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THE IN VITRO ANALYSIS OF tRNA-TRYPTOPHAN
AMBER SUPPRESSOR EXPRESSION IN
Caenorhabditis elegans

by

Rob Mark Linning
Hon.B.Sc. (Biochem), Simon Fraser University, 1988

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biological Sciences

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The In Vitro Analysis of tRNA-Tryptophan Amber Suppressor Expression in Caenorhabditis elegans

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ABSTRACT

In the nematode Caenorhabditis elegans, amber suppressor mutations of different tRNA\textsuperscript{Trp} genes do not suppress amber mutations in different genes equally, even though the mature suprRNA sequences are identical (Kondo et al. JMB 215, 7, 1990). The results suggest either different levels of suprRNA expression, or tissue- and/or developmentally-specific expression of these suprRNAs. The latter may arise through differences in flanking sequences or chromosomal position of the suprRNA gene.

Transcription \textit{in vitro} of individual, cloned suprRNA genes in embryo extracts produces levels of expression roughly paralleling those of the phenotypic assays i.e. a hierarchy with \textit{sup-5} and \textit{sup-7} more efficiently transcribed than the rest. We have now tested the effects of altered flanking sequences on this expression \textit{in vitro}; such experiments might also give us an indication of which templates to test in the future in transgenic animals. The alleles \textit{sup-7}, \textit{sup-24}, and \textit{sup-29} were chosen for deletion analysis since they represented strong, medium, and weak levels of transcription \textit{in vitro} and suppression \textit{in vivo}. For each of these genes, deletions were carried out from the 5' end; an additional series of 3' deletions was also undertaken for \textit{sup-7}. For \textit{sup-24} and \textit{sup-29}, transcriptional activity was sharply reduced only when virtually all of the upstream flanking region had been
replaced with vector sequence. In contrast, \textit{sup-7} activity was significantly diminished with the elimination of an element more distal from the gene (20 to 30 bp away). With the 3' deletions of \textit{sup-7}, loss of expression was seen only when the deletions entered the coding region itself.
"Don't ever become a pessimist, Ira; a pessimist is correct oftener than an optimist, but an optimist has more fun - and neither can stop the march of events."

Lazarus Long
in Time Enough For Love

"Give the future enough thought to be ready for it - but don't worry about it. Live each day as if you were to die next sunrise. Then face each sunrise as a fresh creation and live for it, joyously. And never think about the past. No regrets, ever."

Ibid.

To Tania, the most patient person in the world; Shannon the prettiest little girl in the world; Connor, the goofiest kid in the world; and one more to come.
I would like to thank Dr. B.M. Honda for the opportunity to work in his laboratory; Dr. M.J. Smith for guidance and encouragement; Dr. R. Waterston for the *C. elegans* suppressor clones; L. Willis, H. Vahidi, A. Purac, K. Beckenbach, and I. Scott for invaluable ideas in the lab; C. Mayes for helping me become a Mac user; Shannon, Connor, and Tania for support and fun; my parents and parents-in-law for support and timely babysitting.
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INTRODUCTION

The blueprint for life resides in each cell's DNA and serves as the information databank for almost every aspect of growth and development. This information is transformed into action through a series of processes that are shared by all life. First, the template DNA of a gene is transcribed into its corresponding RNA which may carry out structural or processive functions. In addition, certain "messenger" RNAs may serve as interim templates for the production of the proteins which make up the vast majority of cellular constituents. This two step process, transcription and translation respectively, has been called the central dogma of molecular biology by Crick (1970).

Several refinements have since been added to the central dogma. Segments of proteins containing information for their localization were shown to be cleaved en route to the polypeptide's destination. In 1977 messenger RNAs were shown to be the products of spliced precursors (Berget et al., 1977) overthrowing the assumption of a strict one-to-one relationship between DNA and amino acid sequence. More recently, examples of specific editing of mRNA templates have been uncovered (reviewed in Simpson and Shaw, 1989). Examples of protein splicing have also been unearthed (Kane et al., 1990; Carrington et al., 1985). Despite these incursions the basic principle of the central dogma persists.
A gene may be expressed constitutively, at a certain stage of development, in a given cell type, in response to the needs of growth, or in response to environmental stimuli. Knowledge of the control of this gene expression is essential to the understanding of how the information encoded linearly in DNA is actuated both spatially and temporally.

A gene's expression can theoretically be governed at any point along the pathway described above. Since the transcription of DNA to RNA is the first step, regulation at this point represents both the coarsest and cheapest form of control. As such the process of transcription as well as its regulation has been a prime target for investigation.

Prokaryotic systems, by virtue of their simplicity, have been studied from early on in the history of molecular biology. The use of genetics has allowed the identification of transcriptional regulatory mutations. Biochemical manipulation of the transcriptional machinery permits the determination of the function of specific components at the molecular level (Alberts et al., 1983). This dual, complementary approach provides inroads to study at the in vivo and in vitro levels respectively. A combination of the two approaches is often seen whereby mutant templates are constructed, introduced into hosts, and assayed for their effects.

Transcription in E. coli, the workhorse for prokaryotic studies, is carried out by a single class of RNA polymerases
comprised of several subunits (Chamberlin, 1982). The core enzyme responsible for elongation requires an additional sigma factor as well as specific DNA elements upstream of the transcriptional units for initiation. Two such sequence elements are the TTGACT and TATAAT boxes located 35 and 10 base-pairs 5' to the initiation site respectively (McClure, 1985). A pre-initiation complex is formed with the core enzyme interacting with the TATAAT box and the sigma factor interacting with the TTGACT box. A conformational change results in the unwinding of one turn of the DNA helix centered over the proximal box and subsequent migration of the complex to the initiation site. This situation constitutes the open initiation complex and at this point initiation is possible.

Within the context of cellular energetics, regulation at the initiation of transcription is logical since investment in unwanted product would be wasteful. The lac operon of E. coli was the first well-defined molecular regulatory system and provides an example of control at initiation (Miller and Reznikoff, 1978). The lac repressor binds at a site between the promoter and initiation site thereby preventing the bound complex from moving downstream and forming the open transcription complex. Conversely, the cAMP-catabolite activator protein interacts with DNA upstream of the RNA polymerase binding site and facilitates initiation through protein-protein interactions which alter
neighbouring DNA conformation (Travers, 1987). A variety of control mechanisms exist for the many different operons.

Prokaryotic transcription has been shown to terminate at or shortly after hairpin structures followed by runs of uridyl residues. It is thought that the transcriptional machinery pauses at the hairpin and the DNA-RNA duplex formed immediately thereafter dissociates due to the presence of weak A-U pairings. The coding strand of DNA is now freed to rejoin its complement. Meanwhile, the core enzyme, which has lost its sigma factor immediately after initiation, exhibits less affinity for duplex compared to single-stranded DNA. This process is also the target of regulatory mechanisms such as the antitermination factors encoded by the lambda N and Q genes (Roberts, 1987). Other prokaryotic termination mechanisms also exist. The rho factor recognizes characteristics of nascent RNA and moves to disrupt the transcriptional machinery by effectively pulling away the RNA (Das et al., 1978). In the case of the E. coli trp operon, termination is accomplished via a coupling of the transcriptional and translational processes (Yanofsky, 1987).

The dissection of the transcriptional process in eukaryotes has moved at a much more deliberate pace. This a reflection of the much greater sizes of eukaryotic genomes, the packing of the genetic material into chromatin, and the much greater complexity of eukaryotic transcription components and processes.
For the most part, classical genetic analysis is not available for the isolation of regulatory mutants. Instead, the biochemical approach has been extensively utilized. Eukaryotes were found to require three different but related classes of RNA polymerases (reviewed in Sentenac, 1985). RNA polymerase I is insensitive to high levels of the fungal inhibitor alpha-amanitin and transcribes a precursor RNA which is in turn processed into the 18S, 5.8S, and 26S ribosomal RNAs. RNA polymerase II is sensitive to low levels of alpha-amanitin and transcribes the precursors to messenger RNAs which give rise to the cell's proteins. Finally, RNA polymerase III is sensitive to mid-range concentrations of alpha-amanitin and transcribes small RNAs like 5S, tRNA, U6, 7SL, 7SK, and VA. Alpha-amanitin is a cyclic octapeptide containing several unusual amino acids which binds very tightly to RNA polymerase II preventing transcript elongation (Stryer, 1988).

The class I and II polymerases require promoter sequences which lie upstream of the actual coding region. RNA polymerase I requires two elements (Sollner-Webb and Tower, 1986), a proximal block which interacts with the ancillary polymerase I-specific transcription factors and a more distal block which appears to be necessary under more demanding assay conditions.

RNA polymerase II transcribes the entire complement of the eukaryotic cell's protein-coding genes. A diverse collection of regulatory mechanisms controls their
expression. A 'TATA' box centered at -25 has been implicated in nearly all transcription of class II genes (for an exception see Garritty and Wold, 1990). This element resembles a displaced prokaryotic TATAAA element and has been shown to bind the TFIID transcription factor (Sawadogo and Roeder, 1985; Nakajima et al., 1988). Constitutively expressed genes tend to have GC-rich regions in their promoters. A wealth of other elements and their corresponding binding factors have been identified. For example, the mammalian Spl protein has been shown to be necessary for the transcription of genes containing GC boxes (Dynan and Tjian, 1983). The heat shock transcription factor (HSTF) binds at specific regions of the heat shock gene's promoter (Parker and Topol, 1984). In addition to these elements which lie within a few hundred base-pairs of the transcription initiation site, there exist the so-called enhancer sequences which can influence transcription from great distances (reviewed in Kishu et al., 1991; Muller et al., 1988). Not only found upstream, these enhancers can be found downstream and even within genes themselves. Enhancers are also known to be activated in a tissue-specific manner.

The most extensively studied of the eukaryotic transcriptional systems is that of the class III 5S genes of Xenopus laevis. Surprisingly, when cloned 5S templates were transcribed in vitro it was found that accurate initiation required control sequences embedded within the coding region
This internal control region (ICR) was subsequently found to consist of two discrete elements, the A and C boxes. The ICR is first bound by the factor TFIIIA which then enhances the binding of the A box by TFIIIC. A fully stable pre-initiation complex which persists through many rounds of transcription is formed upon the binding of TFIIIB (Lassar et al., 1983). Polymerase III is then itself bound loosely until initiation. Termination of 5S as well as all class III genes occurs at a run of T's downstream of the gene.

The transcription of other class III genes does not require TFIIIA. In the case of tRNAs a split ICR region is present with A and B boxes which contain bases highly conserved in all tRNAs: U8, A14, G18, and G19 in the A box and G53, T55, C56, A58, and C61 in the B box (Galli et al., 1981). Also, unlike the 5S gene, distances between the two boxes may vary without greatly reducing transcription (e.g. Carrara et al., 1981; reviewed in Geiduschek and Tocchini-Valentini, 1988). A pre-initiation complex on tRNA templates is formed by the sequential binding of TFIIIC then TFIIIB. Recently, a factor consisting solely of RNA (TFIIR) has been shown to be required for 5S and tRNA transcription (Young et al., 1991).

In addition to internal sequence requirements, the transcription of tRNAs has also in many instances been shown to depend on flanking sequences (reviewed in Sharp et al., 1986, and Geiduschek and Tocchini-Valentini, 1988).
bulk of these flanking elements have been found to reside upstream while a very few have been shown to occur downstream. In the case of the 5' elements positive (e.g. Arnold and Gross, 1987), negative (e.g. Hippskind and Clarkson, 1983), and tissue-specific functions have been shown (Young et al., 1986). Combinations of these have also been shown to occur (Sajjadi et al., 1987). Little conservation of these sequences exists, suggesting solely gene-specific modes of regulation.

The role of upstream elements is even more pronounced with the remaining class III genes - U6, 7SL, 7SK, and VAI. In many cases, ICRs appear to be present and necessary while in others 5' sequences seem sufficient (7SK - Murphy et al., 1987). This latter case is reminiscent of the requirements for transcription by RNA polymerase II. In fact, recent investigations have shown that the U6 TATA box is bound by the TFIIID factor (Margottin et al., 1991). It is now believed that the different classes of eukaryotic RNA polymerases may share several transcription factors (reviewed in Folk, 1988; Sollner-Webb, 1988; Murphy et al., 1989; Palmer and Folk, 1990; Wolffe, 1991).

It is now possible to dissect the eukaryotic transcriptional system using the two-pronged in vitro/in vivo approach. Genetic and biochemical methods have been developed for the D. melanogaster and S. cerevisiae model organisms. Polymerase II transcription in the nematode Caenorhabditis elegans has been studied both genetically and
biochemically (Sanford et al., 1983, 1985; Rogalski and Riddle, 1988).

Several attractive features make C. elegans a valuable model organism. The complete cell lineage has been determined, rendering C. elegans invaluable for studies of development. A short 3.5 day life-cycle and the existence of self-fertilizing hermaphrodites for the maintenance of homozygous mutant stocks has permitted straightforward genetic analysis (Brenner, 1974). A small genome size, 100 million base-pairs, and a low proportion of repetitive DNA allows the isolation of specific sequences from recombinant libraries. A physical map has also been begun and is being used as a basis for the sequencing of the genome (Coulson et al., 1986). Transgenic animals can be made via the injection of DNAs into the gonadal syncitium (Mello et al., 1991) thereby allowing the in vivo testing of the functions of cloned sequences. A comprehensive overview of C. elegans biology can be found in Wood et al. (1988).

The background for this thesis can be found in research carried out in the laboratory of Dr. R.H. Waterston involving the characterization of mutations which are able to suppress other mutant phenotypes (reviewed for C. elegans in Hodgkin et al., 1987). During the reversion analysis of a muscle-defective mutant such a suppressor, sup-5 III, was picked up (Waterston and Brenner, 1978). A further study revealed a new suppressor which acted in a similar manner but mapped to a different location - sup-7 X (Waterston,
1981). The mode of action of these suppressors resembled that of nonsense suppressors (for reviews see Celis and Piper, 1981 and Hatfield, 1985). That is, they were thought to affect information transfer since they operated in a general way on similar alleles of a variety of genes. Such nonsense suppressors allow stop codons to be recognized as codons for an amino acid, enabling translation to proceed through the original mutation (Steege and Soll, 1979). This hypothesis was borne out in an experiment where an injected tRNA fraction from a sup-7 strain was shown to suppress an amber allele of the sex determination tra-3 gene (Kimble et al., 1982). The amber suppressor capabilities of the sup-5 and sup-7 genes were further proven by in vitro translation studies on an amber unc-53(e1300) mRNA (Wills et al., 1983). Sup-7 was also shown to encode a tRNA^{Trp}_{UAG} gene with a single base-pair change at the anticodon which was believed to allow read-through of amber translation termination signals (Bolten et al., 1984). A concurrent reversion analysis resulted in the isolation of additional mutations with amber suppressor profiles but varying in the levels of suppressibility (Hodgkin, 1985). To date eight of the twelve members of the C. elegans tRNA^{Trp} gene family have been mutated to amber suppressors. Genomic clones have been sequenced and shown to consist of identical tRNA sequences with the base-pair change described for sup-7. These amber suppressor alleles have also undergone extensive cross-suppression analysis against amber alleles of genes with
known expression profiles (Table 1). The data from these experiments suggest that the suppressors (and their wild-type counterparts) may be expressed in a differential manner (Kondo et al., 1988; Kondo et al., 1990). As such, these genes could represent a rare example of tissue-specific tRNA transcription. In addition, the approach described enables a single gene of a multigene family to be assayed for its individual expression.

The object of this thesis was to begin to investigate the molecular basis for the putative differential expression of these suppressors. Primer extensions were performed in the hope that the initiation sites for the different alleles would differ sufficiently to allow their expression to be followed through hybridization studies. Unfortunately no such differences were seen. Instead, deletions were made into the sup-7, sup-24, and sup-29 clones in order to delineate regulatory elements using transcription extracts. Discrete upstream positive regulatory regions were found to be involved. For sup-7 the region was approximately 20 to 30 base-pairs upstream while the regions modulating the expression of sup's 24 and 29 were much closer to the genes. It is hoped that these altered templates may some day be used for the corresponding in vivo analyses.
Table 1: Summary of Phenotypic Suppression Data

The results of crosses between strains supplying homo- or heterozygous doses for sup's 7, 5, 24, 33, 34, 21, 28, and 29 and strains bearing amber alleles of unc-11, unc-51, unc-24, unc-52, unc-15, dpy-20, lin-1, and tra-3 are shown (after Kondo et al., 1990). Also given are the putative tissues of expression for the amber alleles. The suppression of the unc mutants (resulting in altered movement phenotypes) was assayed using tracking assays. Suppression of dpy-20 was scored by its effect on worm length. lin-1 mutants display multiple vulval protrusions and are egg-laying defective; wild type adult progeny i.e. suppressed individuals were picked and scored. tra-3 is a gene involved in sex determination which when mutant transforms XX hermaphrodites into sterile pseudomales. Revertants for this transformation were scored.
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* + ++ +, maximum suppression observed, not necessarily wild-type; ±, barely detectable suppression; --, no detectable suppression; nt, not tested.*
Materials and Methods

Provided Clones and DNA Isolations

Recombinant plasmid clones of both amber suppressor and wild type counterparts of sup-5, sup-7, sup-24, sup-28, and sup-29 were kindly supplied by Dr. R. Waterston (Kondo et al., 1988). Competent E. coli DH5-alpha cells (Hanahan, 1983) were transformed with the appropriate clones (Golub, 1988) and plated on selective media. Resultant single colonies were used to inoculate large-scale cultures of NZCYM broth which were shaken overnight at 37°C (Maniatis et al., 1982). Plasmid DNAs were isolated via a modified alkaline lysis technique (Maniatis et al., 1982) and purified on cesium chloride - ethidium bromide gradients (Davis et al., 1980).

Large-scale preparations of subcloned fragments were initially carried out as above. Later isolations used a scaled up version of the differential centrifugation protocol of Lee and Rasheed (1990). This method uses a modified ammonium acetate precipitation scheme which removes bacterial DNA, RNA, proteins, heavy metals, detergents and other contaminants. The original protocol was used except that all volumes were scaled up by a factor of ten. After the initial isopropanol precipitation, 700 ul of 2 M ammonium acetate was used to resuspend the pellet. This solution was transferred to a 1.5 ml Eppendorf tube, placed on ice for 10 minutes and spun for 10 minutes at room
temperature in a desktop centrifuge. The supernatant was transferred to a new Eppendorf tube and an equal volume of isopropanol was added. After a ten minute incubation at room temperature the previous centrifugation was repeated. The resultant pellet was washed with 70% ethanol, vacuum dried, and taken up in 100 ul of TE (10 mM Tris-HCl, 0.1 mM EDTA at pH 8.0). Since these templates were later used for in vitro transcriptions, RNase was not added to the final solution. In all cases this latter technique provided templates with higher proportions of super-coiled DNA and lower amounts of RNA compared to the CsCl method when visualized on an ethidium bromide stained agarose gel. No difference was detected between comparable samples from the two methods used for in vitro transcriptions.

Small mini-preparations of plasmid DNAs were carried out using a variety of techniques during the course of this study. Initial isolations employed the alkaline lysis method of Birnboim and Doly (1979). Subsequent procedures included the Miniprep Kit Plus (Pharmacia), the Magic Minipreps DNA Purification System (Promega), and the aforementioned differential centrifugation method of Lee and Rasheed (1990).

Restriction fragments were isolated by fractionating restriction digests on agarose gels and recovering the fragment by electroelution (Maniatis et al., 1982) or through the use of the GeneClean Kit (Bio/Can Scientific Inc.).
Plasmid Subcloning

Gel purified restriction fragments were ligated into appropriately cut Bluescript M13+ (Stratagene Cloning Systems), pUC18, and pUC19 (Yanisch-Pe登 al., 1985; Pharmacia) vectors with T4 DNA ligase. Ligation reactions were used to transform competent E. coli DH5-alpha (for Bluescript) and JM83 (for pUC18 and 19) cells. Confirmation of the identity and orientation of the inserted fragments was achieved by restriction mapping and sequencing. Restriction enzymes were available from Pharmacia and BRL.

Transcription Reactions

Cell-free extracts from C. elegans embryos were obtained as described in Honda et al. (1986). Transcription reactions were carried out in a 25 ul total volume. One hour incubations at 25°C were stopped by the addition of sodium dodecyl sulphate (SDS) to 1% and then phenol-chloroform extracted. The resultant nucleic acids were loaded directly onto 10% polyacrylamide transcription gels containing SDS to 0.01%. After electrophoresis, gels were dried down and autoradiographed on Kodak XK-1 film. The autoradiograph was used as a template for the excision of transcript bands. These bands were Cerenkov counted in Biodegradable Counting Scintillant (Amersham) to quantitate the levels of transcription. This type of counting measures the light emitted by charged particles as they pass from a
transparent medium of low refractive index to a second medium of high refractive index when their velocity in the first medium exceeds the velocity of light in the second (Chase and Rabinowitz, 1967). For each lane a piece of the dried-down gel of equivalent size to that of the band was counted as background and subtracted from the band value.

The effects of template and total DNA concentration were monitored by following the approach of Wilson et al. (1985). First, the transcription of the unaltered sup-7 clone simply as a function of increased template concentration was measured. Transcription was seen to increase linearly with template concentration then begin to level off at approximately 1.5 nM. At very high template concentrations (50 nM and higher) transcription was seen to drop off markedly. From this a sub-saturating fixed concentration (0.4 nM) of template was used to examine the effect of added non-specific DNA (supercoiled Bluescript vector with no insert). The addition of this carrier DNA resulted in an approximate doubling of the template transcription up to a final total DNA amount of 0.3 ug per reaction. Little effect on transcription was seen beyond this point. Finally, a constant total DNA amount (0.3 ug) was maintained while template concentration was varied. In this way the optimal concentration of total DNA and the range of sub-saturating template concentrations was established. For the transcriptions described in this study total DNA concentration was 0.3 ug per reaction and the
template concentration was 0.8 nM. For each template a minimum of three transcriptions were performed. In addition, two different extract batches were used to eliminate the possibility of extract dependent transcription. The wild type undeleted template was assigned a value of 100% to which deleted template levels were compared. A mean value and standard deviation was calculated for each construct.

**Primer Extensions**

Primer extensions on tRNAs generated via in vitro transcriptions were carried out in order to ascertain whether the unprocessed immature transcripts of the various suppressor alleles possessed significantly different 5' extremities (Dingermann and Nerke, 1987). It was hoped that sufficiently disparate transcription initiation sites would allow the expression of individual transcripts to be surveyed by hybridizations with allele-specific primers. After a 25 ul in vitro transcription with cold NTPs the tRNA produced was ethanol precipitated then taken up in 8 ul of doubly distilled water. For sup's 5 and 7, 5 ul of 25 nM template was used in transcriptions while for the remaining more weakly transcribing templates 5 ul of a 50 nM solution was used.

Gamma-\(^{32}\)P labelling of the 5' terminus of the primer was accomplished by a one hour incubation at 37°C of the following mixture: 1 ul of 2 uM primer; 1 ul of 10 times
kinase buffer (0.5 M TrisHCl, 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine, 1 mM EDTA); 1 ul 0.1 M DTT; 5 ul gamma ³²P ATP; 1.5 ul doubly distilled water (ddH₂O); 0.5 ul kinase (Pharmacia, 10 units/ul).

One ul of this labelled primer mix was added to the 8 ul tRNA solution, heated for 2 minutes at 80°C then put on ice. The addition of 1 ul of 3 M NaCl was followed by successive 10 minute incubations at 65°C and 42°C. The solution was allowed to return to room temperature and 40 ul of the following solution was added: 31.8 ul 1 M Tris; 15.5 ul 2 M KCl; 3 ul 1 M MgCl₂; 15 ul each of 10 mM dATP, dCTP, dGTP, and dTTP; 30 ul 100 mM DTT; 12.5 ul DMSO; 97.5 ul doubly distilled water (ddH₂O). Subsequently 1 unit (0.5 ul) of both RNasin (Promega) and AMV reverse transcriptase (Promega) was added and followed by incubations for 30 minutes at 42°C, 15 minutes at 50°C, and 15 minutes at 55°C. The reaction was stopped and the template RNA degraded by the addition of 2.5 ul 0.2 M EDTA and 1 ul 1 mg/ml RNase respectively. After a 15 minute incubation at 37°C the final sample was extracted once with phenol/chloroform (1:1). This was followed by an ethanol precipitation aided by the addition of 1 ul of 4 mg/ml yeast carrier tRNA in 0.1% SDS and 0.1 M NaOAc. Samples were taken up in 2 ul of 98% formamide dye and loaded onto a 10% sequencing gel.

Deletions of Suppressor Subclones
Deletions were performed on the suppressor subclones as per the Exo III - S1 strategy of Henikoff (1984). A variety of strategies were used to generate the deletion constructs. For sup-7, deletions were made from the 5' end of the cloned EcoRI-SalI fragment (see Results section) permitting the isolation of endpoints near or within the gene. In this case the clone was first digested with KpnI allowing the 3' overhang to protect vector sequences from subsequent digestion by exonuclease III. The resultant digest was then phenol-chloroform extracted, ethanol precipitated, and digested by SalI. At this point the digest was run out on a 1.5% agarose gel. The presence of a very small molecular weight fragment consisting of freed polylinker between the KpnI and SalI sites would be evidence of a successful digestion strategy. At this point the Henikoff (1984) protocol would be followed on 5 to 10 ug of digested template. A similar approach was used on each of the other initial templates described in the Results section except that different enzymes with 5' overhangs for the second digest were employed. In the case of the sup-7 3' deletions HindIII was used for the second digestion while HindIII and BamHI were used for the sup-24 and sup-29 deletions series, respectively. In all cases the exonuclease III digestions were carried out at 37°C with 500 units of enzyme. The digestion rate was observed to be roughly 6 to 9 base-pairs per second. Timepoints were taken every 30 seconds until the region of interest was reached at which time fractions
were taken in quick succession. Half of each collected
timepoint was loaded onto an agarose gel to determine the
extent of digestion. Exonuclease and S1 nuclease enzymes
were both obtained from Promega.

*Sequencing of Deletion and Subcloned Plasmid Constructs*

All sequencing techniques used were based on the
enzymatic dideoxy termination strategy (Sanger *et al.*, 1977). Originally DNA sequences were determined from
denatured plasmid templates as described (Hattori and Sakaki, 1986). Later determinations used Miniprep Kit Plus
(Pharmacia) plasmid preparations and Sequenase V. 1.0 and
2.0 (USB) sequencing. When regions of high secondary
structure were encountered, the TaqTrack kit (Promega) as
well as the dimethyl sulfoxide (DMSO) method of Dr. T.
Snutch was attempted. This latter method involved the
mixing of 2 to 4 ug of double-stranded DNA with 2.5 pmol of
T3 or reverse primer in a total volume of 8 ul. One ul of
DMSO was added and the solution was incubated at 95°C for 3
minutes after which the tubes were immediately placed into a
dry ice-ethanol slurry for 5 minutes. After quickly finger-
thawing and spinning the tubes in a microfuge for 1 second,
2 ul of Sequenase sequencing buffer (5X) was added and the
mixture was allowed to sit at room temperature for 5
minutes. 6.3 ul of warm labelling mix (per sample: 1ul DTT;
2 ul diluted labelling mix; 0.8 ul 35S-dATP, 2 ul diluted
Sequenase enzyme; 0.5 ul DMSO) was added and followed by
another 5 minute incubation at room temperature. 3.5 ul of each sample was aliquoted into tubes containing dideoxy termination mixes and incubated at 37°C for 5 minutes. The reactions were stopped by the addition of 4 ul of stop dye and the samples were loaded onto sequencing gels as before.
RESULTS

Comparison of In Vitro Transcriptions

Five suppressor clones were chosen from those supplied by Dr. R. Waterston for comparison of their expression in C. elegans embryonic cell free extracts. These were selected because they represented a range of phenotypic suppression with sup-5 and sup-7 evincing the greatest, sup-24 a moderate, and sup-28 and sup-29 the lowest levels (Kondo et al. 1988; Kondo et al., 1990). Since these were preliminary experiments the in vitro transcription assays were performed simply on a fixed amount of each template (2 nM) with 2 nM of Ce5S1 (a construct containing the C. elegans 1 kb 5S repeat) as an internal standard. Each tRNA band count was normalized with respect to the complementary 5S internal standard. The sup-7 clone was found to be expressed at the highest level and was assigned a value of 100%. Values for the others relative to sup-7 were as follows: sup-5 90%; sup-24 69%; sup-28 26%; sup-29 19%. It should be noted that these determinations were not carried out under the optimized conditions for the deletion endpoints. Figure 1 summarizes this data. Figure 2 gives the sequence of the regions of DNA containing these genes.

Primer Extensions

In order to determine the gene transcription initiation sites, primer extensions were carried out. A 22 base
Figure 1: Comparison of \textit{in vivo} suppression and \textit{in vitro} transcription data.

$T$ represents the relative levels of \textit{in vitro} transcription. $\text{sup-7}$ was taken as 100\% and equated with the +++ \textit{in vivo} scoring value. The \textit{in vivo} data is from Table 1. The numerical denotations on the abscissa correspond to the amber alleles of (1) \textit{unc-13}, (2) \textit{unc-54}, (3) \textit{unc-24}, (4) \textit{unc-15}, (5) \textit{unc-52}, (6) \textit{dpy-20}, (7) \textit{lin-1} and (8) \textit{tra-3}.

\begin{tabular}{cccccccc}
     & + & + & & & & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
T 1 2 3 4 5 6 7 8 & super-5 & | & + & + & & & + \\
     & + & + & & & & + & + \\
     & + & + & & & & + & + \\
     & + & + & + & + & + & + & + \\
T 1 2 3 4 5 6 7 8 & super-7 & | & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
T 1 2 3 4 5 6 7 8 & super-24 & | & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
T 1 2 3 4 5 6 7 8 & super-28 & | & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
     & + & + & + & + & + & + & + \\
T 1 2 3 4 5 6 7 8 & super-29 & | & + & + & + & + & + \\
\end{tabular}
Figure 2: Sequences of Genomic Regions Containing Sup -5, -7, -24, -28, -29

70 base-pairs of 5' (a) and 3' (c) flanking region sequence is given for the above genes. The wild-type mature RNA coding parts are also given (b) and are identical for each gene. The arrow and T represents the change found in each case producing the amber suppressor. After Kondo et al. (1988).
(a)

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(b)

\[
\text{tRNA}^{\text{Trp}} \quad \text{GACTGCTTTGGCGCAATGGTAGCGGCGTTGTCCGACTCCAGNCGAAGGGTTGGCGTTCGATCCGCTCAGTTYGTCA}
\]

(c)

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<td>TTATCAATAAT</td>
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<td>AATGAAAAA</td>
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<td>TTTGCCATTT</td>
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<td>TCTAATCTGCT</td>
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oligonucleotide was used which was complementary to bases 3 to 24 of the mature transcript (5'-GCGCTACCATTGCGCCAAGCAG-3'). In all cases the data from these experiments supported transcription initiation at the first purine (in each case at the -2 position) upstream of the encoded 5' end of the mature transcript (Figure 3). This lack of significant differences in 5' processed transcript precluded the use of allele-specific oligonucleotide probes to follow the expression of individual suppressors.

Optimization of Template Concentration

The optimal template and total DNA concentration for the undeleted sup-7 clone was determined using the three part procedure outlined in the Materials and Methods section. Figure 3a summarizes graphically the dependence of transcriptional strength on template concentration. The final concentrations of template and total DNA chosen for further transcription were 0.8 nM and 0.3 ug/reaction respectively.

Subcloning and Deletions of Sup-7, Sup-24, and Sup-29
1. Sup-7 5' and 3' deletions

The original sup-7 clone (pRW98) supplied by Dr. R. Waterston consisted of a 7.5 kb Hind III fragment cloned into the vector pBR322 (Bolivar et al., 1977). From this a 1.3 kb EcoRI-SalI fragment was subcloned into Bluescript M13+ cleaved with the same enzymes. The SalI site occurs 21
Figure 3: Primer Extension Data

This composite autoradiograph shows the results of primer extensions from an oligo whose 3' terminus hybridized to the +3 nucleotide of the mature transcript (See Materials and Methods section for details). 1, primer extension on sup-5 tRNA; 2, primer extension on the wild-type counterpart tRNA of sup-5; 3, sup-24; 4, parental of sup-24; 5, sup-7; 6, primer extension on tRNA from the in vitro transcription of a template bearing an artificially engineered ochre not amber mutation; 7, sup-28; 8, parental of sup-28; 9, sup-29; A, parental of sup-29; P, unextended primer added to gel at same concentration as for the extended primers; g,a,t,c, are the lanes of a sequencing ladder used to size the extensions. The template for this ladder consisted only of vector DNA. The arrows to the right of the ladder correspond to, from top to bottom, the first upstream purine at -2, the 5' terminal base of the mature transcript, and the base (+3) hybridizing to the 3' terminal base of the extension primer.
Figure 3a: Template Concentration Versus Percent Transcription for Extract Optimizations

The effect on transcription levels of increased template concentration is shown. In each assay the most strongly expressed concentration was assigned the value of 100% to which the other concentrations were compared. The differently shaped data point sets correspond to separate assays. The heavy line represents the linear portion of the concentration dependence. The thin vertical line represents the subsaturating template concentration (0.4nM) used in the subsequent assay of the effect of total DNA concentration on a fixed concentration of template. This graph summarizes the first step of the optimization scheme of Wilson et al., (1985). See the Discussion section for further details.
base-pairs upstream of the encoded 5' end of the mature transcript. This clone was retained as a potential 5' deletion candidate and was also used for further deletions into the sup-7 gene.

A 3.5 kb EcoRI-HindIII fragment which consisted of the above EcoRI-SalI fragment as well as additional upstream flanking sequence was also subcloned into Bluescript M13+. From this a 1.0 kb XbaI fragment was isolated which contained the sup-7 gene as well as 250 bp of 5' and 700 bp of 3' flanking sequences. This subclone was used for 5' deletions into the flanking sequence upstream of the aforementioned SalI site. Figure 4 gives the sequence of the chosen set of 5' deletions into sup-7.

To fashion a clone suitable for 3' deletions into sup-7 the 1.0 kb XbaI fragment was isolated and digested with RsaI, which was known to cut roughly in the middle of the fragment. The two possible XbaI-RsaI fragments were shotgun cloned into Bluescript M13+ cleaved with XbaI and SmaI. The presence of the fragment with the sup-7 was determined by the presence of the SalI site. This construct effectively reduced the 3' flanking sequence to 200 bp and permitted the isolation of a deletion series the sequence of which is given in Figure 5. The presence of a DdeI site just downstream of the B box control region within the gene itself also allowed the isolation of an additional deletion endpoint.

2. Sup-24 5' deletions
Figure 4: Sequences of *Sup-7* 5' Deletions

The sequences of the deletion constructs are shown. The top line gives the wild-type genomic sequence with the one base-pair amber suppressor producing change at the anticodon (underlined). The lines below give the vector sequences introduced during the deletion process. Dots show identity to the original sequence.

```
-50   -40   -30   -20   -10
TTACTCGGTTCATCGTAGCCGTCCATATCATCGCTGTCGACAAGAGAGAATAACAT
AC.AAA.C (Sup7-52)
...AC.CTCA.TAAA.GGAACAAAAG.T (Sup7-32)
.A.AGG.AACA.AAGC.G.GTAC..GG.CCCC.CTC.AG (Sup7-21)
C..TGACCA.G.T.ACGCCAA..CTCGGA..TAAC..CT.AC.AA.GGGA.C (Sup7-10)
GA.ACA.C.ATGAC.A.GATTACGCCAAGCTCGGAATTAAC..CT.A..GGA.CA.A
CA.T.TCACA..GGAAACAGCTAT..ACCATGATTAC..CAAGCT.GGA.TTA.CCCT..C
..GTGT..AATTG.GAGC.GATAACAATT.CA...A.GAAA.AG.T.TGACC.TG.TT.C
A.G..TCCGG.T.GTA.GTT.TGT.GAAT.G.G.GC.GATAAC.ATTTCAACAC.GG.A.C

+10   +20   +30   +40   +50   +60
GACTGTGCTGGCGCAATGGTAGCCGTCCATCTCAGATCGAAAGGTTGGCGTTGCATCC
AG (Sup7+3)
T.AA.GGAACCAA (Sup7+13)
.C.AAGC.C.GAATTAACCCT.A.TAAA.GGAAC (Sup7+35)
AG..ATGACCATG.T.ACGCCAAGC.CG..A.TA.CCCTC.CTAAAG..AACAAA (Sup7+56)

+70   +80   +90   +100   +110
GCTCAGTGGTCA-AATTTTTTGTTTTTTAAGTAGTAATATAATACAATTTAAATCAAAT
Figure 5: Sequences of Sup-7 3' Deletions

The sequences of the deletion constructs are shown. The top line gives the wild-type genomic sequence with the one base-pair amber suppressor producing change at the anticodon (underlined). The lines below give the vector sequences introduced during the deletion process. Dots show identity to the original sequence. Note that the +107 and +174 endpoints occur 35 and 102 base-pairs downstream, respectively, from the 3' end of the mature transcript.

```
-50 -40 -30 -20 -10
TTACTCGGGTCACTCCTAGCCTGCTCCATTATCATGCTGCTGACAAAAAGAGAGAATAACAT

+10 +20 +30 +40 +50 +60
GACTGCTTTGCACATGATGCGCCGTCTGCTGACCTCTAGATCGAAAGTTGCGGTTCGATCC
CAGCTTTTTGTTCCC.TTA.T.AGG.TTAA.TCCGA.CTT..CGTAATCA.G.TCAT
(Sup7-3'+4) (Sup7-3'+33) AGCT.TTGTTCCC.TTAGTGAG.G.TA

+70 +80 +90 +100 +110
GCTCAGTGGTCA-AATTTTTTTTTTTAAAGTACATATATAATACAAATTATTAATCCAAAT
AGCTGT.CCTG-TG.GAAA.TG..A.CCGC.CAC...TCC.C..CA..CGAGCCGGA
AT..C.A.C.TG-GCG.AA.CA.GG.CAT..CT..TTCC.GTGTG..A..GT.ATCCGC.
G..GG.-TCCAC.AGT.C.AGAGC.GCCGCCAACCAGGCTGGAGCTCCAATTCCGC
(Sup7-3'+66) (Sup7-3'+107) CAGC..TTGTTC
(Sup7-3'+174)
```
The supplied sup-24 clone (pKJ312) consisted of a 14 kb HindIII fragment in Bluescribe M13+. From this a 0.7 kb NsiI-EcoRI fragment containing the sup-24 gene was isolated, blunted, and ligated into Bluescript M13+ cleaved with SmaI. Once the orientation with the 5' end of the gene proximal to the KpnI site of the polylinker was established a series of deletions into the 5' flanks of sup-24 was performed. The sequences of the deletion clones of this series is shown in Figure 6.

3. Sup-29 5' deletions

A 1.0 kb NsiI-HindIII fragment containing the sup-24 gene was isolated from the supplied clone pKJ508 which consisted of a 4.3 kb HindIII fragment cloned into Bluescribe M13+. This fragment was blunted and subcloned into the SmaI site of Bluescript M13+. With only 180 base-pairs of 5' flanking sequence present this construct was used for 5' deletions into the upstream flanks of sup-24. In addition, a NarI site 16 base-pairs upstream of the 5' end of the encoded mature transcript was used to subclone a deletion endpoint. The sequences of the 5' deletions into the regions near sup-29 are given in Figure 7.

In Vitro Transcriptions of Deletion Series

The conditions for the in vitro transcriptions of the described deletions series are given in the Materials and Methods section. Table 2 gives the percentage raw data for the Cerenkov counted excised bands for each construct. In
Figure 6: Sequences of Sup-24 5' Deletions

The sequences of the Sup-24 5' deletion series is shown. The top lines represent the wild-type sequence with the one base-pair change at the anticodon which produces the amber suppressor (underlined). Dots represent those bases of the introduced vector sequence which are equivalent to those found in the wild-type.

-50 -40 -30 -20 -10
TATTCTCTCCTTTCTCTCTACCCCTTCGATCCTAGATGACAAAGGAAATGT
(Sup24-79)
G.A  (Sup24-57)
A.A.TAAC...CA  (Sup24-47)
GGAAT.AA...C.CACTAAAGGAAC (Sup24-35)
.GA.TA.G...AAG...GGA.TTAACCTCAC.A.AG.GAC.A.A.C  (Sup24-13)
G.AA.AGCTA.GA.CA.GATTA.GCCA.ACTCGGAATT.ACCCTCACT....G..C
(Sup24-3)

+10 +20 +30 +40 +50 +60
GACTGCTTTGCGCAATGTAGCGGTTCCGACTCTAGATCGAAAGGTTGCGTCCGATCC

+70 +80 +90 +100 +110 +119
GCTCAGTGGTCGA-AAGTTTTTTTTTTTTCTAAATTTGAGTAAAAACTTTAAAGTTATCAA
Figure 7: Sequences of Sup-29 5' Deletions

The sequences of the sup-29 5' deletion series are shown. The top line represents the wild-type sequence. Dots represent the bases of the introduced vector sequences that are equivalent to those found in the wild-type sequence.

```
-50 -40 -30 -20 -10
AATGATTCGTAATGAGCAGCAAGGTTCCTGACTGGAAGTTGCGCCGGCCGAATCGT-GAC
(Sup29-97)
(Sup29-73)
..C  (Sup29-57)
..A.GGAA.AA..GC  (Sup29-45)
.GGTCGA.GC..TC..TA...TT.A.A..GAATTCCTGAGCCC  (Sup29-16)
C.CAGGAAACAGC.AT.ACCATGAT.ACG.CA.GCTCGGAA.TAAC..TCATC...GG.A-AC
(Sup29+2)
```
Table 2: Raw Percentage Value Data for Transcriptions of Deletion Constructs

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* These percentage values represent values corrected downwards by 54% to account for the longer transcript size. See Discussion.

- The absence of numerical values here reflect either the lack of a standard deviation value for the assigned 100% levels or the presence of a zero or subzero band count after background correction.
each case the construct with the least amount of DNA deleted was assigned a value of 100%. The remaining deletions endpoints were assigned percentage values relative to this. A minimum of three assays were performed for each endpoint. Figures 8 to 10 present photographs of representative gels for each deletion series. Figures 11 to 13 graphically represent this data showing the extent of remaining original DNA below and the transcription level of each deletion endpoint relative to the wild type undeleted template.

Recloning of the Sup-7 SalI (S75'-21) Clone

Because of the complete lack of transcription of the subcloned EcoRI-SalI fragment of sup-7 the region containing and surrounding the gene was sequenced. This revealed no sequence changes from what was expected. To rule out the possibility of specific vector sequences killing expression ("vector poisoning") the EcoRI-SalI fragment was recloned into both pUC18 and 19 cut with those enzymes. Assays of midipreps of these templates also revealed a lack of transcription (data not shown).
Figure 8: Transcriptions of Sup-7 5' Deletions

Plates A and B represent overexposed and underexposed versions of the same autoradiograph. Lanes 1, 2, 3, 4, 5, 6, 7, 8, and 9 are transcriptions of Sup-7 5' deletion endpoints -91, -52, -32, -21, -10, +3, +12, +35, and +56 respectively. The arrows to the right of the upper panel are directed to the faint bands in lane 7 which are not seen in the lower exposure.
Figure 9: Transcriptions of Sup-7 3' Deletions

A representative autoradiograph of the transcription of sup-7 3' deletions is shown. Lanes 1, 2, 3, 4, and 5 correspond to transcriptions of endpoints sup-7 +174, +107, +66, +33, and +4 respectively. The arrows to the right of the panel indicate the higher molecular weight transcripts seen for the +66 endpoint.
A representative autoradiograph of the transcription of sup-24 and sup-29 5' deletions is shown. Lanes 1, 2, 3, 4, 5, and 6 correspond to transcriptions of endpoints sup-29 -97, -73, -57, -45, -16, and +2 respectively. Lanes 7, 8, 9, A, B, and C correspond to transcriptions of endpoints sup-24 -79, -57, -47, -35, -13, and -3 respectively. The arrows to the right of the panel refer, from top to bottom, to the origin of the gel, the faint slightly higher molecular weight transcript of the sup-24 -3 endpoint, and the remaining mature transcripts.
Figure 11: Graphical Representation of Sup-7 5' and 3' Deletion Transcriptions

A graphical representation of the data in Table 2 for the transcriptions of the sup-7 5' and 3' deletions is shown. Each data point (X for 5' and o for 3') is the mean of the collected values with the error bar supplying the standard deviation. The lines below the graph represent those genomic sequences remaining after the deletions were performed. The top line gives the 5' and 3' ends of the regions encoding the mature transcript, the A and B boxes, and the Tn termination site.
Sup7-91
Sup7-52
Sup7-32
Sup7-21
Sup7-10
Sup7+3
Sup7+12
Sup7+35
Sup7+56
Sup7+4
Sup7+33
Sup7+66
Sup7+107
Figure 12: Graphical Representation of Sup-24 5' Deletion Transcriptions

A graphical representation of the data in Table 2 for the transcriptions of the sup-24 5' deletions is shown. Each data point is the mean of the collected values with the error bar supplying the standard deviation. The lines below the graph represent the extent of genomic sequences remaining after the deletions were performed.
Figure 13: Graphical Representation of Sup-29 5' Deletion Transcriptions

A graphical representation of the data in Table 2 for the transcriptions of the sup-29 5' deletions is shown. Each data point is the mean of the collected values with the error bar supplying the standard deviation. The lines below the graph represent the extent of genomic sequences remaining after the deletions were performed.
Discussion

The object of this thesis was to determine the molecular basis for the putative temporal and/or tissue-specific expression of the tRNA\(^{Trp}\)\(_{UAG}\) gene family of *Caenorhabditis elegans* described in Kondo *et al.* (1988) and Kondo *et al.* (1990). The cross-suppression tests carried out in these studies have been mentioned in the Introduction along with the basis for the claim of differential expression. One other instance of differential tRNA expression is that of the tRNA\(^{Ala}\) in the silk gland of the silkworm *Bombyx mori* (Young *et al.*, 1986). The capability of isolating large quantities of silkgland polymerase III competent extract has allowed the molecular dissection of this gene's expression and the identification of upstream regulatory regions. In *C. elegans*, however, extracts from specific tissues or organs are not available due to the small size of the organism. The existence of suppressor mutations may provide a vehicle for isolating a single gene's expression away from the background expression of the other family members.

To begin this study the original suppressor allele clones of the five tRNA\(^{Trp}\)\(_{UAG}\) genes (sup-5, sup-7, sup-24, sup-28, and sup-29) described in Kondo *et al.*, (1988) were assayed in our embryonic cell free extracts. The levels of expression roughly paralleled the extent of phenotypic suppression seen in the genetic studies. This presented
clear evidence of varied levels of expression between different members of the gene family. While of interest, it should be remembered that these assays were carried out in only one type of extract and that nothing could be said about differential expression in specific tissues or stages of development. Since the coding regions of each of these genes are identical and many examples exist of 5' polymerase III regulatory elements, the upstream flanking regions were examined. Some sequence similarity between the strongly expressed sup-5 and sup-7 genes was seen. 9 of 13 bases between positions -27 and -39 were shared. For the two most weakly transcribing genes (sup-28 and sup-29) there was a marked conservation of sequence very near the 5' end of the gene which tailed off quickly with distance. These two genes may represent the product of a recent duplication event. Limited divergence of flanking sequences after this event would result in the similarity of sequence and transcriptional levels seen. More subtle mutations might result in the more pronounced suppression profiles observed. Figure 14 compares the upstream flanking sequences. Finally, three suppressor clones were chosen for upstream deletion studies - sup-7, sup-24, and sup-29. These represent strongly, moderately, and weakly transcribing/suppressing alleles respectively. Sup-7 was chosen over sup-5 because in all cases it appeared to be the most highly expressed allele. As such it was also chosen for a series of deletions from the 3' direction. Sup-29 was
Figure 14: Comparison of 5' Flanking Sequences for Sup's 5, 7, 28, and 29

A. The line designated Sup-7 gives the 60 base-pairs of 5' flanking sequence for that gene. The line immediately below, designated Sup-5, supplies the sequence of the corresponding sequence for the Sup-5 gene. Identities between the two sequences are represented by dots in the lower line.

B. The 60 base-pairs of 5' flanking sequence for the Sup-28 and Sup-29 genes are compared in the same fashion as panel A.
A.

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<td>T...T..T.</td>
<td>G.A.ACTGCA</td>
<td>..CTTTCTC</td>
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B.

<table>
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chosen over sup-28 because it was consistently the most weakly expressed. In a large number of the cross-suppression studies sup-28, despite its lower in vitro transcription level, greatly resembled sup-24.

Primer extensions were done to determine the 5' extremities of the unprocessed transcripts of the suppressor and wild type alleles. If one or more of these transcription sites differed sufficiently in spacing from the mature 5' end it may have been possible to design allele-specific oligonucleotide probes to follow the expression of a single gene. This could have been done by probing Northern blots of stage-specific RNA isolations. Additionally, it is now possible to perform in situ hybridizations using short oligonucleotides (M. Chalfie, personal communication). Unfortunately, insufficient differences in the lengths of extension products was seen. In all cases the initiation site corresponded to the first (-2) purine upstream of the mature transcript's 5' end. This result, coupled with the similarity of termination signals seen in the immediate 3' flanking sequences shown in Figure 2, suggests the production of similarly sized primary transcripts for these genes. Similar primary transcript sizes would argue for transcriptional regulation rather than regulation at the level of processing.

Before the deletion constructs were assayed in vitro, the procedure of Wilson et al. (1985) was followed to optimize both template and total DNA concentrations. The
mechanics of this approach was outlined in the Materials and Methods section but the underlying principles will be discussed here. First, the level of transcription with respect to increasing template concentration (sup-7) was determined. The curve arising from this is a composite of the effects of both rising template and total DNA concentrations. To ascertain the contributions of the latter increasing amounts of non-specific vector DNA was added to a fixed sub-saturating concentration of template (0.4 nM). Finally, the optimal total DNA concentration (0.3 ug/reaction) was held constant and the behaviour of an increasing concentration of template DNA was observed. A suitable subsaturating template concentration of 0.8 nM was obtained from this. For the pioneering work done in Bombyx mori the total DNA concentration was kept in the range 0.2 to 0.4 ug per reaction while the saturating template concentration was encountered at approximately 1 nM (Wilson et al., 1985).

In vitro transcription assays of the deletion endpoints were carried out under the conditions just described. For simplicity these results will be discussed in a piecemeal fashion:

(i) 5' deletions within the sup-7 gene;

The 5' deletion endpoint at +3 within the mature transcript encoded region exhibits sharply reduced transcription due to the excision of an upstream positive regulatory element (to be discussed later). Also seen was
what appeared to be a continuum of slightly higher sized transcripts. This could be due to the fact that the vector replacement sequence introduced adjacent to the deletion endpoint consists of a stretch of 13 purines with one cytosine at the new -4 position. It is possible that the transcriptional machinery is choosing several different purines as its starting point. The deletion endpoint at +12 disrupts the A box internal control region by removing the canonical +8 thymidine residue. A very low level of transcription is, however, seen albeit at a band size higher than usually seen. This is consistent with other studies where the A box is altered (Wilson et al., 1985). It is believed in these cases that a "pseudo A box" is sought by the transcriptional machinery as well as alternate initiation sites (Geiduschek and Tocchini-Valentini, 1988).

In order to initiate transcription at the site seen, it is possible that the removal of the +8 thymidine has reduced the original A box to the status of a pseudo A box and consequently affected initiation. The deletion endpoint at +35 is at the anticodon region of the mature transcript. No A box is present but a highly over-exposed gel (data not shown) reveals a very faint band for this construct migrating at a somewhat higher molecular weight. This could be explained by the presence of a very weak pseudo A box and new initiation sites further upstream. No such pseudo-box, however, was uncovered in a search of the sequence. The final deletion endpoint occurs at +56 within the B box.
internal control region. No transcription is detected, which is not surprising considering none of the 5' flanks and internal elements remain.

(ii) 3' deletions within the sup-7 gene;

The sup-7 3' +66 deletion endpoint occurs just before the B box is encountered. A pair of large transcripts was seen. In this case all the essential 5' and internal control elements are present except for the original termination signal. It is possible that the decrease in level of transcription (by approximately 76% corrected value) may be due to more than the simple elimination of the wild-type termination site. In its place a run of 4 thymidine residues (corresponding to nucleotides 600 through 603 of Bluescript M13+), present approximately 90 base-pairs downstream of the endpoint by virtue of vector replacement, may be acting as a termination site. By sequence analysis these larger transcripts were estimated at 150 bases in size. Since the mature wild-type transcript is 72 bases in length a 54% downward correction factor was implemented to adjust these lanes' counts. The remaining two deletions within the gene occur at +4 and +33. The former retains only the first four bases of the mature transcript and none of the internal control regions. As might be expected no transcription is observed. The +33 endpoint is at the anticodon and removes the B box and termination site. Such truncated genes have been shown initiate correctly but the levels of transcription are extremely low (Carrara et al.,
1981; Johnson et al., 1984; Wilson et al., 1985). To see these low levels, high template concentrations beyond those of this study or lowered electrolyte concentrations are needed (Dingermann et al., 1983; Wilson et al., 1985).

(iii) Deletions into the 3' flank of sup-7:

Two deletion endpoints into the downstream flanking regions of sup-7 were isolated. These occurred at +107 (35 base-pairs past the end of the mature transcript) and +174 (102 from end). In each case transcription levels were indistinguishable from wild type under our assay conditions. Wilson et al. (1985) reported that as many as 50 base-pairs of downstream sequence are required for full transcription and suggest that this larger control region may be general for all tRNAs. No other similar requirements have been reported for tRNAs but this may be due to the fact that the majority of interest in extragenic control elements for tRNAs has been directed toward upstream sequences. Thus while our results may be in contrast to their findings, in our study the closest deletion endpoint to the gene was still 35 base-pairs away. It is therefore conceivable that any decrease in transcription for this construct was simply not detected in our assay or that the deletion had not yet entered a 3' region of importance.

(iv) Deletions into the 5' flank of sup-7:

The deletions into the upstream region of sup-7 which terminated at -91, -52, and -32 could not be distinguished statistically within the framework of our analysis. For the
deletion ending at -21 a precipitous drop in level of transcription to zero was seen. At first glance this suggested the excision of a strong positive regulatory element between -32 and -21. A low level of transcription was seen with the -10 deletion. Oddly, however, for both the -10 and +3 deletions the vector replacement sequences have very little similarity to the wild type sequence in the -21 to -32 region. Perhaps the transcriptional machinery is recognizing something beyond simple sequence identity. Examples exist of sequences upstream of polymerase III genes that are important not by virtue of a specific sequence but by the presence of a stretch of AT-richness (K. Sprague, and P. Fruscoloni, personal communications). There is a small cluster of adenosines and thymidines between positions -33 and -29 which are restored in the -10 construct. This cluster is not restored in the +3 construct. Another explanation is that vector replacement sequence is "poisoning" a basal level of transcription set by signals downstream of position -21. To examine this possibility the EcoRI-SalI fragment which makes up this construct was recloned into pUC18 and 19. Again no transcription was seen (data not shown). A comparison of the vector replacement sequences gave no obvious similarities which might have caused a poisoning effect. Again perhaps something beyond simple sequence recognition is involved.

(v) Deletions into the 5' flanks of sup-24:
5' deletion endpoints at -57, -47, -35, and -13 exhibited close to wild type transcription although a slight drop in the means of transcription levels was seen as the gene was approached. A large drop was seen for the -3 endpoint. This was surprising given the fact that seven of the ten bases deleted between the -13 and -3 endpoints were restored by vector sequence. The simplest explanation would involve a specific positive regulatory element residing in this region. It is also possible that the transcriptional machinery recognizes what it believes to be an inhibitory signal in the introduced vector sequences.

(vi) Deletions into the 5' flank of sup-29;

The situation here was much the same as for sup-24. Transcription dropped off markedly only at the +2 endpoint. A faint transcript of wild-type size is detectable which suggests weak but similar initiation. This again suggests a control element near the 5' end of the gene. For the other deletions transcription remained high.

The results of this thesis represents an introductory look into the transcriptional requirements for three genes of the tRNA^Trp_UAG family of C. elegans. In each case, for sup-7, 24, and 29 an upstream positive element was implicated in the control of tRNA expression. It is also possible that these stretches of flanking sequence may be involved in the putative differential expression of these genes. A wide variety of experiments can be done to further
analyse the mechanisms of these gene expression. The site-specific mutagenesis of sites believed to be signals for the transcriptional machinery would clearly delineate precise sequence elements required. Similarly, gel shift assays done on given DNA regions would clearly indicate whether those stretches are implicated in the transcriptional process. On a broader scale it may be possible to set up an in vivo system to study the expression of this gene family. Advances in DNA microinjection technology for *C. elegans* may permit the introduction of reporter genes such as beta-galactosidase containing amber mutations into strains bearing the various suppressor alleles. In this way differential expression of the suppressors would be revealed by a tissue-specific activation of the reporter gene. Alternatively, the injection and stable incorporation of single copies of altered suppressor constructs might permit the in vivo analysis of clearly delineated sequences when transformed strains are crossed to worms bearing amber alleles of known expression.

These results would contribute to the growing collection of sequences known to regulate the expression of polymerase III genes. This study looked at the sequence requirements of three members of a multigene tRNA family. If the suspected differential nature of their expression is borne out in later experiments, the information obtained here may be useful for the determination of underlying mechanisms.
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