provided by Dr. J.R. Smiley, Department of Pathology, McMaster University. This plasmid contains HSV-1 thymidine kinase (TK) gene sequences disrupted by a *Bam*H1 linker.

2.1.5 Eukaryotic Reporter Vectors

The pRSVCAT expression vector encodes the *E. coli* Tn9 chloramphenicol acetyltransferase (CAT) gene under transcriptional control of the Rous sarcoma virus long terminal repeat (Gorman *et al*, 1982). Plasmids pRSVCAT(am), (oc), (op) are derivatives of pRSVCAT in which serine codon 27 of the CAT gene has been mutated to UAG, UAA, and UGA nonsense codons, respectively (Capone *et al*, 1986). Plasmid pRSV*βgal* encodes the *E. coli lacZ* gene, expressing β-galactosidase (*βgal*), while pRSV*βgal*(am) encodes a *lacZ* gene containing a suppressible amber nonsense mutation. The *βgal* plasmids were provided by Bob Rosenberger, National Institute for Medical Research, London, UK.

2.1.6 Eukaryotic Expression Vectors

Plasmids pUCtS Su^+(am), (oc), (op) encode the amber, ochre, and opal suppressor derivatives, respectively, of a human serine tRNA gene (Capone *et al*, 1985, 1986). The retrovirus shuttle vector pZIPNEOSV(X) encodes a simian virus 40 (SV40) replicon containing the bacterial neomycin phosphotransferase gene (Cepko *et al*, 1984). Plasmid pZIPNEOSV(am) is a derivative of pZIPNEOSV(X) in which the 900 bp *Sau*3A fragment containing the human amber su^+ $tRNA^{Ser}$ gene from plasmid pUCtS Su^+(am) was inserted in the unique *Bam*H1 site (Sedivy *et al*, 1987). Similarly, plasmids pZIPNEOSV(oc), (op) encode the ochre and opal su^+ tRNA genes, respectively (J.P. Capone, unpublished). Plasmid pLTRtsA58 encodes a temperature-sensitive (*ts*) allele of the SV40 large T antigen under transcriptional control of the Rous sarcoma virus long

terminal repeat (Sedivy *et al*, 1987). Plasmid pSV2NEO encodes the neomycin phosphotransferase gene under transcriptional control of the SV40 early promoter (Southern and Berg, 1982). Plasmid pSV2HIS encodes the *Salmonella typhimurium* histidinol dehydrogenase gene under transcriptional control of the SV40 early promoter (Hartmen and Mulligan, 1988) and was provided by Dr. D.C. Johnson. Plasmid pRSVIns encodes the *E. coli* lactose operon (*lac*) repressor, which has been modified by the addition, to the carboxy terminus, of the nuclear targeting signal from the SV40 large T antigen (Hu and Davidson, 1991).

2.1.7 Mammalian Cell Lines

Mammalian cell lines used for transfections and virus work were BSC-40 and Vero African green monkey kidney cells, and HeLa human cervical carcinoma cells. Virus work was also carried out in VD60 cells, which were derived from Vero cells and express high levels of HSV-1 glycoprotein D (gD) upon infection with HSV-1 (Ligas and Johnson, 1988), and AM12 cells, which were derived from BSC-40 cells and conditionally express a human amber su^+ $tRNA^{Ser}$ gene (Sedivy *et al*, 1987). HeLa S-3 cells, a suspension cell derivative of the HeLa cell line, were used in the preparation of nuclear extracts to examine transcription *in vitro* by polIII. BSC40, Vero, and HeLa cells were obtained from the American Type Culture Collection, USA. VD60 cells were provided by Dr. D.C. Johnson.

2.1.8 Mammalian Viruses

HSV-1 strain KOS (Smith, 1964) was used for chemical mutagenesis and marker transfer experiments. F-gDβ, a recombinant HSV-1 virus in which the gD gene has been disrupted by insertion of the *E. coli lac* Z gene (Ligas and Johnson, 1988), and PAA^{r}5, a DNA polymerase mutant HSV-1 virus (Hall *et al*, 1984), were used in marker transfer experiments. F-gDβ was provided by Dr. D.C. Johnson and HSV-1 Kos and the PAA^{r}5 derivative were provided by Dr. J.R. Smiley, Department of Pathology, McMaster University.

2.1.9 Oligonucleotides

The oligonucleotides described in Table 2.1 were used in the generation of constructs (section 2.2.1) or for DNA sequence analyses as indicated, and were synthesized by the Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University.

*Xba*1 linker 5'-TCTAGA, *Bam*H1 linker 5'-CGGATCCG, and the pUC18 multiple cloning site (MCS) were purchased from Pharmacia Inc. (Canada). The *Xba*1 and *Bam*H1 linkers were used in the construction of plasmids pB-*βgal*(am) and pTK-*βgal*(am), respectively. The pUC18 MCS was used to introduce unique cloning sites both upstream and downstream of the su$^+$ tRNA gene coding region. *Hind*III linker 5'-CAAGCTTG was purchased from New England Biolabs Inc. and was used in the construction of plasmid pTK-ICP6LUC. The su$^+$ tRNA gene sequencing primer 5'-AACATATATTCCTCATGGGA (annealing position nucleotide (nt) -47 to nt -28) was

Table 2.1 Oligonucleotides

Oligonucleotide	Sequence	Purpose
AB355 AB356	5'-CTTCCTAGGCCCCGC (lower strand) 5'-GGGGCCTAGGAAGACGT (upper strand)	*AatII*/*SacII* HSV-1 gD gene cassette containing an in-frame amber nonsense codon
AB164	5'-CTAGCTAGCTAG	linker containing in-frame amber nonsense codons for creating a stop codon within the *E. coli lacZ* gene
AB882	5'-ATTGTGAGCGCTCACAAT	Symmetric *E. coli lac* operator sequence used for construction of tRNA/operator clones (Brown *et al*, 1987)
AB883	5'-CTAGATTGTGAGCGCTC- ACAAT	*E. coli lac* operator sequence with *XbaI* sticky ends for construction of tRNA/operator clones
AB1465	5'-TGTGGAATTGTGAGCGC- TCACAATTCCACA	Extended symmetric *E. coli lac* operator sequence with a higher affinity for *lac* repressor than wild-type operator sequence (Sadler *et al*, 1983); used for construction of tRNA/operator clones
AB2151 AB2152	5'-CGAATCCTGCCGACTAC- (upper strand) GGCCGATATCTTTTTTCCC 5'-GGGAAAAAAAGATATCG- (lower strand) GCCGTAGTCGGCAGGATT	*BstI*/*SmaI* su$^+$ tRNA gene cassette used to introduce unique *EagI*, *EcoRV*, and *SmaI* cloning sites downstream of the tRNA gene coding region
AB586	5'-GGATTTCCTCTACCCGAG	Su$^+$ tRNA gene sequencing primer (annealing position nt 99 to nt 116)

obtained from Dr. U.T. RajBhandary, Centre for Cancer Research, and Department of Biology, Massachusetts Institute of Technology. The HSV-1 gD gene sequencing primer 5'-GTACTCCATGACCGTGA (annealing position (reverse strand) nt 660 to nt 644) was obtained from Dr. D.C. Johnson.

2.2 Methods

2.2.1 Construction of Plasmids

Generation of all constructs was carried out according to standard molecular biology protocols as defined by Ausubel *et al*, (1989) and Sambrook *et al*, (1989) and according to product manufacturer's specifications. Enzymes were purchased from Gibco/BRL, New England Biolabs Inc., Pharmacia Inc., Promega Corp., and Boehringer Mannheim. Supplementary techniques include the use of SpinBind™ DNA extraction units (FMC BioProducts) and GeneClean™ kits (BIO/CAN Scientific Inc.) for the purification of DNA framents from agarose gels, and Qiagen columns (Qiagen Inc.) for the large scale preparation of plasmid DNA. The integrity of clones was confirmed through DNA sequence analysis using the Sequenase™ DNA seqencing kit (United States Biochemical Corporation). Plasmid DNA concentration was quantitated by fluorescence spectroscopy. The following constructs are not described in the Results/Discussion section (refer to Appendix, section 7).

2.2.1.1 *E. coli LacZ* Gene Nonsense Codon Derivatives (Appendix 7.2)

Plasmid pR-*βgal*(am) was constructed as follows: the ICP6/*lacZ* gene cassette (Goldstein and Weller, 1988), a fusion between the HSV-1 ICP6 gene (ICP6 promoter

and amino-terminal 59 amino acids) and the *E. coli lacZ* gene coding region, was obtained from Dr. D.C. Johnson in plasmid pD6p. The ICP6/*lacZ* cassette was cloned into pUC18 as a *Bam*HI fragment to generate pICP6*βgal*. An oligonucleotide cassette containing amber nonsense codons within all 3 reading frames (AB164; Table 2.1) was blunt-end ligated into the unique *Eco*RV site (nt 1128) within the *lacZ* gene to generate plasmid pR-*βgal*(am).

Plasmid pB-*βgal*(am) was constructed as follows: the *Xba*I site in pICP6*βgal* was destroyed by blunt-ending the *Xba*I sticky ends with Klenow DNA polymerase followed by plasmid recircularization. Plasmid pB-*βgal*(am) was generated by blunt-end ligating a 6 nt *Xba*I linker (5'-TCTAGA) into the unique *Bal*I site of the ICP6 gene, thereby creating an in-frame amber nonsense codon, 2 codons downstream of the ICP6 gene translation initiation codon ATG.

Plasmid pTK-*βgal*(am) was constucted as follows: a *Bam*HI linker 5'-CGGATCCG was ligated to the *Nru*I blunt end of pRSV*βgal*(am). This DNA was digested with *Bam*HI yielding a *Bam*HI RSV*βgal*(am) cassette which was cloned into the unique *Bam*HI site of plasmid pTKSB generating pTK-*βgal*(am).

2.2.1.2 Amber Su^+ tRNA Gene Marker Transfer Construct (Appendix 7.3)

Plasmid pTK-Su^+(am) was constructed by inserting the *Sau*3AI fragment of pUCtS Su^+(am), containing the su^+ tRNA gene, into the unique *Bam*HI site of pTKSB.

2.2.1.3 HSV-1 ICP6 Gene/Reporter Gene Fusion Constructs (Appendix 7.4)

Plasmid pSS-ICP6β*Gal* was constructed by cloning the *Bam*HI fragment of pICP6β*Gal*, containing the ICP6β*Gal* gene cassette, into the *Bgl*II site of plasmid pSS17B.

Plasmid pTK-ICP6LUC was constructed as follows: plasmid pSVOA-LΔ5' encodes the firefly luciferase gene (DE WET *et al*, 1987) and was provided by Dr. R.A. Rachubinski, Department of Biochemistry, McMaster University. The *Hind*III/*Sma*I fragment, containing the luciferase gene, was cloned into the *Hind*III/*Hinc*II sites of pUC18 to generate pLUC. The *Hind*III linker 5'-CAAGCTTG was blunt-end ligated into the *Sma*I site of pLUC to generate pLUC-SH. Plasmid pICP6, which retains the ICP6 gene sequences, was constructed from pICP6β*gal* through an *Xho*I/*Sal*I collapse. The *Hind*III fragment of pLUC-SH, containing the luciferase gene, was cloned into the *Hind*III site of pICP6 to generate pICP6LUC, which encodes the 59 terminal amino acids of the HSV-1 ICP6 gene fused to the luciferase gene under the transcriptional control of the ICP6 gene promoter. The *Bam*HI fragment of pICP6LUC, containing the ICP6LUC gene cassette, was cloned into the *Bam*HI site of pTKSB to generate pTK-ICP6LUC.

2.2.1.4 Additional *E. coli Lac* Operator/tRNA Gene Constructs

2.2.1.4.1 Cloning Vectors (Section 5)

Plasmid pUC-O was generated from pUCtS Su^+(am) through a *Pvu*II collapse thereby deleting both the endogenous plasmid-borne *lac*O sequence and the su^+ tRNA gene.

Plasmid pUCtS Su^+(am)-O was generated by cloning the *Hinc*II/*Sma*I fragment from pUCtS Su^+(am), containing the su^+ tRNA gene, into the *Pvu*II site of pUC-O.

Plasmid pUCtS93 was generated as follows: the region spanning the unique *Bst*I site within the coding region of the su^+ tRNA gene and the *Sma*I site present downstream of the termination sequence in pUCtS Su^+(am) was replaced with an oligonucleotide cassette (AB2151/AB2152; Table 2.1) to introduce unique *Eag*I, *Eco*RV, and *Sma*I cloning sites downstream of the tRNA gene coding region. The *Hinc*II/*Eco*RI fragment, containing the tRNA gene, was blunt-ended with Klenow DNA polymerase and ligated into the *Pvu*II site of pUC-O to generate pUCtS93.

Plasmid pUCtS93MCS was generated by inserting the pUC18 MCS, which was blunt-ended with Klenow DNA polymerase, into the unique *Eco*RV site of pUCtS93 at position nt 88 (relative to the first nt (+1) of the coding region), downstream of the tRNA gene coding region.

Plasmid pMCtS was generated by inserting the blunt-ended pUC18 MCS into the unique *Sna*BI site of pUCtS Su^+(am)-O directly upstream of the tRNA gene coding region at position nt -1.

2.2.1.4.2 Upstream *Lac*O/Su^+ tRNA Gene Construct (Appendix 7.1)

Plasmid pSVHIS-Su^+(am) was constructed by inserting the *Sau*3AI fragment of p*Lac*OtS(am4) (section 4.4), containing the su^+ tRNA gene, into the *Bam*HI site of pSV2HIS.

2.2.1.4.3 Downstream *Lac*O/Su$^+$ tRNA Gene Constructs (Section 5.2)

Plasmid pt*SLac*O(am7) was isolated as a cloning artifact, in the construction of pt*SLac*O(am8) (section 5.2), in which the *lac*O sequence was partially duplicated.

Plasmid ptS-2*Lac*O(am8) was constructed by inserting the *lac*O oligonucleotide AB1465 (Table 2.1) into the unique *Eco*RV site of pt*SLac*O(am8) (section 5.2), thereby generating two proximal *lac*O sequences positioned downstream of the tRNA gene coding region.

Plasmid pt*SLac*O(am9) was generated by inserting the *lac*O oligonucleotide AB1465 (Table 2.1) into the unique *Sca*I site of pUCtS Su$^+$(am)-O at position nt 154, downstream of the tRNA gene coding region.

2.2.2 Maintenance and Manipulation of Mammalian Cells and Viruses

2.2.2.1 Maintenance of Mammalian Cells

Cell lines were maintained as monolayers at 37°C in Dulbecco's modified Eagle medium (Vero and BSC40 cells), α-minimal essential medium (VD60 and HeLa cells), or in suspension in Joklik's medium (HeLa S-3 cells). Media were supplemented with 10% calf serum, or 10% fetal bovine serum (HeLa cells) or 5% fetal bovine serum (HeLa S-3 cells), either 1% L-glutamine or 2% L-glutamine (HeLa S-3 cells), 1% penicillin-streptomycin, and for HeLa S-3 cells, 1% vitamin and 1% non-essential amino acid solutions. AM12 cells were maintained as monolayers at 39°C in supplemented Dulbecco's modified Eagle medium containing 800 μg G418/ml. Medium was purchased from the Cancer Research Group, Department of Pathology, McMaster University.

To detach cells from plates for subculture, cells were first washed with sterile PBS (NaCl 136.8mM, KCl 2.7mM, $Na_2HPO_4.7H_2O$ 8mM, KH_2PO_4 1.5mM) followed by trypsinization (trypsin 1X; diluted in PBS). HeLa S-3 cells were maintained at cell densities between 2 X 10^5 cells/ml and 1 X 10^6 cells/ml, and were subcultured through dilution.

2.2.2.2 Virus Infection of Mammalian Cells and Plaque Isolation

Virus infections were carried out as follows: medium from cell monolayers was aspirated and replaced with serum-deficient medium containing virus at a defined multiplicity of infection (moi), sufficient to just cover the monolayer. The cells were incubated with virus with occasional shaking for 2 hours to allow virus adsorbtion. Fresh supplemented medium was then added to the cells. In the isolation of virus plaques, supplemented medium contained immune serum globulin (0.05%, final). Individual virus plaques were recovered by picking with sterile toothpicks and then transferred onto cell monolayers grown on 96-well trays. HSV-1 superinfection of transfected cells in transient expression assays was carried out 24-48 hours post-transfection (moi of 10 pfu/cell).

2.2.2.3 Virus Stocks

Virus stocks were prepared as follows: 10-15cm diameter plates of cells were infected with virus at a moi of 0.1 plaque forming units (pfu)/cell. The cells were harvested and recovered by centrifugation (10 min; 4°C; 3000 RPM; IEC rotor 216, IEC Centra-8R Centrifuge (USA)) after total cytopathic effect (cpe) (2-3 days). The pellet was

resuspended in 10 mls of supplemented medium, subjected to 3 freeze-thaw cycles, and probe sonicated 3X at 4°C for 15 seconds. Cellular debris was removed through centrifugation (10 min; 3000 RPM; 4°C), and the supernatant was aliquoted and stored at -80°C.

2.2.2.4 Plaque Assays

Virus stocks were quantitated for infectivity by performing plaque assays. Cells were infected with serial dilutions of the virus stock and incubated for 2 hours. Supplemented medium, containing immune serum globulin (0.05%, final), was then added to the monolayers. The resulting plaques (2-3 days post virus infection (pvi)) were counted to yield the virus titre (pfu/ml). A record of virus plaque assays was maintained by removing the medium from the cells and staining the monolayer with crystal violet stain (0.1g/100ml [water:absolute ethanol (3:1)]).

2.2.3 Viral DNA Preparation

The preparation of highly purified HSV-1 DNA, which was subjected to nitrous acid mutagenesis, was as described by Sandri-Goldin *et al*, (1981). The final viral DNA concentration was approximately 2.0 mg/ml.

The preparation of HSV-1 DNA used in marker transfer experiments was as follows: 10-10 cm diameter plates of Vero cells were infected at a moi of 10 pfu/cell. Upon reaching total CPE (2-3 days pvi), the cells were harvested and collected by centrifugation (10 min; 3000 RPM; 4°C; IEC rotor 216). The pellet was washed several

times with PBS and then resuspended in 10 ml of 0.2 M EDTA (pH 8.0). SDS and proteinase K were added to a final concentration of 0.5% and 100 μg/ml, respectively, and the pellet was incubated overnight. The mixture was then extracted very gently with phenol four times, and dialyzed successively for 3-4 days against 100 volumes of 0.1X SSC buffer (15 mM NaCl, 1.5 mM trisodium citrate). Viral DNA was stored at 4°C.

The integrity of viral DNA in the production of virus particles was established through DNA transfection (section 2.2.4.3).

2.2.4 Transfer of DNA into Mammalian Cells

The introduction of DNA into mammalian cells was by DNA transfection using the calcium phosphate co-precipitation technique (Graham and van der Eb, 1973).

2.2.4.1 Transient Transfections

Transient transfection assays in gene expression analyses were performed in BSC40 cells using a total of 18 μg of DNA (made up with salmon testes DNA as required) per 6 cm diameter plate of cells at 80% confluency. Briefly, plasmid DNA (and salmon testes DNA) was added to an Eppendorf tube containing 250 mM $CaCl_2$ (stock 2.0 M) and sufficient water to give a final volume of 250 μl. The solution was vortexed as 250 μl of 2X HBSS pH 7.12 (0.28 M NaCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4.7H_2O$) was added dropwise over 90 seconds. The DNA/calcium phosphate precipitate was allowed to form at room temperature for 30 minutes (min) and then added dropwise to the cells. Cells were shocked 4 to 6 hours post transfection with serum-

deficient medium containing 10% DMSO. The cells were washed 3X with PBS, upon which fresh supplemented medium, containing 20 mM isopropylthio-β-D-galactoside (IPTG) as indicated (stock 1 M in PBS; filter sterilized), was added.

2.2.4.2 Stable Transfections

Stable transfections for the generation of defined mammalian cell lines were carried out using 20-40 μg of plasmid DNA/10 cm diameter plate of cells as described in section 2.2.4.1. The cells were split to sub-confluency (usually ratio 1:5) 48 hours post transfection. The cells were then allowed to grow for another 24 hours at which time fresh supplemented medium, containing 600 μg/ml-800 μg/ml G418 (stock 100 mg/ml in 100 mM Hepes pH 7.4; filter sterilized), was added. The cells were maintained under selection with G418 until individual colonies appeared (usually 2-3 weeks). Individual colonies were either pooled or isolated using cloning cylinders and expanded under selection in preparation for characterization.

2.2.4.3 Transfection of HSV-1 DNA

Transfection of HSV-1 DNA, carried out in chemical mutagenesis experiments and in the generation of recombinant HSV-1 viruses, was essentially as described in section 2.2.4.1 with the exception that viral DNA-containing tubes were gently flicked rather than vortexed to facilitate the formation of the calcium phosphate/DNA precipitate. The optimum volume of viral DNA used for transfections in marker transfer experiments and in viral DNA mutagenesis was determined by transfecting a range of viral DNA

aliquots (usually 5-200 μl) onto cells. The volume of viral DNA yielding 50-100 plaques or 500-1000 plaques per 10 cm diameter plate was used in such experiments, respectively.

2.2.5 Random Chemical Mutagenesis of HSV-1 DNA

Nitrous acid mutagenesis of HSV-1 DNA was carried out essentially as described by Sandri-Goldin *et al*, (1981). Briefly, 10 μl aliquots of pure viral DNA (20 μg, section 2.2.3) were incubated in a 20 fold excess (200 μl) of nitrous acid reaction mixture (0.1 M sodium acetate buffer pH 4.52, containing 0.05 M $NaNO_2$ and 2 X 10^{-4} M spermine) at 37°C from 0 to 30 min. Reactions were terminated by adding a 10 fold excess (1.2 ml) of ice-cold 2X HBSS pH 7.73 containing 10 μg salmon testes DNA/ml. Mutagenic time point 0 min corresponds to the immediate inactivation of nitrous acid upon addition to viral DNA. 840 μl of water was added and the formation of a calcium phosphate precipitate was initiated by adding 150 μl 2.0 M $CaCl_2$ dropwise with gentle shaking. The precipitate was allowed to form at room temperature for 40 min and then added dropwise to AM12 cells (3-6 cm plates) at 33°C (AM12 cells were pre-incubated 12 to 24 hours at 33°C). All transfected monolayers were harvested when total CPE was reached on cell monolayers corresponding to a single mutagenic time point. Virus stocks were prepared from these cells and titred on AM12 cells at 33°C to generate a HSV-1 nitrous acid inactivation curve.

2.2.6 Generation and Identification of HSV-1 Recombinant Viruses

2.2.6.1 HSV-1 Marker Transfer Experiments

The construction of defined HSV-1 recombinants was carried out through marker transfer into the HSV-1 TK gene, gD gene, and gI gene loci. Co-transfection of HSV-1 DNA and 1-10 μg of the appropriate marker transfer construct (linearized and uncut plasmid DNA) was carried out as described in section 2.2.4.3. When total CPE was reached, the cells were harvested and virus stocks were prepared. Selection for virus recombinants was then carried out.

2.2.6.2 Selection for Marker Transfer into the HSV-1 Thymidine Kinase Gene

Potential HSV-1 TK gene recombinants generated from marker transfer experiments were identified by infecting cells with serial dilutions of the virus in the presence of 200 μM acycloguanosine (stock 20 mM, in 200 μM NaOH) and immune serum globulin (0.05%, final) in the overlay medium (section 2.2.2.2). Individual virus isolates (2-4 days pvi) were plaque purified under selection.

2.2.6.3 Selection for Marker Transfer into the HSV-1 Glycoprotein D and Glycoprotein I Gene Loci:X-Gal/Agarose Overlays

A colorimetric assay for the detection of *βgal* expressing virions was carried out as follows: cells were infected with serial dilutions of virus stock in the presence of immune serum globulin (0.05%, final). Medium was aspirated from the cells when plaques became just visible (36-48 hours pvi) and replaced with 5 to 10 mM of an X-gal/agarose overlay (an equal volume of 2X supplemented Dulbecco's modified Eagle

medium, containing 600 μg/ml X-gal (stock 250 mg/ml dimethylformamide) preheated at 37°C, and a sterile solution of 1% agarose preheated at 42°C). After allowing the overlay to solidify for 45 min at room temperature, the plates were returned to the incubator. Colorimetric reactions were allowed to proceed for 18-40 hours and agarose plugs containing virus plaques were picked into cell monolayers using sterile Pasteur pipets.

2.2.7 Preparation of Mammalian Cell Extracts

2.2.7.1 Transient Expression Assays

Cells from transfection experiments were harvested 48 hours post transfection. Cells from transfection/HSV-1 superinfection experiments, and cells infected in the characterization of HSV-1 recombinants (moi of 10 pfu/cell), were harvested 12-24 hours pvi. Cells were washed 3X in PBS and then incubated at room temperature for 5 min in TEN buffer (40 mM Tris-Cl pH 7.4, 1 mM EDTA, 0.15 M NaCl). Cells were scraped into Eppendorf tubes and recovered through centrifugation for 5 min at 14000 RPM using an Eppendorf microfuge. Mammalian cell extract preparation was as described (Capone *et al*, 1986) and modified (Capone, 1988).

2.2.7.2 HeLa S-3 Cell Nuclear Extracts

HeLa S-3 cell nuclear extracts for the examination of tRNA gene transcription *in vitro* were prepared and dialyzed against buffer D (20 mM HEPES pH 7.9, 0.2 mM EDTA, 20% (vol/vol) glycerol, 0.1 M KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF) as

previously described (Dignam *et al*, 1983). The final extract protein concentration was 7 to 10 mg/ml.

2.2.7.3 ^{35}S-Methionine Labelled HSV-1 Infected Cell Extracts

Cell extracts from transfection/HSV-1 superinfection experiments (6 cm-diameter plates) were prepared by first harvesting cells as described in section 2.2.7.1, followed by resuspension in extraction buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.5% deoxycholate, 1% NP40, and 0.5 mM PMSF). Cells from HSV-1 infection experiments (96-well dish) were harvested at the termination of individual labelling periods, as described. Cell extracts were prepared by directly solubilizing the cells in extraction buffer, after washing 3X with PBS. Extracts were further processed through 3 cycles of water bath sonication and freeze-thaw. Cellular debris was removed by microfugation for 5 min at 14000 RPM.

2.2.8 Determination of Reporter Gene Activity

Mammalian cell extracts from transient expression assays were prepared as described in section 2.2.7.1.

2.2.8.1 Assay for Chloramphenicol Acetyltransferase Activity

Determination of CAT protein activity through the separation of acetylated derivatives of chloramphenicol using thin layer chromatography was as previously described (Capone *et al*, 1986; Capone, 1988). Determination of CAT protein activity

using the liquid scintillation method was as described (Neuman *et al*, 1987) and modified (Eastman, 1987).

2.2.8.2 Assay for Luciferase Activity

Determination of luciferase activity was carried out using the Luciferase Assay System (Promega Corporation) according to manufacturer's specifications, and either a luminometer or a scintillation counter.

2.2.8.3 Assay for β-Galactosidase Activity

βgal activity was determined essentially as described (Sato *et al*, 1986).

2.2.9 Analysis of tRNA Gene Transcription *In Vitro*

The standard 20 μl transcription reaction contained 80 to 100 μg of nuclear extract (section 2.2.7.2), 10 mM HEPES (pH 7.9), 50 mM KCl, 10% (vol/vol) glycerol, 8 mM creatine phosphate, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.25 mM PMSF, 2.5 mM $MgCl_2$, 0.4 mM each ATP, CTP, and UTP, 40 μM GTP, 2 μCi of ^{32}P-GTP, and 0.1 μg of template DNA. Reactions were supplemented with 2.5 μg/ml purified *E. coli lac* repressor (diluted in 20 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM EDTA, 500 mM NaCl, 50% (vol/vol) glycerol from a 1 mg/ml stock in the same buffer), 50 mM IPTG, 300 μg heparin/ml, and 1.2 mM unlabelled GTP as indicated. IPTG was prepared in water, while heparin was prepared in buffer D (Dignam *et al*, 1983). Control reactions lacking these components were

supplemented with the appropriate buffer. Transcription reactions were carried out at 30°C according to the conditions indicated. Reactions were terminated by the addition of 10 vol of 0.3 M sodium acetate (pH 5.0)-0.5% SDS followed by phenol/chloroform extraction. RNA transcription products were precipitated in ethanol.

2.2.10 Analysis of HSV-1 Infected Cell Polypeptides

2.2.10.1 ^{35}S-Methionine Labelling of Cells

^{35}S-methionine metabolic labelling of cells was carried out 35-40 hours post-transfection (in transfection/HSV-1 superinfection experiments) or from 3-20 hours pvi (in HSV-1 infection experiments) (moi of 10-20 pfu/cell), as described. Cells were washed 3X in PBS and incubated in labelling mix (methionine-deficient minimal essential medium supplemented with 1% penicillin/streptomycin cocktail, 1% L-glutamine, 10% dialyzed fetal bovine serum, 10% α-minimal essential medium, and ^{35}S-methionine (0.1 μCi/μl)) for various labelling periods, as described.

2.2.10.2 Immunoprecipitation of HSV-1 Polypeptides

Aliquots of HSV-1 infected cell extracts (section 2.2.7.3) were normalized for radionucleotide incorporation ($5X10^5$ - $5X10^7$ cpm) and mixed with the appropriate titre of antibody and 1ml of PBS for 3 hours at 4°C. Rabbit anti-HSV-1 glycoprotein C (gC), gD, and TK polyclonal antibodies were provided by Dr. D.C. Johnson. Rabbit anti-HSV-1 antibody is a polyclonal serum which recognizes all major HSV-1 glycoproteins (Dakopatts). Protein A-sepharose beads (100 μl; prepared in extraction buffer; section

2.2.7.3) were added and mixed for 1 hour at 4°C. The beads were washed 10X with extraction buffer containing 0.3% SDS, resuspended in an equal volume of 2X SDS loading buffer (Sambrook *et al*, 1989), and then boiled for 5 min. The supernatant was recovered by microfugation for 5 min at 14000 RPM.

2.2.11 Analysis of tRNA Gene Transcripts and HSV-1 Polypeptides using Polyacrylamide Gel Electrophoresis

2.2.11.1 tRNA Gene Transcription Products

tRNA transcripts generated *in vitro* (section 2.2.9) were analysed by denaturing electrophoresis on 10% polyacrylamide-7 M urea gels (constant power; 12-14 Watts/16cm gel), prepared in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na_2-EDTA) followed by autoradiography. tRNA transcripts were solubilized in 1X loading buffer (0.6X TBE, 7 M urea, 10% vol/vol glycerol, 0.01% xylene cyanol, and 0.01% bromophenol blue). Autoradiographs were quantitated using a Hoefer scanning densitometer. Transcript sizes were approximated by using a DNA sequencing ladder for size markers.

2.2.11.2 ^{35}S-Labelled HSV-1 Infected Cell Polypeptides

Aliquots of HSV-1 infected cell extracts (section 2.2.7.3), normalized for radionucleotide incorporation ($5X10^5$ - $5X10^7$ cpm), and immunoprecipitated HSV-1 polypeptides, were analysed on 10% SDS-polyacrylamide gels using a discontinuous buffer system as described (Sambrook *et al*, 1989), followed by autoradiography. Polypeptide relative molecular mass (M_r) was determined using ^{14}C-labelled protein standards.

2.2.12 Analysis of Cellular and Viral DNA by DNA Blot and Hybridization

2.2.12.1 Preparation of Cellular and HSV-1 DNA

2.2.12.1.1 Total DNA Preparation from Mammalian Cells

Cells were washed 3X in PBS, trypsinized, and recovered through microfugation at 14000 RPM for 5 min at 4°C. Cell pellets were resuspended in STE buffer (10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA) (1 ml/10 cm-diameter dish) and incubated at 65°C for 2 hours upon addition of SDS, proteinase K, and RNase A to a concentration of 0.5%, 500 μg/ml, and 100 μg/ml, respectively. DNA was phenol/chloroform extracted several times and precipitated in ethanol.

2.2.12.1.2 Preparation of Extra-Chromosomal Cellular and Viral DNA

Uninfected mammalian cells were recovered as described (section 2.2.12.1.1). Infected cells (moi of 10 pfu/cell) were harvested upon reaching total CPE (usually 24 hours pvi) after washing in PBS (section 2.2.12.1.1) or by dislodging the cells using a pipetman. Preparation of extra-chromosomal DNA was carried out as described (Hirt, 1967). Cell pellets were resuspended in a solution of 10 mM Tris-Cl pH 7.6, 10 mM EDTA (0.5 ml) and then gently mixed with SDS (0.5%) until lysis. Lysed cells were then gently mixed with NaCl (1 M, final) and incubated 12-18 hours at 0°C. Cellular debris was removed through microfugation at 4°C for 10 min at 14000 RPM, and the supernatant was phenol extracted several times. Extra-chromosomal DNA was precipitated with ethanol and recovered through centrifugation. DNA was solubilized in TE buffer (10 mM Tris-Cl pH 7.8, 1 mM EDTA) containing RNase A (100 μg/ml).

2.2.12.2 Southern Blot Analysis

Southern blot analyses for the characterization of mammalian cell lines and HSV-1 viral recombinants were carried out as described (Davis *et al*, 1986) using cellular and viral DNA preparations (section 2.2.12.1). DNA probes were generated by ^{32}P-labelling of agarose gel-purified DNA fragments by primer extension using a random hexadeoxyribonucleotide oligolabelling kit (Pharmacia Inc.), and purified using a sephadex G-50 spin column (Sambrook *et al*, 1989).

RESULTS AND DISCUSSION

3. Mammalian Systems of Nonsense Mutation Suppression in the Study of Eukaryotic Gene Expression

This work represents a preliminary undertaking towards the utilization of su^+ tRNAs in the genetic analysis of mammalian viruses through the development and use of mammalian systems of nonsense mutation suppression. Specifically, the aim of this work was to further our understanding of eukaryotic class II gene expression and regulation. The approach adopted involved further defining the coding potential of HSV-1 through the isolation and characterization of both random and defined nonsense mutants using a mammalian nonsense suppressor cell line as the permissive host in a simple host-range screening procedure. The ability of su^+ tRNAs to abrogate the effects of otherwise-lethal nonsense mutations forms the basis of a conditional gene expression system and hence, a genetic screen for the identification of essential genes. The central tenet underlying this approach is the propensity of random nonsense codons or mutations to be preferentially suppressed over natural termination sites (section 1.2).

3.1 Conditional-Lethal Mutations

The classical means by which essential genes of an organism are marked and characterized is through the study of conditional-lethal mutations. Temperature-sensitive mutations arise through the induction of missense mutations which confer a thermo-labile

phenotype upon the encoded polypeptide products. These proteins exhibit a compromised structure and/or function at a nonpermissive temperature, while maintaining normal function at a permissive temperature. Hence, the physiological effect of the mutation may be examined through growth at temperature extremes. The alternate class of conditional lethal mutations are nonsense mutations which, as described (section 1.1), cause premature translation termination and hence, the generation of truncated, often nonfunctional polypeptides. The polarity nature of nonsense codons in determining the severity of protein dysfunction can be ascribed to the position of the nonsense codon in the mRNA (Newton *et al*, 1965). That the deleterious effects of nonsense mutations may be alleviated through su^+ tRNA-mediated translational readthrough of the nonsense codon, forms the basis of a conditional system by which the physiological effects of nonsense mutations may be examined.

The application of nonsense mutations in genetic studies offers a number of potential advantages over that of *ts* mutations. In mammalian systems, the utility of *ts* mutations is limited by an inherent leakiness, a manifestation of the temperature constraints at which mammalian cells may be cultured. However nonsense mutations, in general, effect a tight, null phenotype. The altered polypeptide can often be identified as a truncated species on polyacrylamide gels, while the location of a nonsense mutation may be approximated by determining the size of the truncated species. Furthermore, polypeptides encoded by mRNAs harbouring nonsense mutations are readily amenable to structure/function analyses through the insertion of a variety of amino acids at the nonsense codon by using nonsense su^+ tRNAs which maintain different amino acid specificities (section 1.3).

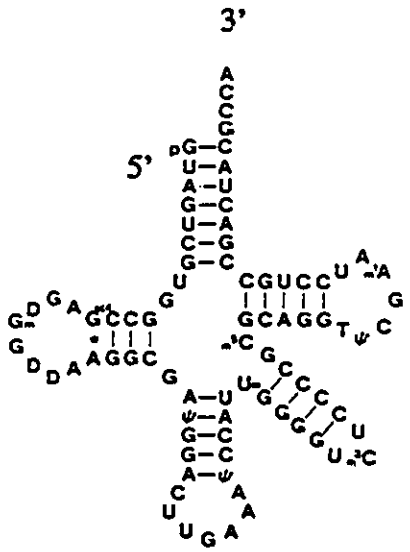
The utility of su^+ tRNAs in examining the effects of otherwise-lethal nonsense mutations is subject to a number of prerequisites: 1) suppression activity must be adequate to mediate production of a sufficient level of the suppressed polypeptide to ensure restoration of biological activity. Suppression activity itself is dependent upon the level of su^+ tRNA and the efficiency of suppression at a particular nonsense codon (section 1.2); 2) the amino acid inserted in response to a nonsense codon must be compatible with protein stability and structure/function at that site. Amino acid compatibility thus represents a direct determinant of nonsense codon suppressibility; 3) restoration of biological activity is also dependent upon the ability to maintain adequate levels of a nonsense codon-containing mRNA (section 1.1).

3.2 Mammalian Nonsense Suppressor Cell Lines

The recent availability of mammalian su^+ tRNA genes has facilitated the advent of mammalian systems of nonsense mutation suppression through the establishment of mammalian cell lines expressing defined engineered su^+ tRNA genes. Early attempts towards generating such suppressor cell lines were limited by the poor levels of nonsense suppression activity that were achieved (Hudziak *et al*, 1982; Young *et al*, 1983; Ho *et al*, 1986). It was concluded that mammalian cells are unable to tolerate high constitutive levels of su^+ tRNA gene expression. Implicit with this observation is the existence of a threshold level of suppression activity, up to which the function of natural termination sites can remain unimpaired. Hence, a system was developed in which high level suppression activity was rendered conditional. This system is based on the rapid and controlled amplification of a human su^+ $tRNA^{Ser}$ gene.

The initial step towards the development of such mammalian nonsense suppressor cell lines involved mutation of a human $tRNA^{Ser}$ gene to generate amber, ochre, and opal nonsense su^+ tRNA gene derivatives (Figure 3.1; Capone *et al*, 1985). These su^+ tRNA genes were linked to a SV40 replicon, pZipNeoSV(X), and are functionally expressed (Sedivy *et al*, 1987; J.C. unpublished). Suppression activity is easily quantitated as a function of CAT protein activity generated through su^+ tRNA-mediated translational readthrough of the corresponding pRSVCAT gene nonsense codon derivative in co-transfection assays in mammalian cells (Capone *et al*, 1986). Indeed, nonsense suppression activity derived from such pZipNeoSV(am), (oc), and (op) su^+ tRNA gene constructs, and those encoded within a pUC plasmid backbone (section 2.1.6), is both quantitative and highly selective (Figure 3.2). Suppression of the CAT gene amber mutation is dependent upon an amber su^+ tRNA (compare lanes 2 and 12 with lanes 3 and 11). Similarly, suppression of the CAT gene ochre and opal nonsense mutations is dependent upon the corresponding su^+ tRNAs (compare lanes 4 and 6 with lane 5, compare lanes 8 and 9 with lane 7, repectively).

Mammalian amber nonsense suppressor cell lines were established upon stable introduction of the SV40 replicon-linked amber su^+ tRNA gene into the BSC40 monkey kidney cell line, along with a *ts* allele of the SV40 large T antigen (Sedivy *et al*, 1987) (Figure 3.3). At 33°C, the permissive temperature for T antigen, replication of the SV40 replicon-containing su^+ tRNA gene is initiated and, upon excision from the chromosome, continues as an episomal species to generate approximately 2000 to 4000 copies of the linked su^+ tRNA gene per cell. This massive increase in su^+ tRNA gene copy number



Anticodon	
$tRNA^{Ser}$ gene	5'-TGA-3'
su^+ $tRNA^{Ser}$ gene (amber)	5'-CTA-3'
su^+ $tRNA^{Ser}$ gene (ochre)	5'-TTA-3'
su^+ $tRNA^{Ser}$ gene (opal)	5'-TCA-3'

Figure 3.1 **Sequence and structure of human nonsense suppressor $tRNAs^{Ser}$.** A human $tRNA^{Ser}$ gene was mutated to amber, ochre, and opal nonsense su^+ tRNA gene derivatives using oligonucleotide-directed site-specific mutagenesis. The anticodon of the unaltered $tRNA^{Ser}$ gene and that of each nonsense su^+ tRNA gene derivative is shown. Adapted from Capone *et al*, 1985.

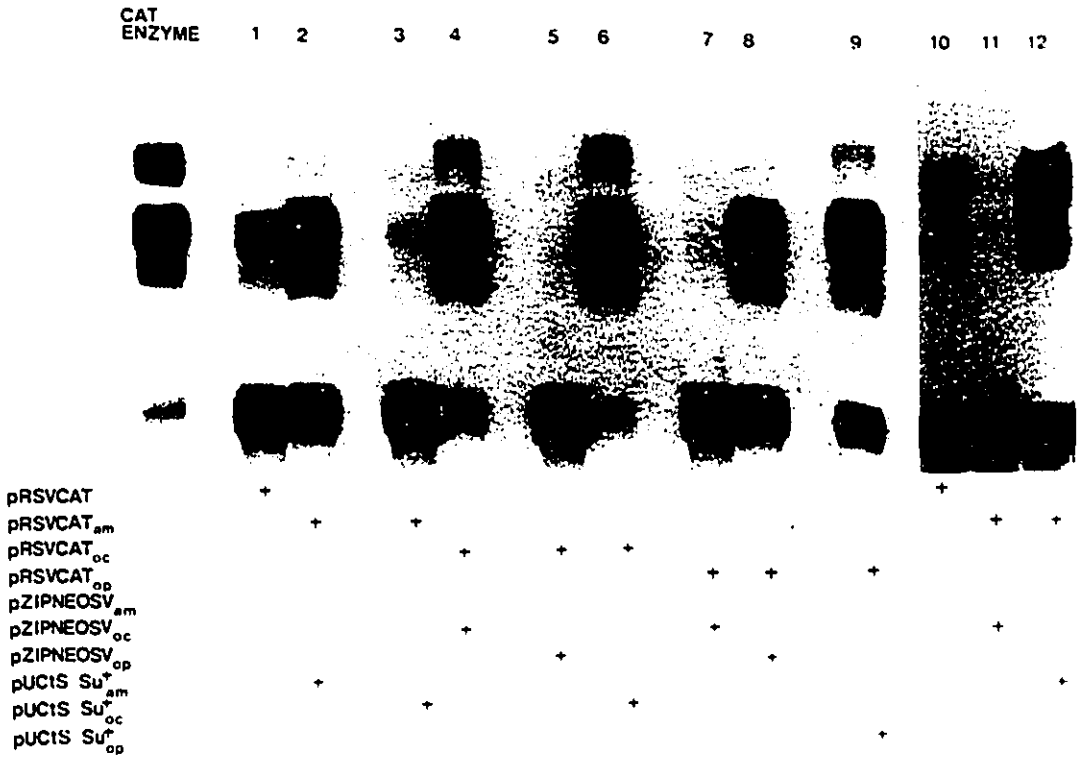


Figure 3.2 **Su$^+$ tRNA nonsense suppression activity in mammalian cells.** BSC40 cells were transfected with various combinations of CAT gene nonsense codon derivatives and nonsense su$^+$ tRNA gene constructs. Assay for CAT protein activity through separation of acetylated chloramphenicol derivatives by thin layer chromatography was as described (Capone *et al*, 1986; Capone, 1988), followed by autoradiography. This figure comprises a composite of autoradiographs derived from separate experiments (lanes 1-9, and lanes 10-12). However, note that suppression efficiencies are comparable (compare lane 1 with lane 10). The pUCtS constructs encode the nonsense suppressor derivatives of a human serine tRNA gene within a pUC plasmid backbone (Capone *et al*, 1985, 1986), while the pZIPNEOSV constructs encode the nonsense su$^+$ tRNA genes within a SV40 replicon backbone (Sedivy *et al*, 1987; J.P. Capone, unpublished). (CAT enzyme, chloramphenicol acetyltransferase (Pharmacia)).

effectively results in a very high nonsense suppression efficiency which, for one isolated cell line (AM12 cells), approaches 70% (Sedivy *et al*, 1987; Figure 3.4, compare lanes b and c). Suppression activity is virtually negligible at 39.5°C, the nonpermissive temperature for T antigen (Figure 3.4; compare lanes d and e). This system of conditional gene amplification has thus facilitated the development of a mammalian cell line which can be rapidly induced to express high levels of a human amber su^+ $tRNA^{Ser}$ gene. These cells can be maintained indefinitely under conditions of minimal suppression activity, while a simple temperature shift to 33°C serves to generate high levels of su^+ tRNA gene expression. At 33°C, growth cessation is observed however, these cells can survive for at least 2 weeks, a period sufficient to allow manipulation of most mammalian viruses. AM12 nonsense suppressor cells thus represent the permissive host in a simple host-range screening system for the isolation of mammalian virus nonsense mutants.

The utility of nonsense suppressor cell lines in genetic analyses of mammalian viruses is potentially great. Defined nonsense mutations may be introduced into the viral genome and examined within the context of the virus. Indeed, the isolation of numerous conditionally-lethal virus nonsense mutants, harbouring mutations in a variety of genes, would be dependent upon a single complementing cell line. Moreover, the generation of a battery of nonsense suppressor cell lines would facilitate structure/function analyses. Towards this end, much work has been devoted towards expanding the availability of mammalian nonsense suppressor cell lines (Appendix 7.1).

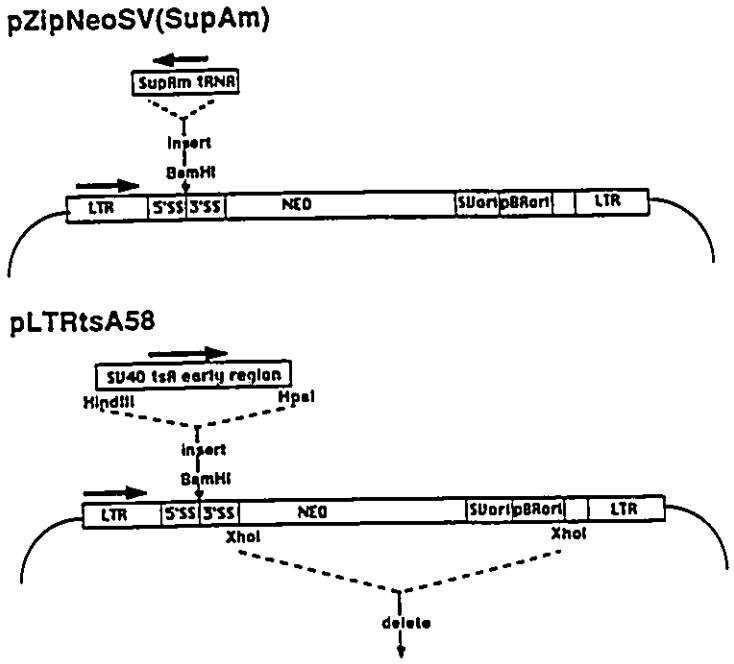


Figure 3.3 **SV40 replicon-mediated conditional amplification of amber su$^+$ tRNA^{Ser} gene copy number.** A human amber su$^+$ tRNA^{Ser} gene (Capone *et al*, 1985, 1986) was cloned into the retrovirus shuttle vector pZIPNEOSV(X) which encodes a SV40 replicon containing the bacterial neomycin phosphotransferase gene (Cepko *et al*, 1984) to generate pZIPNEOSV(am). Similarly, a *ts* allele of the SV40 large T antigen was cloned into pZIPNEOSV(X) to generate pLTRtsA58, upon removal of the neomycin phosphotransferase gene and the SV40 origin of replication. These constructs were stably introduced into monkey kidney BSC40 cells to generate AM12 nonsense suppressor cells. Adapted from Sedivy *et al*, 1987.

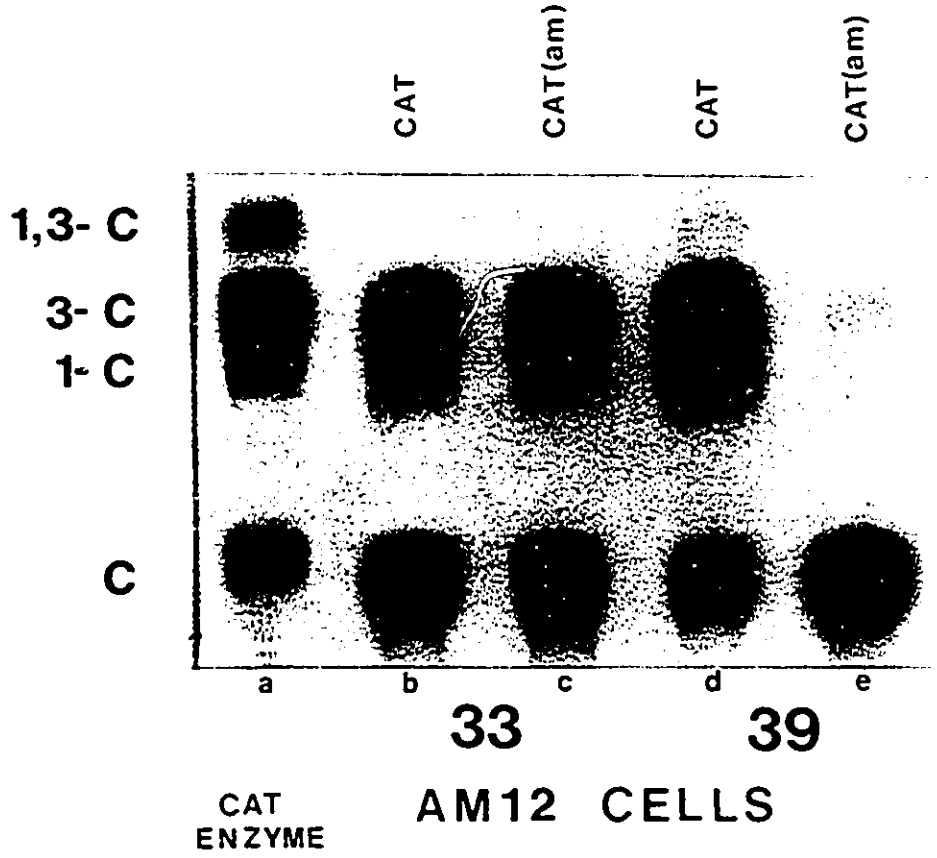


Figure 3.4 **Determination of the efficiency of suppression of AM12 nonsense suppressor cells: CAT assay.** AM12 cells were transfected with pRSVCAT and pRSVCAT(am) at 33°C and 39°C. Assay for CAT protein activity through separation of acetylated chloramphenicol derivatives by thin layer chromatography was as described (Capone *et al*, 1986; Capone, 1988), followed by autoradiography. (CAT, pRSVCAT; CAT(am) pRSVCAT(am); C, chloramphenicol; 1-C, 1-acetyl chloramphenicol; 3-C, 3-acetyl chloramphenicol; 1,3-C, 1,3-diacetyl chloramphenicol; CAT enzyme, chloramphenicol acetyltransferase (Pharmacia)).

3.3 HSV-1: The Model System

The recent establishment of mammalian nonsense suppressor cell lines has afforded the opportunity, for the first time, of isolating conditional-lethal nonsense mutants of animal viruses. Amber nonsense suppressor cell lines have facilitated the isolation and propagation of amber nonsense mutants of small, rapidly replicating viruses that maintain a genome with a limited coding capacity, such as polio virus (Sedivy *et al*, 1987), vesicular stomatitis virus (White and McGeoch, 1987), and adeno-associated virus (Chejanovsky and Carter, 1989). However, such experiments were somewhat biased since amber termination codons are not utilized by these viruses to effect cessation of polypeptide synthesis. Hence, the feasibility of adopting mammalian nonsense suppressor cell lines as a generally applicable means of procuring the genetic analysis of a variety of animal viruses has not been established. Specifically, little is known regarding the capacity of suppressor cell lines to support the growth of nonsense mutants of large animal viruses.

To address these issues, HSV-1 was chosen as a model system since HSV-1 is a large animal virus which undergoes a complex replication cycle. HSV-1 is particularly well suited to serve as the model system for such a genetic analysis since much of the vast HSV-1 genome has been unmarked by conditional-lethal mutations. Furthermore, HSV-1 employs a complex scheme of gene regulation and hence serves as a useful model system for the study of eukaryotic gene expression and regulation.

3.3.1 HSV-1 Gene Expression and Regulation

HSV-1 genes are coordinately regulated and are expressed in an ordered cascade as at least three temporally distinct classes (Figure 3.5). The first class of genes to be expressed upon virus infection are the immediate early genes, which are then followed by early gene and then late gene expression. There are five immediate early genes, four of which play a role in the activation of both early and late gene transcription. Moreover, the α4 immediate early gene product, ICP4, can auto-regulate its own expression and that of other immediate early genes, while the α27 immediate early gene product, ICP27, is involved in the negative regulation of immediate early genes and some early genes. Hence, immediate early genes are thought to effect the transition from early gene to late gene expression. Early genes encode factors required for DNA replication and nucleotide metabolism while late genes encode structural polypeptides required for virus assembly. Late gene expression is dependent upon productive DNA replication (Everett, 1987; Wagner, 1991).

3.3.2 Random Chemical Mutagenesis of HSV-1

The HSV-1 genome comprises approximately 152 kbp's, a GC content of 68%, and encodes 72 ORFs with 70 distinct polypeptides (McGeoch *et al*, 1988). However, much of the HSV-1 genome is mutationally silent since only approximately 25-30 complementation groups have been identified through induction of *ts* mutations (Schaffer *et al*, 1978; Chu *et al*, 1979). Moreover, only two of the five HSV-1 immediate early genes, α4 and α27, have been marked by *ts* mutations (Wagner, 1991). A thorough

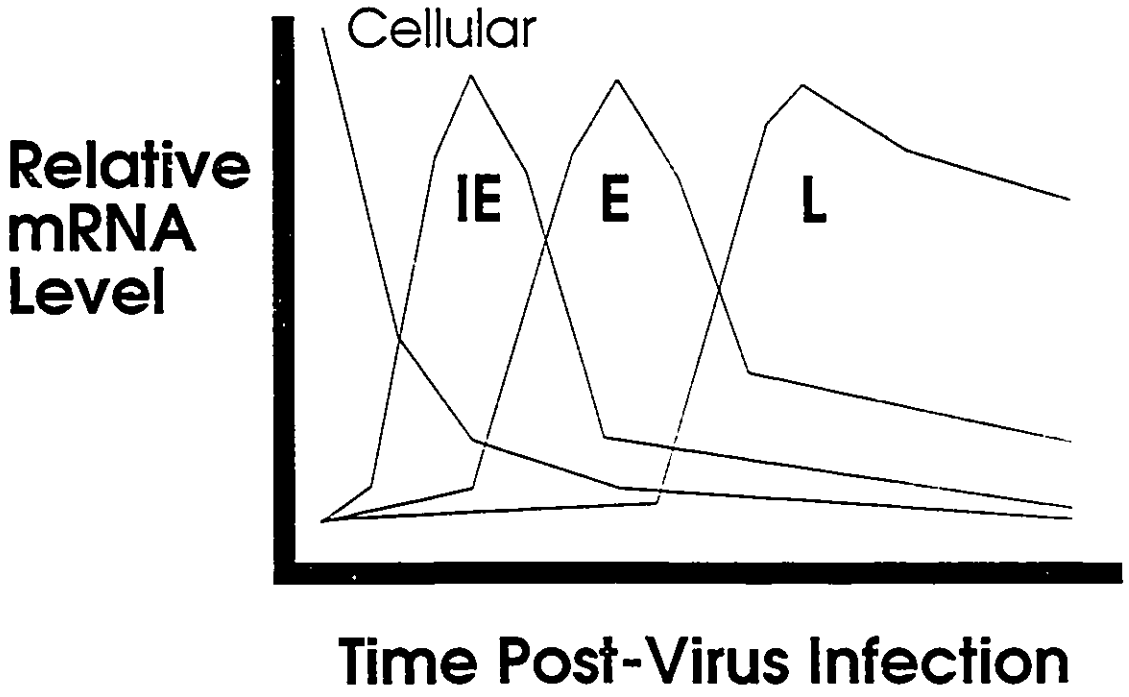


Figure 3.5 **HSV-1 gene expression is temporally and coordinately regulated.** The transition from HSV-1 IE to E to L gene expression over the course of the HSV-1 lytic cycle, with the concomitant inhibition of cellular gene expression, is described as a function of the shift in the relative level of mRNA. HSV-1 IE gene transcription peaks at approximately 3 hours post infection, while HSV-1 E and L gene transcription peaks at approximately 5-7 and 9-12 hours post infection, respectively. (IE, immediate early; E, early; L, late).

understanding of the structural and functional organization of the HSV-1 genome is dependent upon the identification of all viral genes and hence, in the absence of complementing cell lines, the ability to introduce conditional-lethal mutations into all areas of the HSV-1 genome. 80% of HSV-1 *ts* mutations have been induced through the T-specific mutagen, 5-bromodeoxyuridine (Schaffer *et al*, 1978). Since the HSV-1 genome is GC rich, C-specific mutagens may induce mutations within genes unmarked by 5-bromodeoxyuridine and hence, further the characterization of the coding potential of the HSV-1 genome. Mutagens which cause C to T transitions may facilitate the induction of nonsense mutations since nonsense codons are rich in A and T residues. An increase in the availability of conditional-lethal mutants of HSV-1, through the establishment of a set of HSV-1 nonsense mutants, will facilitate the genetic analysis of HSV-1. HSV-1 nonsense mutants defective in gene regulation are of particular interest.

3.4 Treatment of HSV-1 DNA with Nitrous Acid

Nitrous acid reacts principally with 1° amines to mediate the deamination of amino group-containing bases (A,G, and C). Hence, nitrous acid can effect bi-directional G/C to A/T base pair transitions (Zimmermann, 1977), and is particularly useful since it is highly mutagenic on double-stranded DNA in the presence of certain alcohols, phenols or 1° amines such as spermine (Thomas *et al*, 1979).

Highly purified HSV-1 viral DNA was prepared and mutagenized *in vitro* with nitrous acid essentially as described (Sandri-Goldin *et al*, 1981). Viral DNA was highly infectious since 5 μl aliquots (approximately 10 μg) consistently yielded 350-400 viral

plaques per 10 cm dish of cells in transfection assays (data not presented). 10 μl viral DNA aliqouts (approximately 20 μg) were subjected to nitrous acid exposure from 0 to 30 min. Upon inactivation of the nitrous acid, the viral DNA was transfected directly onto AM12 cells, which were preincubated at 33°C for 48 hours. This period is sufficient to allow for the accumulation of su^+ tRNA molecules. Replication on AM12 cells serves to resolve mutational heterozygotes and hence facilitates the segregation of wild type and nonsense mutant virus from the remaining pool of defective virions. After seven days, cell monolayers derived from transfected viral DNA corresponding to nitrous acid exposure time point 0 min exhibited total cpe. All plates were harvested and virus stocks were prepared and titred. Treatment of viral DNA with nitrous acid for 15 and 20 min reduced viral infectivity to approximately 1% and 0.2% survival, respectively (Figure 3.6). Under these conditions, defective virions likely arose on average through a single mutagenic hit per genome. Exposure of viral DNA to nitrous acid for extended periods thereafter did not further decrease virus yield hence, defective genomes were likely subjected to multiple mutagenic events. Viral infectivity derived from control reactions, in which viral DNA was incubated in water at room temperature or mutagenic reaction mixture lacking nitrous acid, for 30 min, was similar to that derived from mutagenic time point 0 min (data not presented). These results suggest that loss of viral infectivity was indeed due to nitrous acid-mediated mutagenic events, rather than due to viral DNA heat lability or the effects of other components of the reaction mixture, respectively.

AM12 cells preincubated at 33°C were infected with virus stocks derived from exposure of viral DNA to nitrous acid for 15 and 20 min, at a density of 50 plaques per

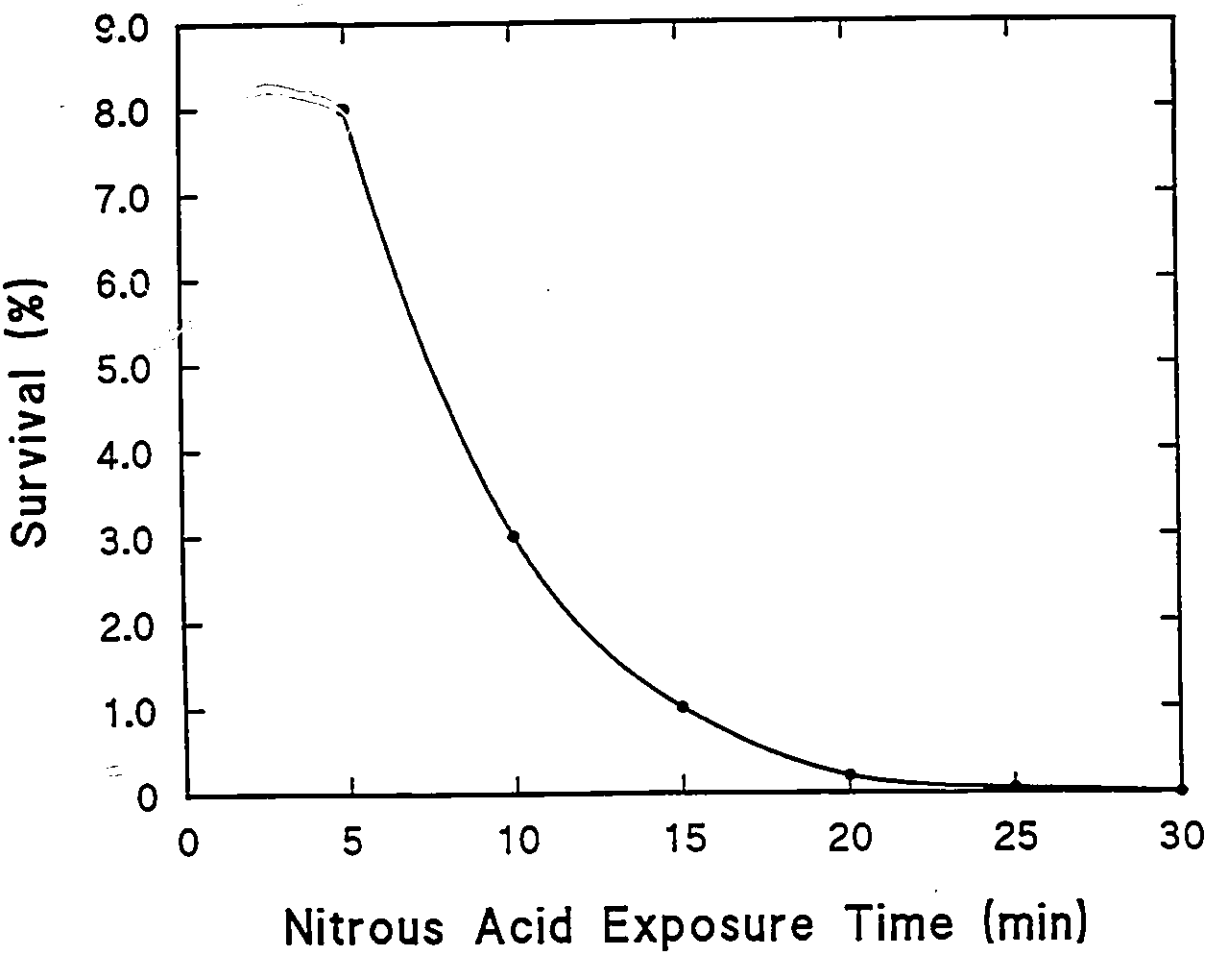


Figure 3.6 **Nitrous acid inactivation of HSV-1.** Pure HSV-1 DNA (20 μg) was incubated in nitrous acid reaction mixture (0.1M sodium acetate buffer pH 4.52, containing 0.05M $NaNO_2$ and 2 X 10^{-4}M spermine) at 37°C from 0 to 30 min. Reactions were terminated by adding ice-cold 2X HBSS pH 7.73 containing 10μg/ml salmon testes DNA. Mutagenized viral DNA was transfected into AM12 cells preincubated at 33°C. Cells were harvested when monolayers corresponding to one mutagenic time point reached total cpe. Virus yield was determined on AM12 cells at 33°C and normalized against that derived from virus stocks corresponding to mutagenic time point 0 min, which is expressed as 100% survival.

10 cm plate to facilitate the isolation of individual progeny virions. Virus plaques were picked onto AM12 cells and allowed to grow to total CPE at 33°C. An enrichment step for the isolation of potential HSV-1 nonsense mutants was carried out by picking only plaques which appeared small and turbid in nature. Poliovirus nonsense mutants grow very slowly on AM12 cells, a manifestation of the less than 100% suppression efficiency of AM12 cells and hence, virus plaques are smaller and appear much later than those of wild type poliovirus. Moreover, plaques of poliovirus nonsense mutants appear turbid or heterogeneous in nature due to the variable efficiency of suppression from cell-to-cell and since only approximately 50% of AM12 cells are induced for su^+ tRNA gene amplification (Sedivy *et al*, 1987). Indeed, some cells within a plaque are refractory to infection. Potential HSV-1 nonsense mutants were identified by replica-transferring isolates onto AM12 cells and BSC40 cells at 33°C to determine host range. Importantly, there is no apparent difference in the growth characteristics of wild type HSV-1 on AM12 cells and non-suppressor cells at 33°C or 39°C (Table 3.1; Figure 3.7). These results suggest that high levels of an amber nonsense su^+ tRNA have no deleterious affect on HSV-1 replication, at least in the short term.

3.5 Host-Range Screening for HSV-1 Nonsense Mutants

Hydroxylamine is a C-specific mutagen which causes unidirectional C to T base transitions (Freese *et al*, 1961). Initially, hydroxylamine was chosen for mutagenesis experiments since hydroxylamine is readily mutagenic on both intact HSV-1 virions and partially denatured HSV-1 viral DNA. (Chu *et al*, 1979). Greater than 800 plaques

derived from such experiments were screened for host range on AM12 cells and BSC40 cells at 33°C (data not presented). Similarly, greater than 400 plaques derived from nitrous acid mutagenesis of HSV-1 duplex DNA were isolated and screened for host-range. Several isolates from these mutagenesis strategies initially exhibited a growth restriction to AM12 cells under conditions of suppression however, upon subsequent analyses, this growth restriction was not maintained. This apparent leakiness may reflect nonsense codon reversion events, a manifestation of an inadequate level of suppression activity by the suppressor cells to complement virion growth defects; alternatively, low level contamination by wild type virus originating from initial plaque isolations may account for the observed growth on non-suppressor cells. Indeed, HSV-1 can remain tightly membrane-associated. Virus isolates were probe sonicated during all stages of plaque purification and subsequent analyses to minimize wild type virus contamination.

Mutagenesis with nitrous acid, however, facilitated the identification of a number of interesting progeny virions. Isolates A7'(A9-2) and E4(A4-1) were initially identified through a growth restriction to AM12 cells under conditions of suppression. However, upon 6 to 7 rounds of plaque purification, mutant A7'(A9-2) merely exhibits a growth enhancement of 100 fold on AM12 cells at 33°C compared to growth on BSC40 or Vero cells. Viral plaques are not observed at 39°C suggesting that this isolate carries a mutation in an essential gene and moreover, that growth of this mutant on the non-suppressor cells is not due to a reversion event. Similarly, mutant E4(A4-1) has lost any growth advantage on AM12 cells, under conditions of suppression, upon several rounds of plaque purification since this virus now grows equally well on all monkey kidney cells

Table 3.1 **Growth characteristics of HSV-1 mutants.**

	33°C			39°C
HSV-1 MUTANT	AM12 CELLS	BSC40 CELLS	VERO CELLS	MAMMALIAN CELLS[f]
A7'(A9-2)	- 100 fold growth enhancement[a,e] - heterogeneous population of plaques - large, diffuse, symmetrical - rounded cell aggregates	- small plaques, rounded cell aggregates	- small plaques, rounded cell aggregates	- no virus growth
E4(A4-1)	- 10^3-10^4 fold growth enhancement[b,e] - heterogeneous population of plaques - large, symmetrical - small, rounded cell aggregates	- 10^3-10^4 fold growth enhancement[b,e] - heterogeneous population of plaques - large, symmetrical - small, rounded cell aggregates	- 10^3-10^4 fold growth enhancement[b,e] - heterogeneous population of plaques - large, symmetrical - small, rounded cell aggregates	- irregular plaques, extensive clumps of rounded cell aggregates
E4'(E2-1)-1	- 10^3-10^4 fold growth enhancement[c,e] - *syn* plaques	- 10^3-10^4 fold growth enhancement[c,e] - *syn* plaques	- 10^3-10^4 fold growth enhancement[c,e] - *syn* plaques	- irregular plaques, extensive clumps of rounded cell aggregates
KOS	- large, diffuse, symmetrical plaques[d] - central cell clearing	- large, diffuse, symmetrical plaques[d] - central cell clearing	- large, diffuse, symmetrical plaques[d] - central cell clearing	- large, diffuse, symmetrical plaques[d] - central cell clearing

[a] Mutant virus yield 1 x 10^8 PFU/ml. Growth enhancement relative to titre on BSC40 and Vero cells at 33°C.

[b] Mutant virus yield 1.5 x 10^9 PFU/ml. Growth enhancement relative to titre on AM12, BSC40, and Vero cells at 39°C.

[c] Mutant virus yield 2 x 10^8 PFU/ml. Growth enhancement relative to titre on AM12, BSC40, and Vero cells at 39°C.

[d] HSV-1 KOS yield 1-2 x 10^9 PFU/ml at 33°C. The virus reproducibly grew to a 2-4 fold higher titre at 39°C.

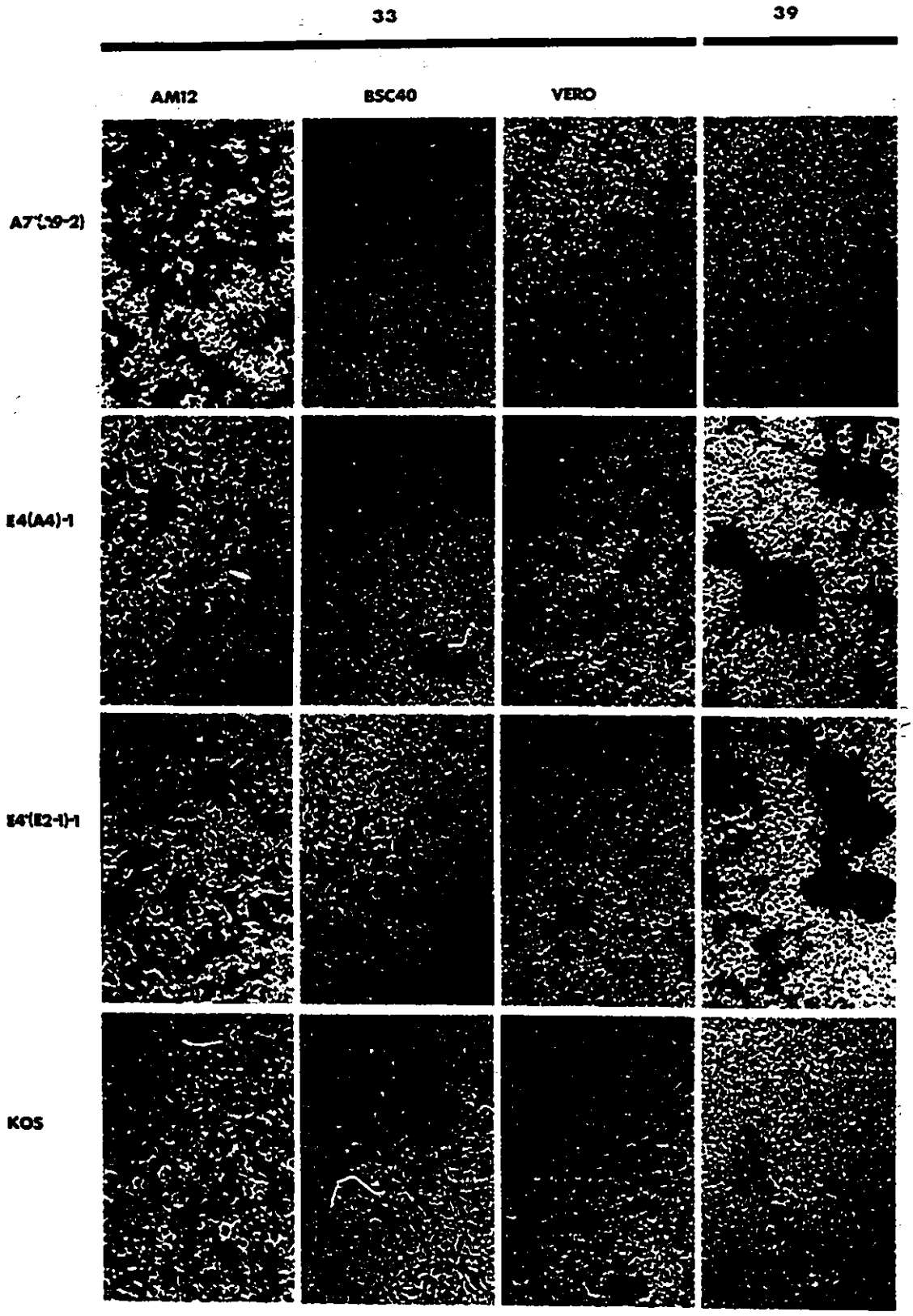
[e] Relative titres are maintained whether derived from direct plaque assay or upon quantitation of virus yields from infected cell monolayers.

[f] Virus growth characteristics were identical for AM12, BSC40, and Vero cells at 39°C.

at 33°C. However, growth of this mutant virus at 39°C is reduced by 3 to 4 orders of magnitude. Mutant E4'(E2-1)-1, isolated as a plaque purification derivative of E4(A4-1), exhibits a similar growth enhancement at 33°C. Growth properties of mutant HSV-1 isolates are summarized in Table 3.1, Figure 3.7.

Mutant virus A7'(A9-2) produces a heterogeneous population of plaques on AM12 cells at 33°C; the majority of plaques are very large and symmetrical, much like wild type virus, while a small percentage of plaques constitute tiny aggregates of rounded cells. These plaques may represent growth of the mutant virus on AM12 cells which poorly express the su^+ tRNA gene. Indeed, that an unusually long time is required for plaques to appear on AM12 cells (5 to 6 days; in contrast, wild type virus plaques can be observed after 2 to 3 days), may reflect an inadequate level of suppression activity to efficiently propagate this mutant virus. However, there is an apparent growth enhancement of this mutant under conditions of suppression since plaques on non-suppressor cells (BSC40 and Vero) require a slightly longer period to appear (6 to 7 days) and moreover, all plaques are very small and irregular in shape (much like the minority of small plaques observed on AM12 cells). These "pile-up" plaques resemble rounded cell aggregates and may indicate that the compromised infectivity of this mutant virus on non-suppressor cells may involve a defect in a function required for cell-to-cell spread.

Figure 3.7 **Plaque morphology of HSV-1 mutants.** BSC40 cells, Vero cells, and AM12 nonsense suppressor cells were infected with serial dilutions of HSV-1 strain KOS and HSV-1 mutants A7'(A9-2), E4(A4-1), and E4'(E2-1)-1 at 33°C and 39°C. Cell monolayers were stained approximately 5-7 days post virus infection, except for HSV-1 KOS infected cells (2-3 days). Viral plaque magnification was the same in all cases. At 39°C, virus plaques exhibited an identical morphology regardless of cell type. Shown at 39°C are infected cell monolayers of BSC40 cells (A7'(A9-2)), AM12 cells (E4(A4-1), E4'(E2-1)-1), and Vero cells (HSV-1 KOS).

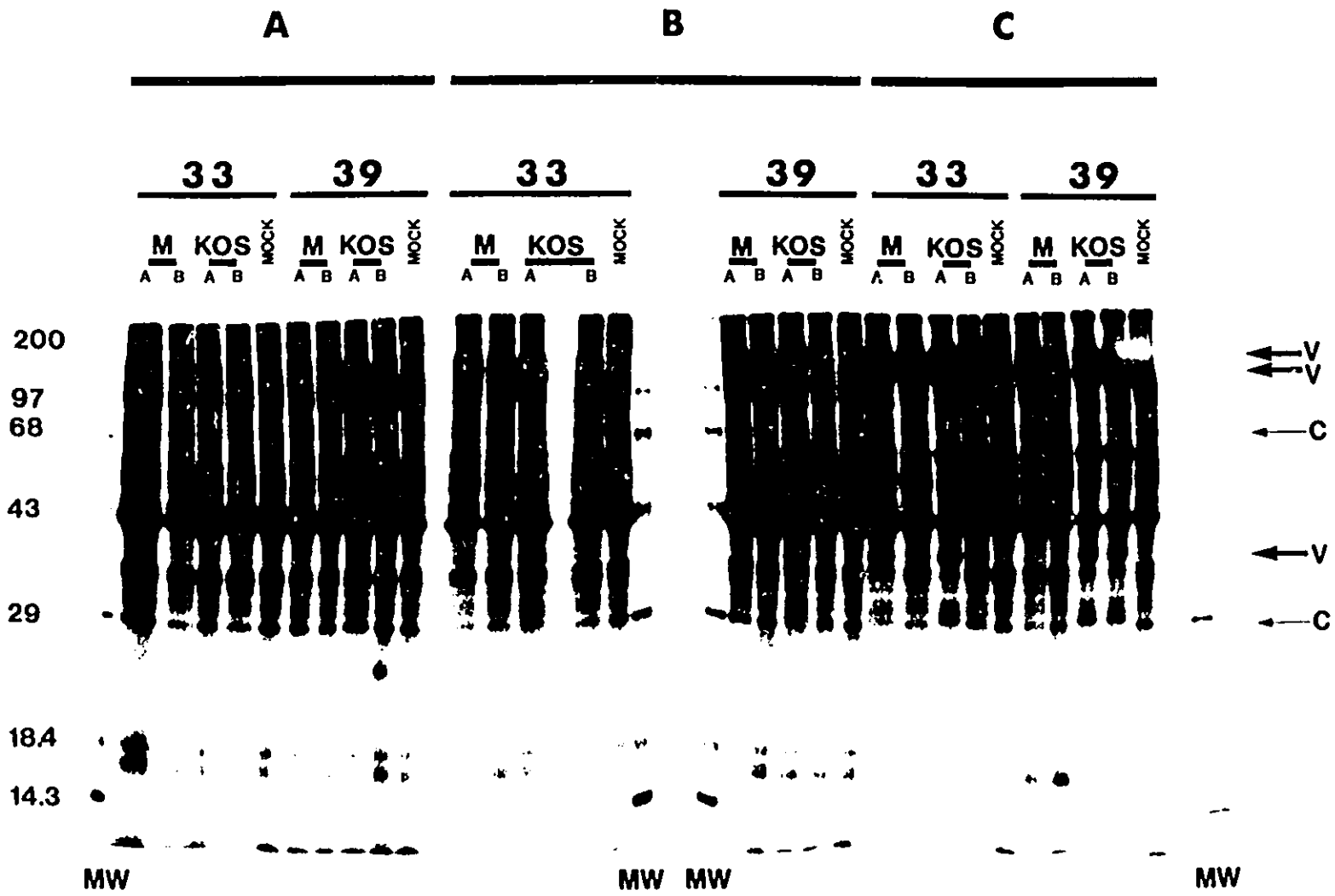

33
39
AM12
BSC40
VERO
A7(:9-2)
E4(A4)-1
E4(E2-1)-1
KOS

Mutants E4(A4-1) and E4'(E2-1)-1 exhibit no apparent difference in plaque morphology when grown under conditions of suppression or on non-suppressor cells at 33°C. Mutant E4(A4-1) plaques are heterogeneous in population; some large, symmetrical plaques are observed, in addition to smaller irregular plaques consisting of rounded cell aggregates. The ratio of these smaller plaques to the large symmetrical plaques is much higher under conditions of suppression. The nature of this disparity is not known. Mutant E4'(E2-1)-1 exhibits the syncytial (*syn*) cell fusion phenotype (Spear, 1992) when grown at 33°C. However, "plaques" from mutants E4(A4-1) and E4'(E2-1)-1 grown on AM12, BSC40, or Vero cells at 39°C resemble extensive clumps of rounded cell aggregates which then lift off the plate over time. Interestingly, the cell monolayer remains intact. Although these mutations remain undefined, these isolates may carry a *ts* mutation within a gene encoding a product involved in mediating viral cell-to-cell spread. It has recently been demonstrated that HSV-1 glycoproteins gE and gI facilitate virus cell-to-cell spread (Dingwell *et al*, 1994). Only partial complementation for this mutation may occur at 33°C, thereby accounting for the heterogeneous population of plaques observed on monolayers at this temperature. The *syn* phenotype of mutant E4'(E2-1)-1 may itself be *ts* since the *syn* phenotype is not observed at 39°C. Alternatively the *ts* mutation may be dominant over that yielding the *syn* phenotype, hence giving rise to plaques which appear as aggregates of rounded cells. The *syn* phenotype may have arisen through a second-site mutation. Indeed, several HSV-1 proteins, including the products of the UL20, UL24, UL27, and UL53 genes, can effect the *syn* phenotype (Hutchinson *et al*, 1993).

3.6 Investigation of the Expression of Mutant HSV-1 Polypeptides

To investigate the nature of the defect in the replication cycle of HSV-1 mutant A7'(A9-2), the only potential nonsense mutant isolated, the virus polypeptide profile in infected cells was examined. To determine if the expression of a specific class of genes was compromised, or if the observed phenotype was due to a defect in the expression of a single gene product, infected cells were labelled with ^{35}S-methionine for 3-6 hours, 7-10 hours, and 11-15 hours post virus infection (pvi). These labelling periods correspond to the periods of peak expression of immediate early gene products, early gene products and late gene products, respectively, and hence essentially encompass the entire virus replication cycle (Figure 3.5). For the two earliest labelling periods, there is no apparent difference between both the levels and the complement of polypeptides derived upon mutant virus A7'(A9-2) and wild type HSV-1 KOS infection of AM12 cells or BSC40 cells at 33°C or 39°C (Figure 3.8, Panels A and B). These results suggest that mutant virus immediate early and early gene expression is not compromised. The appearance of viral-specific polypeptides becomes readily distinguishable over the background of cellular polypeptides at 7-10 hours pvi. The polypeptide profile reflects the accumulation of viral gene products over the replication cycle, and the ensuing elimination of cellular polypeptides through the virus-encoded host cell shut-off function (Figure 3.8, Panel B, viral- and cellular-specific polypeptides denoted by arrows; compare to mock infection). For labelling period 11-15 hours pvi, there is no significant difference in mutant virus-derived and wild type virus-derived polypeptide profiles on AM12 or BSC40 cells at 33°C. However, at 39°C, the majority of labelled polypeptides derived from mutant virus

Figure 3.8 **HSV-1 infected cell polypeptide profile.** AM12 cells and BSC40 cells were infected (moi of 10 pfu/cell) with HSV-1 strain Kos and mutant A7'(A9-2) at 33°C and 39°C. Cells were labelled with ^{35}S-methionine (0.1μCi/μl) from 3-6 hours (Panel A), 7-10 hours (Panel B), and 11-15 hours (Panel C) pvi. Infected cell extracts were prepared at the end of each labelling period, normalized for ^{35}S-methionine incorporation, and analysed on a 10% SDS-polyacrylamide gel, followed by autoradiography. Mock infection was of AM12 cells. (M, HSV-1 mutant A7'(A9-2); A, AM12 cells; B, BSC40 cells; MW, ^{14}C-labelled protein molecular weight standards (kDa)).


A
B
C
33
39
33
39
33
39
M
KOS
MOCK
A B A B
200
97
68
43
29
18.4
14.3
V
V
C
V
C
MW
MW MW
MW

infection are cellular polypeptides, while the majority of those derived from wild type virus infection are viral-specific (Panel C; viral- and cellular-specific polypeptides denoted by arrows; compare to mock infection). Hence, expression of a number of mutant virus late gene products is absent or reduced at 39°C, an effect independent of cell type. These results suggest that mutant A7'(A9-2) is defective in late gene expression.

Immunoprecipitation experiments of markers for true early and late gene expression confirm these results. HSV-1 thymidine kinase (TK), a marker for early gene expression, was immunoprecipitated from both wild type virus and mutant A7'(A9-2) infected AM12 and BSC40 cells at 33°C and 39°C. The cells were subjected to an extended labelling period of 5-20 hours pvi. Quantitative levels of HSV-1 TK protein are clearly present in mutant and wild type virus infected cells under all conditions, except in mock infected cells (Figure 3.9, TK (M_r approximately 45 kDa)). These results demonstrate that the mutant virus can efficiently express early genes. Similarly, at 33°C, there is no significant difference in the level of glycoprotein C (gC), a marker for late gene exression, derived from AM12 cells or non-suppressor cells infected with mutant or wild type virus (Figure 3.9, gC (M_r approximately 120kDa)). The apparent enhancement in the level of gC on AM12 cells at 33°C is not reproducible and may reflect a variability in the density of AM12 and BSC40 cells at the time of virus infection and hence, an enhanced efficiency of AM12 cell infection. The results demonstrate that the mutant virus can undergo late gene expression at 33°C. In contrast, however, gC is not expressed by the mutant virus at 39°C, although quantitative levels of gC are clearly

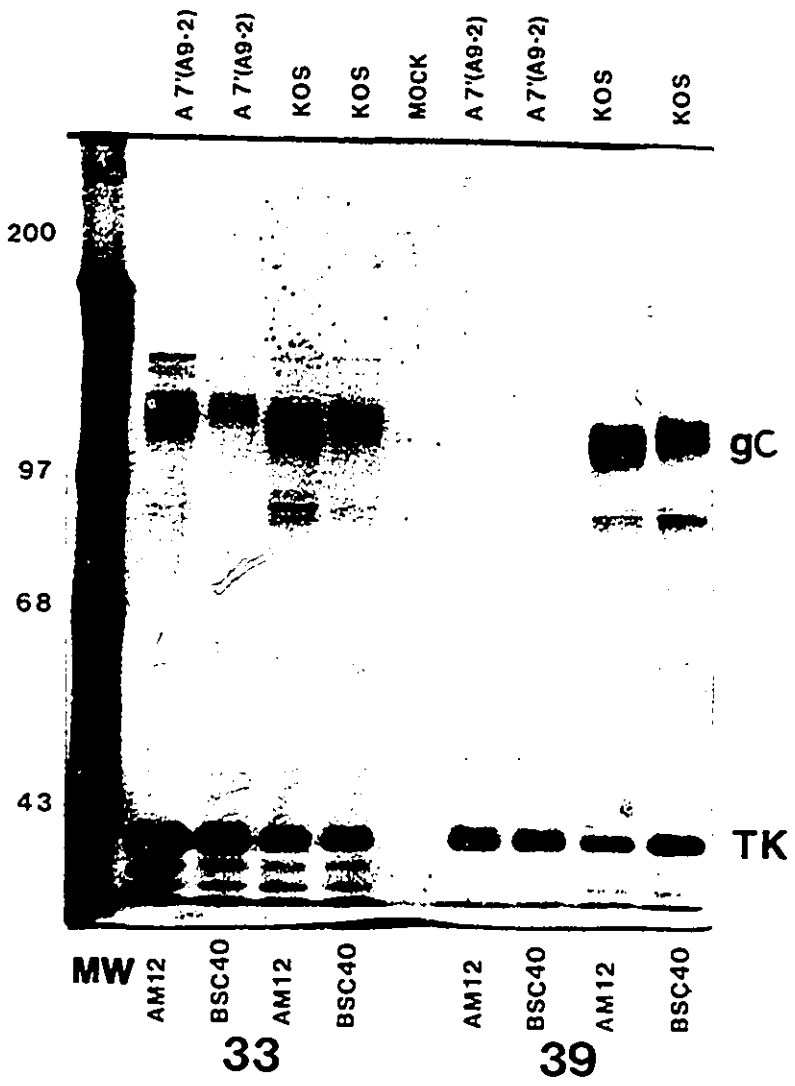


Figure 3.9 **Immunoprecipitation of HSV-1 thymidine kinase and glycoprotein C from virus-infected cells.** AM12 cells and BSC40 cells were infected (moi of 20 pfu/cell) with HSV-1 strain KOS and mutant A7'(A9-2) at 33°C and 39°C. Cells were labelled with ^{35}S-methionine (0.1μCi/μl) from 5-20 hours pvi. Infected cell extracts were prepared 20 hours pvi, normalized for ^{35}S-methionine incorporation, and subjected to immunoprecipitation reactions using rabbit anti-HSV-1 TK and anti-HSV-1 gC polyclonal antibodies. Immunoprecipitation products were resolved on a 10% SDS-polyacrylamide gel, followed by autoradiography. Mock infection was of AM12 cells. (MW, ^{14}C-labelled protein molecular weight standards (kDa)).

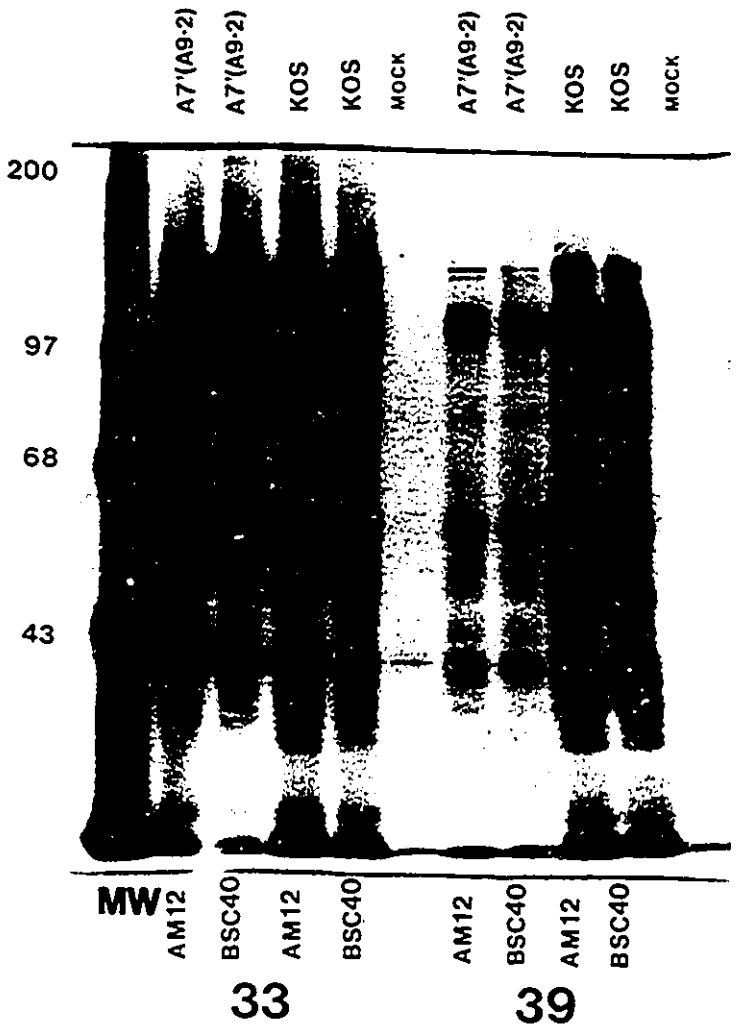


Figure 3.10 **Immunoprecipitation of HSV-1 glycoproteins from virus-infected cells.** AM12 cells and BSC40 cells were infected (moi of 20 pfu/cell) with HSV-1 strain KOS and mutant A7'(A9-2) at 33°C and 39°C. Cells were labelled with ^{35}S-methionine (0.1μCi/μl) from 5-20 hours pvi. Infected cell extracts were prepared 20 hours pvi, normalized for ^{35}S-methionine incorporation, and subjected to immunoprecipitation reactions using rabbit anti-HSV-1 antibody (Dakopatts, Denmark), a polyclonal serum to HSV-1 glycoproteins. Immunoprecipitation products were resolved on an 8% SDS-polyacrylamide gel, followed by autoradiography. Mock infection was of AM12 cells. (MW, ^{14}C-labelled protein molecular weight standards (kDa)).

present in wild type virus infected cells. The differential mobility of gC under the various conditions reflects the heterogeneity in the state of glycosylation of this protein. The results demonstrate that mutant virus A7'(A9-2) is defective in late gene expression. Indeed, immunoprecipitation experiments using a polyclonal serum against HSV-1 glycoproteins and the same infected cell extracts, demonstrate that mutant virus expression of a whole spectrum of HSV-1 glycoproteins, which are late gene products, is dramatically reduced at 39°C (Figure 3.10).

These results demonstrate that, while expression of both the immediate early and early class of HSV-1 genes is apparently normal, mutant virus A7'(A9-2) is defective in the expression of the late class of HSV-1 genes. A mutation may lie within a gene encoding a product involved in the regulation of HSV-1 late gene expression. The ICP4, ICP27, ICP0, and ICP22 immediate early gene products are involved in the activation of both early and late genes, while ICP4 and ICP27 also play a role in the negative regulation of immediate early genes and some early genes (section 3.3.1). Given that an alteration in the immediate early and early gene polypeptide profile was not detected (Figure 3.8, Panels A and B), and moreover, that the mutant virus expressed quantitative levels of the thymidine kinase early gene (Figure 3.9), hence it is unlikely that mutant virus A7'(A9-2) carries a mutation within an immediate early gene. It should be noted, however, that a rare mutation in the ICP27 gene can result in the selective inhibition of late gene expression (Everett, 1987; Wagner, 1991). The results are consistent with the compromised function of an individual early gene in mutant A7'(A9-2). Indeed, that the expression of a whole spectrum of HSV-1 glycoproteins was impaired (Figure 3.10), may

indicate a defect in viral DNA replication, a process critical to HSV-1 late gene expression. A carboxy-terminal nonsense mutation in an early gene, or a missense mutation, may account for the apparent absence of an alteration to the mutant virus early gene polypeptide profile (Figure 3.8, Panel B). HSV-1 encodes a number of polypeptides involved in DNA replication and nucleotide metabolism; a mutation within such an early gene may account for the observed phenotype.

3.7 Characterization of HSV-1 Mutant A7'(A9-2): Overview

Mutant A7'(A9-2) exhibits a growth enhancement under conditions of suppression. This mutant virus maintains a 100 fold higher titre on AM12 cells at 33°C compared to that on non-suppressor cells. The virus also maintains an enhanced infectivity; virus is able to spread from cell-to-cell more efficiently, consequently plaques are very large and symmetrical. These observations are consistent with a defect in viral DNA replication, and also late gene expression. Unlike wild type virus, however, the mutant virus requires an unusually long period to plaque, suggesting that a growth defect is not completely complemented by the nonsense suppressor cells. The mutation lies within an essential gene perhaps involved in DNA replication and is extremely stringent since the virus is unable to grow at 39°C. Hence, based solely on growth characteristics, isolate A7'(A9-2) represents a potential HSV-1 nonsense mutant. A nonsense mutation localized to the carboxy terminus of an essential gene may direct the synthesis of a truncated polypeptide which is able to maintain moderate function on non-suppressor cells at 33°C. Such a phenomenon may mediate the growth of the virus, although compromised, on non-

suppressor cells. Restoration of biological activity through the synthesis of full length polypeptide upon growth on AM12 nonsense suppressor cells would account for the observed growth enhancement. A truncated polypeptide which is thermo-labile may account for the absence of virus growth at 39°C, although no such temperature-sensitive polypeptide was detected upon examination of the mutant virus polypeptide profile (Figure 3.8). The mutation in virus isolate A7'(A9-2) remains undefined.

3.8 Defined HSV-1 Nonsense Mutants

The ability of mammalian nonsense suppressor cells to support the growth of HSV-1 nonsense mutants has not yet been established. Indeed, very few HSV-1 nonsense mutants are available, consequently little is known regarding viral growth characteristics (plaque morphology, reversion frequency) on nonsense suppressor cells. The prospect of introducing defined nonsense mutations into any ORF, and subsequently re-introducing the mutation into the viral genome by complementing ensuing viral growth defects through propagation on nonsense suppressor cell lines, represents a potentially powerful approach to defining genome structure/function. To establish the feasibility of adopting such an approach for the propagation of mammalian virus nonsense mutants, and to examine the growth characteristics of HSV-1 nonsense mutants on nonsense suppressor cell lines, a defined nonsense mutation was introduced into the essential glycoprotein D (gD) gene of HSV-1. gD maintains a major structural role in the virus and is required in stoichiometric amounts. The rationale in choosing the gD gene was the supposition that nonsense suppressor cells which are able to support the growth of a HSV-1 gD gene

nonsense mutant, should be able to propagate any nonsense mutant virus, especially those harbouring mutations in genes encoding products required in catalytic amounts. Hence, the propagation of a HSV-1 gD gene nonsense mutant represents a very stringent evaluation of the suppression efficiency of AM12 cells.

Codon 110 of the gD gene was chosen as the site for mutagenesis since: 1) codon 110 is proximal to the amino terminus, thus the truncated polypeptide is likely to be non-functional; 2) codon 110 normally encodes serine, thereby eliminating the possibility of amino acid incompatibility upon growth on AM12 cells, which express a su$^+$ tRNA^{Ser} gene; and 3) codon 110 is within a nucleotide context which may favour translational readthrough (section 1.2). An amber nonsense codon was introduced into the gD gene using cassette mutagenesis. Briefly: the *Hind*III/*Kpn*I fragment of plasmid pSS17L, a marker transfer vector containing the HSV-1 gD gene and flanking sequences (section 2.1.4), was cloned into the *Hind*III/*Kpn*I sites of plasmid pUC118 to generate pUC118gD. Plasmid pUC118gD(am) was constructed by replacing the gD gene *Sac*II/*Aat*II fragment (nt 562 to nt 578), generated through an *Aat*II/partial *Sac*II digest, with a *Sac*II/*Aat*II oligonucleotide cassette (AB355/AB356; Table 2.1) to create an in-frame amber nonsense codon at serine codon 110. The *Hind*III/*Kpn*I fragment of pUC118gD(am), containing the mutated gD(am) gene, was re-introduced into the *Hind*III/*Kpn*I sites of pSS17L to generate pSS17LgD(am). The novel amber mutation generated a diagnostic *Sty*1 restriction site, not present in pSS17L (Figure 3.11, Panel A; note the conversion of the 546 bp pSS17L *Sty*1 fragment to two smaller (304 bp and 242 bp) pSS17LgD(am) *Sty*1 fragments). Nucleotide sequence analysis established the

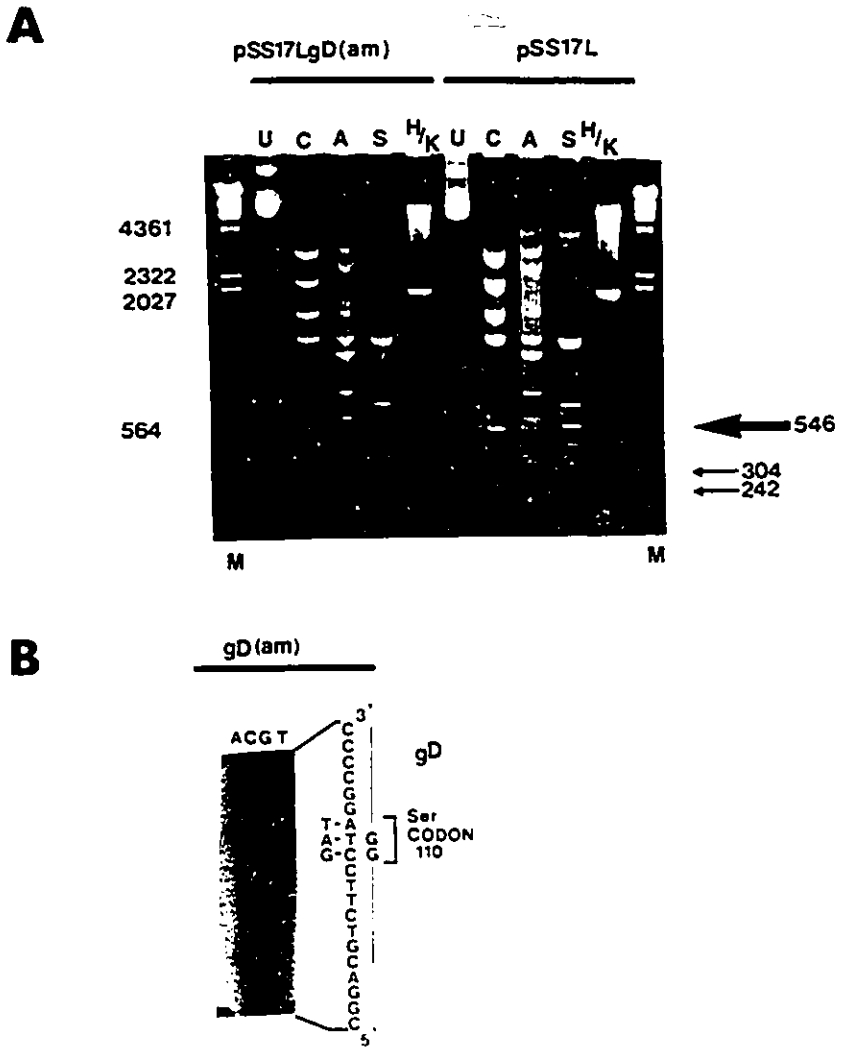


Figure 3.11 **Characterization of the amber nonsense mutation in the HSV-1 glycoprotein D gene.** (A) Restriction endonuclease analysis of pSS17LgD(am). Plasmid pSS17L, encoding an unaltered gD gene, and plasmid pSS17LgD(am), encoding the gD(am) mutation, were digested with various restriction endonucleases. Restriction fragments were resolved on a 0.8% agarose gel. (B) Autoradiograph and nucleotide sequence of the gD(am) gene template strand and the corresponding sequence of the unaltered gD gene. The amber nonsense mutation 5'-TAG-3'at codon 110 is indicated. (U, uncut; C, *Sac*II; A, *Aat*II; S, *Sty*1; H, *Hind*III; K, *Kpn*I; M, nucleic acid standards (bps)).

integrity of the two nucleotide substitutions (Figure 3.11, Panel B).

Transfer of the gD(am) gene derivative to the normal gD locus in the HSV-1 genome was attempted by co-transfecting pSS17LgD(am) with mutant virus F-gDβ DNA onto VD60 cells, which express gD upon HSV-1 infection. F-gDβ is a recombinant virus in which the gD gene has been replaced by a *lac*Z gene cassette and hence, forms blue plaques when grown on complementing VD60 cells covered with an X*gal*/agarose overlay (Ligas and Johnson, 1988). Loss of *lac*Z gene activity is scored as white plaques on X*gal*/agarose overlays; subsequent growth of such a virus isolate on wild type non-complementing cell lines would be indicative of a recombination event in which the gD gene has been targeted to its normal locus. Although transfer of the unmutated gD gene resulted in the isolation of several white wild-type progeny virions in control reactions, several attempts to transfer the gD(am) gene mutation to the HSV-1 genome were unsuccessful. A number of white plaques were isolated on VD60 cells and subsequently tested for host-range through growth on AM12 cells and non-suppressor cells at 33°C however, no progeny virions exhibited a growth restriction to AM12 cells. Southern analyses of several potential HSV-1 gD(am) gene recombinants originally scored as white plaques upon co-transfection of VD60 cells, demonstrated that these isolates were wild-type; no gD(am) gene recombinants were identified (data not presented). It is conceivable that growth of F-gDβ virus on VD60 cells resulted in a low level of wild-type virus contamination due to a recombination event with the cell-endogenous gD gene, thereby accounting for the growth of wild type progeny virions on AM12 cells. However, successful generation of a gD(am) gene recombinant cannot be excluded, and may have

been overlooked if the efficiency of nonsense suppression by AM12 cells was inadequate to complement a gD gene null growth defect. A selection may have developed for the generation of wild type virus through reversion of the gD gene amber mutation. Indeed, marker transfer of the gD(am) gene mutation to the HSV-1 gD locus through direct co-transfection of AM12 cells under conditions of suppression, was unsuccessful. Such an approach represents a more stringent strategy for transfer of the gD(am) gene mutation, since fulfilment of such a recombination event is dependent upon efficient complementation by the nonsense suppressor cells. The appearance of plaques on AM12 cells would indicate a recombination event however, no progeny virions were recovered. The results may indicate that AM12 nonsense suppressor cells are unable to support the growth of a gD gene amber nonsense mutant of HSV-1.

3.9 Mammalian Systems of Nonsense Mutation Suppression: Outlook

AM12 cells have facilitated the isolation of a number of interesting HSV-1 mutants. A7'(A9-2) is of particular interest since this isolate is apparently defective in the regulation of HSV-1 late gene expression. However, the isolation of HSV-1 nonsense mutants has been extremely labour intensive. Only one potential HSV-1 nonsense mutant has been identified upon screening greater than 1200 isolates. Indeed, the inability to isolate a gD(am) gene recombinant virus suggests that the nonsense suppression efficiency of AM12 cells may be inadequate to support the growth of HSV-1 nonsense mutants; this in itself may transcend into an unusually high reversion frequency. Hence, mammalian nonsense suppressor cell lines may be of limited utility for genetic analyses

involving large animal viruses.

In contrast, mammalian nonsense suppressor cell lines have facilitated the isolation and propagation of conditional-lethal nonsense mutants of polio virus (Sedivy *et al*, 1987), vesicular stomatitis virus (White and McGeoch, 1987), and adeno-associated virus (Chejanovsky and Carter, 1989). Hence, utilization of suppressor cells may be restricted to the genetic analysis of small DNA and RNA viruses. The nature of the apparent disparity in the capacity of mammalian suppressor cells to support the growth of viral nonsense mutants is not understood, but may reflect a less stringent requirement by small mammalian viruses for absolute fidelity in gene expression. The complex replication cycle of large animal viruses may be less tolerant of gene products with compromised activity. Regardless, the further development of mammalian systems of nonsense mutation suppression is warranted (Appendix 7.1, 7.2, 7.3).

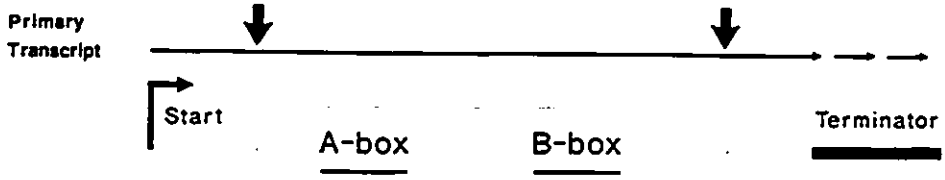
4. Conditional Expression of a Mammalian Nonsense Su^+ tRNA Gene

There are a number of disadvantages inherent to the SV40 replicon-based system adopted to confer conditional su^+ tRNA gene expression in the generation of AM12 cells (section 3.2). Induction of su^+ tRNA gene expression in these cells is based on gene amplification and hence, once the su^+ tRNA gene copy number has been amplified, expression cannot be modulated. The ability to conveniently regulate suppression activity at defined stages throughout the replication cycle of a viral nonsense mutant would be extremely valuable. Induction of high level suppression activity in AM12 cells is achieved through a temperature shift which invariably results in altered cell growth. As well, since not all AM12 cells are competent for gene amplification, cell-to-cell suppression efficiency is variable. Moreover, use of this system of conditional gene expression is limited to cells permissive for SV40 virus replication. Hence, in view of these limitations, an alternate and more generally applicable means of regulating the expression of a su^+ tRNA gene was developed.

4.1 tRNA Gene Transcription

Class III genes, such as tRNA genes, are transcribed by polIII. tRNA genes employ primarily intragenic promoter elements consisting of the highly conserved, intragenic A-block and B-block, which are sufficient for recognition and binding by transcription factor (TF) IIIC (Figure 4.1). The factor requirements and ordered assembly of functional transcription complexes, competent to support productive initiation by polIII, are well defined in yeast (Geiduschek and Kassavetis, 1992). Yeast TFIIIC is

A



tRNA Gene

B

```
                                                    ***
5' - CATGGGAATA TATCCAGGTT GTTGAAGGAG
     GTACCCTTAT ATAGGTCCAA CAACTTCCTC

                        A-Box
     GTACGTAGTC GTGGCCGAGT GGTTAAGGCG
     CATGCATCAG CACCGGCTCA CCAATTCCGC

          Anticodon
     ATGGACTCTA AATCCATTGG GGTCTCCCCG
     TACCTGAGAT TTAGGTAACC CCAGAGGGGC

               B-Box
     CGCAGGTTCG AATCCTGCCG ACTACGGCGT
     GCGTCCAAGC TTAGGACGGC TGATGCCGCA

     Terminator
     GCTTTTTTTA CTCTCGGGTA GAGGAAATCC
     CGAAAAAAAT GAGAGCCCAT CTCCTTTAGG
```

Figure 4.1 **Eukaryotic tRNA gene transcription unit.** (A) Transcription of eukaryotic tRNA genes is directed by the intragenic A-box and B-box promoter elements. Transcription initiates upstream of the tRNA gene and terminates a variable distance downstream to generate a primary tRNA transcipt. Sequential 5' and 3' processing events (denoted by the upside-down arrows) liberate the mature-size tRNA gene transcript. The tRNA gene coding region is denoted by the open box. The heterogeneous nature of transcription termination at the terminator site is denoted by the broken arrows. (B) Nucleotide sequence of the human amber su^+ tRNA gene showing the concensus A-box and B-box sequence, the anticodon, and the transcription termination signal. The major sites of initiation of transcription are denoted by asterisks (Tapping *et al*, 1994). The tRNA gene coding region is highlighted.

multi-component, consisting of at least 5 to 6 polypeptides (Gabrielsen *et al*, 1989; Bartholomew *et al*, 1990; Parsons and Weil, 1990) with an estimated total mass of 510 to 670 kDa (Schultz *et al*, 1989). Unlike yeast TFIIIC, human TFIIIC can be resolved into two functional components, TFIIIC1 and TFIIIC2 (Yoshinaga *et al*, 1987), however the exact polypeptide nature of human TFIIIC remains controversial. TFIIIC serves as the assembly factor for TFIIIB by directing its association with class III gene promoters through protein-protein interactions. Yeast TFIIIB consists of two distinct 70 kDa and 90 kDa polypeptides (Kassavetis *et al*, 1991; Bartholomew *et al*, 1991) and associates 35 to 40 nts upstream of the transcription start site of a yeast tRNA gene, in a sequence non-specific manner (Kassavetis *et al*, 1989). However, interaction with promoter-bound TFIIIC is thought to effect a structural transition within TFIIIB, unmasking a high affinity DNA binding site, resulting in the formation of a very stable complex which remains competent to support transcription by polIII through multiple rounds. The inherent stability of polIII transcription complexes is reflected in their ability to remain refractory to challenge by secondary templates, electrolytes and polyanions, and template dilution (Wolffe, 1991). In yeast, TFIIIB is the initiation factor for class III genes, sufficient for recruitment and positioning of polIII over the transcription start site, thereby mediating accurate initiation (Kassavetis *et al*, 1990). Transcription initiates at a purine residue 4 to 12 nts upstream of the tRNA gene coding region and terminates at a stretch of 4 or greater T nts located a variable distance downstream, to generate a primary transcript (Figure 4.1). Sequential processing events, which include trimming of the 5' leader and 3' extension sequences, then liberate the mature tRNA transcript

(Geiduschek and Tocchini-Valentini, 1988; Gabrielsen and Sentenac, 1991; Geiduschek and Kassavetis, 1992).

tRNA genes in eukaryotes are represented as multigene families. While the basis for this redundancy in genomic information is not understood, it is evident that individual members of tRNA gene families can be differentially expressed and subject to developmental regulation (Sprague *et al*, 1987; Dingermann *et al*, 1988; Kondo *et al*, 1988, 1990). Thus, regulation of tRNA abundance, (in conjunction with a defined mRNA codon usage), can function to impart translational control over gene expression (Ikemura, 1981; Bennetzen and Hall, 1982). The mechanisms which underly tRNA gene regulation cannot be ascribed directly to the highly conserved intragenic promoter elements; rather, differential tRNA gene expression observed within a particular tRNA isoacceptor gene family may be directed by extragenic flanking sequences, which lack significant sequence conservation. Indeed, upstream and downstream extragenic sequences can impart a modulatory effect on tRNA gene transcription in yeast and higher eukaryotes. DNA conformation and/or gene- or species-specific DNA-binding factors may mediate these effects (Sharp *et al*, 1985; Gabrielsen and Sentenac, 1991; Kunkel, 1991; Sprague, 1992).

4.2 Transcriptional Regulation of Class III Genes

Regulation of class III gene expression at the level of transcription has been demonstrated only in higher eukaryotes. Such instances include the activation (Gaynor *et al*, 1985; Aufiero and Schneider, 1990) and repression (Clark *et al*, 1991; Sollerbrant

et al, 1993) of class III gene transcription by viral-encoded factors, the cell cycle-dependent repression of transcription by polIII (Gottesfeld *et al*, 1994), the down-regulated transcription of rodent-specific B2 genes during F9 embryonal carcinoma cell differentiation (White *et al*, 1989), the inactivation of class III genes in *Xenopus* embryos during the gastrula-neurula transition (Andrews *et al*, 1991), and the developmentally regulated transcription of *Xenopus* oocyte-type and somatic-type 5S ribosomal RNA (rRNA) (Wolffe and Brown, 1988) and $tRNA^{Tyr}$ (Stutz *et al*, 1989) genes. Regulated tRNA gene transcription has also been demonstrated in the cellular slime mold (Dingermann *et al*, 1988) and underlies the tissue-specific expression of $tRNA^{Ala}$ genes in the silk-gland of *Bombyx mori* (Sprague *et al*, 1977; Young *et al*, 1986). Moreover, multiple copies of a $tRNA^{Gly}$ gene in *Bombyx mori* are subject to differential transcription (Fournier *et al*, 1993).

The molecular mechanisms which mediate transcriptional regulation of such tRNA genes remain poorly defined and hence, are not easily adopted as a facile means of manipulating the expression of a mammalian su^+ tRNA gene. However, well characterized and proven strategies of conferring conditional gene expression lie in heterologous systems and may afford similar utility in the regulation of mammalian class III genes.

4.3 Prospects/Potential Strategies for the Regulation of a Mammalian Su^+ tRNA Gene

4.3.1 Inducible Hybrid Class II/Class III Gene Promoters

Several well characterized inducible polII promoter elements are available and which have successfully imparted conditional class II gene expression in heterologous systems. Promoter activity can be modulated in response to a number of external stimuli including heat shock (Topol *et al*, 1985), hormones (Hynes *et al*, 1981; Karin *et al*, 1984a), and heavy metals (Brinster *et al*, 1982; Karin *et al*, 1984b, 1987). Moreover, stringent control of gene expression has been afforded through combination of viral, cellular, and bacterial regulatory signals (Hu and Davidson, 1990; Labow *et al*, 1990).

A potentially effective strategy towards the generation of an inducible tRNA gene would thus incorporate an inducible class II gene promoter element as a means of conferring regulation upon transcription by RNA polIII. The underlying assumption in the construction of such composite promoters is the maintenance of polII promoter element functional integrity when placed within a class III gene promoter context. Indeed, there is now precedent for functional conservation among the transcriptional apparatus of all eukaryotic RNA polymerases (Sollner-Webb, 1988; Murphy *et al*, 1989; Kunkel, 1991).

Transcription of several class III genes, including some tRNA (Larson *et al*, 1983; Carbon and Krol, 1991), 5S rRNA (Tyler, 1987), and 7SL RNA (Ullu and Weiner, 1985) genes, and the Epstein-Barr virus EBER genes (Howe and Shu, 1989) is dependent upon upstream sequence elements in addition to classical intragenic class III gene promoter elements. Furthermore, intragenic sequences are entirely dispensable for

transcription by polIII of vertebrate U6 small nuclear RNA (snRNA) and human 7SK RNA genes; rather, transcription is directed solely through extragenic upstream elements (Murphy *et al*, 1987; Das *et al*, 1988; Kleinert and Benecke, 1988) which are analogous to those which constitute the promoters of certain class II genes. Basal level transcription of the U6 snRNA and 7SK RNA genes is directed through two essential promoter elements, a TATA-like element (Murphy *et al*, 1987; Kunkel and Pederson, 1988) predominantly found upstream of many class II genes, and a proximal sequence element, whereas transcriptional activation is mediated through a distal sequence element. Interestingly, the proximal and distal sequence elements are found upstream and direct transcription of the polII-transcribed snRNA genes (Bark *et al*, 1987; Carbon *et al*, 1987; Kunkel and Pederson, 1988; Murphy *et al*, 1989) and other class II genes (Kleinert *et al*, 1990). Moreover, activation of a $tRNA^{(Ser)Sec}$ gene is dependent upon an SPH motif (Myslinski *et al*, 1992, 1993), a stimulatory element common to several class II genes.

Such class II and class III genes exhibit not only extensive conservation of gene structure, but also an overlapping requirement for common regulatory factors such as the octamer binding factor (Carbon *et al*, 1987; Murphy *et al*, 1989), the proximal sequence element binding protein (Bernues *et al*, 1993), activating transcription factor (Howe and Shu, 1989; Bredow *et al*, 1990) and transciption factor Sp1 (Howe and Shu, 1989; Lescure *et al*, 1992). Basal polII transcription factor TFIIA (Waldschmidt and Seifart, 1992; MeiBner *et al*, 1993) and a TFIIB-related factor (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-De-Leon *et al*, 1992) have been implicated in mediating transcription by polIII. The TATA element binding protein (TBP), which is a subunit of

TFIID, the integral preinitiation complex assembly factor for transcription by polII, is required for transcription of both TATA element-containing U6 genes (Lobo *et al*, 1991; Margottin *et al*, 1991; Simmen *et al*, 1991; Waldschmidt *et al*, 1991) and conventional class III genes which lack polII promoter elements (White *et al*, 1992). Furthermore, *in vivo* analyses in yeast have demonstrated that TBP is a central component of the transcriptional machinery of all eukaryotic RNA polymerases (Cormack and Struhl, 1992; Schultz *et al*, 1992). Indeed, TBP is actually an integral component of the polI- and polIII-specific transcription initiation factors SL1 (Comai *et al*, 1992) and TFIIIB (Lobo *et al*, 1992; White and Jackson, 1992a), respectively. TBP and the TFIIB-related factor constitute the 70 kDa component of yeast TFIIIB (Kassavetis *et al*, 1992b), while human TFIIIB comprises TBP and at least two TBP-associated factors (TAFs), TAF-172 and TAF-L (Taggart *et al*, 1992).

Although intragenic promoter elements are dispensable for transcription of the vertebrate U6 snRNA and human 7SK RNA genes, a requirement is maintained for TFIIIB (Moenne *et al*, 1990; Waldschmidt *et al*, 1991). Hence, for these class III genes, protein-protein interactions are fundamental in establishing the polIII transcriptional machinery, and are apparently mediated entirely through class II gene promoter elements and their cognate regulatory factors. RNA polymerase specificity is directed primarily by the TATA element and promoter element spacing (Mattaj *et al*, 1988; Lobo and Hernandez, 1989; Waibel and Filipowicz, 1990; Lescure *et al*, 1991). Indeed, promoter structure serves to define the complement of TAFs, including TFIIB (-like factor), and therefore the nature of the transcription complex which ultimately confers RNA

polymerase selection (Pugh and Tjian, 1992; Sharp, 1992; White and Jackson, 1992b; Rigby, 1993; Willis, 1993). Conversely, subunits unique to each eukaryotic RNA polymerase may also direct promoter selection (Palmer and Folk, 1990). Interestingly, some genes are transcribed by both polII and polIII however, the nature of the underlying transcription complexes remain undefined (Chung *et al*, 1987; Mattaj *et al*, 1988; Huang *et al*, 1994).

Hence, transcriptional regulation in higher eukaryotes can be ascribed, in part, to a vast interplay of molecular mechanisms common to each RNA polymerase. This is perhaps not surprising in view of the structural conservation among eukaryotic RNA polymerases (Allison *et al*, 1985; Sentenac, 1985; Memet *et al*, 1988). It is thus conceivable that artificial hybrid class II/class III gene promoters, which incorporate an inducible class II gene promoter element and a basal polIII promoter (or the equivalent which imparts polIII specificity), may confer transcriptional control upon a class III gene and hence, may afford a novel strategy for the regulation of mammalian su^+ tRNA genes. Indeed, there is precedent for such an approach since upstream elements can confer regulation upon eukaryotic tRNA genes (Larson *et al*, 1983; Myslinski *et al*, 1992, 1993). Moreover, heat shock-induced polIII transcriptional activation of the G8 cytoplasmic RNA gene from *Tetrahymena thermophila* may arise through upstream heat shock response motifs analogous to those found in class II genes (Hallberg *et al*, 1992). However, the utility of hybrid promoter systems in manipulating su^+ tRNA gene expression for use in mammalian cells (section 3.2) may be limited by the inherent leakiness of inducible class II gene promoters, and further compromised by modest levels

of induction and inducing agent-associated pleiotropic effects. Regardless, hybrid promoters represent a potentially useful strategy for the regulation of class III genes, which otherwise remain difficult to control.

4.3.2 Thermal Regulation of Su^+ tRNA Gene Expression

Thermal regulation of su^+ tRNA gene activity has been mediated through both tRNA transcription unit-internal extragenic mutations, which essentially render production of functional su^+ tRNA *ts* for processing (Marschalek *et al*, 1990), and intragenic mutations, which apparently compromise tRNA stability and hence abolish functional suppression at elevated temperatures (Eggertsson and Soll, 1988). While such approaches have mediated stringent su^+ tRNA gene regulation in yeast and *E. coli*, the *ts* phenotype is undesirable for application in mammalian cells (section 4). However, thermal regulation of su^+ tRNA gene expression conveyed by such tRNA structural characteristics may be of greater general applicability than of the SV40 replicon-based system adopted in the construction of AM12 cells, since control of tRNA synthesis is not restricted by cell type (section 3.2).

4.3.3 Transcriptional Regulation of a Su^+ tRNA Gene by RNA PolII

The availability in *E. coli* of well characterized, tightly regulated promoters has permitted the conditional expression of a su^+ tRNA gene as an extended transcript (Steege and Horabin, 1983). The corollary strategy in mammalian cells, transcription by polII of a su^+ tRNA gene which has been linked to a heterologous inducible class II gene

promoter, necessitates abrogating tRNA gene recognition by polIII, a formidable task in view of the intragenic nature of tRNA gene promoters and the strict sequence requirements in maintaining tRNA structural and functional integrity. The utility of such an approach is dependent upon faithful transcription termination and processing, and may be subject to previously discussed limitations associated with inducible polII promoter systems (section 4.3.1).

4.3.4 *E. coli Lac* Operator/Repressor System

One such highly characterized promoter system in *E. coli*, that of the *lac* operon, adopts a negative control mechanism to effect stringent regulation of genes responsible for lactose metabolism. The *lac* repressor protein binds with great specificity and affinity to its cognate *lac* operator sequence (*lac*O) which overlaps the *lac* promoter site, and in so doing, prevents polymerase elongation, effectively inhibiting *lac* gene transcription (Lee and Goldfarb, 1991). Repressor binding can be allosterically regulated with the *lac* inducer IPTG, which decreases the affinity of *lac* repressor for operator DNA, mediating repressor displacement and renewed transcription. Hence, the lactose operon is subject to both stringent repression and high inducibility (Barkley and Bourgeois, 1980).

The stringency in regulation of the *E. coli lac* operon is unrivalled by the available eukaryotic systems of conditional gene expression hence, the *lac* operator/repressor system represents a potentially useful strategy for the regulation of gene expression in mammalian cells. Indeed, the *lac* operator/repressor system has conferred inducibility upon a number of class II genes in transient transfection assays

(Brown *et al*, 1987; Hu and Davidson, 1987), and within the context of mammalian chromatin (Figge *et al*, 1988) and the viral genome (Fuerst *et al*, 1989; Rodriguez and Smith, 1990). Transcription of class II genes by polII is directed essentially by extragenic promoter elements. Positioning of repressor protein proximal to such promoter elements is thought to interfere with the assembly or stability of the polII transcription complex through promoter occlusion or steric hindrance and hence, inhibit transcription initiation; introduction of IPTG results in the restoration of gene expression. Similar strategies in a variety of systems have afforded a means of conditional gene expression (Smith *et al*, 1988; Deuschle *et al*, 1989; Wilde *et al*, 1992).

However, unlike class II genes, class III genes such as tRNA genes maintain intragenic promoter elements, the manipulation of which invariably results in the alteration of the tRNA gene product. Concurrent to our attempts towards the development of an alternate system of regulating the expression of a su^+ tRNA gene came the demonstration that a component of the yeast polIII transcriptional machinery assembled on a tRNA gene, namely TFIIIB, functionally interacts with flanking sequences upstream of the transcription start site (Kassavetis *et al*, 1989; Huibregtse and Engelke, 1989). Implicitly, it was reasoned that positioning of a *lac*O sequence directly upstream of a mammalian tRNA gene, and hence overlapping the initiation site, may interfere with the formation of an active polIII transcription complex upon repressor binding, or with initiation itself, and thereby inhibit tRNA gene transcription. Since *lac* repressor has been adapted for expression in a variety of mammalian cells and furthermore, since mammalian cells are refractory to repressor-associated pleiotropic effects, it was reasoned

that the *lac* operator/repressor system may serve as a novel and particularly useful means of regulating the expression of a mammalian su$^+$ tRNA gene (Syroid *et al*, 1992).

4.4 Regulated Expression of a Mammalian Nonsense Suppressor tRNA Gene Using the *E. coli Lac* Operator/Repressor System

To determine whether the *lac* operator/repressor system could be adopted to render inducibility upon a mammalian su$^+$ tRNA gene, the *lac*O sequence was introduced at various positions upstream of a human amber su$^+$ tRNA^{Ser} gene (Figure 4.2). Indeed, flanking sequences upstream of this su$^+$ tRNA gene can be altered without adversely affecting tRNA gene expression *in vivo* and *in vitro* (Tapping *et al*, 1993). Plasmid p*Lac*OtS(am1) was constructed from pUCtS Su$^+$(am) by inserting, through blunt-end ligation, the symmetrical 18-residue-long *lac*O oligonucleotide AB882 (Table 2.1) into the unique *Sna*BI site at position nt -1 directly upstream of the su$^+$ tRNA gene coding region, thereby placing the centre of the *lac*O site at position nt -9 relative to the tRNA gene coding sequence. Plasmid p*Lac*OtS(am2) was prepared from p*Lac*OtS(am1) by inserting, through blunt-end ligation, the same *lac*O oligonucleotide into the unique *Sca*I site at position nt 154 downstream of the tRNA gene. Plasmid p*Lac*OtS(am3) contains the *lac*O oligonucleotide AB883 (Table 2.1) inserted within the unique *Xba*I site at position nt -32 upstream of the su$^+$ tRNA gene coding region, thereby placing the relative centre of the *lac*O site at position nt -46. p*Lac*OtS(am3) was constructed from a derivative of pUCtS Su$^+$(am) in which sequences upstream from position nt -32 were deleted and replaced with an *Xba*I linker (ptS-32X; Tapping *et al*, 1993). Plasmid p*Lac*OtS(am4) is identical to p*Lac*OtS(am1) except that it contains the 30-residue-long

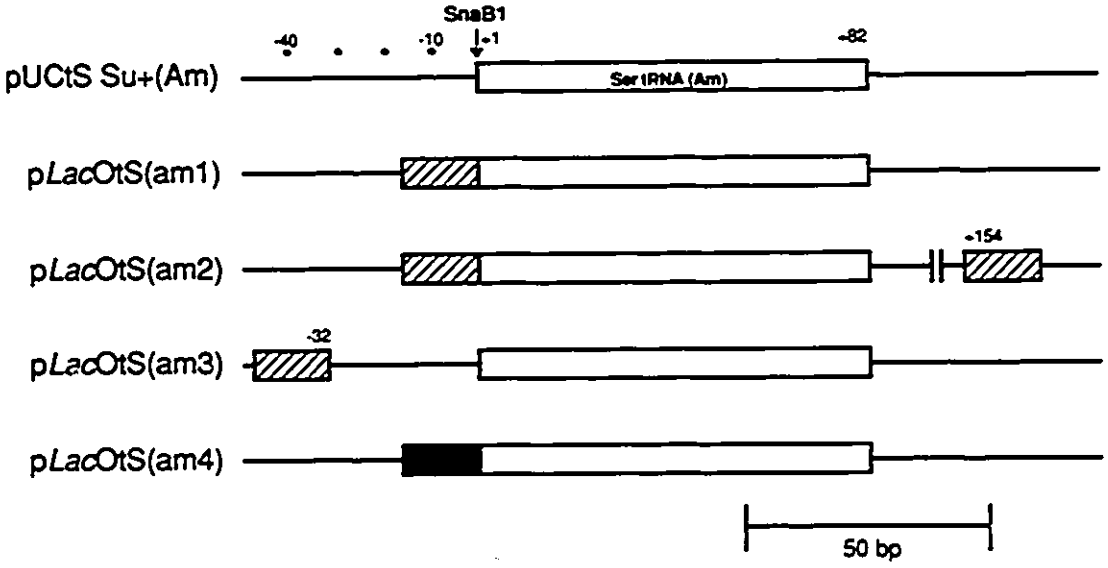


Figure 4.2 **Structure of *lac* operator-containing su$^+$ tRNA^{Ser} genes.** The structure of the wild-type amber su$^+$ tRNA gene is shown at the top. p*Lac*OtS(am1) contains a single operator, 5'-ATTGTGAGCGCTCACAAT, blunt-end ligated into the unique *Sna*B1 site at position nt -1. p*Lac*OtS(am2) contains an additional operator site inserted into the unique *Sca*I site at position nt 154. p*Lac*OtS(am3) contains the operator cloned into a unique *Xba*1 (GTCTAGAC) linker inserted at position nt -32. The oligonucleotide was identical to that described above except that it was designed to contain *Xba*I-compatible ends. Sequences upstream from the *Xba*I site are vector derived. p*Lac*OtS(am4) contains an extended operator sequence, 5'-TGTGGAATTGTGAGCGCTCACAATTCCACA, cloned into the *Sna*B1 site.

symmetrical *lac*O oligonucleotide AB1465 (Table 2.1) at position nt -1, thereby placing the centre of the *lac*O site at position nt -15 relative to the coding sequence. This *lac*O sequence has a higher affinity for *lac* repressor than does the wild type *lac*O sequence (Sadler *et al*, 1983). Restriction endonuclease analyses established the presence of a novel *lac*O sequence-derived *Hae*II site(s) (Figure 4.3, compare novel restriction fragments in lane H for constructs p*Lac*OtS(am1), p*Lac*OtS(am2), and p*Lac*OtS(am4) with those found in lane H for construct pUCtS Su^+(am). Similarly, compare novel *Hae*II-derived fragment in lane H for construct p*Lac*OtS(am3) with that found in lane H for construct ptS-32X). *Lac*O sequence insertions at *Sna*BI and *Sca*I restriction sites abolished recognition by the corresponding endonucleases (Figure 4.3, lanes S and C, respectively). Nucleotide sequence analyses confirmed the integrity of *lac*O constructs (data not presented).

4.4.1 Regulated Expression of a Mammalian Su^+ tRNA Gene *In Vivo*

4.4.1.1 *Lac* repressor can inhibit su^+ tRNA gene expression

The effect on su^+ tRNA gene expression of *lac* repressor bound to its cognate *lac*O site positioned immediately upstream and proximal to the tRNA gene coding region in p*Lac*OtS(am1) was examined *in vivo* through co-transfection of BSC40 cells with pRSVIns, a plasmid that expresses the *lac* repressor, modified at the carboxyl terminus through addition of the SV40 large T antigen nuclear localization signal (Hu and Davidson, 1991), and pRSVCAT(am). Determination of suppression efficiency through quantitation of su^+ tRNA-mediated CAT protein activity is a direct measure of functional

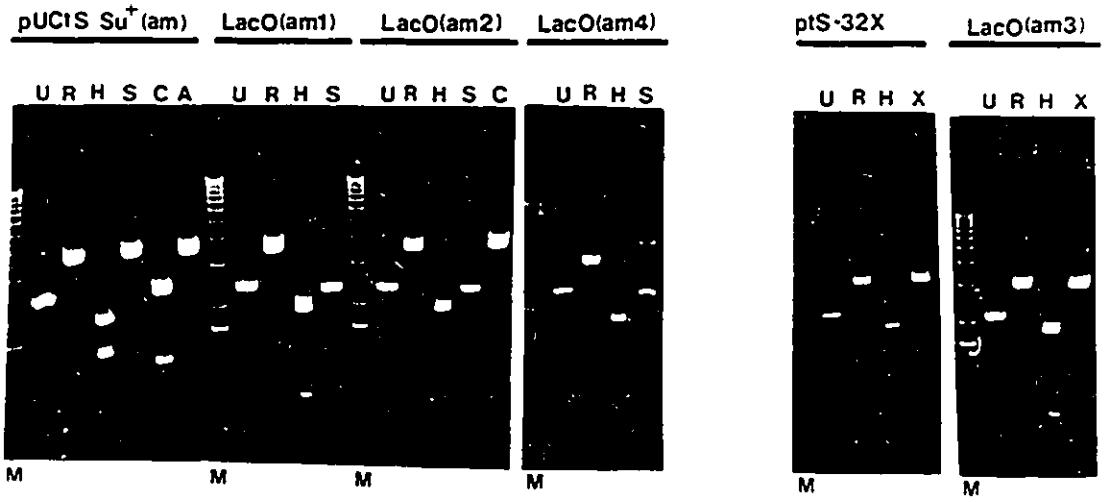


Figure 4.3 **Restriction endonuclease analysis of *lac* operator-su$^+$ tRNA gene derivatives.** *Lac*O-su$^+$ tRNA gene constructs p*Lac*OtS(am1), p*Lac*OtS(am2), and p*Lac*OtS(am4), in addition to p*Lac*OtS(am3), and the plasmids from which they were derived, pUCtS Su$^+$(am) and ptS-32X, respectively, were digested with various restriction endonucleases. Restriction fragments were resolved on 0.8% agarose gels. (U, uncut; R, *Eco*R1; H, *Hae*II; S, *Sna*B1; C, *Sca*1; A, *Afl*III; X, *Xba*1; M, nucleic acid standards (bps)).

su^+ tRNA gene expression (section 3.2). As demonstrated in Figure 4.4, Panel A, insertion of the *lac*O sequence at position nt -1 in p*Lac*OtS(am1) had no significant affect on expression of functional su^+ tRNA in comparison to that derived from the unaltered su^+ tRNA gene, a relationship which was maintained over a wide range of *lac*O-su^+ tDNA concentrations (Figure 4.5, Panel A). These observations reaffirm the view that upstream extragenic sequences of most tRNA genes bestow only minor influences towards transcriptional activity (Sharp *et al*, 1985). Inclusion of pRSVIns in the transfection had no affect on the expression of the wild type su^+ tRNA gene in pUCtS Su^+(am), but reduced functional expression of the p*Lac*OtS(am1) derivative by 75-85%, demonstrating that *lac* repressor can effect the inhibition of nonsense suppression activity when bound to a *lac*O sequence positioned directly upstream of a su^+ tRNA gene. Indeed, repressor bound to an endogenous plasmid-borne *lac*O site approximately 500 nts upstream of the wild type su^+ tRNA gene in pUCtS Su^+(am) (Capone *et al*, 1986), also present within the *lac*O-su^+ tRNA derivatives, had no affect on the level of suppression activity (Figure 4.4. Panel A), which in fact, was similar to that derived from a plasmid in which this naturally occurring *lac*O site was deleted (pUCtS Su^+(am)-O; section 2.2.1.4.1; data not presented). The contribution of this upstream *lac*O site to repressor-mediated inhibition of su^+ tRNA gene expression was therefore minimal. The results demonstrate that *lac* repressor protein can selectively inhibit su^+ tRNA gene expression when appropriately positioned upstream of the gene.

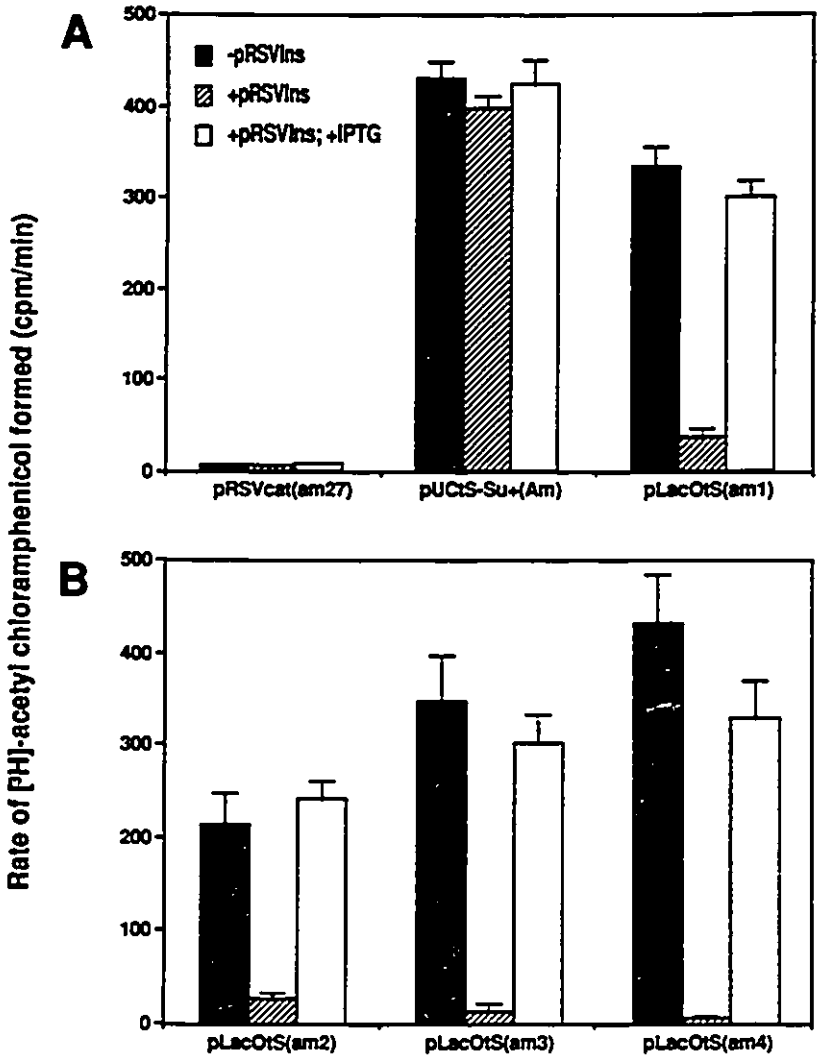


Figure 4.4 **Selective and reversible inhibition by *lac* repressor of phenotypic su$^+$ tRNA expression *in vivo* of *lac* operator-linked tRNA genes.** (A) BSC40 cells (60-mm-diameter plates) were cotransfected in duplicate with 5 μg of pRSVCAT(am), 1 μg of su$^+$ tRNA plasmids, and 1 μg of pRSVIns, as indicated. Where indicated, cells were incubated in the presence of 20 mM IPTG for the course of the transfection. CAT protein activity was determined 48 hours later, using the scintillation counting method (Eastman, 1987; Neuman *et al*, 1987) and measuring the rate of formation of ^{3}H-acetyl chloramphenicol from the slope of the line by linear regression analysis. The results are averages from three independent experiments (+/- standard error of the mean). (B) The various *lac* operator-linked tRNA genes (0.25 μg) were transfected as described above. For p*Lac*OtS(am3) and p*Lac*OtS(am4), suppression activity was reduced by 95 to 98% by pRSVIns in comparison with the nonrepressed state. For p*Lac*OtS(am2), activity was reduced by 75 to 80%. The results are averages (+/- range) from duplicate transfections. Corresponding symbols are as shown in Panel A.

4.4.1.2 Optimization of *lac* repressor-mediated inhibition of su^+ tRNA gene expression

To optimize *lac* repressor-mediated inhibition of su^+ tRNA gene expression, several additional *lac*O-su^+ tRNA derivatives were constructed (Figure 4.2) and the effect of *lac* repressor on suppression activity was examined *in vivo* (Figure 4.4, Panel B). The rationale underlying the construction of p*Lac*OtS(am2), in which a second *lac*O site was introduced into p*Lac*OtS(am1) at position nt 154 downstream of the tRNA gene, is based on the observation that spatially separated *lac*O sites can facilitate the cooperative binding of *lac* repressor tetramer through looping out of intervening DNA, resulting in the formation of a very stable protein/DNA complex (Hsieh *et al*, 1987; Kramer *et al*, 1987; Flashner and Gralla, 1988). Cooperative binding of *lac* repressor and thus, the requisite tRNA gene positioning within such a repressor-mediated DNA loop, may afford more stringent inhibition of su^+ tRNA gene expression. Indeed, precedent for such a DNA looping mechanism in mediating *E. coli* RNA polymerase transcriptional regulation, through an effect on the initial transcribing complex, has been established both *in vivo* and *in vitro* (Choy and Adhya, 1992). However, although suppression activity derived from this construct was reduced (50%) compared to that from the unaltered su^+ tRNA gene or p*Lac*OtS(am1) (Figure 4.4, Panel A), there was no change in the relative level of inhibition in the presence of repressor.

p*Lac*OtS(am3) was generated to investigate the effect of varying the position of the *lac*O site upstream of the su^+ tRNA gene coding region; the relative centre of *lac*O sequence in this construct is 37 nts further upstream at position nt -46, compared to that of p*Lac*OtS(am1), at position nt -9. As with p*Lac*OtS(am1), *lac*O insertion in

p*Lac*OtS(am3) did not overtly affect su$^+$ tRNA gene expression compared to that of wild-type pUCtS Su$^+$(am). Interestingly, *lac* repressor mediated a 90% to 95% reduction in suppression activity (Figure 4.4, Panel B). Hence, *lac* repressor positioned 32 nts upstream of the tRNA gene coding region can still inhibit su$^+$ tRNA gene expression, and apparently, can do so more efficiently than when positioned directly proximal to the tRNA gene coding region in p*Lac*OtS(am1).

Insertion of a higher affinity *lac*O site in p*Lac*OtS(am4) improved the repressor-mediated reduction of suppression activity by approximately 98% (Figure 4.4, Panel B). It is not known whether the enhanced efficiency of inhibition of su$^+$ tRNA gene expression represents a contribution from higher affinity *lac* repressor binding or is a manifestation of altered *lac*O site positioning (centre position nt -15).

4.4.1.3 Su$^+$ tRNA gene expression can be allosterically regulated using IPTG

The efficient regulation of su$^+$ tRNA gene expression is also dependent upon the ability to reliably elevate su$^+$ tRNA levels. The ability to relieve repressor-mediated inhibition of su$^+$ tRNA gene expression was examined in co-transfection assays in the presence of IPTG. As shown in Figure 4.4, Panel A, IPTG could effect the quantitative reversal of repressor-mediated inhibition of suppression activity by p*Lac*OtS(am1). IPTG alone did not affect su$^+$ tRNA gene expression (data not presented). Similarly, inhibition of su$^+$ tRNA gene expression by p*Lac*OtS(am2) was quantitatively relieved with IPTG however, derepression of suppression activity by p*Lac*OtS(am3) and p*Lac*OtS(am4) with IPTG was only approximately 85% and 75%, respectively (Figure 4.4, Panel B). The

basis for the limited derepression exhibited for p*Lac*OtS(am3) and p*Lac*OtS(am4) is not known.

4.4.1.4 The efficiency of inhibition of su$^+$ tRNA gene expression is a function of *lac* repressor/*lac*O-tRNA gene stoichiometry

The development of any system of conditional gene expression necessitates defining the parameters which dictate the efficiency of regulation. The efficiency of *lac* repressor-mediated inhibition of su$^+$ tRNA gene expression *in vivo* is likely a function of the stoichiometry between repressor molecules and *lac*O-tDNA target sites, and the dissociation rate of DNA-bound repressor. To address this issue, the effect of varying the amount of p*Lac*OtS(am1) on the level of nonsense suppression activity, while maintaining a constant amount of pRSVIns, was examined *in vivo* in co-transfection assays in the presence and absence of IPTG. As demonstrated in Figure 4.5, Panel A, suppression activity was a linear function of tDNA concentration up to approximately 1 μg of transfected plasmid. Over the same *lac*O-tDNA concentration range, suppression activity was reduced 75% to 85% in the presence of pRSVIns, while inhibition was quantitatively relieved in the presence of IPTG. However, with an increasing ratio of p*Lac*OtS(am1) to pRSVIns (5:1 by mass), *lac* repressor-mediated inhibition was not observed and likely can be ascribed to excess *lac*O-tDNA titrating out available repressor molecules. The results demonstrate that the ratio of *lac*O-tRNA gene copy number to repressor protein is indeed important for efficient *lac* repressor-mediated su$^+$ tRNA gene regulation.

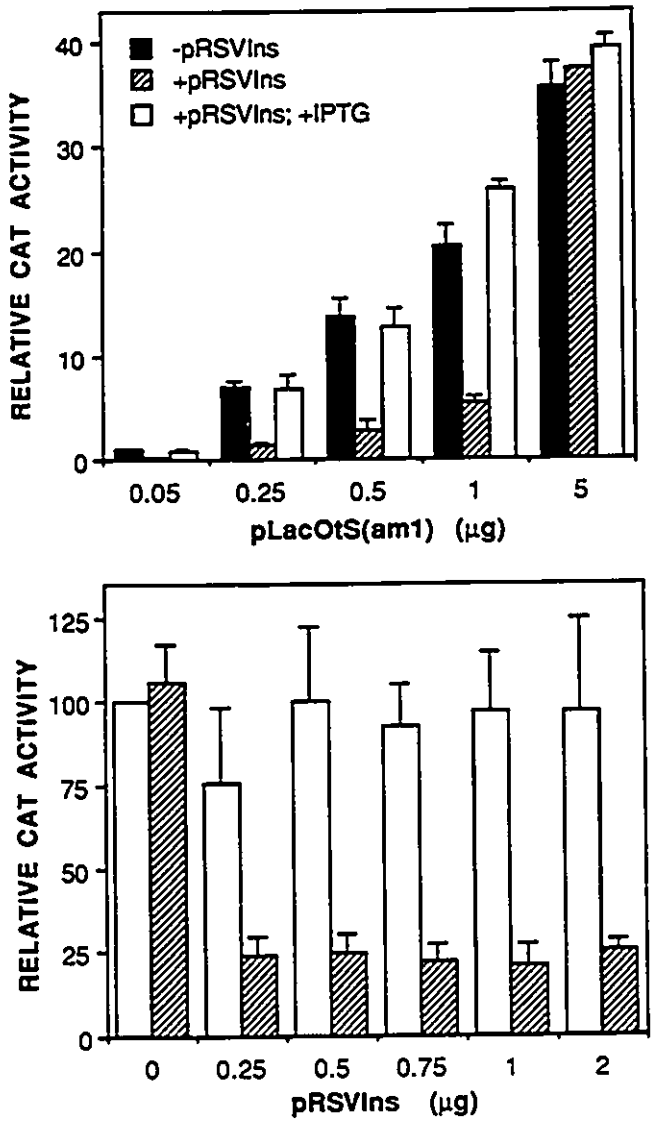


Figure 4.5 **Effects of increasing tRNA and *lac* repressor plasmid concentration on *lac* repressor-mediated inhibition of transcription.** (A) BSC40 cells were transfected with 5 μg of pRSVCAT(am), 1 μg of pRSVIns, and various amounts of p*Lac*OtS(am1) in the presence or absence of 20 mM IPTG, as indicated. CAT protein activity was determined as for Figure 4.4. The results are averages from 3 independent experiments (+/- standard error of the mean) normalized against the CAT protein activity obtained from transfection of 0.05 μg of tDNA plasmid in the absence of pRSVIns, which was taken as 1. (B) Cells were cotransfected with 5 μg of pRSVCAT(am), 0.5 μg of p*Lac*OtS(am1), and various amounts of pRSVIns, as indicated. The CAT protein activity was averaged from three independent experiments (+/- standard error of the mean) and normalized to the value for transfections carried out in the absence of pRSVIns, but in the presence of IPTG, which was taken as 100%. Corresponding symbols are as shown in Panel A.

Similarly, the effect on suppression activity of maintaining a constant concentration of p*Lac*OtS(am1) while varying the amount of *lac* repressor was examined (Figure 4.5, Panel B). Maximum inhibition of suppression activity was derived from the lowest amount of pRSVIns transfected (0.25μg); increasing pRSVIns concentration thereafter does not further reduce su$^+$ tRNA gene expression. The inability to completely eliminate suppression activity may thus reflect a lag period in the synthesis and accumulation of sufficient repressor molecules to assemble all *lac*O sites, hence permitting su$^+$ tRNA gene expression. However, more stringent su$^+$ tRNA regulation than that achieved with p*Lac*OtS(am1) has been observed (Figure 4.4, Panel B).

4.4.1.5 Regulated Expression of a Mammalian Su$^+$ tRNA Gene *In Vivo*: Summary

The results demonstrate that the appropriate positioning of *lac* repressor upstream of a su$^+$ tRNA gene can effectively mediate inhibition of tRNA gene expression. Moreover, suppression activity can be allosterically regulated with IPTG. The *E. coli lac* operator/repressor system can confer stringent regulation upon a mammalian nonsense su$^+$ tRNA gene.

4.4.2 Transcriptional Regulation of a Mammalian tRNA Gene *In Vitro*

4.4.2.1 *Lac* repressor can inhibit tRNA gene transcription *in vitro*

Lac repressor-mediated inhibition of su$^+$ tRNA gene expression *in vivo* likely emanates from an aberrant initiation or inhibition of tRNA gene transcription. To address this question directly, the *lac*O-tDNA constructs were transcribed *in vitro* in HeLa cell

nuclear extracts in the presence or absence of purified *lac* repressor and IPTG. As shown in Figure 4.6, there was little difference in the *in vitro* transcriptional activities of the *lac*O-tDNA derivatives compared to that of the wild-type tRNA gene (variation of 10% to 30%, except for p*Lac*OtS(am2) in which template activity was reduced 30% to 50%; compare lane a with lanes e, i, m, and q). These results are consistent with those derived *in vivo*, in which introduction of a *lac*O site within extragenic sequences upstream of the tRNA gene had little affect on phenotypic su^+ tRNA gene expression (Figure 4.4).

Preincubation of the tRNA genes with purified *lac* repressor prior to the addition of nuclear extract and nucleoside triphosphates (NTPs) resulted in the selective and stringent inhibition of transcription of the *lac*O-tDNA derivatives (Figure 4.6). Repressor had no affect on transcription of the wild-type tRNA gene (compare lane a with c) even in the presence of a 10-fold-higher concentration of *lac* repressor protein (data not presented). Inhibition of transcription was absolute for all *lac*O-tDNA derivatives containing the *lac*O sequence at position nt -1 under the conditions described (compare lanes e, i, and q with lanes g, k, and s, respectively). However, inhibition of transcriptional activity of p*Lac*OtS(am3), in which the *lac*O sequence is located further upstream at position nt -32, was less efficient and reduced only approximately 50% to 70% (compare lane m with o). Interestingly, the efficiency of *lac* repressor-mediated inhibition of su^+ tRNA gene expression derived from p*Lac*OtS(am3) *in vivo* was similar to that of the other *lac*O-tDNA derivatives (Figure 4.4). The nature of the disparity in the efficiency of inhibition of phenotypic expression of p*Lac*OtS(am3) is not known but may reflect a difference in the stability of the repressor-DNA complex *in vivo* and *in vitro*.

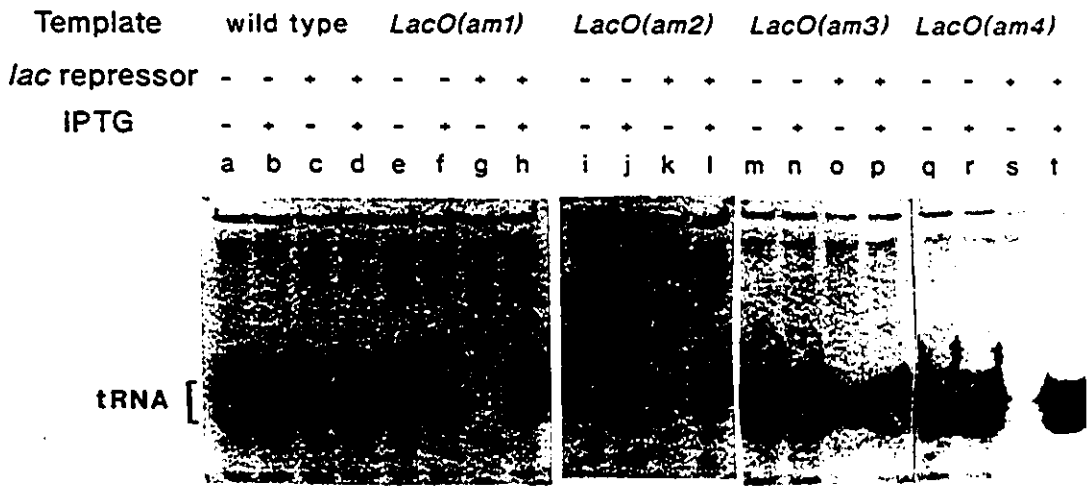


Figure 4.6 **Inhibition by *lac* repressor of transcription of *lac* operator-linked tRNA genes *in vitro*.** pUCtS Su^+(am) (lanes a to d) or the various *lac* operator-linked tRNA gene plasmids (lanes e to t) were transcribed *in vitro*, using HeLa cell nuclear extracts. Where indicated, the templates were preincubated with 50 ng of purified *lac* repressor (Stratagene) in the presence or absence of 60 mM IPTG for 10 min at 25°C prior to addition of nuclear extract and NTPs. Transcription reactions were incubated at 37°C for 2 hours and analyzed by gel electrophoresis. Autoradiographs were quantitated by densitometry. The figure is a composite of the same gel in which lanes i to l were exposed twice as long as the other lanes in order to normalize the intensity of the control lanes.

4.4.2.2 tRNA gene transcriptional activity can be allosterically regulated *in vitro* using IPTG

Lac repressor-mediated inhibition of *lac*O-tRNA gene transcription was efficiently relieved upon addition of IPTG to the transcription reactions (Figure 4.6, lanes h, l, p, and t). The level of derepression ranged from 40% to 90% compared to control transcriptions. IPTG alone had no affect on tRNA gene transcription (lanes b, f, j, n, and r).

4.4.2.3 Transcriptional Regulation of a Mammalian tRNA Gene *In Vitro*: Summary

These results demonstrate that *lac* repressor can effect the complete inhibition of tRNA gene transcription *in vitro*, and moreover, that IPTG can efficiently restore transcriptional activity. *Lac* repressor-mediated inhibition of su^+ tRNA gene expression *in vivo* occurs at the level of transcription.

4.4.3 Regulated Expression of a Mammalian Nonsense Suppressor tRNA Gene Using the *E. coli Lac* Operator/Repressor System: Conclusions

A novel means of regulating the expression of a mammalian nonsense su^+ tRNA gene has been developed. A sequence-specific DNA binding protein has been adopted to confer transcriptional regulation upon the expression of a human su^+ tRNA gene. The *E. coli lac* repressor, bound to its cognate *lac*O site appropriately positioned upstream of the su^+ tRNA gene, can stringently and reversibly block tRNA gene expression both *in vivo* and *in vitro*. This work represents the first example of a generally applicable system for the regulation of a mammalian class III gene.

Such an inducible su$^+$ tRNA gene may serve as a regulatory switch for the conditional expression of eukaryotic protein-coding genes and hence, may foster rapid progress in the development of mammalian su$^+$ tRNA genetics. Indeed, the ability to conveniently manipulate su$^+$ tRNA gene expression may permit the examination of viral gene function over the course of an infection, and of cellular gene function at defined stages of the cell cycle. Furthermore, the utility of the *lac* operator/repressor system in conferring regulated gene expression may be extended further towards regulation of other genes transcribed by polIII.

4.5 Conditional Expression of a Mammalian Nonsense Su$^+$ tRNA Gene: Outlook

The utility of the *lac* operator/repressor system in conferring conditional gene expression in mammalian cells may be limited by the slow and inefficient action of IPTG, and hence the modest and variable levels of induction which ensue. The *E. coli* tetracycline operator/repressor system has recently been adapted for controlling gene expression in mammalian cells, and in contrast to the *lac* operator/repressor system, is subject to rapid gene induction (Gossen and Bujard, 1992). Indeed, the tetracycline operator/repressor system has conferred regulation upon a *Dictyostelium discoideum* su$^+$ tRNA gene in yeast (Dingermann *et al*, 1992a); moreover, the scope of this approach has been demonstrated in *Dictyostelium discoideum* with the establishment of a su$^+$ tRNA-based system of conditional gene expression (Dingermann *et al*, 1992b).

The tremendous potential of nonsense su$^+$ tRNA technology for the advancement of mammalian viral and cellular genetics has yet to be realized. The preeminent obstacle

towards this end in mammalian cells is apparently the ability to maintain su^+ tRNA gene expression sufficiently low to minimize su^+ tRNA-associated cellular toxicity (Hudziak *et al*, 1982) and paradoxically, the ability to render su^+ tRNA gene expression sufficiently high to mediate su^+ tRNA competition for nonsense codon recognition over the release factor (section 1.2), thereby ensuring restoration of biological activity. Such constraints have imposed the requisite stringency in su^+ tRNA gene regulation in the establishment of mammalian nonsense suppressor cell lines (section 3.2; Appendix 7.1). As such, the *lac* operator/repressor system has afforded a degree of su^+ tRNA gene regulation which necessarily must be further improved. That tRNA gene transcription can be completely inhibited *in vitro* by purified *lac* repressor suggests that complete abrogation of su^+ tRNA gene expression in mammalian cells may be attainable, perhaps through prior establishment of cell lines which constitutively express *lac* repressor (Figge *et al*, 1988; Hu and Davidson, 1991). The challenge lies in increasing overall suppression activity while maintaining a repressor/*lac*O site stoichiometry which permits both inhibition of su^+ tRNA gene expression and efficient su^+ tRNA gene induction (Figure 4.5). A strategy which is potentially useful, within the context of generating mammalian nonsense suppressor cell lines, lies in increasing the copy number of both the *lac*O-su^+ tRNA gene and the *lac* repressor gene through methotrexate-dihydrofolate reductase gene-mediated DNA amplification (Kaufman and Sharp, 1982).

The task in establishing a sufficiently large dynamic range in suppression activity to render su^+ tRNA technology suitable for use in mammalian cells, may be best achieved by adopting a strategy which combines an active mechanism to confer

transcriptional repression upon a su^+ tRNA gene to effect low basal level expression, and a separate mechanism to activate su^+ tRNA gene expression to greatly increase suppression activity. Indeed, such an approach has resulted in the generation of a number of high level inducible polII promoter systems in which transcription activity may be elevated up to five orders of magnitude. One such system has coupled *lac* repressor-mediated inhibition of basal gene expression with activated transcription to impose tight gene regulation; simultaneous derepression with positive induction provides high level gene induction (Hu and Davidson, 1990). An alternate approach has incorporated the highly specific DNA binding properties of prokaryotic repressor proteins and the potency of a strong eukaryotic transcriptional activator in the generation of hybrid transactivators to conditionally stimulate transcription from a minimal polII promoter (Labow *et al*, 1990; Baim *et al*, 1991; Gossen and Bujard, 1992).

The prospect of adopting similar strategies to facilitate the flexible manipulation of su^+ tRNA gene expression in mammalian cells, while simultaneously maximizing the dynamic range in suppression activity, demands further defining the factor requirements and sequence elements which underly basal and regulated transcription by mammalian polIII. Indeed, the extent of mechanistic conservation maintained by the eukaryotic RNA polymerases must ultimately be established. Specifically, examining the ability to adapt the well defined mechanisms of regulating transcription by polII to function within a polIII promoter context, will bear direct relevance in evaluating the feasibility of adopting a repression-activation mechanism to effect a large dynamic range of su^+ tRNA gene expression and thus, nonsense suppression activity. Moreover, the ability to selectively

compromise su^+ tRNA gene expression while maintaining su^+ tRNA functional integrity may facilitate the generation of silent polIII promoters which then may be rendered responsive to high level transcriptional activation. Nevertheless, while efficient regulation of su^+ tRNA gene expression may be attained, the ability to reversibly manipulate suppression activity may remain limited by the inherent longevity of the tRNA gene product itself.

Su^+ tRNA technology in prokaryotes and yeast has facilitated a wide variety of biological and biochemical analyses; however, su^+ tRNAs have been invaluable in their role in forming the basis of genetic screens (section 1.3). Alternative and perhaps more generally applicable strategies than the construction of mammalian suppressor cell lines for the isolation and propagation of animal virus nonsense mutants may involve the generation of recombinant viruses which express su^+ tRNA genes. A su^+ tRNA gene inserted within the genome of a virus which undergoes a lengthy replication cycle, such as adenovirus, could be used to propagate a virus nonsense mutant which replicates much more quickly. A replication-defective transducing virus may be ideal. Furthermore, the stringent regulation of su^+ tRNA gene expression afforded by the *lac* operator/repressor system may permit the generation of inducible nonsense su^+ tRNA gene transducing viruses. Such recombinant viruses may serve as recipient viruses for introduction of defined nonsense mutations into the viral genome. Such an approach would eliminate the requirement for complementing cell lines and would be useful for the genetic analysis of a variety of mammalian viruses. Indeed, such su^+ tRNA transducing viruses may mediate mammalian cellular genetic analyses as well. Due to the high replicative capacity of

mammalian viruses, introduction of the *lac* repressor gene within the viral genome will be required to maintain repressor/*lac*O site stoichiometry (Figure 4.5), as noted for vaccinia virus (Fuerst *et al*, 1989). Indeed, preliminary attempts towards development of such a system suggest that the requirement for stringent su^+ tRNA gene regulation in mammalian cells is maintained within the context of the viral genome (Appendix 7.3). Such su^+ tRNA gene transducing viruses may be useful for the genetic analysis of viruses for which suppressor cell lines are unavailable, or if attempts to generate a wide range of mammalian nonsense suppressor cell lines remain unsuccessful (Appendix 7.1).

5. Mechanisms of Transcription by Mammalian RNA PolIII

5.1 Analysis of Transcription Initiation by Mammalian RNA PolIII

5.1.1 Prelude: Mechanism of *Lac* Repressor-Mediated Inhibition of tRNA Gene Transcription

The *E. coli lac* operator/repressor system has afforded a novel means of efficiently regulating the expression of a mammalian nonsense su^+ tRNA gene (section 4.4.1; Syroid *et al*, 1992). Conception of this approach was based on the supposition that *lac* repressor protein bound upsteam of a tRNA gene, and thus overlapping the initiation site, may interfere with the formation of an active polIII transcription complex or directly prevent initiation and hence, abolish tRNA gene transcription (section 4.3.4). Indeed, that repressor-mediated inhibition of su^+ tRNA gene expression was at the level of transcription was established by examining tRNA gene transcription *in vitro* (section 4.4.2). Preincubation of the *lac*O-tDNA templates with purified *lac* repressor prior to addition of HeLa cell nuclear extract resulted in the complete abrogation of tRNA gene transcription. However, this experiment did not fully address the molecular mechanism(s) by which *lac* repressor was able to effect the complete inhibition of tRNA gene transcription.

5.1.1.1 *Lac* repressor inhibits transcription of tRNA genes assembled into stable preinitiation complexes

To further define the nature of *lac* repressor-mediated inhibition of transcription, the effect of repressor on tRNA gene transcription, using p*Lac*OtS(am1) as the tRNA gene template, was examined both prior to and after stable transcription complex

assembly. Consistent with results shown in Figure 4.6, preincubation of tRNA template with repressor prior to addition of nuclear extract and NTPs resulted in the selective inhibition of transcription of p*Lac*OtS(am1) while having no affect on the transcription of pUCtS Su^+(am) (Figure 5.1; compare lanes a and b with c and d, respectively). This finding suggests that once *lac* repressor is tightly bound to DNA, it cannot be readily displaced by polIII or cognate transcription factors. Similarly, when the *lac*O-tDNA template was preincubated simultaneously with repressor and nuclear extract prior to the addition of NTPs, transcription was also inhibited (lanes e and f). These results suggest that repressor protein is able to efficiently compete with the polIII transcription components for template recognition, thus preventing formation of a functional transcription complex. A prediction which then follows is that fully formed polIII transcription complexes may become refractory to challenge by repressor, in view of the inherent stability of class III gene transcription complexes (section 4.1). Moreover, the high local polypeptide concentration thought to remain associated with the intragenic promoters of tRNA genes through multiple rounds of initiation (see Figure 5.3; Geiduschek and Kassavetis, 1992) may render the *lac*O site inaccessible to recognition by *lac* repressor. Hence, subsequent to stable complex formation, the tRNA gene may be refractory to repressor-mediated inhibition. To examine this possibility, the *lac*O-tDNA template was preincubated with nuclear extract to mediate formation of a stable polIII preinitiation complex. The conditions of preincubation were defined as those which precluded the transcription of a secondary challenge template added subsequent to the test template (R. Tapping, unpublished; Lassar *et al*, 1983). Interestingly, addition of

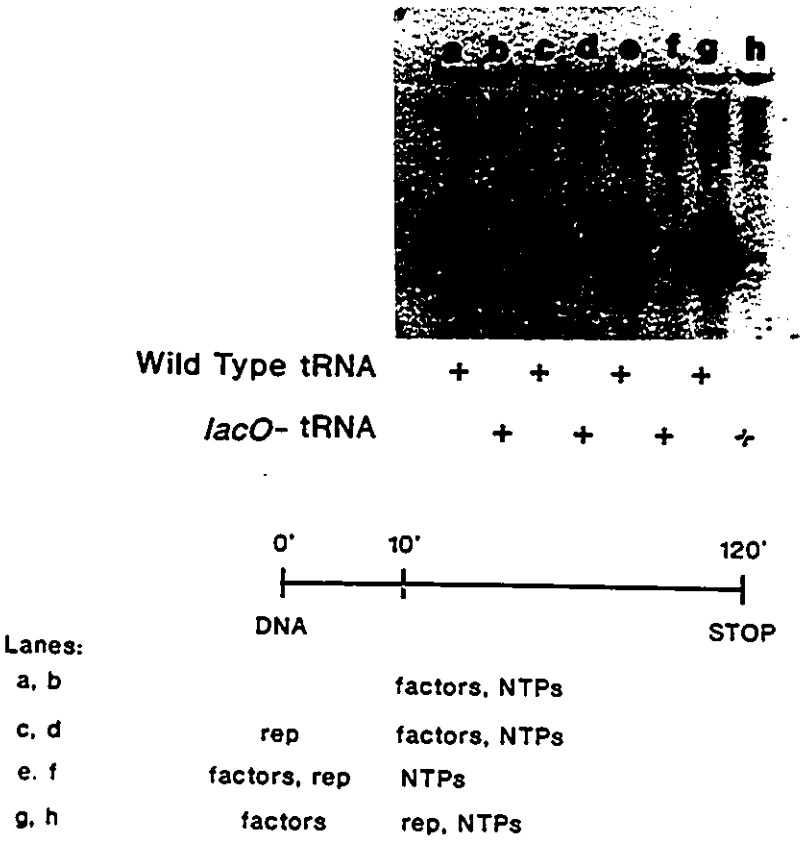


Figure 5.1 **Evidence that *lac* repressor prevents transcription of tRNA genes already assembled into a preinitiation complex.** Preincubation reactions were performed as diagrammed. Lanes a and b are control transcription reactions using pUCtS su^+(am) and p*Lac*OtS(am1), respectively. In lanes c and d, templates (0.1 μg) were preincubated with *lac* repressor (50 ng) prior to the sequential addition of nuclear extract and NTPs. In lanes e and f, templates were preincubated with nuclear extract and *lac* repressor for 10 min prior to the addition of NTPs. In lanes g and h, templates were preincubated with nuclear extract for 10 min (a time sufficient to allow formation of the preinitiation complex) prior to the addition of *lac* repressor and NTPs. In this case, NTPs were added 1 min after repressor addition. Reaction mixtures were incubated for 2 hours at 37°C following the addition of NTPs. Transcription products were analyzed by gel electrophoresis, and subjected to autoradiography.

repressor to such preinitiation complexes resulted in the complete inhibition of tRNA gene transcription (lanes g and h). Similar results were obtained when the preincubation period ranged from 5 to 60 min prior to repressor and NTP addition (Figure 5.2, Panel A). The results demonstrate that repressor is capable of inhibiting transcription of *lac*O-tRNA genes that are assembled into stable preinitiation complexes.

5.1.1.2 *Lac* repressor prevents multiple rounds of transcription from actively transcribing complexes

To determine whether repressor can inhibit further transcription from ongoing reactions, the effect on tRNA gene transcription of repressor addition subsequent to initiation was examined. In Figure 5.2, Panel B, transcription from p*Lac*OtS(am1) was initiated for various times prior to the addition of repressor. Each reaction was allowed to proceed for 90 min following NTP addition. Repressor addition subsequent to initiation reduced but did not completely inhibit tRNA gene transcription. Transcriptional activity was proportional to the time interval which preceded repressor addition, and moreover, coincided to that derived from control reactions carried out in parallel in which transcription was terminated prematurely at the corresponding time intervals (Figure 5.2, Panel C). Interestingly, although overall transcriptional activity was identical, the level of mature-size transcript in Panel B, was greater than that derived from the corresponding time interval in the control reactions in Panel C. This reflects the additional period in which maturation of primary transcripts was allowed to proceed subsequent to repressor addition. The results demonstrate that *lac* repressor can prevent multiple rounds of transcription from actively transcribing complexes.

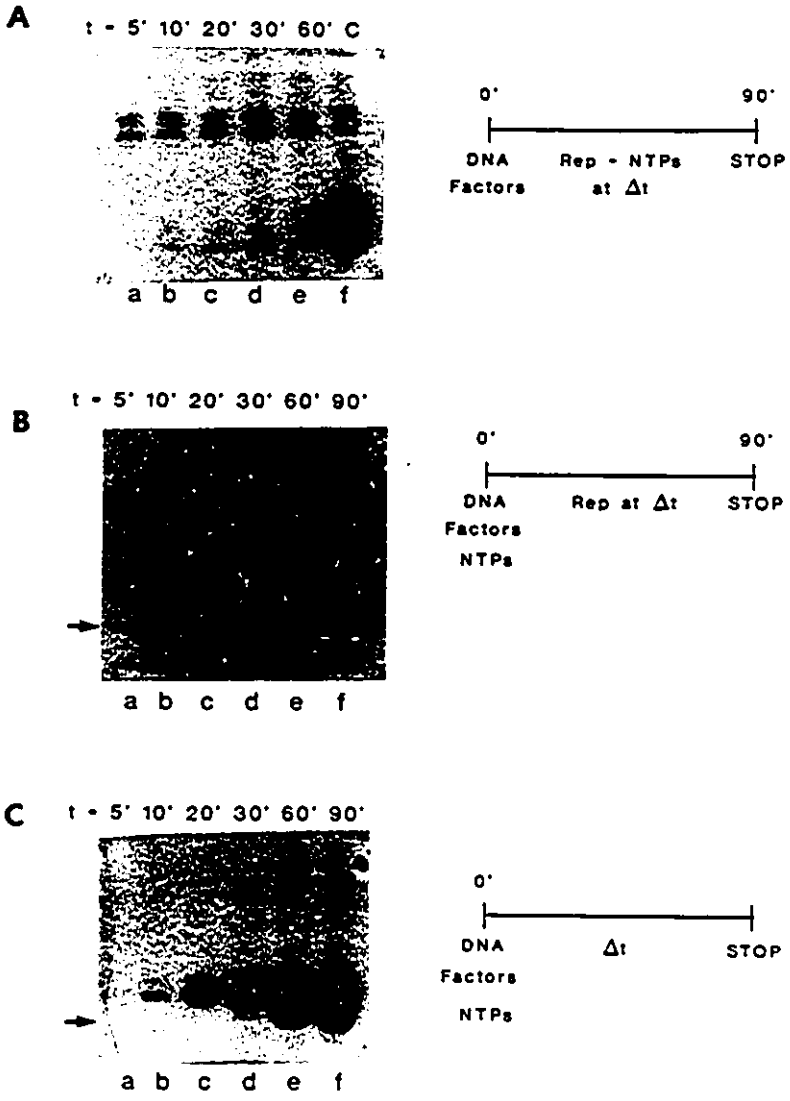


Figure 5.2 **Prevention of reinitiation of transcription by *lac* repressor.** Transcription reactions were carried out as diagrammed. (A) p*Lac*OtS(am1) (0.2 μg) was incubated with nuclear extract for various times, as indicated, prior to the sequential addition of *lac* repressor (Rep; 50 ng) and NTPs. There was a 1 min interval between the addition of repressor and NTPs. Reaction mixtures were incubated for 90 min following the addition of NTPs. Lane c of Panel A is a 90 min control transcription reaction carried out in the absence of *lac* repressor. (B) p*Lac*OtS(am1) was incubated with nuclear extract in the presence of NTPs. *Lac* repressor was added at various times thereafter, as indicated. Reaction mixtures were incubated 90 min from the time of NTP addition. (C) p*Lac*OtS(am1) was incubated with nuclear extract and NTPs, and transcription was terminated at the times indicated with 10 volume of 0.3 M sodium acetate (pH 5.0) containing 0.5% SDS. Arrows denote mature-size tRNA transcript. Actual experiment by R. Tapping.

5.1.1.3 Mechanism of *Lac* Repressor-Mediated Inhibition of tRNA Gene Transcription: Overview

Lac repressor can inhibit transcription of a tRNA gene containing a *lac*O site positioned immediately upstream of the tRNA coding region regardless of the transcriptional state of the gene. Prebound repressor protein may interfere with the assembly of the preinitiation complex through promoter occlusion or prevent essential protein factor interactions through steric hindrance. Alternatively, repressor may directly preclude access by polIII to the site of initiation. It is also conceivable that repressor-imposed structural alterations or incomplete transcription complex assembly may transcend into compromised complex stability. Indeed, the association of both the 90 kda and 70 kda components of yeast TFIIIB is required to reconstitute the functional integrity and stability of yeast polIII transcription complexes (Kassavetis *et al*, 1990, 1991). That repressor can inhibit transcription of preassembled complexes, both prior to initiation and during ongoing transcription, indicates that repressor can act at a step subsequent to the formation of a stable transcription complex. This finding is particularly interesting considering the stability associated with polIII transcription complexes, which remain competent to support transcription through multiple rounds (section 4.1). Repressor may prevent transcription initiation (or re-initiation) by interfering with recruitment of polIII; alternatively, repressor may disrupt a requisite structural transition(s) such as polIII-dependent open promoter complex formation (Kassavetis *et al*, 1992a) or perhaps TFIIIB- and TFIIIC-induced promoter bending (Leveillard *et al*, 1991). The observations are also consistent with an inhibitory mechanism in which modification of the initial transcribing complex by *lac* repressor prevents polIII exit from the initiation site, resulting in abortive

RNA synthesis. Indeed, such a mechanism may underlie *lac* repressor-mediated transcriptional inhibition in prokaryotes (Lee and Goldfarb, 1991) and may be of bearing here, in view of the structural conservation maintained between prokaryotic and eukaryotic RNA polymerases (Allison *et al*, 1985). It is also conceivable that repressor may disrupt the transcription complex through displacement of a component(s) required for initiation. That ongoing transcription can be inhibited by *lac* repressor *in vitro*, supports the contention that the low level of suppression activity derived *in vivo* in transient assays represents a lag in plasmid-borne repressor synthesis (section 4.4.1); presumably, accumulation of sufficient repressor molecules to associate with all *lac*O sites mediates inhibition of the remaining transcribing complexes, thereby preventing further su^+ tRNA gene expression. These findings are of particular relevance and suggest that, tRNA stability notwithstanding, strict regulation of su^+ tRNA gene expression may be attainable (section 4.5).

Hence, *lac* repressor protein may function through several mechanisms to inhibit tRNA gene transcription. The experiments described cannot distinguish between these possibilities which themselves are not necessarily mutually exclusive. It would be informative to identify the target(s) of repressor and any ensuing structural alterations in the transcription complexes which render transcriptional inactivity. Understanding the nature of transcription inhibition by *lac* repressor may aid in defining the molecular mechanisms which underly transcription by polIII.

5.1.2 Examination of Transcription by RNA PolIII: Historical Perspective

Early studies of transcription by polIII have focused primarily on defining the promoter elements and protein factors responsible for directing transcription initiation. The steps involved in the assembly of polIII transcription complexes and their inherent properties have been examined using both crude extracts and partially purified components through a variety of molecular and biochemical analyses, including template commitment, competition, and transcription inhibition studies (Lassar *et al*, 1983; Schaack *et al*, 1983; Sharp *et al*, 1983; Logan and Ackerman, 1988; Kovelman and Roeder, 1990). More recently, the protein-DNA interactions and corresponding structural alterations involved in establishing the multi-component polIII transcription complex have been examined through DNA protection and protein-crosslinking analyses (Kassavetis *et al*, 1989, 1990; Bartholomew *et al*, 1991, 1993). These studies have led to an understanding of the spatial arrangement and topology of polIII transcription complexes (Figure 5.3) and have been conducted almost exclusively in yeast, in which purification of polIII transcription factors, and the subsequent cloning of a number of individual components, has been readily carried out (Geiduschek and Kassavetis, 1992). Importantly, yeast factors efficiently assemble into active transcription complexes *in vitro*, an inherently critical property of the yeast system, prerequisite for the assignment of functional relevance to such biochemical analyses.

However, in contrast, purification of mammalian polIII transcription factors and formation of active transcription complexes in mammalian systems has been inefficient. Moreover, the lack of a viable genetic system has hindered the identification and cloning

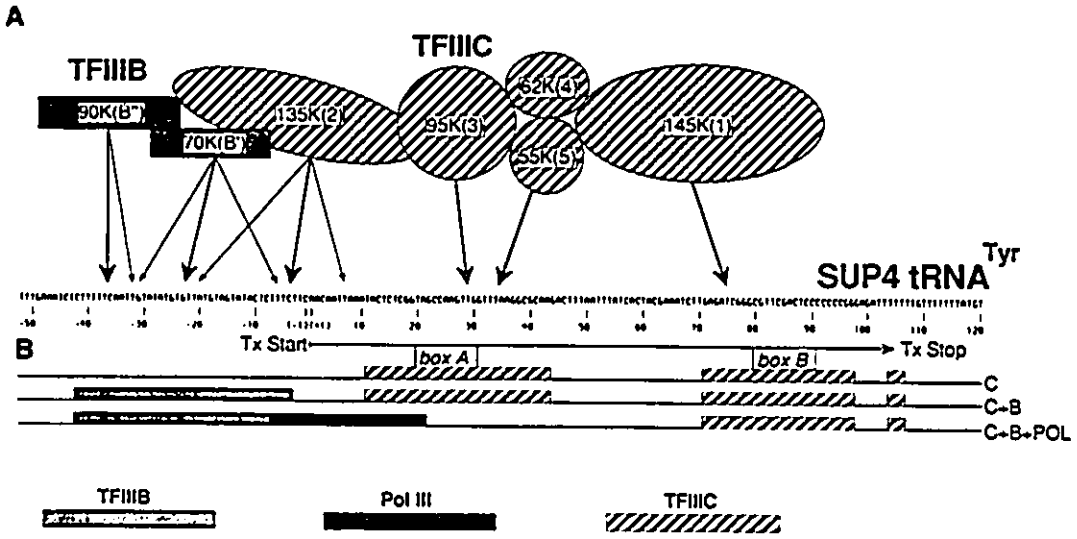


Figure 5.3 **Representation of the structure of a yeast tRNA gene transcription complex.** The sequence of a $tRNA^{Tyr}$ gene (non-coding strand), in addition to the site of transcription initiation and termination, and the intragenic A-box and B-box promoter elements are indicated. (A) Representation of the locations of individual polypeptides of TFIIIC and TFIIIB derived from photocrosslinking data. Arrows indicate the DNA sites that are proximal to individual factor subunits. (Thick arrows denote DNA locations where photocrosslinking is most efficient, while thin arrows denote DNA sites at which photocrosslinking is weaker). (B) Summary of DNase I footprinting data, indicating the degree of DNase I footprints at different stages of transcription complex assembly, and the sites on the tRNA gene which interact with individual components of the transcription complex. Adapted from Geiduschek and Kassavetis, 1992.

of individual components. As such, the application of biochemical analyses similar to those which have been invaluable in defining the composition and properties of polIII transcription complexes in yeast, has been problematic in mammalian systems. Hence, mammalian polIII transcription complexes remain poorly characterized. Studies of this nature are particularly important since class III genes in higher eukaryotes can be subject to transcriptional regulation (section 4.2).

5.1.3 The *Lac* Operator/Repressor System in the Study of Transcription Initiation by Mammalian RNA PolIII

A novel approach has been adopted to study the molecular mechanisms which underly mammalian polIII transcription, based on the observation that *lac* repressor can differentially inhibit tRNA gene transcription *in vitro*, depending upon its position upstream of the tRNA gene (Figure 4.6). When repressor protein was prebound at position nt -46 in p*Lac*OtS(am3) prior to HeLa cell nuclear extract addition, tRNA gene transcription was reduced only 50 to 70%, but was completely inhibited when repressor was prebound at positions nt -9 and nt -15 in p*Lac*OtS(am1) and p*Lac*OtS(am4), respectively (Figure 4.6; compare lane o with lanes g and s). Moreover, *lac* repressor prebound at position nt -46 in p*Lac*OtS(am3) directed the synthesis of shortened primary transcripts (Figure 4.6; compare lanes m with o). This may reflect repressor-mediated steric constraints on complex assembly upstream of the gene, and perhaps a corresponding alteration in initiation site selection by polIII. In view of these findings, it was reasoned that, by altering the position of the *lac*O site upstream of the tRNA gene, *lac* repressor protein may serve as a useful reagent to examine various stages of polIII

transcription complex assembly and hence, aid in defining the spatial arrangement and properties of mammalian polIII transcription complexes. It was reasoned that such an approach may afford insight into the mechanisms which govern mammalian class III gene transcription (Tapping *et al*, 1994).

Towards this end, additional *lac*O-tDNA derivatives were generated, progressively moving the *lac*O site in the 5' flanking region further upstream of the tRNA gene (Figure 5.4). Plasmids p*Lac*OtS-35 and p*Lac*OtS-52 were generated by blunt-end ligating *lac*O oligonucleotide AB1465 (Table 2.1) into the unique *Hinc*II site at position nt -20 and the unique *Sma*I site at position nt -37 upstream of the tRNA gene respectively, of pMCtS. Plasmid p*Lac*OtS-43 was constructed by blunt-end ligating *lac*O oligonucleotide AB1465 (Table 2.1) into the unique *Xba*I site of pMCtS, which was generated through an *Xba*I collapse, and subsequent blunt-ending with Klenow DNA polymerase. Plasmid p*Lac*OtS-37 was constructed by inserting *lac*O oligonucleotide AB883 (Table 2.1) into the unique *Xba*I site of pMCtS. The 3' end of the *lac*O sequence in p*Lac*OtS-43 and -37 is positioned at nt -28 upstream of the tRNA gene coding region. Construct numbering denotes the centre of the *lac*O sequence; hence p*Lac*OtS(am1), p*Lac*OtS(am3), and p*Lac*OtS(am4) (Figure 4.2) become p*Lac*OtS-9, p*Lac*OtS-46, and p*Lac*OtS-15, respectively. Restriction endonuclease analyses have confirmed the presence of the novel, *lac*O sequence-derived *Hae*II site in each *lac*O-tDNA derivative (data not presented; refer Figure 4.3). Nucleotide sequence analyses have confirmed the integrity of all *lac*O-tDNA derivatives (data not presented).

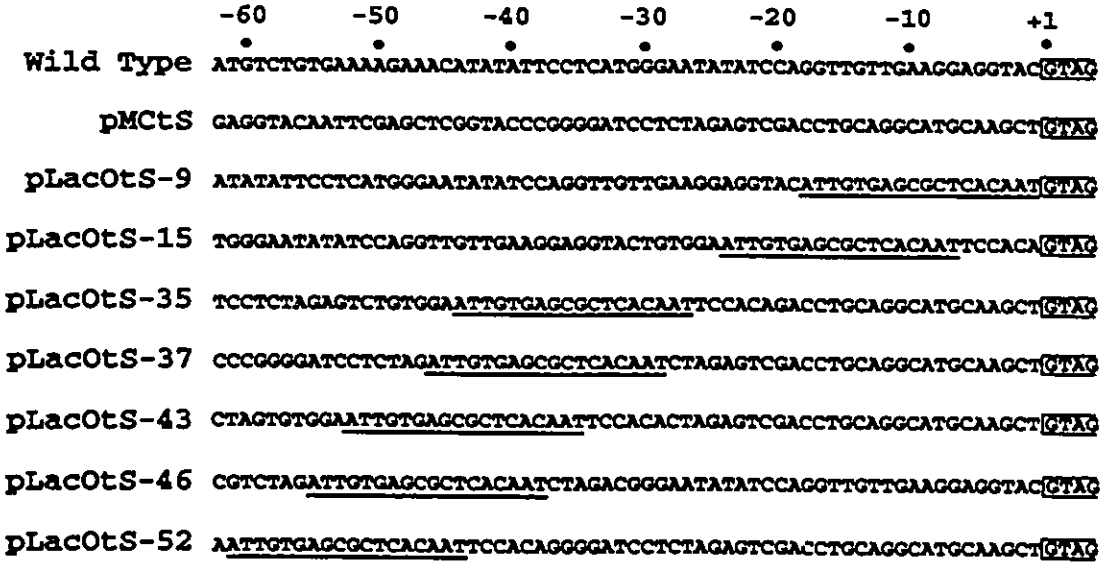


Figure 5.4 **Structure of *lac* operator-containing tRNA^{Ser} genes.** The open box represents the 5' end of the mature tRNA and the underlined sequences correspond to the *lac* operator site. Plasmid numbering corresponds to the position of the central nucleotide of the *lac* operator sequence relative 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. pMCtS is the parental plasmid of p*Lac*OtS-35, -37, -43, and -52. Constructs pMCtS, p*Lac*OtS-35, and p*Lac*OtS-52 were generated with the assistance of Johanna Reichert.

Experiments were directed towards examining the nature of repressor-mediated inhibition of tRNA gene transcription. The effect on transcription of varying the position of *lac* repressor protein upstream of the tRNA gene was examined upon repressor addition prior to and subsequent to stable complex assembly, or during ongoing transcription, in studies similar to those described in Figure 5.1 and Figure 5.2. Such a protocol addresses the ability of repressor protein to inhibit transcription complex assembly, to disrupt preformed complexes or prevent initiation, or to disrupt actively transcribing complexes or prevent reinitiation, respectively. Such order-of-addition experiments, in conjunction with kinetic, template commitment and competition, and transcription inhibition analyses, have afforded significant insight into the spatial arrangement of mammalian polIII transcription complexes and have identified a number of distinct functional and structural properties inherent to these complexes (Tapping *et al*, 1994; Tapping, in preparation). This work was a collaborative effort. The actual experiments were performed by R. Tapping. A descriptive review of this work is presented within this thesis.

5.1.3.1 Mammalian polIII transcription complexes extend at least 35 nts upstream of the tRNA gene

Repressor prebound directly upstream of the tRNA gene at *lac*O site positions nt -9, -15, -35, and -37 completely inhibited tRNA gene transcription by preventing the stable association of at least one limiting component of the mammalian transcription complex through promoter occlusion or steric hindrance. Repressor prebound at positions nt -46 (see p*Lac*OtS(am3), Figure 4.6) and nt -43 greatly diminished, but did not

completely preclude tRNA gene transcription. The level of transcriptional inhibition decreased as repressor was progressively moved further upstream of the tRNA gene from position nt -43 to nt -46 until repressor had no affect on transcription when prebound at position nt -52. Inhibition of tRNA gene transcription was due to compromised complex stability rather than a decrease in the rate of assembly, and presumably reflects a structural alteration(s) to the complex due to repressor-imposed spatial constraints.

These findings indicate that the mammalian polIII transcription complex extends at least 35 nts upstream of the tRNA gene, given that *lac* repressor tetramer footprints 25 bps of DNA (Schmitz and Galas, 1979) and maintains dimensions of approximately 80 Angstroms square (Ohshima *et al*, 1975; Zingsheim *et al*, 1977). These results are in general agreement with those which describe the extended upstream interactions of yeast polIII transcription complexes. In a similar study to that described herein, *tet* repressor, centred at position nt -46 upstream of a su^+ tRNA gene, had no affect on tRNA gene expression in yeast and in *Dictyostelium discoideum* (Dingermann *et al*, 1992a,b). Moreover, footprinting analyses demonstrate that the yeast polIII transcription complex assembled on a tRNA gene extends 35 to 40 nts upstream of the start site, an interaction involving TFIIIB (Kassavetis *et al*, 1989).

5.1.3.2 Flanking sequences directly upstream of the human tRNA gene remain accessible to DNA binding proteins throughout transcription

Order-of-addition experiments with *lac* repressor demonstrate that sequences immediately upstream of the tRNA gene coding region, and thus overlapping the initiation site, remain accessible to DNA binding proteins throughout stable transcription

complex assembly, prior to initiation and through multiple rounds of transcription. Introduction of repressor subsequent to stable complex formation had no affect on tRNA gene transcription derived from *lac*O-tDNA derivatives in which the cognate *lac*O sites were located upstream of position nt -15. The results suggest that these upstream sequences may be refractory to binding by repressor due to stable interaction with components of the transcription complex. Indeed, nuclease protection analyses of yeast polIII transcription complexes assembled on a tRNA gene demonstrate that upstream sequences are protected throughout transcription, while sequences encompassing the initiation site remain largely unprotected (Kassavetis *et al*, 1989, 1990). However, it is also conceivable that binding of repressor protein to *lac*O sites positioned upstream of nt -15 is permitted, but which has no affect on tRNA gene transcription once a stable complex has formed. Repressor interference with the association of a component of the polIII transcription machinery which maintains an exclusive role in complex assembly, playing no direct part in subsequent initiation, may account for the apparent resistance of preassembled complexes to repressor-mediated transcription inhibition when the cognate *lac*O sites are located upstream of position nt -15. Such a function has been ascribed to yeast TFIIIC *in vitro* (Kassavetis *et al*, 1990), although TFIIIC may also serve to alleviate chromatin-mediated repression of polIII transcription (Burnol *et al*, 1993).

5.1.3.3 The site of transcription initiation by mammalian polIII is flexible and can be re-directed

A particularly interesting finding of this work was that residual transcription derived from *lac*O-tDNA derivatives in which repressor was prebound at positions nt -46 (for example, see p*Lac*OtS(am3), Figure 4.6) and -43 was associated with shortened primary transcripts. Indeed, mapping of such transcripts indicates that transcription has initiated from novel downstream sites. Release of repressor with IPTG, subsequent to incubation with HeLa cell nuclear extract and hence transcription complex formation, not only restored transcriptional activity but also the synthesis of normal length transcripts. This presumably reflects the re-established use of the natural initiation site by polIII. These results are in direct contrast to those derived through similar experiments carried out in yeast (Leveillard *et al*, 1993). The GCN4 protein, positioned over the binding sequence for TFIIIB upstream of a tRNA gene prior to complex assembly, effectively mediated the repositioning of TFIIIB and hence, the site of initiation by polIII, to a downstream site. However, upon protein release, use of the altered start site was maintained. These results comply with the extreme stability of polIII transcription complexes which, in yeast, can be ascribed to the remarkably rigid association of TFIIIB (Kassavetis *et al*, 1990). Restoration of the normal site of initiation upon release of *lac* repressor may reflect the reversal of a repressor-mediated structural alteration to the mammalian transcription complex which, in itself, confers complex instability and can account for the observed reduction in transcriptional activity. Indeed, by analogy to the role of TFIIIB as the initiation factor for transcription by polIII in yeast, repressor positioned at nt -46 upstream of a human tRNA gene may act to compromise the

functional integrity of mammalian TFIIIB, when prebound prior to the formation of an active mammalian polIII transcription complex. As such, repressor may interfere with the assembly, stability or function of any component(s) of mammalian TFIIIB, including TBP, TAF-172, and TAF-L, and thus subsequent initiation by polIII. TAF-L may represent the primary target of repressor since this factor is thought to be loosely associated with TBP (Taggart *et al*, 1992). Alternatively, mammalian polIII transcription complexes may be less stable than those in yeast, perhaps the manifestation of a less stable interaction by mammalian TFIIIB (Carey *et al*, 1986; Jahn *et al*, 1987; Wu, 1989). Mammalian TFIIIB may possess the ability to be maintained within a flexible conformation, able to reconfigure and resume use of the natural site of initiation upon repressor release. A structural transition similar to that which is thought to unmask a high affinity DNA binding site in yeast TFIIIB (Geiduschek and Kassavetis, 1992), may not occur in mammalian TFIIIB. The results may indicate that mammalian polIII preinitiation complexes remain in an adaptable state. Alternatively, it is possible that restoration of the natural site of initiation by polIII, upon removal of *lac* repressor, may reflect RNA synthesis originating from newly assembled transcription complexes. Indeed, the nature of such complexes which support normal transcription upon repressor release has not been defined. However, it remains conceivable that these observations simply reflect a repressor-imposed alteration(s) to the positioning or function of polIII itself which, by nature, is loosely associated with the transcription complex. Since this work was carried out using crude HeLa cell nuclear extracts, these possibilities could not be differentiated.

5.1.3.4 The *Lac* Operator/Repressor System in the Study of Transcription Initiation by Mammalian RNA PolIII: Conclusions

The results demonstrate that mammalian polIII transcription complexes extend at least 35 nts upstream of a human tRNA gene and moreover, that flanking sequences directly upstream of the tRNA gene remain accessible to DNA binding proteins throughout multiple rounds of transcription. A number of distinct functional and structural properties of mammalian polIII transcription complexes have been identified. Interestingly, the site of transcription initiation by mammalian polIII, and perhaps the positioning of a component(s) of the mammalian polIII transcription complex upstream of the tRNA gene, is flexible and can be re-directed.

Hence, at the outset, in addition to maintaining common sequence and protein requirements, the yeast and mammalian polIII transcriptional apparati apparently exhibit conservation in the overt spatial arrangement of polIII transcription complexes. The results, however, may also underscore some species-specific differences in the properties of mammalian and yeast polIII transcription complexes.

5.1.4 Analysis of Transcription Initiation by Mammalian RNA PolIII: Outlook

A novel strategy has been devised to examine mechanistic aspects of transcription by mammalian polIII, which otherwise has remained largely refractory to study through conventional approaches. A sequence-specific DNA binding protein has facilitated the preliminary functional characterization of the promoter disposition and properties of mammalian polIII transcription complexes. *Lac* repressor can effect the selective and reversible disruption of different stages underlying productive transcription initiation.

This approach has afforded insight into not only the DNA-protein interactions involved in establishing and maintaining the mammalian polIII transcriptional machinery, but also the protein-protein interactions, and may potentially reveal any conformational changes involved in the generation of active mammalian polIII transcription complexes. The utility of this approach will become realized upon purification of such complexes, in addition to the purification of active mammalian polIII transcription components; indeed, the resolution derived from such a strategy will become further improved when carried out in conjuction with more conventional biochemical analyses. The results illustrate a number of potential mechanisms whereby upstream extragenic sequences, and perhaps the corresponding DNA binding protein factors, may modulate tRNA gene transcription *in vivo* (Tapping *et al*, 1992).

5.2 Analysis of RNA Chain Elongation and Termination by Mammalian RNA PolIII

5.2.1 Sequence-Specific DNA Binding Proteins and Elongation by RNA PolIII: Prospects for a Regulatory Strategy and a Means of Examining Mechanisms of Transcription

The *E. coli lac* operator/repressor system has afforded a means of efficiently controlling the *in vivo* expression of a human su^+ tRNA gene (Syroid *et al*, 1992; section 4.4.1) by imparting regulation upon transcription by polIII (section 4.4.2). Moreover, the *lac* operator/repressor system has mediated an examination of the mechanisms of transcription initiation by mammalian polIII (Tapping *et al*, 1994; section 5.1). *Lac* repressor protein, appropriately positioned upstream of the tRNA gene, can differentially interfere with the formation of an active mammalian polIII transcription complex and thereby, differentially inhibit transcription initiation, *in vitro*. Moreover, *lac* repressor can prevent further transcription from actively transcribing complexes by interfering with subsequent rounds of re-initiation. The mechanisms invoked to account for the ability of repressor protein to inhibit tRNA gene transcription (section 5.1.1) concur with current views regarding the molecular mechanisms which underly the transcriptional regulation of class II gene expression, in that any step leading to productive initiation maintains the capacity to serve as a control point (Conaway and Conaway, 1991). Indeed, regulation of transcription initiation is a primary mode of gene control in both eukaryotes and prokaryotes. As such, studies of transcription have focused largely on resolving the essential steps which culminate in the initiation of RNA synthesis.

In contrast, the dynamic events which constitute transcription elongation and termination remain less well defined, but which nevertheless, represent potential sites at

which gene expression may be regulated. Indeed, transcription attenuation is a well established regulatory mechanism in prokaryotes, and functions through imparting conditional elongation and termination upon a polymerse ternary complex (Friedman *et al*, 1987; Yanofsky, 1988). However, control of RNA synthesis at the level of transcription elongation has only recently become recognized as an important mechanism for gene control in eukaryotes (Spencer and Groudine, 1990; Kerppola and Kane, 1991; Richardson, 1993). Transcription elongation can be modulated by intrinsic pause sites or conditional factor-mediated blocks that can either result in polymerase stalling, in which case the ternary complex can remain stable and competent for resumed elongation, or in premature termination, in which the transcription complex dissociates and the shortened transcript is released. Such a regulatory mechanism efficiently controls the amount of mature transcript produced.

Sequence-specific DNA binding proteins can function in both prokaryotes and eukaryotes to regulate RNA polymerase elongation. Aside from acting solely in the inhibition of transcription initiation, *lac* repressor protein also functions in blocking elongation (Flashner and Gralla, 1988; Gralla, 1989) and in mediating transcription termination by *E. coli* RNA polymerase (Sellitti *et al*, 1987), both physiologically important events in mediating the stringent regulation of the *lac* operon. Moreover, specific blocks to polII elongation by viral (Skarnes *et al*, 1988; Bertin *et al*, 1992) and cellular (Connelly and Manley, 1989; London *et al*, 1991; Roberts *et al*, 1992) DNA-binding proteins have been described for a number of eukaryotic class II genes, and are thought to serve important regulatory roles. Furthermore, termination of transcription by

polI (Kuhn *et al*, 1990; McStay and Reeder, 1990) and mitochondrial RNA polymerase (Christianson and Clayton, 1988; Kruse *et al*, 1989) is mediated by sequence-specific DNA-binding termination factors. There is no evidence, however, that elongation or termination by polIII is subject to regulation through such strategies.

A consequence of the intragenic nature of tRNA gene promoters is the formation of a multi-component transcription complex which, for yeast tRNA genes, encompasses virtually the entire tRNA coding region and extends into extragenic flanking sequences (Figure 5.3; Geiduschek and Kassavetis, 1992). Mammalian polIII transcription complexes likely exhibit a similar promoter disposition given the conservation in promoter elements, factor requirements, and in the order of assembly of such components into active polIII transcription complexes, in addition to the apparent similarities in the upstream spatial arrangement of mammalian tRNA gene transcription complexes (section 5.1.3). Hence, an intriguing aspect of the transcription of class III genes that maintain intragenic promoters is the ability of polIII to elongate through transcription components which bind downstream of the initiation site and which are thought to remain stably associated through multiple rounds of transcription (Wolffe, 1991). Implicit with this observation, and bearing direct relevance to understanding class III gene regulation, is the question of whether a DNA-binding protein can block, and thus function to modulate, elongation by polIII. Access to such intragenic sites may be restricted or factor binding may preferentially interfere with the initiation of transcription, in view of the steric constraints imposed on polIII transcription complex assembly (section 5.1.3).

Pausing and/or clustering of RNA polymerase molecules in transcription-unit internal sites has been noted for a number of class II genes and may serve to maintain a store of active ternary complexes, poised for renewed RNA synthesis upon alleviation of an elongation block (Skarnes *et al*, 1988; Chen *et al*, 1990; Rougvie and Lis, 1990; Spencer and Groudine, 1990; Kerppola and Kane, 1991). While the outright size of class II genes affords the capacity to accomodate numerous DNA-binding proteins and polymerase molecules on transcribed sequences, the extent to which polIII molecules can reside on small tRNA gene templates is not known. The feasibility of a regulatory mechanism which functions to prime class III genes for RNA synthesis by maintaining a multitude of paused active polIII ternary complexes has not been established. However, it remains conceivable that the conditional arrest of a single polIII ternary complex and/or subsequent termination may adequately serve to attenuate transcription by polIII.

Class III genes in higher eukaryotes are subject to differential regulation, through mechanisms thought to act primarily at transcription initiation (sections 4.1, 4.2). Interestingly, transcription of a *Xenopus laevis* $tRNA^{Lys}$ gene apparently terminates prematurely in a tissue-specific manner however, the biological relevance of such conditional terminator usage has not been established (Mazabraud *et al*, 1987). Hence, it is not yet known whether class III genes are subject to regulation at the level of transcription elongation. This is an intriguing prospect for the control of tRNA genes, since the highly conserved nature of tRNA gene promoter elements (section 4.1) may pose a limit on the capacity of regulatory strategies which function to modulate initiation. To address whether class III genes can be regulated at the level of transcription

elongation, the *lac* operator/repressor system was adopted as a means to position, in a conditional manner, a DNA-binding protein in the path of elongation by polIII. Examining whether *lac* repressor can block elongation by polIII may be of direct relevance to the apparent ability of polIII to elongate though its own multi-component transcription complex, and may afford insight into the mechanisms which govern elongation and termination by polIII. Furthermore, studies of this nature may aid in understanding how elongating polymerase molecules respond to nucleoprotein complex obstacles (Syroid and Capone, in press).

5.2.2 The *Lac* Operator/Repressor System in the Study of Transcription Elongation and Termination by Mammalian RNA PolIII

Additional *lac*O-tDNA derivatives were generated, through placement of the *lac*O sequence at two separate positions between the 3' end of the tRNA gene coding region and the natural termination sequence, or directly downstream from the termination sequence (Figure 5.5). Extragenic sites were selected to lessen the probability of repressor protein interference with transcription complex assembly or initiation (section 5.1.1). Plasmids pt*SLac*O(am5) and pt*SLac*O(am6) were generated by inserting the *lac*O oligonucleotide AB1465 (Table 2.1) into the unique *Eco*RV site at position nt 88 downstream of the tRNA gene coding region, and the *Sma*I site at position nt 101 downstream of the termination site, respectively, in pUCtS93. Plasmid pt*SLac*O(am8) was generated by inserting the *lac*O oligonucleotide AB1465 (Table 2.1) within the unique *Eag*I site (which was blunt-ended with Klenow DNA polymerase) in pUCtS93. This places the 5' end of the *lac*O sequence at position nt 85 downstream of the tRNA

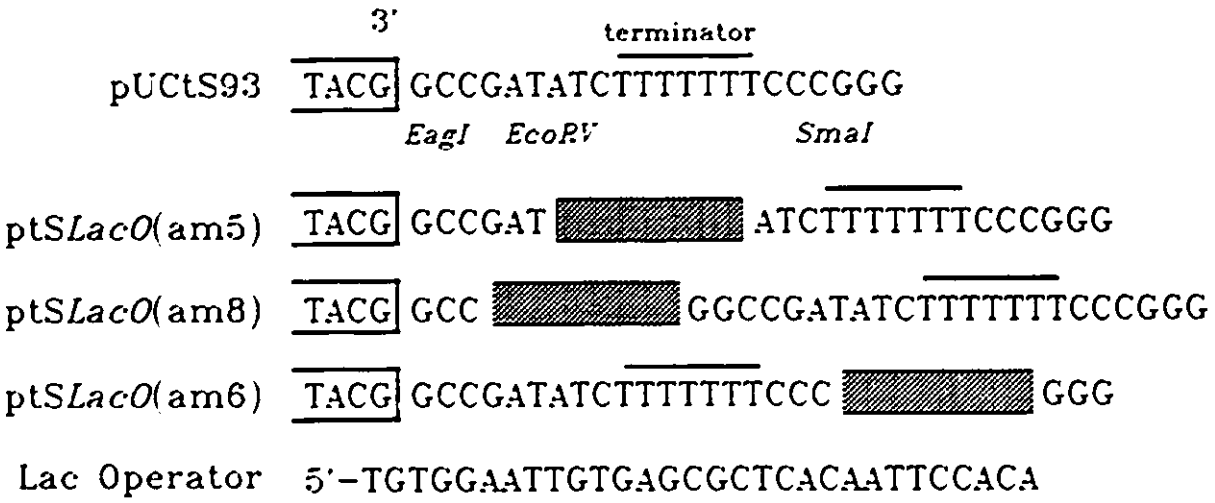


Figure 5.5 **Structure of *lac* operator-containing tRNA^{ser} genes.** The human tRNA^{ser} gene was modified by cassette mutagenesis in order to incorporate the indicated restriction sites at the 3' end of the gene. The position of the *lac* operator is represented by the shaded box while the line identifies the termination site. The boxed nucleotides correspond to the 3' end of the mature tRNA gene coding region.

gene coding region. Restriction endonuclease analyses have confirmed the presence of the novel, *lac*O sequence-derived *Hae*II site in each *lac*O-tDNA derivative (data not presented; refer Figure 4.3). The integrity of each *lac*O-tDNA derivative was confirmed through nucleotide sequence analyses (Figure 5.5).

5.2.2.1 *Lac* repressor inhibits transcription of a tRNA gene when positioned downstream of the coding region

The effect of *lac* repressor protein on tRNA gene transcription was examined *in vitro*. Insertion of the *lac*O sequence downstream of the tRNA gene coding region had no affect on overall template activity or RNA processing compared with the control tRNA gene (Figure 5.6, Panel A, compare lane a with lanes d, g, and j). For the transcription unit-internal *lac*O sequence insertions, ptS*Lac*O(am5) and ptS*Lac*O(am8), the extended size of the precursor transcripts was consistent with the additional length of the inserted oligonucleotide and termination of transcription at the normal site. However, in the presence of *lac* repressor, transcription of these templates was reduced by 75 to 95% (compare lanes d and e, and lanes g and h). Moreover, in addition to the reduced levels of normal precursor products and mature tRNA, two novel intermediate-sized transcripts were generated with both ptS*Lac*O(am5) and ptS*Lac*O(am8) (X and X', lane e; Z and Z', lane h, respectively). Transcription inhibition and the synthesis of novel truncated products was not observed in reactions carried out in the presence of IPTG (lanes f an i) or when a template which lacked a *lac*O sequence was used (lane b), demonstrating that these effects were dependent upon template-bound *lac* repressor.

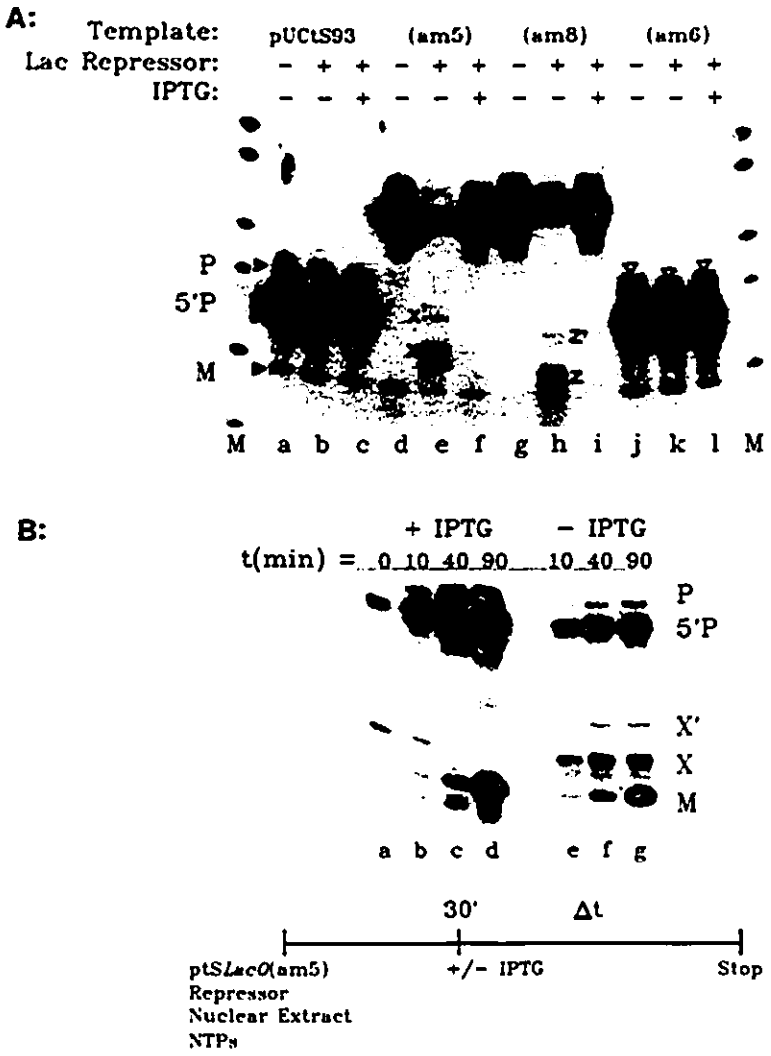


Figure 5.6 ***Lac* repressor reversibly inhibits transcription and blocks elongation by RNA polymerase III *in vitro*.** (A) Transcription reactions using the different tRNA gene templates were carried out in HeLa cell nuclear extracts for 30 min in the presence or absence of purified *lac* repressor and IPTG as indicated. P, 5' P, and M designate the transcription products of the control template, pUCtS93, and correspond to the precursor tRNA, the 5' processed intermediate, and the mature-size tRNA transcript (85 nts following 3' CCA addition), respectively. This pattern is similar for all of the templates. X', X (approximately 101 nts, 93 nts respectively) and Z', Z (approximately 97 nts, 89 nts respectively), represent novel repressor-dependent truncated transcripts. The arrowheads accent the small repressor-dependent shortening of the precursor transcripts observed with ptS*Lac*O(am6). Size markers (lane m) are in nucleotides. (B) Transcription reactions using ptS*Lac*O(am5) were scaled up and carried out as indicated in the schematic diagram. Samples were removed and processed following the addition of IPTG (lanes a-d) or water (lanes e-g) at the times (min) indicated.

In contrast, repressor bound immediately downstream of the termination site in pt*SLac*O(am6) had no affect on the level of transcription. However, the primary and 5' end processed transcription products were quantitatively shortened by 2-3 nts (data not presented; compare lanes j and k), an effect not observed in the presence of IPTG (lane l). Hence, repressor positioned proximal to the 3' boundary of the termination site functions exclusively to direct the premature termination of transcription by polIII, while apparently having no affect on polymerase recycling. *Lac* repressor placed further downstream of the termination site (section 2.2.1.4.3) had no affect on tRNA gene transcriptional activity or on the size of the transcription products (data not presented).

The relative size and abundance of the two novel repressor-dependent truncated products generated with pt*SLac*O(am5), (the major product X and less abundant product X', approximately 93 nts and 101 nts, respectively), is consistent with X being the 5' processed product of the precursor X'. Indeed, the kinetics of synthesis of the truncated transcription products follows that of the normal, full length species (Figure 5.8, lanes d to j; compare synthesis of products P and 5'P with products X' and X, respectively). The centre of the *lac*O sequence in pt*SLac*O(am8) is 3 nts further upstream compared with that in pt*SLac*O(am5) and hence the corresponding products, (Z and Z', approximately 89 nts and 97 nts, respectively) were shorter (compare lanes e and h). The truncated transcripts X'and Z' map to the 5' border of the *lac*O sequence (data not presented) therefore, synthesis is dependent upon the position of the *lac*O sequence. *Lac* repressor footprints approximately 25 nts of *lac*O DNA (Schmitz and Galas, 1979). These results are consistent with a block to polIII elongation at the boundary of the

repressor/operator complex and suggest that the active site of polIII is close to the leading edge of the transcription complex. Similar observations were noted upon arresting elongation by polI (Kuhn *et al*, 1990), polII (Deuschle *et al*, 1990) and *E. coli* RNA polymerase (Sellitti *et al*, 1987) through a *lac* repressor-mediated block. These results demonstrate that elongation by polIII can be blocked by a sequence-specific DNA-binding protein. Furthermore, arrest of polIII elongation between the tRNA gene coding region and the termination site effectively inhibits overall transcriptional activity.

5.2.2.2 *Lac* repressor-mediated inhibition of tRNA gene transcription is reversible

To determine if the block to tRNA gene transcription could be reversed, IPTG was added to ongoing transcription reactions using pt*SLacO*(am5) and the products were analysed at various times thereafter (Figure 5.6, Panel B). The addition of IPTG following 30 min of tRNA synthesis in the presence of repressor resulted in both the disappearance of the truncated species and the renewed synthesis of precursor transcripts and accumulation of mature size tRNA (compare lanes a with lanes b to d). These results demonstrate that the repressor-mediated block to transcription was indeed reversible. Interestingly, although the steady state levels of the truncated species remained unchanged in the absence of IPTG (lanes e to g), full length precursor transcripts and mature tRNA continued to slowly accumulate during the course of the reaction (see below).

Disappearance of the truncated transcripts following release of the block to polymerase elongation with IPTG may be a consequence of their extension to the full

length precursor transcripts or alternatively, their release from stalled complexes and/or 3' end processing to mature tRNA. To examine the fate of truncated transcripts derived from paused ternary complexes following alleviation of the block, reactions were carried out as in Figure 5.6, Panel B, except that heparin was added just prior to IPTG addition (Figure 5.7, Panel A). In yeast extracts, heparin permits further elongation from preformed transcription complexes which have initiated and have undergone limited elongation, however subsequent rounds of reinitiation in addition to post-transcriptional processing is prevented (Kassavetis *et al*, 1989). In HeLa cell extracts, heparin also limits transcription to a single round (Kovelman and Roeder, 1990; Figure 5.7, Panel A, compare lanes a to e; note that only 3' end processing is inhibited). As shown in Figure 5.7, Panel A, there was no change in the level of the truncated transcripts upon addition of heparin, either in the presence or absence of IPTG (lanes g to j and lanes k to n, repectively). Moreover, when excess unlabelled GTP was used rather than heparin, there was a gradual reduction in the level of truncated transcripts, which was more pronounced in the presence of IPTG (Figure 5.7, Panel B, compare lane a with lanes b to d and lanes e to g). The increase in the level of mature tRNA reflects the maintenance of functional 3' end processing under these conditions. Taken together, these findings suggest that the IPTG-dependent disappearance of the truncated transcripts which had accumulated during a prolonged repressor-mediated block to transcription elongation (Figure 5.6, Panel B), is largely due to their release and/or 3' end processing to mature tRNA, rather than renewed extension to full length products.

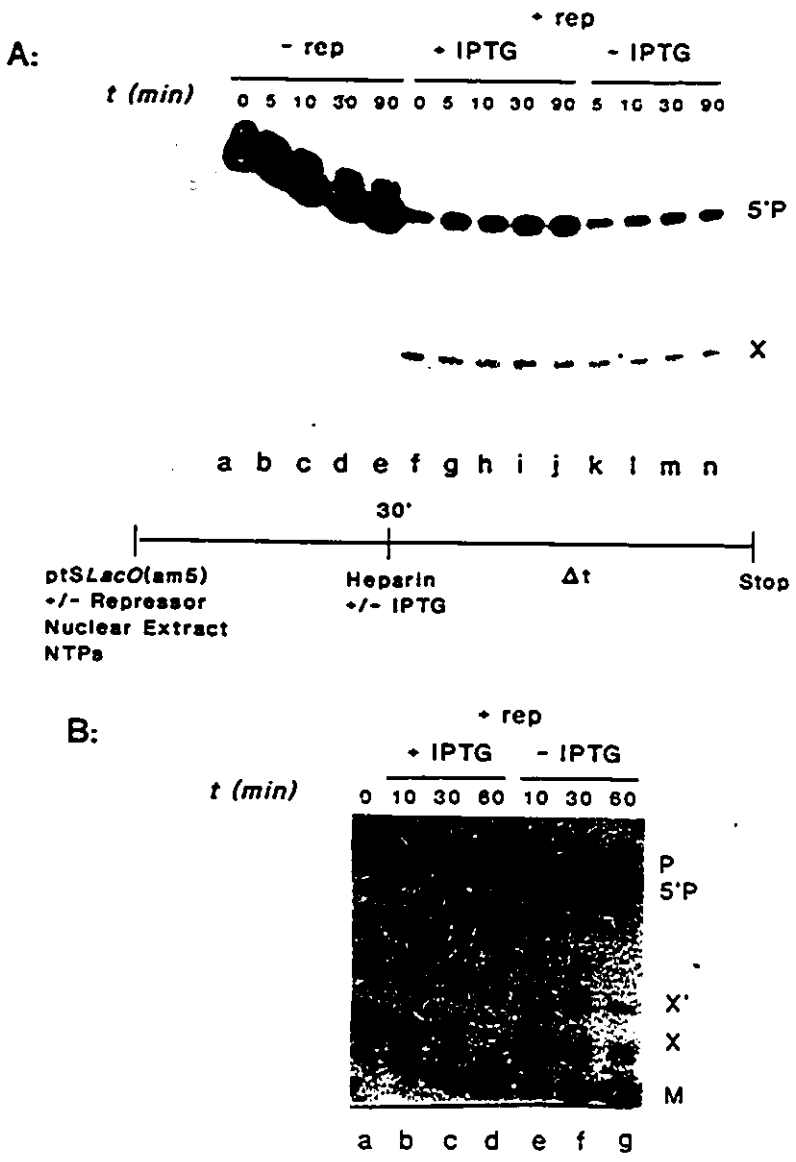


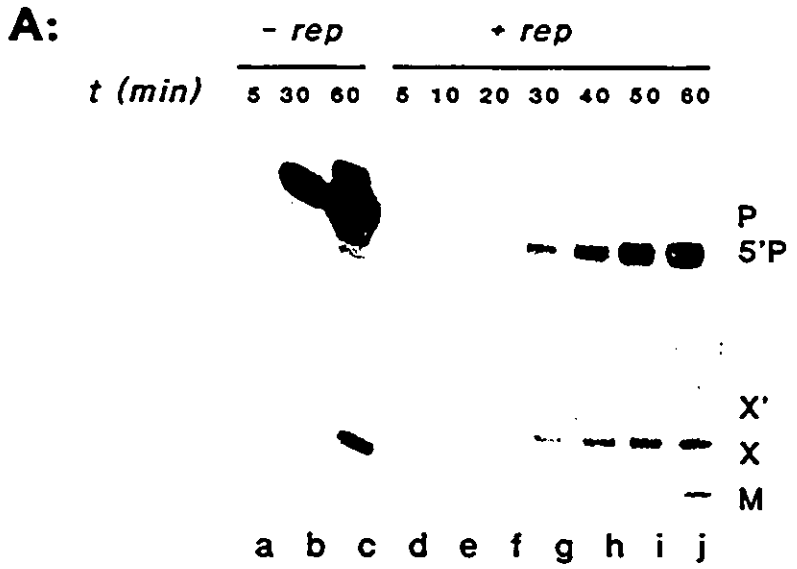
Figure 5.7 **Truncated transcripts cannot be extended following a prolonged block to elongation.** (A) Transcription reactions with pt*SLacO*(am5) were scaled up and carried out in the presence of repressor according to the schematic diagram. After 30 min of incubation, reactions were supplemented with heparin and either IPTG (lanes f-j) or water (lanes k-n) and incubation was continued for the times (min) indicated. Lanes a-e are control lanes carried out in the absence of repressor, demonstrating that the addition of heparin prevents re-initiation. (B) Reactions were identical to those above except that unlabelled GTP was used in place of heparin. Reactions were supplemented after 30 min with a 30 fold excess of unlabelled GTP and either IPTG (lanes a-d) or water (lanes e-g) and further incubated for the indicated times.

Unexpectedly, there was a moderate increase (2 fold) in the level of extended precursor transcripts following addition of IPTG (Figure 5.7, Panel A, lanes f to j). That heparin prevents reinitiation suggests that this increase in transcription may represent renewed elongation from polIII molecules which were allowed to initiate transcription but which were prevented from further elongation because of steric constraints imposed by an arrested polIII complex further downstream. In yeast, TFIIIB alone, positioned upstream of a tRNA gene, is required to recruit and position polIII over the site of initiation, and remains stably associated following advancement of the polymerase through multiple rounds of reinitiation (Kassavetis *et al*, 1990). Since mammalian polIII transcription complexes likely exhibit a similar promoter disposition (section 5.1.3), it is conceivable that mammalian TFIIIB may function through a similar fashion in support of transcription initiation by polIII. Hence, a tRNA gene transcription unit which contains a block to polIII elongation at a distal position may have sufficient capacity to accommodate the productive interaction of an additional polymerase molecule farther upstream at the site of initiation. Renewed extension of initiated transcripts may be contingent upon alleviation of the distal block to polIII elongation with IPTG. Consistent with this view, the level of both the truncated species and the precursor transcripts remained relatively unchanged in the absence of IPTG (Figure 5.7, Panel A, lanes k to n). A similar IPTG-dependent increase in the amount of normal precursor product, which could not be accounted for by a corresponding decrease in the levels of truncated products, was observed upon addition of excess unlabelled GTP in place of heparin (Figure 5.7, Panel B, compare lanes b to d with lanes e to g). These results suggest that

small class III genes such as tRNA genes may accomodate more than one RNA polymerase molecule.

5.2.2.3 RNA polIII can read through template-bound *lac* repressor

Interestingly, the block to polIII elongation mediated by *lac* repressor was not quantitative; truncated transcripts accounted for only approximately 50% of total transcription after 30 min of synthesis (Figure 5.6). Indeed, transcription of normal full length precursor products was maintained even in the presence of much higher concentrations of repressor protein (data not presented). An investigation of the kinetics of synthesis of transcription products derived from ptS*Lac*O(am5) demonstrates that both the appearance and accumulation of the truncated and precursor transcripts coincide at early times (Figure 5.8). However, upon further incubation, the truncated species reach steady-state levels while the precursor transcripts and mature tRNA continue to slowly accumulate (note Figure 5.6, Panel B, lanes e to f). Synthesis of full length precursor transcripts in the presence of a repressor-mediated block to polIII elongation may originate from templates that escape interaction with repressor. Alternatively, it is conceivable that synthesis of both the repressor-dependent truncated species and the normal precursor products are coupled in some manner. To examine these possibilities, single-round transcription assays were undertaken using ptS*Lac*O(am5) by adding heparin and NTPs after first allowing formation of a stable polIII transcription complex (Figure 5.9, Panel A). Inclusion of heparin prior to stable complex assembly completely abolishes transcription (lanes b and e) but allows a single round of transcription upon



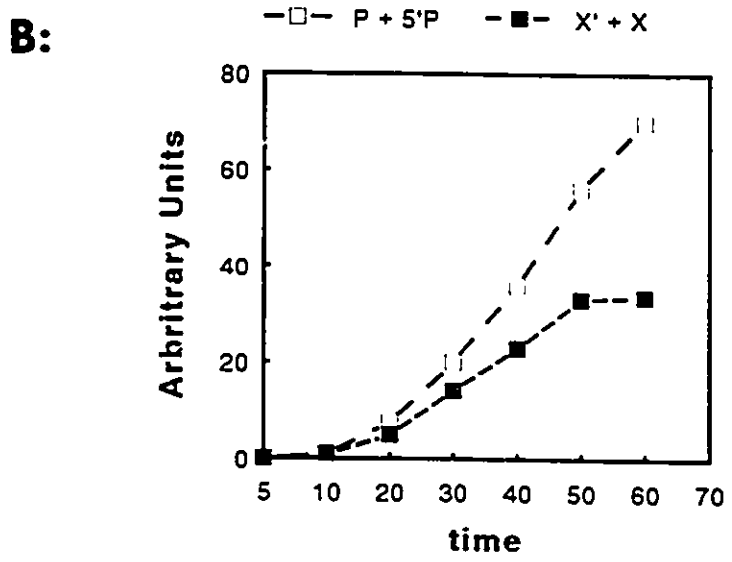


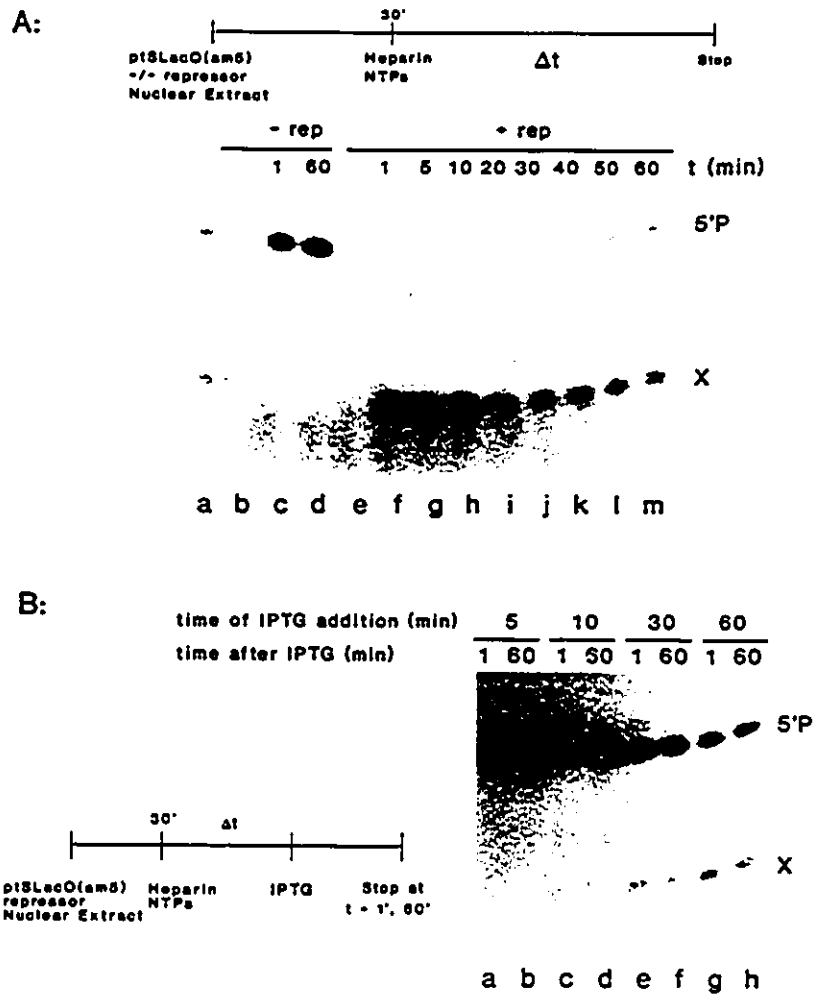
Figure 5.8 **Full length transcripts continue to be synthesized in the presence of an elongation block.** (A) Transcription reactions using ptS*Lac*O(am5) were scaled up and incubated in the absence (lanes a-c) or presence (lanes d-j) of *lac* repressor. Samples were removed and processed at the times indicated. (B) The amount of truncated transcripts (X' + X) and precursor transcripts (P' + 5'P) was quantitated by densitometric scanning of the autoradiograph in (A) (lanes d-j).

addition subsequent to preinitiation complex formation (transcript 5'P, compare lanes c and d). In the presence of repressor, the sole product synthesized within the first 5 to 10 min following heparin and NTP addition was the 5' end processed truncated transcript (X, lanes f to h). Moreover, the absolute level of single-round transcription in the absence or presence of repressor was identical (compare 5'P and X in lanes c and f, respectively). These results suggest that repressor bound downstream of the tRNA gene coding region does not affect transcription complex assembly or initiation and furthermore, that repressor binds quantitatively to all templates which are competent for transcription. These results discount the possibility that normal precursor products originate from templates that may have escaped interaction with repressor.

Upon further incubation, the 5' end processed precursor transcript began to accumulate while there was a concomitant decrease in the level of X (lanes i to m). These results establish a precursor-product relationship between the repressor-dependent truncated transcript and the extended product and demonstrate that at least some of the paused polIII ternary complexes can directly overcome a repressor-mediated block to elongation. Hence, the full length precursor transcripts synthesized in the presence of a *lac* repressor-mediated obstacle to elongation by polIII are due to direct transcriptional readthrough.

The truncated transcripts which had accumulated during a prolonged block to elongation were incapable of further extension (Figure 5.6, Panel B; see text) and moreover, only approximately 40 to 50% of the truncated transcripts synthesized under single round transcription assay conditions could be elongated to the full length precursor

Figure 5.9 **Paused ternary complexes can read through template-bound repressor and are competent for renewed elongation upon removal of the block.** (A) Transcription reactions were carried out according to the schematic diagram. Samples were removed and processed following the addition of heparin and NTPs at the times (min) indicated. Preincubation was for 30 min at 25°C. Lanes b and e are 60 min reactions which show that inclusion of heparin prior to complex assembly inhibits transcription, whereas addition of heparin after complex assembly allows initiation but prevents re-initiation (compare lanes c and d). In the presence of template-bound repressor, X is the first transcript to appear (lanes f-h) and is partially chased to full length 5' processed product (lanes i-m). Lane a is from a standard transcription reaction carried out using pt*SLac*O(am5) in the presence of repressor to indicate the positions of the normal 5' processed precursor transcript and the repressor-dependent transcript X. This reaction was diluted 20 fold prior to loading to normalize the band intensities with respect to the other lanes. (B) Transcription reactions were carried out according to the schematic diagram and were essentially as in (A) except that IPTG was added at the times indicated and incubation was continued for either 1 min or 60 min. The results show that truncated products associated with paused ternary complexes can be quantitatively extended at early (lanes a-d) but not at late times (lanes e-h). Autoradiographs in (A) and (B) were over-exposed compared to previous figures in order to visualize the low level of radionucleotide incorporation under these conditions.


A:
30'
ptSLacO(sm6)
+/- repressor
Nuclear Extract
Heparin
NTPs
Δt
Stop
- rep
+ rep
1 60
1 5 10 20 30 40 50 60
t (min)
5'P
X
a b c d e f g h i j k l m
B:
time of IPTG addition (min)
5 10 30 60
time after IPTG (min)
1 60 1 60 1 60 1 60
5'P
X
30'
Δt
ptSLacO(sm6)
repressor
Nuclear Extract
Heparin
NTPs
IPTG
Stop at
t = 1', 60'
a b c d e f g h

product (Figure 5.9, Panel A). Hence, the ability of arrested ternary polIII transcription complexes to remain competent for elongation was examined more closely. In Figure 5.9, Panel B, IPTG was added to repressor-arrested polIII complexes at various times following addition of heparin and NTPs, and incubation was allowed to continue for either 1 min or 60 min. Complexes which had been arrested for up to 10 min prior to IPTG addition were fully capable of rapidly and quantitatively extending nascent truncated transcripts to the normal 5' processed precursor product (lanes a to d). These results indicate that at early times, all truncated transcripts are associated with paused ternary complexes that are poised for renewed elongation upon removal of the repressor-mediated block. However, for times thereafter, the ability to extend these nascent chains was progressively reduced (lanes e to h), suggesting that a prolonged factor-mediated block to polIII elongation may result in the destabilization of arrested complexes or release of nascent chains, a process which is functionally analogous to termination. It is conceivable that prolonged factor-mediated pausing of polIII may generate stable dead-end ternary complexes that are functionally incompetent for elongation, analogous to those observed upon halting *E. coli* RNA polymerase (Arndt and Chamberlin, 1990). The overall inhibition of transcription that is observed in the presence of repressor may be due to the formation of such stable abortive complexes and/or to a lag in the dissociation of stalled ternary complexes and hence, subsequent rounds of transcription reinitiation. Since repressor bound downstream of the tRNA coding region had no affect on transcription complex assembly or initiation (Figure 5.9, Panel A), transcript release and polIII recycling immediately upon reaching the elongation block would not be expected

to affect overall levels of transcription. Indeed, *lac* repressor positioned immediately downstream of the natural termination site directed premature transcription termination while having no affect on the level of transcription (Figure 5.6, Panel A, lanes j and k).

Taken together, the results suggest that the steady-state level of synthesis of the repressor-dependent truncated transcripts under multiple round transcription conditions (note Figure 5.8), reflects a balance between depletion of the truncated transcripts through both transcriptional readthrough and termination/processing, resulting in the accumulation of the extended precursor transcripts and mature tRNA, respectively, and replenishment through subsequent rounds of reinitiation. Precursor transcripts continue to accumulate since they are depleted only through processing reactions, which become saturated under multiple round transcription conditions (data not presented).

5.2.2.4 The *Lac* Operator/Repressor System in the Study of Transcription Elongation and Termination by Mammalian RNA PolIII: Conclusions

The results demonstrate that elongation by mammalian polIII can be reversibly blocked by a sequence-specific DNA binding protein, resulting in the formation of paused polIII ternary complexes which effectively mediate the inhibition of transcriptional activity. Alternatively, arrest of elongation by polIII can also result in premature transcription termination. Furthermore, a subset of paused polIII ternary complexes maintains the ability to directly transcribe through a factor-mediated block to elongation. Interestingly, paused mammalian polIII ternary complexes can acquire different functional properties that are dictated by the position and the period for which they are stalled in the tRNA gene transcription unit.

5.2.3 Analysis of RNA Chain Elongation and Termination by Mammalian RNA PolIII: Implications

5.2.3.1 Attenuation of Transcription by RNA PolIII

This work demonstrates, for the first time, that elongation by polIII can be reversibly blocked by a protein obstacle *in vitro*. Hence, the conditional stalling of polIII may represent a functional regulatory mechanism in which class III genes are primed for expression. Further support for such a prospect comes from findings which suggest that small class III genes can accommodate greater than one molecule of polIII at a time (Figure 5.7). Moreover, that the formation of paused polIII ternary complexes, through a factor-mediated block to polIII elongation, results in the dramatic reduction of overall transcriptional activity, further suggests that sequence-specific DNA binding proteins may function *in vivo* to attenuate transcription of class III genes.

The evolutionary inception of such a strategy in the regulation of tRNA genes may be dependent upon adherence to a number of requisite conditions, imposed by the intragenic nature of tRNA gene promoters and the small size of the tRNA gene transcription unit. Accessibility of a factor to its cognate DNA binding site within the tRNA gene transcription unit must be maintained, while having no affect on transcription complex assembly or initiation. This is a demanding condition given the expanse of the polIII transcription complex (Figure 5.3). Moreover, the utility of a regulatory mechanism which adopts the conditional pausing of polIII to mediate attenuated tRNA gene transcription is also dependent upon precluding synthesis of the 3' end of the tRNA gene coding region and thus, liberation of the mature tRNA transcript through post-transcriptional processing. Stringent regulation of transcription may be afforded by

stalling polIII through an intrinsic pause site or a factor-mediated block at a coding region-internal position, thereby blocking RNA chain elongation prior to synthesis of the 3' end of the tRNA coding region. However, the capacity for nucleotide diversity, in establishing intrinsic pause sites or factor binding motifs at tRNA coding region-internal positions, may be limited since sequence alterations must conform to the stringent constraints on tRNA gene sequence in maintaining the functional and structural integrity of the tRNA product, in addition to the transcriptional properties of the gene. Moreover, association of a factor with intragenic sequences would likely affect polIII transcription complex formation (section 5.1.1).

Alternatively, binding of a factor to transcribed sequences downstream of the 3' end of the tRNA coding region which, except for the site of termination, are nonessential for transcription, may serve to regulate elongation by polIII. Indeed, *lac* repressor protein can access such sequences both prior to (Figure 5.9, Panel A) and following (data not presented) preinitiation complex assembly without affecting transcription complex formation or initiation. This result may delineate the 3' extremities of the mammalian polIII transcription complex to within 10 nts downstream of the tRNA gene coding region (see section 5.1.3). *Lac* repressor positioned downstream of the tRNA gene coding region effectively blocked elongation by polIII however, synthesis of the full length primary pre-tRNA transcript was not completely prevented (Figure 5.6, Panel A). This can be ascribed to the ability of a subset of paused polIII ternary complexes to undergo direct transcriptional readthrough of DNA-bound *lac* repressor (Figure 5.9, Panel A). Subsequent trimming of 5' leader and 3' extension sequences of the primary pre-tRNA

readthrough transcript results in the generation of mature tRNA transcript. Regardless, blocking elongation by polIII dramatically reduced overall transcriptional activity (Figure 5.6, Panel A).

Synthesis of mature tRNA transcript may also be attributed directly to 3' end processing of truncated transcripts derived from stalled polIII complexes (Figure 5.7, Panel A). Truncated transcripts synthesized in the presence of a repressor-mediated block to elongation extend to the boundary of the *lac*O sequence downstream of the tRNA gene coding region (Figure 5.6, Panel A; data not presented) and, as such, are subject to processing. The nature of truncated transcripts derived from such stalled complexes is not known. Dissociation from paused polIII ternary complexes may render truncated transcripts amenable to processing. Alternatively, it is also conceivable that such truncated transcripts remain nascent but which themselves may directly serve as substrates for 3' end processing. A block to polIII elongation through binding of a factor immediately 3' proximal to the tRNA coding region may preclude synthesis of this region outright, thereby preventing liberation of the mature tRNA transcript through a 3' end processing event. It is interesting to note that the 3'-proximal nucleotides of nascent polII RNA transcripts may remain polymerase-associated within an RNA binding site (Rice *et al*, 1991). The sustained association with polIII upon polymerase stalling, of the region of the nascent transcript comprising the 3' end of the tRNA gene coding region, may preclude release of mature tRNA transcript through a processing event by rendering the nascent transcript refractory to access by processing enzymes. In this manner, the appropriate positioning of a factor-mediated block to polIII elongation may effectively

prevent synthesis of mature tRNA through a processing pathway.

Interestingly, transcription *in vitro* of *lac*O-tDNA derivatives in which two spatially separated *lac*O sequences have been positioned between the tRNA coding region and the termination site (section 2.2.4.3), results in the synthesis of truncated transcripts, intermediate in size between primary and mature transcripts, in a repressor-independent manner (data not presented). These effects were not observed upon insertion of a single *lac*O sequence, suggesting that RNA secondary structure may mediate polymerase stalling. Indeed, RNA secondary structure in nascent mRNA transcripts mediates the attenuation of transcription of a number of class II genes (Spencer and Groudine, 1990; Richardson, 1993).

An artificial and potentially feasible approach to manipulate tRNA gene transcription at the level of elongation may employ a synthetic intron encoding the cognate recognition site for a sequence-specific DNA-binding protein. Looping out of the intron-containing DNA-binding factor would presumably restore the function of the tRNA gene intragenic promoter, thereby allowing preinitiation complex assembly, and facilitating the positioning of a factor-mediated block to polIII elongation at a gene-internal site. Intron-containing pre-tRNAs are found predominantly in yeast, but have been identified in a variety of eukaryotes (Deutscher, 1984; Phizicky and Greer, 1993). Heterologous introns inserted into normally intronless yeast (Winey *et al*, 1989) and mammalian (Drabkin, 1988) tRNA genes are efficiently removed and have no affect on functional tRNA gene expression.

In summary, positioning of *lac* repressor 18 nts and 21 nts downstream of the

tRNA coding region (*lac*O centre in ptS*Lac*O(am8) and ptS*Lac*O(am5), respectively; Figure 5.5), does not completely inhibit synthesis of mature tRNA transcript *in vitro*. A block to polIII elongation at these positions may result in either the reversible pausing of polymerase, in which polIII transcription complexes retain the ability to undergo direct transcriptional readthrough or renewed elongation upon removal of the block, or in the irreversible stalling of polIII, in which complexes no longer maintain the capacity to elongate and the transcripts derived ultimately undergo processing to the mature tRNA transcript. Regardless of the outcome, it is evident that the conditional arrest of polIII elongation *in vitro*, by virtue of the temporal dependence associated with transcript processing and/or transcriptional readthrough, results in the inhibition of tRNA gene transcriptional activity. DNA binding factors may adequately serve in the attenuation of transcription by polIII and hence, in the regulation of class III genes. However, such a regulatory mechanism in the control of class III gene expression has yet to be identified. It is interesting to note, however, that a tract of four T residues located at a coding region-internal position on a *Xenopus laevis* $tRNA^{Lys}$ gene, apparently can differentially mediate premature termination by polIII *in vivo*, and thus may function to control tRNA gene expression (Mazabraud *et al*, 1987).

5.2.3.2 RNA PolIII Elongation Through the Multi-Component PolIII Preinitiation Complex

Transcriptional readthrough of *lac* repressor by polIII may represent an active process whereby polIII transiently displaces or traverses template-bound repressor protein. However, it is conceivable that the passive dissociation of repressor may also

afford renewed elongation by polIII (repressor binds to a single wild type *lac*O sequence with a dissociation constant of 1 X 10^{-13}M and a half life of 30 min at 24°C *in vitro* (Barkley and Bourgeois, 1980)). Determining the fate of repressor protein upon readthrough by polIII may aid in defining the molecular mechanisms by which RNA polymerases transcribe through DNA binding proteins and furthermore, may bear relevance to understanding the capacity of polIII molecules to elongate through polIII transcription complexes.

Several RNA polymerases can transcribe through DNA binding proteins *in vitro*, however, the response to such obstacles in the path of elongation has been variable. Prokaryotic RNA polymerases can undergo transcriptional readthrough (Horowitz and Platt, 1982; Pavco and Steege, 1991), termination (Deuschle *et al*, 1986) or stable arrest (Sancar *et al*, 1982; Pavco and Steege, 1990) when subjected to a factor-mediated block. Moreover, while elongation by polI (Kuhn *et al*, 1990), polII (Deuschle *et al*, 1990; Kuhn *et al*, 1990), and polIII (Figure 5.6, Panel A) can be blocked by DNA-bound *lac* repressor, a subset of paused polIII ternary complexes maintains the ability to elongate through repressor protein (Figure 5.9, Panel A). Interestingly, unlike SP6 RNA polymerase and polII, in which transcription is unobstructed (Lorch *et al*, 1987; Losa and Brown, 1987), polIII is unable to transcribe through an assembled nucleosome *in vitro* (Morse, 1989). Furthermore, whereas transcription by polI can be efficiently terminated by the DNA-binding factor TTF-1, elongation by polII, polIII and several prokaryotic RNA polymerases is unimpeded, implicating a role for specific protein-protein interactions between polI and TTF-1 in mediating termination of polI transcription (Kuhn

et al, 1990). The nature of the disparity in the reponse of polIII (and other RNA polymerases) to factor-mediated blocks to elongation is not understood. The ability of RNA polymerases to overcome factor-mediated blocks to elongation may reflect an inherent property of the polymerase ternary complex, although the molecular mechanisms by which this property becomes manifest have not been identified. While RNA polymerase elongation through heterologous DNA binding proteins is likely devoid of requisite protein-protein interaction, the ability of polIII to faithfully transcribe through its own multi-component transcription complex may lie in specific contacts between polIII and components of the transcription complex. Indeed, elongation by polIII through yeast TFIIIC assembled on a tRNA gene intragenic promoter is orientation-specific (Bardeleben *et al*, 1994; see below). Alternatively, the ability of the polIII transcription complex to withstand passage by polIII may reflect an inherent property of the complex itself.

Class III gene transcription complexes, by definition, are inherently stable, competent to support renewed initiation through multiple rounds of transcription (section 4.1). Indeed, the functional integrity of *Xenopus* 5S RNA gene transcription complexes is maintained through at least 40 rounds of RNA synthesis (Bogenhagen *et al*, 1982); moreover, such complexes apparently exhibit a dynamic stability in form, since they can withstand repeated passage by a prokaryotic RNA polymerase without disruption (Wolffe *et al*, 1986). Hence, polIII transcription complexes may be predisposed to permitting passage of RNA polymerase, perhaps by undergoing a conformational change in response to an elongating polymerase. For tRNA genes, individual components of the polIII transcription complex, such as TFIIIC, may be transiently dissociated and/or maintained

through protein-protein interactions with TFIIIB, permitting elongation by polIII through the intragenic TFIIIC DNA binding domain (Huibregste and Engelke, 1989). It is also possible that successive binding of TFIIIC between the A box and B box may facilitate elongation by polIII (Wolffe, 1991).

However, the nature of class III gene transcription complexes upon elongation by polIII is poorly understood. Protein crosslinking studies *in vitro* and footprinting analyses both *in vivo* and *in vitro* clearly demonstrate that TFIIIB remains stably associated upstream of a yeast tRNA gene during transcription (Huibregtse and Engelke, 1989; Kassavetis *et al*, 1990; Bartholomew *et al*, 1990, 1993; Geiduschek and Kassavetis, 1992). The fate of TFIIIC which, in yeast, encompasses essentially the entire tRNA gene coding region, is less clear. These studies suggest that TFIIIC or components of TFIIIC may also remain associated with the tRNA gene during elongation by polIII however, this association may reflect protein-protein interactions rather than direct interaction of TFIIIC with DNA. Interestingly, yeast TFIIIC is completely dispensable for multiple rounds of initiation by polIII *in vitro*, once TFIIIB has been stably assembled (Kassavetis *et al*, 1990). Hence, the inherent stability of the polIII transcription complex may primarily relate to a functional stability manifested as a sustained competence to support repeated rounds of initiation, rather than the maintenance of preinitiation complex structural integrity through elongation. Indeed, it is conceivable that components of the transcription complex such as TFIIIC in yeast, which apparently function exclusively in the assembly of initiation factor TFIIIB, may dissociate during the elongation phase (Kassavetis *et al*, 1990). Recent experiments *in vitro* demonstrate that TFIIIC, bound to

the intragenic promoter of a yeast tRNA gene, has little affect on the rate of elongation by polIII and is readily displaced, suggesting that TFIIIC may place no restriction on gene activity *in vivo* (Bardeleben *et al*, 1994; Matsuzaki *et al*, 1994). These results, and those which demonstrate that TFIIIA is displaced by an elongating polymerase when bound alone to the *Xenopus* 5S RNA gene intragenic promoter (Campbell and Setzer, 1991), suggest that TFIIIC (and TFIIIA) may not remain template-associated during polIII transcription. However, in these studies, the fate of such factors during elongation by polIIII was not addressed within the context of a fully formed, active polIII transcription complex. Indeed, while TFIIIC binds tightly to DNA (Baker *et al*, 1986), the extreme stability of tRNA gene transcription complexes can be ascribed to the association of both TFIIIB and TFIIIC (Jahn *et al*, 1987; Kassavetis *et al*, 1989). Conversely, it is interesting to speculate that regulation of the association of TFIIIC with its intragenic binding site may act to modulate elongation by polIII. Indeed, the sustained association of TFIIIC with the class III gene promoter may serve in transcription unit occlusion from DNA binding proteins and chromatin assembly and thus, TFIIIC may function in the maintenance of transcriptional activity *in vivo* (Burnol *et al*, 1993; Bardeleben *et al*, 1994). In summary, the molecular mechanisms which underly transcription elongation by polIII through class III gene intragenic promoters, and the ensuing fate of TFIIIC, remain poorly defined.

5.2.3.3 Ternary Transcription Complexes of RNA PolIII

Ternary transcription complexes of *E. coli* (Arndt and Chamberlin, 1990; Krummel and Chamberlin, 1992a,b; Erie *et al*, 1993) and eukaryotic (Linn and Luse, 1991; Kassavetis *et al*, 1992a; Marshall and Price, 1992) RNA polymerases are dynamic entities that can undergo both structural and functional transitions during elongation. Structural differences in ternary complexes in *E. coli* can serve as potential targets for regulation through recognition by accessory factors which act to mitigate the response to termination signals (Mason and Greenblatt, 1991; Roberts, 1993). Indeed, accessory factors that interact directly with or modify RNA polymerase ternary complexes may be required during normal elongation to prevent premature polymerase arrest at intrinsic sites (Kerppola and Kane, 1991). Interestingly, the response of polII ternary complexes to termination sequences can be influenced by the nature of the promoter (Hernandez and Weiner, 1986; Hernandez and Lucito, 1988), thereby implicating a role for promoter-specific modifications to early transcribing complexes in establishing the transcription elongation properties of RNA polymerases. As well, the consequence of premature pause sites on elongation by polII may also be influenced by proximity to the site of initiation (Krumm *et al*, 1992; Roberts and Bentley, 1992), and may reflect structural alterations, to the initial promoter-defined polII ternary complex, which serve to modulate RNA polymerase processivity through sequences farther downstream. In light of these findings, it is interesting to speculate that newly initiated polIII ternary complexes, positioned proximal to the site of initiation on a tRNA gene transcription unit, maintain an enhanced capacity to elongate through the polIII transcription complex. The propensity of the polIII

ternary complex to read through blocks to elongation may progressively decrease thereafter, through structural transitions or the cycling of accessory factors. While there is no evidence in support of such a view, it may be informative to assess the ability of polIII to transcribe through a factor-mediated block at more distal sites.

RNA polIII ternary complexes are thought to undergo structural transitions upon elongation arrest, thus comprising an assortment of dynamic structural states (Bartholomew *et al*, 1993). That the ability of stalled polIII ternary complexes to undergo elongation through DNA-bound *lac* repressor was a temporally dependent event (Figure 5.9, Panel A), may represent a slow rate of transition between such structural states and the ability of only one such conformation to promote elongation through a factor-mediated block. The ability of paused polIII ternary complexes to remain competent for transcription through *lac* repressor was also dependent upon the period for which polIII was paused since only a subset of polIII ternary complexes were competent for elongation through *lac* repressor (Figure 5.9, Panel A). This may also reflect the properties of functionally distinct complexes or alternatively, a temporal dependence associated with the ability of paused polIII ternary complexes to remain competent for elongation. Truncated transcripts derived from polIII ternary complexes subjected to repressor-mediated pausing for short periods remained nascent and were quantitatively extended following removal of repressor (Figure 5.9, Panel B). However, for extended periods of polIII arrest, the ability of paused polIII transcription complexes to undergo renewed elongation following removal of repressor progressively decreased. This may reflect a kinetic competition between elongation and termination pathways (von Hippel and Yager,

1991, 1992) or the destabilization of ternary complexes. The nature of these complexes remains undefined, however it is conceivable that prolonged factor-mediated pausing of polIII ternary complexes results in structural transitions or alterations which lead to the production of stable, abortive complexes which are functionally incompetent for renewed elongation, analogous to *E. coli* dead-end ternary complexes (Arndt and Chamberlin, 1990). The dissociation, from paused polIII ternary complexes, of accessory factors normally required for elongation and/or transcriptional readthrough may underly such structural alterations.

Several factors involved in modulating elongation by both *E. coli* RNA polymerase and polII have been identified, including those which mediate elongation through premature termination or pause sites (Kerppola and Kane, 1991; Kassavetis and Geiduschek, 1993; Richardson, 1993). The GreA and GreB proteins can render dead-end ternary transcription complexes in *E. coli* competent for renewed elongation (Borukhov *et al*, 1992, 1993), while TFIIS, thought to interact transiently with polII at pause sites (Sluder *et al*, 1989; Bengal *et al*, 1991; Archambault *et al*, 1992), can facilitate elongation through both intrinsic arrest sites (Reines *et al*, 1989; SivaRaman *et al*, 1990) and a factor-mediated block (Reines and Mote, JR., 1993). These elongation factors confer a 3' to 5' exonuclease activity upon a paused RNA polymerase ternary complex (Borukhov *et al*, 1992, 1993; Izban and Luse, 1992; Reines, 1992; Wang and Hawley, 1993), promoting the hydrolytic cleavage of nascent transcripts as a prerequisite to their renewed extension. The question of whether ribonucleolytic transcript cleavage represents an activity inherent to the RNA polymerase that requires activation by such elongation

factors, or is an activity intrinsic to these factors themselves, has not yet been resolved (Kassavetis and Geiduschek, 1993). A 3' to 5' exonuclease activity associated with highly purified yeast polIII ternary complexes has recently been identified, and may be intrinsic to polIII itself, since transcript hydrolysis is apparently independent of a dissociable TFIIS-like factor (Whitehall *et al*, 1994). Indeed, it is conceivable that such an activity may mediate elongation by polIII through TFIIIC (Bardeleben *et al*, 1994). It is not yet known whether nascent RNA chains associated with stalled mammalian polIII ternary complexes can undergo 3' to 5' hydrolytic retraction. Yeast TFIIS was unable to effect the renewed extension of a mammalian polIII ternary complex which was subjected to *lac* repressor-mediated pausing, although the functional integrity of this factor was not established (data not presented). It is notable, however, that repressor blockage of mammalian polIII elongation results in the generation of truncated species that appear heterogeneous in size (species X, Z; Figure 5.6, Panel A).

5.2.3.4 Termination of Transcription by Mammalian RNA PolIII

Termination of transcription by polIII is directed primarily by a stretch of T residues in the noncoding DNA strand (section 4.1), although termination efficiency may be influenced by sequences flanking the termination signal (Bogenhagen and Brown, 1981; Mazabraud *et al*, 1987). Presumably, instability of the U:A hybrid RNA/DNA tract (Martin and Tinoco, Jr., 1980; Wang and Folk, 1994) results in the destabilization of the nascent RNA transcript, thereby affording transcript dissociation and polymerase recycling through subsequent rounds of initiation. Although DNA-binding proteins effect

termination of transcription by mitochondrial RNA polymerase (Christianson and Clayton, 1988; Kruse *et al*, 1989) and polI (Kuhn *et al*, 1990; McStay and Reeder, 1990), no such factors have been implicated in mediating termination by polIII. RNA polIII maintains intrinsic termination activity since accurate termination can occur in the absence of accessory factors (Cozzarelli *et al*, 1983; Watson *et al*, 1984; Matsuzaki *et al*, 1994).

However, the molecular mechanisms by which elongating polIII ternary complexes terminate transcription are less well understood. Indeed, little is known regarding the interaction between the polIII ternary complex and the termination signal, although accessory factors are clearly involved in the mammalian system. The eukaryotic transcription termination factor La, which associates with the U-rich 3' termini of nascent polIII transcripts, exhibits nucleic acid-dependent ATPase/dATPase and helicase activities, and catalyzes the ATP-dependent dissociation of the nascent RNA/DNA duplex (Bachmann *et al*, 1990). While La protein is required for efficient transcript release and polymerase recycling (Gottlieb and Steitz, 1989a,b; Maraia *et al*, 1994), polIII itself may be directly responsible for termination site recognition and subsequent polymerase pausing (James and Hall, 1990; James *et al*, 1991). Indeed, a termination event is dependent upon the fate of the nascent RNA transcript, unlike termination signal recognition. Thus polymerase release and termination signal recognition by polIII can be experimentally uncoupled and may be mechanistically distinct processes (Campbell and Setzer, 1992).

Interestingly, depletion of La antigen from HeLa cell nuclear extracts results in the dramatic reduction of overall polIII transcriptional activity and can be ascribed to the reversible stalling of polIII just upstream of the termination site (Gottlieb and Steitz, 1989a,b). La protein influences transcript completion since transcripts derived from residual synthesis are shortened by 1 to 5 nts. These results are reminiscent of those derived upon imposing a factor-mediated block to polIII elongation between the tRNA gene coding region and the termination site (Figure 5.6, Panel A), and support the view that impaired or delayed recycling of polIII may underly *lac* repressor-mediated inhibition of tRNA gene transcriptional activity. Modulation of polIII recycling thus represents a potential mechanism by which class III gene transcription may be attenuated.

Polymerase pausing is thought to be prerequisite to transcription termination (Platt, 1986). However, arrest of elongation by polIII does not necessarily lead to termination of transcription since truncated transcripts derived from polIII ternary complexes which have been transiently paused by *lac* repressor can be quantitatively extended following alleviation of the block. However, prolonged factor-mediated pausing of polIII results in the generation of ternary complexes which, although remaining stable, are incompetent for renewed elongation (Figure 5.9, Panel B). This process is functionally equivalent to termination since truncated transcripts are eventually processed to mature tRNA transcript (Figure 5.7). In stark contrast to the effects of positioning *lac* repressor between the tRNA gene coding region and the termination site, *lac* repressor positioned downstream of the termination site mediated premature polIII transcription termination, but otherwise had no affect on nascent transcript release, polymerase

recycling, or re-initiation, since overall transcriptional activity was unaffected (Figure 5.6, Panel A). Transcripts were approximately 2 to 3 nts shorter than normal precursor transcripts, in an IPTG-reversible manner, suggesting that repressor-imposed steric contraints on polIII elongation resulted in polymerase pausing just upstream of the termination site. Such polIII ternary complexes then undergo rapid dissociation, in direct contrast to those paused further upstream. These results, and those previously discussed, suggest that mammalian polIII ternary complexes can acquire different functional properties upon factor-mediated elongation arrest.

The disparity in the fate of mammalian polIII ternary complexes upon arrest at different positions within the tRNA gene transcription unit is not understood. It is conceivable that pausing of polIII just upstream of the termination signal may be influenced by the proximity of the natural termination sequence. RNA polIII may still have access to the termination signal even under spatial constraints imposed by bound *lac* repressor. Alternatively, associated termination factors such as the La protein may confer termination signal recognition unto the polIII ternary complex. These results may indicate that termination signal recognition occurs at the leading edge of the polIII ternary complex, but that the precise point of termination by paused polIII is flexible, since termination of transcription was premature. However, factor-mediated termination of polIII transcription may be mechanistically distinct from normal termination, which requires recognition of, and pausing at, the normal termination signal. Indeed, *lac* repressor bound downstream of the termination signal effected premature termination, apparently independent of U:A hybrid RNA/DNA tract formation. It is possible,

however, that RNA transcript 3' end formation may be a manifestation of rapid processing rather than a true termination event, although extended precursor transcripts have not been detected (data not presented).

Alternatively, premature termination by polIII, upon arrest directly upstream of the termination signal, may be a function of the distance from the site of initiation, rather than of proximity to the termination signal. RNA polIII that is paused at this distal site may be rapidly displaced by a second elongating polIII molecule, originating from a second productive initiation event. In contrast, RNA polIII molecules paused further upstream of the termination signal apparently allow the assembly and initiation of a second polymerase molecule (Figure 5.7), but may preclude a functional transition from newly initiated polIII ternary complexes to productive elongating polIII ternary complexes (Kassavetis *et al*, 1992a) through polymerase-imposed steric constraints, thereby averting polymerase dissociation. Such initiation phase to elongation phase transitions are best understood in *E. coli* (Gill *et al*, 1990). It is conceivable, however, that sequence context alone, immediately upstream of the termination signal, may cause the polIII ternary complex to assume a conformation which is destabilized upon pausing.

5.2.4 Analysis of RNA Chain Elongation and Termination by Mammalian RNA PolIII: Outlook

The results demonstrate that a sequence-specific DNA-binding protein can be used to examine the molecular mechanisms which govern elongation and termination of transcription by mammalian polIII. The ability to selectively pause polIII at defined locations within the class III gene transcription unit, through a *lac* repressor-mediated

block to elongation, affords a novel and potentially useful strategy to generate homogeneous populations of discrete mammalian polIII ternary complexes and hence, to study their functional and biochemical properties. Indeed, the composition of polIII ternary complexes remains poorly defined. Resolving the complement of auxillary factors required to sustain elongation by polIII is of particular importance. Moreover, such a factor-mediated "tag" on arrested polIII ternary complexes may form the basis of a functional assay for the identification and characterization of factors which act in trans to modulate transcription termination by polIII, and those which direct the response of polIII ternary complexes in the face of obstacles to elongation. Such a system may complement more conventional approaches towards the biochemical analysis of polIII transcription complexes, which have been of limited utility in mammalian systems (Geiduschek and Kassavetis, 1992). This work represents a preliminary investigation of the molecular mechanisms by which polIII elongates through its own transcription complex and may be relevant to understanding how RNA polymerase molecules transcribe through DNA-bound templates.

The molecular mechanisms which underly regulated eukaryotic class III gene expression are poorly defined. These results demonstrate that elongation and termination by mammalian RNA polIII can be modulated *in vitro* by a DNA binding protein. Hence, although biological relevance has yet to be borne out, conditional factor-mediated blocks to elongation by polIII may serve to attenuate transcription of class III genes *in vivo*. The results warrant the search for regulatory mechanisms which function to control class III genes at the level of transcription elongation.

6. SYNOPSIS

Gene regulation is ultimately the fundamental process which directs the normal development and preservation of an organism. In eukaryotes, delineating the mechanisms by which class II genes are controlled has thus far received the most attention. However, interest regarding the mechanisms which underly polIII transcription has been renewed in recent years, with the demonstration in higher eukaryotes that class III genes can be subject to regulated transcription. Transcription by polIII is best understood in yeast, however, relatively little is known of the mechanisms which constitute both basal and regulated transcription by polIII in higher eukaryotes. Indeed, such investigations in mammalian systems have been rather problematic (Geiduschek and Kassavetis, 1992). This thesis represents work directed towards the development and use of novel approaches for the study of eukaryotic class II and class III gene expression.

The *E. coli lac* operator/repressor system has afforded a novel means of investigating the mechanisms of transcription by mammalian polIII. Indeed, *lac* repressor protein has served as a useful reagent to examine the initiation, elongation, and termination phases of transcription by polIII. Such an approach has advanced our understanding of the promoter disposition of mammalian class III gene transcription complexes which, otherwise, have been difficult to study. As well, the functional properties of both mammalian polIII preinitiation complexes and elongating polIII ternary

complexes have been rendered amenable to study. The utility of this system may be extended further by serving as a novel approach to isolate such transcription complexes, and consequently, to examine their biochemical properties. This work has illustrated a number of plausible mechanisms whereby DNA binding proteins may confer transcriptional regulation upon class III genes. Indeed, the *lac* operator/repressor system has afforded a novel strategy for the efficient manipulation of su^+ tRNA gene expression in mammalian cells. Such an inducible su^+ tRNA gene may facilitate the establishment of additional mammalian nonsense suppressor cell lines. Mammalian nonsense suppressor cell lines have been useful in the genetic analysis of small RNA and DNA animal viruses, and have mediated the isolation of a number of interesting mutants of H^r -1, including one such mutant which is apparently defective in the regulation of class II gene expression. The results of this work may help realize the immense potential of nonsense su^+ tRNAs in the advancement of mammalian viral and cellular genetics.

7. APPENDIX

Mutagenic Systems for the Study of Eukaryotic Gene Expression and Regulation

Much effort has been devoted towards the development of novel mutagenic systems for the study of eukaryotic gene expression and regulation in mammalian systems, and improving the utility of those currently available. Although development of the mutagenic systems described below has been problematic, the potential to serve as general tools for the advancement of both mammalian viral and cellular genetics is maintained.

7.1 Mammalian Nonsense Suppressor Cell Lines

The application of nonsense su^+ tRNAs to mammalian viral and cellular genetics may be expanded through establishment of a wide spectrum of mammalian nonsense suppressor cell lines. Nonsense suppressor cell lines of different origin would permit the study of a wide range of animal viruses, while cell lines expressing su^+ tRNAs of different amino acid specificity would facilitate structure/function analyses. Maintaining a battery of nonsense suppressor cell lines, including those of amber, ochre, and opal codon specificity, would therefore expand the utility of this host-range screening system for the identification of viral nonsense mutants, and render such mutants amenable to biochemical analyses (section 3.1).

The establishment of mammalian nonsense suppressor cell lines is contingent upon the ability to efficiently control su^+ tRNA gene expression (section 3.2). The SV40 replicon-based system of mediating high level induction of nonsense suppression activity (section 3.2) was adopted to establish ochre and opal nonsense suppressor cell lines in BSC40 cells (data not presented). In addition, the introduction of ochre su^+ tRNA gene concatenates was undertaken in HeLa cells, which serve as the permissive host for a variety of animal viruses. Stable transfection of cell lines was carried out with the appropriate su^+ tRNA gene, plasmid pLTRtsA58 (BSC40 cells), and plasmid pSV2NEO. Approximately 200 clonal lines were isolated, expanded, and evaluated for nonsense suppression activity. Several ochre and opal nonsense suppressor cell lines were identified and characterized. Growth curves were generated, and Southern analyses were conducted to determine the genome copy number and amplification kinetics of the su^+ tRNA gene. Such cell lines were stable over 30 passages in culture, however, nonsense suppression activity was apparently lost thereafter. It is conceivable that cells able to express high levels of su^+ tRNA were outcompeted by those which maintained poor levels of suppression activity.

The difficulties in generating mammalian nonsense suppressor cell lines bear testament to the intolerability of mammalian cells to nonsense suppression activity. Stringent control of su^+ tRNA gene expression must be maintained to establish the utility of su^+ tRNAs for genetic analyses in mammalian cells.

The *lac* operator/repressor system has afforded a means of efficiently regulating the expression of a mammalian su^+ tRNA gene (section 4.4). This system represents an

alternative and potentially useful strategy for the generation of additional mammalian nonsense suppressor cell lines and moreover, may be adopted for use in a variety of different mammalian cells. The generation of such suppressor cell lines may be mediated through stable co-transfection of mammalian cells with a *lac*O-su$^+$ tRNA gene derivative (such as plasmid p*Lac*OtS(am4)), a plasmid encoding *lac* repressor protein (plasmid pRSVIns), and a plasmid encoding a selectable marker (plasmid pSV2NEO). Alternatively, the prior establishment of a *lac* repressor-expressing cell line may facilitate the subsequent stable introduction of the *lac*O-su$^+$ tRNA gene derivative. Indeed, the *lac*O-su$^+$ tRNA gene derivative, plasmid p*Lac*OtS(am4), has been linked to a selectable marker in preparation for such an approach (plasmid pSVHIS-Su$^+$ (am); section 2.2.1.4.2). The accumulation of *lac* repressor molecules prior to introduction of the su$^+$ tRNA gene, may ensure stringent inhibition of su$^+$ tRNA gene expression and hence, may mediate stable incorporation into the mammalian cell genome.

7.2 HSV-1 *LacZ* Gene Nonsense Mutant Transducing Viruses

Very little is known regarding the level of nonsense suppression activity derived from AM12 cells during an actual HSV-1 infection. Hence, generation of HSV-1 transducing viruses which express nonsense codon derivatives of the *E. coli lac*Z gene was undertaken. *βgal* activity is readily detectable in mammalian cells when expressed from within the context of the viral genome (section 2.2.6.3). Such a recombinant virus would constitute a simple and rapid assay for the identification of mammalian nonsense suppressor cell lines, and would serve as an essential control for the suppression

efficiency of nonsense suppressor cells, carried out in parallel to routine host-range screening analyses (section 3.5). Moreover, such a virus would permit a preliminary examination of the reversion frequency of virally-encoded nonsense mutations when grown on nonsense suppressor cells.

Several *lacZ* gene nonsense codon derivatives under transcriptional control of the HSV-1 ICP6 gene promoter were constructed (section 2.2.1.1). However, *βgal* activity was minimal and apparently not a function of su^+ tRNA gene expression, as determined upon HSV-1 superinfection of transiently transfected AM12 and BSC40 cells. Moreover, the ability to suppress the amber nonsense mutation encoded within pRSV*βgal*(am) (section 2.1.5) was lost upon introduction of a DNA cassette, containing this hybrid gene, into the HSV-1 marker transfer vector, pTKSB, to generate pTK-*βgal*(am) (section 2.2.1.1). It is possible that an artifact in cloning may have resulted in the inactivation of the indicator gene. Alternatively, it is also conceivable that the Rous sarcoma virus-derived promoter cannot function from within the context of HSV-1 sequences. However, these possibilities were not addressed. The utility of this system was not examined.

7.3 HSV-1 Nonsense Suppressor tRNA Transducing Virus

Much effort was directed towards expanding the application of nonsense su^+ tRNAs in mammalian viral and cellular genetics, through the development of an alternative and novel mammalian system for the characterization of nonsense mutations. Indeed, the application and potential utility of nonsense su^+ tRNA transducing viruses has been discussed (section 4.5). Early attempts towards the development of such a

system involved introduction of an unaltered su^+ tRNA gene into the HSV-1 genome. It was of interest to determine whether such a recombinant virus was viable and the levels of suppression that could be achieved. Moreover, from a transcriptional perspective, it was of interest to examine the expression kinetics of a heterologous class III gene encoded within the context of the viral genome. Hence, an amber su^+ tRNA gene was inserted into the HSV-1 marker transfer vector, pTKSB (section 2.2.1.2) and introduced into the thymidine kinase locus of HSV-1 derivative, PAA'5. 11 thymidine kinase minus HSV-1 recombinants were isolated and Southern analyses established the integrity of the su^+ tRNA gene. However, nonsense suppression activity could not be detected, as determined upon viral superinfection of pRSVCAT(am)-transfected mammalian cells. It is conceivable that su^+ tRNA gene point mutations may have been incurred such that the expression and/or function of the su^+ tRNA product may have been compromised. Alternatively, it is possible that the su^+ tRNA gene may have been downregulated within the context of the HSV-1 genome. Large mammalian viruses may not tolerate high levels of nonsense suppression activity (section 3.9). A selection may have ensued for the abrogation of su^+ tRNA gene function. This view led to the development of an inducible mammalian nonsense su^+ tRNA gene (section 4.4). Interestingly, similar difficulties were encountered in attempting to establish an opal nonsense su^+ tRNA SV40 recombinant virus, although the amber and ochre derivatives were generated (Capone *et al*, 1985).

7.4 Genetic Selection for HSV-1 Genes Encoding Transcriptional Regulatory Proteins

Much effort was expended towards the development of a mutagenic system which would mediate the directed selection of mutations in genes of HSV-1 regulatory proteins. This system is based on the generation of a double recombinant virus which separately encodes two different indicator genes under the transcriptional control of identical test promoters. Since each indicator gene encodes products which are readily amenable to simple and well characterized assays (see below), progeny virions from chemically mutagenized stocks exhibiting a simultaneous loss or decrease of indicator gene function are easily scored. A mutant of this nature would be expected to be deficient in some function necessary to direct transcription off of the test promoter, rather than reflect simultaneous indicator gene mutation events. This system would essentially afford a more focussed examination of viral polypeptides involved in the regulation of specific genes or classes of genes, given the coordinate regulation of HSV-1 genes (section 3.3.1). Indeed, this approach is generally applicable to any animal virus, and is particularly attractive since the assay system is conveniently built into the virus, facilitating further characterization of mutations within the context of the viral genome. Marker rescue for the mapping of mutations is easily carried out through restoration of indicator gene activity.

Towards construction of such a mutagenic system, the HSV-1 ICP6 gene promoter was chosen as the test promoter and inserted upstream of the *E. coli lac*Z and the firefly luciferase indicator genes, and subsequently introduced into separate HSV-1 marker transfer vectors (section 2.2.1.3). Functional indicator gene activity was

confirmed upon HSV-1 superinfection of transiently transfected mammalian cells. Several strategies were adopted for marker transfer into the glycoprotein I gene and thymidine kinase gene loci of the HSV-1 genome. HSV-1 recombinants expressing high levels of luciferase activity were isolated and stable; however, those recombinants also expressing *βgal* activity were apparently unstable, since *βgal* activity was lost over passage. A double recombinant virus could not be isolated and thus, the utility of this sytem could not be examined.

8. BIBLIOGRAPHY

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