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

by

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

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ABSTRACT

The goal of this research was to study expression of the $tRNA_{Trp}$ gene family in the nematode *Caenorhabditis elegans*. Eight of the 12 members, identical in coding sequence, were previously recovered as amber suppressor alleles, and suppression efficiency of amber mutations in different genes was different for individual $suptRNA_{Trp}$ genes (Kondo, *et al.*, 1990; 1988). The results suggested that members of the $tRNA_{Trp}$ gene family were differentially expressed, possibly in a tissue- or development stage-specific manner. Transcription *in vitro* of individual, cloned suptRNA genes in embryo extracts produces levels of expression roughly paralleling strength of expression in the phenotypic assays (Linning, 1992).

An advantage of working with tRNA genes from *C. elegans* is the availability of individual sup genes; this allows us to study expression of individual family members *in vivo*, using suppression of an amber mutated reporter gene. An amber site was introduced by unique site elimination site-directed mutagenesis (Deng and Nickoloff, 1992) near the N-terminus of a *lacZ* gene driven by *C. elegans* heat shock gene *hsp16-48* promoters (Stringham, *et al.*, 1992). The resulting single amber mutation was verified by DNA sequencing and expression testing in *E. coli*. It was then introduced as a reporter gene into transgenic worms (Fire, 1986; Mello *et al.*, 1991). The presence of transgene arrays was verified by PCR using primers from *C. elegans hsp16-48* and *E. coli lacZ* respectively. *Sup7, sup24, sup28* and *sup29* strains were chosen because they represented strong, medium and weak levels of transcription *in vitro* and of suppression *in vivo*. Suppression of the *lacZ* amber was studied by *in situ* histochemical staining techniques (Fire *et al.*, 1990). One strain of *sup29/sup29* transformed with an amber *lacZ* was obtained; other sup worms were too weak to survive the germline microinjection. Heterozygote *sup7/+*, *sup24/+*, *sup28/+* and *sup29/+* worms were generated by appropriate crosses to w.t. worms carrying *lacZam*. In nervous system, the suppression efficiency order is *sup7/+* > *sup24/+*. No suppression was observed in *sup28/+*, *sup29/+* and *sup29/sup29* worms. In

pharynx muscle cells, the suppression order is *sup7/+* >> *sup29/sup29* > *sup24/+* > *sup28/+* > *sup29/+*. No suppression was observed in *sup29/+* worms. In body muscle cells, the order is *sup24/+* ≥ *sup28/+* ≥ *sup29/sup29* > *sup7/+* ≥ *sup29/+*. In pharynx hypodermis cells, the order is *sup7/+* > *sup24/+* ≥ *sup28/+* > *sup29/sup29* > *sup29/+*. In body hypodermis cells, the order is *sup24/+* ≥ *sup28/+* ≥ *sup29/sup29* > *sup7/+* ≥ *sup29/+*. In intestinal cells, the order is *sup7/+* > *sup29/sup29* ≥ *sup24/+* > *sup28/+* > *sup29/+*.

These results suggest that suptRNA genes may be expressed tissue-specifically. Making stable integrated *lacZ* amber strains will allow me to further confirm these results, and pursue possible molecular mechanisms.

Dedication

Dedicated to my parents
and my other parents with love.

Acknowledgements

I would like to thank my supervisor Dr. B. M. Honda for the opportunity to work in his laboratory and also for his guidance. I am also thankful to my committee supervisors, Dr. D L. Baillie and Dr. B. P. Brandhorst for guidance and support, Dr. E.P.M. Candido (UBC, Vancouver) and Dr. E. Stringham (UBC, Vancouver) for giving me the reporter gene pPCZ1, D. Jones (UBC, Vancouver) for suggestions about staining technique; Caenorhabditis Genetics Centre and Dr. J. Hodgkin for providing me the sup strains; my former lab mates: A. Purac, P. Davison, S. Swenson and my present lab mates: V. Lloyd, M. Keramati, F. Mantiri, C. de Hoog, M. Singh; J.E. Schein for constructing a transgenic sup 29 strain; H. Stewart, D. Collins, R. Rosenbluth, S. Jones, K. Beckenbach, and R. Linning for their help and support. I am especially thankful to my parents and my other parents for their love, encouragement and understanding.

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INTRODUCTION

One of the important issues in molecular biology is how genes are regulated developmentally and spatially. Much of this regulation occurs at the transcriptional level.

Transcription in Eukaryotes

Historically, eukaryotic transcription units have been subdivided into three classes, each transcribed by a different RNA polymerase. This classification is based on the differential sensitivities of RNA polymerase I, II, and III to the drug α-amanitin. In animal cells, RNA polymerase I is insensitive, RNA polymerase II is generally very sensitive, and RNA polymerase III is only moderately sensitive to α-amanitin. RNA polymerase I synthesizes pre-rRNA transcripts. In sharp contrast to RNA polymerase I, RNA polymerase II is responsible for the regulated synthesis of a multitude of different transcripts in all eucaryotic cells. RNA polymerase II acts on genes specifying certain small nuclear RNAs (U1, U2, U3, etc.) as well as on every known protein-coding gene. RNA polymerase III transcribes a large number of genes to produce a variety of small untranslated RNA molecules, many with essential cellular functions, *e.g.* 5S RNA, tRNA, U6 small nuclear RNA, and 7SL RNA (Geiduschek and T-Valentini, 1988; Gabrielsen and Sentenac, 1991; Geiduschek and Kassavetis, 1992). The DNA sequences required for initiation of synthesis by RNA polymerase I and RNA polymerase II usually lie almost entirely outside of the region copied into RNA, but it was quite unexpected to learn that some promoters for RNA polymerase III are located inside of the genes themselves (Bogenhagen *et al*, 1980, Lassar *et al.*, 1983).

Promoters of Genes Transcribed by RNA polymerase III

The genes transcribed by RNA polymerase III can be classified according to the cis-acting sequences required for transcription. Both the type-1 (5S RNA) and type-2 (tRNA) genes contain intragenic promoter elements whereas the type-3 (U6 RNA, only found in vertebrate systems) genes contain extragenic promoter elements. In addition, there are an increasing number of genes that either do not completely conform to these classes or contain additional elements to specify transcription. These include the Epstein-Barr virus-encoded RNA gene (Howe and Shu, 1989), the *Xenopus* selenocysteine tRNA gene (Carbon and Krol, 1991), and the human 7 S RNA gene (Bredow *et al.*, 1990). Figure 1 shows the three types of class III promoters.

The promoters of most RNA polymerase III-transcribed genes include discontinuous intragenic structures, termed internal control regions (ICRs). These are composed of essential sequence blocks separated by nonessential nucleotides. The type I (5S RNA) ICRs are comprised of two functional domains: an A-block and a second domain consisting of an intermediate element and a C block. Most Class III genes, including tRNA, VA, EBER, vRNA, 7SL, Alu, 4.5S, B1, and B2 genes, have type II ICRs: these again have two domains, an A-block and a B-block. The A-blocks of types I and II are homologous. The A-block is located much further from the start site in type I than it is in type II promoters. Determination of the start site in class III genes is directed by box A, and the level of expression is directed by box B. As well as the ICR, extragenic sequences can also affect the strength of type I and II promoters. Sequences immediately upstream from the start site have also been found to be important for transcription of many class III genes, probably because TFIIIB binds this region (Kassavetis, *et al.*, 1990; 1989). However, with type III promoters, such as those of the vertebrate U6 and 7SK genes, transcription is independent of intragenic elements and is dictated solely by 5' flanking regions (Murphy, *et al.*, 1987; Das, *et al.*, 1988; Lobo and Hernandez, 1989).

ICR sequences are highly conserved between different genes and different species. This is likely to reflect a strong conservation of the general transcription factors employed

by pol III. In contrast, the flanking sequences of type I and II promoters frequently show little or no conservation, although they can often have powerful modulatory effects. This suggests that the flanking sequences are more likely to be recognised by gene- or species-specific factors, or that their cognate factors have very flexible DNA-binding specificity.

Figure 1 Schematic illustration of promoters

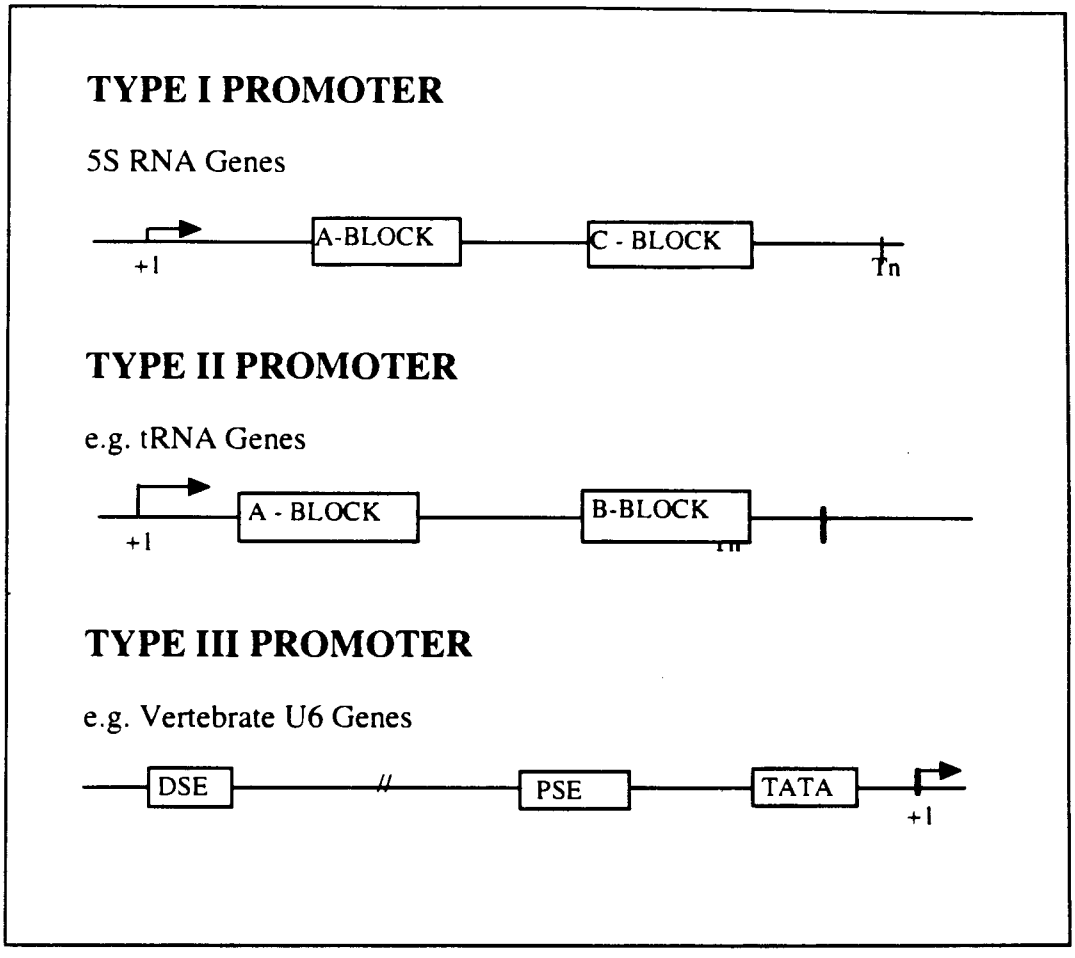


Diagram depicting the three types of promoter arrangement that are utilized by class III genes. The site of transcription initiation is indicated by +1 and the site of termination is indicated by Tn. Promoter elements are shown as open boxes. Abbreviation: DSE, distal sequence element; PSE, proximal sequence element. (after R. J. White).

Transcriptional Factors Involved in Transcription by RNA polymerase III

Both general and specific transcription factors as well as RNA polymerase III are required for the accurate and efficient transcription of genes with internal promoter sequences. TFIIIA is specifically required for transcription of 5S RNA genes and was the first eukaryotic transcription factor to be purified to homogeneity (Engelke *et al.*, 1980). It was also the first eukaryotic transcription factor to be cloned (Ginsberg *et al.*, 1984).It is complexed with 5S RNA to form an abundant cytoplasmic 7S storage particle (Picard and Wegnez, 1979; Honda and Roeder, 1980; Pelham and Brown, 1980). The cloning of a corresponding cDNA and elucidation of the primary sequence of the *Xenopus* factor (Ginsberg *et al.*, 1984) led to a model for the arrangement of the amino-terminal 80% of this protein into 9 zinc finger motifs of approximately 30 amino acids each (Miller *et al.*, 1985). TFIIIA has also been purified from HeLa cells (Seifart *et al.*, 1989, Moorefield and Roeder, 1994) and *S. cerevisiae* (Wang and Weil, 1989). Human TFIIIA produces zinc-dependent footprints on 5S genes that are similar to those generated by *Xenopus* TFIIIA. Like the *Xenopus* TFIIIA, yeast TFIIIA contains nine zinc fingers with invariant cysteine and histidine pairs (Archambault *et al.*, 1992; Woychik and Young, 1992).

TFIIIB and TFIIIC were originally identified for RNA polymerase III transcription (Segall *et al.*, 1980; Shastry *et al.*, 1982). Much progress has recently been made in the characterization of the protein components of these factors. TFIIIB is the central class III initiation factor since it alone serves to recruit the polymerase and specify the site at which transcription begins (Kassavetis, et al., 1990). Since TBP , cloned BRF and a 90-kD polypeptide could reconstitute yeast TFIIIB (Kassavetis, *et al.*, 1992), these molecules appear to represent the sole essential components of yeast TFIIIB. TFIIIC is one of the largest and most complex transcription factors to have been studied. The structures of TFIIIC subunits in yeast have been best studied. Six distinct subunits were found in TFIIIC (Willis, 1993). TFIIIC plays a primary role in the process of transcription complex assembly by binding to the intragenic promotor sequences of tRNA genes or to the preformed 5S DNA•TFIIIA complex. Once bound, TFIIIC acts as an assembly factor to

allow the binding of the initiation factor TFIIIB to an upstream gene position. TFIIIB then recruits RNA polymerase III and directs accurate initiation of transcription (Kassavetis, *et al.*, 1990, 1992).

TBP (TATA-binding protein) is a general factor required by pols I, II, III transcription. Margottin *et al.* (1991) first discovered that TBP is utilized by both pol II and III. It was first purified and then cloned from yeast. TBP genes were cloned from humans, mice, frogs, flies, plants, plasmodia and protozoa (review, White, 1994). The 27-38 kD polypeptide has a variable N-terminus and conserved C-terminus which has the TATA box -binding function. Since TBP is required by all pols I, II, and III, White (1994) proposed a model: TBP achieves its polymerase specificity by associating with distinct sets of TAFs (**T**BP - **A**ssociated **F**actors) in order to assemble polymerase-specific complexes. The recent study showed that carboxyl-terminal part of BRF interacts with TBP (Huet, *et al.*, 1994, Chaussivert *et al.*, 1995) and with the C34 subunit of pol III (Werner, *et al.*, 1993).

Other transcriptional factors like TFIIA, TFIID and TFIIIR have also been described. TFIIA has been shown to stimulate transcription of VA, tRNA, 5S and U6 genes (Waldschmidt and Seifart, 1992; Meissner *et al.*, 1993). TFIIA can bind directly to TBP, and this property has been exploited for its affinity purification (Ranish and Hahn, 1991; Usuda *et al.*, 1991; Cortes *et al.*, 1992). In the case of U6, TFIIA served to stimulate binding of TBP to the TATA box (Waldschmidt and Seifart, 1992). For TATA-less class III genes, Meissner *et al.* (1993) suggested that it may serve as an anti-inhibitor to prevent repressors from binding to TBP. TFIID is required for pol III transcription in *Bombyx mori* , while TFIIIR was found subsequently to be artifact, an isoleucine tRNA in silkworm with only an indirect effect on transcription (Young *et al.*, 1991; Dunstan *et al.*, 1994; review, Söll and Rajbhandary, 1995).

Transcriptional Regulation of tRNA genes

tRNA promoters (type II) are split into two essential and highly conserved regions of 10 bp each, the A- and B-blocks, that are generally separated by 30-40bp. Though the

A- and B-blocks constitute the essential promoter elements of a tRNA gene, additional internal or flanking sequences can often have modulatory effects. A consensus of TGGCNNAGTGG for the A-block and GGTTCGANNCC for the B-block was derived (Galli *et al.*, 1981). The position of the start site is fixed primarily relative to the A-block rather than the B-block (Baker *et al.*, 1987; Fabrizio *et al.*, 1987).

In eukaryotes, generally, ten to several hundred genes for each tRNA species are present in the haploid genome, depending on the organism examined. In organisms with lower numbers of tRNA gene copies, identical genes are usually widely dispersed and have flanking sequences that are totally unrelated--even in those immediately adjacent 5' and 3' regions that are transcribed as part of their respective tRNA precursor molecules. In *S. cerevisiae*, each tRNA is coded by eight tRNA genes on average. However, each amino acid is not represented by equal numbers of tRNA genes. Those amino acids that are relatively more abundant in proteins tend to be represented by more tRNA genes. Members of a given gene family are usually located on different chromosomes.

Differential regulation of tRNA genes is necessary for the adaptation of the tRNA population to different codon frequencies and amino acid utilization in different cell types. Only very few cases have been reported: For example, in *Bombyx mori*, a $tRNA_{Ala}$ gene is expressed exclusively in the silk gland cells (Sprague *et al.*, 1977); and the similar expression of silk-gland-specific $tRNA_{Ala}$ gene was also found in spiders (Candelas *et al.*, 1990). The transcripts of a particular, as yet uncharacterized, *Dicyostelium discoideum* $tRNA_{Val}$ gene are detected during early development but not during growth (Dingermann et al., 1988). A $tRNA_{Gln}$ suppressor gene is specifically induced upon retroviral infection in mouse (Kuchino et al., 1987) and oocyte-specific $tRNA_{Tyr}$ genes are activated stage specifically during *Xenopus laevis* development (Stutz et al., 1989, Reynolds, 1995). The members of a $tRNA_{Trp}$ gene family in *C. elegans* showed different suppression efficiencies *in vivo* (Kondo, *et al.*, 1990, 1988) and transcriptional levels *in vitro* (Linning, 1992). Although detailed mechanisms are still unknown, regulation seems to occur at the transcriptional level. Positional effect might be taken into consideration. For instance, the inhibitory influence of an upstream negative element can be diminished by short deletions or insertions that alter its distance from the coding sequence of a

Drosophila $tRNA_{Lys2}$ gene (DeFranco *et al.*, 1981). A yeast tRNA gene is repressed following its insertion into the silent HMR mating-type locus (Schnell and Rine, 1986).

Since ICR sequences are highly conserved whereas flanking regions show little homology, the latter are obvious candidates for mediating selective transcriptional control. Variations in 5' flanking sequences result in the differential expression of members of the *Xenopus* $tRNA_{Tyr}$ gene family (Gouilloud, *et al.*, 1986), which display strong developmental regulation (Stutz, *et al.*, 1989). The 5'-flanking sequence, from -34 to -11 is required for transcription of the silkworm $tRNA_{Ala2}$ gene in silkworm extracts, but is dispensable for transcription in a *Xenupus* system (Larson *et al.*, 1983). Homologous sequences which include a TATA motif occur at corresponding regions of silkworm $tRNA_{Gly}$ and 5S genes, where they are also required for transcription (Young *et al.*, 1986). A silkgland-specific $tRNA_{Ala1}$ gene lacks these sequences and is less actively transcribed; the use of chimerae generated by swapping upstream regions between the constitutive and silkgland-specific $tRNA_{Ala}$ genes demonstrated that the distinctive transcriptional properties are conferred by the 5'-flanking sequences. The recent results suggest that TFIIIB interacts with the upstream sequences and TBP is a discriminatory component in this fraction (Sullivan *et al.*, 1994, Young *et al.*, 1996).

5'-flanking sequences are likely to have some influence upon the transcription of most tRNA genes. The 5' elements that affect transcription are generally located within about 80bp of the initiation site, and much shorter separations are most commonly found. In yeast $tRNA_{Leu3}$, an element between -15 to -1 was found to stimulate transcription both *in vivo* and *in vitro* (Raymond *et al*, 1983, 1985; Johnson and Raymond, 1984). *Xenopus* selenocysteine tRNA $tRNA^{(Ser)Sec}$ is highly dependent upon 5'-flanking sequence (Carbon and Krol, 1991; Lee *et al.*, 1989). There is little or no sequence homology conserved between the 5'flanking regions of different tRNA genes, even of different genes that encode the same tRNA isoacceptor species. Although there are some instances in which limited homologies are shared between the upstream regions of several tRNA genes of a particular species [e.g. a partially conserved pentanucleotide in yeast (Raymond, 1985)], such sequences are not well conserved between different species. One exception is that the presence of an upstream A/T-rich region. Rooney and Harding (1988) found that

sequences between -53 and -31 account for the 5-fold greater transcriptional activity of murine $tRNA_{Asp2}$ relative to $tRNA_{Asp1}$; this region is 78% A/T in $tRNA_{Asp2}$ and only 45% A/T in $tRNA_{Asp1}$. Thus, distinct flanking regions in tRNA genes modulate the transcriptional level.

Normally, transcription of tRNA genes *in vivo* is difficult to study, because the precursor molecules are transient and the large pool of mature, stable tRNAs hinders measurement of newly synthesized molecules. Nonsense suppression has proven to be an extremely useful tool for elucidating the nature of biological phenomena, as well as for genetic interactions *in vivo*. Classically, the use of nonsense suppression has been limited to phage, prokaryotes and single-celled eukaryotes. Genetic screens have succeeded in finding nonsense suppressors in the nematode *C. elegans* (Waterston and Brenner 1978; Waterston 1981; Kondo *et al.*, 1988, 1990). Nonsense suppression in *C. elegans* is a generally useful technique, with more than 20 different phenotypically suppressible genes available, affecting almost every tissue in the animal (Hodgkin *et al.*, 1987). All nonsense suppressors thus far isolated in *C. elegans* have been suppressors of amber mutations (Hodgkin, *et al.*, 1987). Furthermore, all that have been characterized have been members of the $tRNA_{Trp}$ genes in nematodes, and mutations in eight of them have been isolated as amber suppressors (Kondo *et al.*, 1990; 1988). The pattern of suppression of the panel of amber nonsense mutations suggests that different members of the $tRNA_{Trp}$ gene family may be expressed at different levels, and possibly in a cell-type specific manner (Kondo, *et al.*, 1990). They found that suppressor $tRNA_{Trp}$ expressed from different loci gives distinctive patterns of informational suppression. Since the coding regions are identical, the variation in suppression ability must be due to differences in the level or the tissue specificity of suppressor gene expression. Since some of the amber-mutated genes being suppressed are cell type-specific genes, the different spectrum of suppression efficiencies of suppressors suggests cell type-specific expression of the $tRNA_{Trp}$ genes exists. *In vitro* analysis of $tRNA_{Trp}$ genes was carried out by Linning (1992). The *in vitro* transcription assay showed that *sup-7* expressed at the highest level, values for others relative to *sup-7* were: *sup-5* 90%, *sup-24* 69%, *sup-28* 26%, *sup-29* 19%. Discrete upstream positive regulatory regions were found to be involved in the expression of *sup-7*, *sup-24* and *sup-*

29 by deletion analysis. It was found that approximately 20-30 base-pairs of upstream sequences modulated the expression of *sup-7* whereas the sequences of *sup-24* and *sup-29* were much closer to the genes.

My research is focused on how individual members of a tRNA gene family are regulated, and whether this mechanism can be more generally applied to genes transcribed by RNA polymerase III. In order to understand this problem further, an amber site was introduced by unique site elimination site-directed mutagenesis (Deng and Nickoloff, 1992) near the N-terminus of a *lacZ* gene driven by *C. elegans* heat shock gene *hsp16-48* promoters (Stringham, *et al.*, 1992). The resulting single amber mutation was verified by DNA sequencing and expression testing in *E. coli*. It was then introduced as a reporter gene into transgenic worms (Fire, 1986; Mello *et al.*, 1991). The presence of transgene arrays was verified by PCR using primers from *C. elegans hs16-48* and *E. coli lacZ* respectively. *Sup7, sup24, sup28* and *sup29* strains were chosen because they represented strong, medium and weak levels of transcription *in vitro* and of suppression *in vivo*. Suppression of the *lacZ* amber was studied by *in situ* histochemical staining techniques (Fire *et al.*, 1990). One strain of sup29 transformed with an amber *lacZ* was obtained; other sup worms were too weak to survive the germline microinjection. Heterozygote *sup7/+, sup24/+, sup28/+* and *sup29/+* worms were generated by appropriate crosses to w.t. worms carrying *lacZam*. In nervous system, the suppression efficiency order is *sup7/+* > *sup24/+*. No suppression was observed in *sup28/+, sup29/+* and *sup29/sup29* worms. In pharynx muscle cells, the suppression order is *sup7/+* >> *sup29/sup29* > *sup24/+* > *sup28/+* > *sup29/+*. No suppression was observed in *sup29/+* worms. In body muscle cells, the order is *sup24/+* ≥ *sup28/+* ≥ *sup29/sup29* > *sup7/+* ≥ *sup29/+*. In pharynx hypodermis cells, the order is *sup7/+* > *sup24/+* ≥ *sup28/+* > *sup29/sup29* > *sup29/+*. In body hypodermis cells, the order is *sup24/+* ≥ *sup28/+* ≥ *sup29/sup29* > *sup7/+* ≥ *sup29/+*. In intestinal cells, the order is *sup7/+* > *sup29/sup29* ≥ *sup24/+* > *sup28/+* > *sup29/+*.

These results suggest that suptRNA genes may be expressed tissue-specifically. In tissues tested, *sup 29* is always weakest. Others are interchanged in strength. Making

stable integrated *lacZ* amber strains will allow us to further confirm these results, and pursue possible molecular mechanisms.

MATERIALS AND METHODS

Maintenance of Strains and Genotypes of sup strains used

Caenorhabditis. elegans strains were handled and cultured on nematode growth medium (NGM) plates seeded with *Escherichia coil* strain OP50 at 21°C, essentially as described by Brenner (1974). The genetic nomenclature follows Horvitz *et al.* (1979).

The genotype and description of *C. elegans* strains used are summarized in Table I. The strains were supplied by Caenorhabditis Genetics Centre (University of Minnesota).

Table I: *C. elegans* Strains studied

Strain	Genotype	Description and culture conditions	Reference
DR497	unc-13 (e450) I; daf-7 (m62) III; sup-7 (st5) X.	Both unc-13 and daf-7 are suppressed by sup-7. sup-7 should be grown between 22.5°C and 24°C.	Made by Golden J.
CB 4425	tra-3 (e1107) sup-24 (st354) IV.	Wild type hermaphrodite phenotype, since tra-3 is fully suppressed.	Kondo *et al.*, 1988
CB4435	dpy-20 (e2017) sup-24 (st354) IV.	Extreme Dpy suppressed to weak Dpy or non-Dpy phenotype by amber suppressor.	Kondo *et al.*, 1988
CB3874	dpy-20 (e2017); sup-28 (e2058) X.	Partly or completely suppressed Dpy.	Hodgkin, 1985
CB3737	sup-29 (e1986) tra-3 (e1903) IV.	WT phenotype. tra-3 amber mutant carrying homozygous linked amber suppressor	Hodgkin, 1985

Site-directed mutagenesis

The site-directed mutagenesis techniques used were based on the unique site elimination strategy (Deng and Nickoloff, 1992). A USE mutagenesis kit (Pharmacia Biotech) was used, except that purification of the DNA by spin column chromatography through Sepharose CL-6B was omitted; T_4 Kinase (10u/µl, GIBCO BRL) was used for phosphorylation of the primers; and the heat shock temperature for transformation of *E. coli* was changed to 37°C instead of 42°C. Plasmid DNA was treated with *EcoRV*. Mutated plasmid would lack this restriction site and therefore remain circular, whereas wild type DNA would be linearized by this digestion. The digested DNA mixture was used to transform a repair-defective *BMH 71-18 mutS* (Zell and Fritz, 1987) strain of *E. coli*. Transformation with mutant plasmid DNA was favoured since the transformation efficiency was 100-1000-fold greater with circular than with linearized DNA (Conley and Saunders, 1984). Plasmid DNA was extracted following the instructions of the Magic or Wizard Minipreps kits (Promega) and subjected to a second round of *EcoRV* digestion and transformed into *E. coli DH5α*. Potential mutated plasmid DNA was isolated following the method called "Easypreps" which was described by Berghammer *et al.* (1993). 700 µl overnight culture was collected and centrifuged at 13,000 rpm for 1-2 min. The pellet was resuspended in 70 µl lysis buffer (10 mM Tris-Hcl, pH 8.0; 1 mM EDTA; 15% wt/vol sucrose; 2 mg/ml lysozyme; 0.2 mg/ml pancreatic RNase; 0.1 mg/ml bovine serum albumin [BSA], stored at -20°C). The suspension was left at room temperature for 5 min and heated to 99°C (use PCR machine) for 60-90 sec, then chill it on ice for 1 min and centrifuged at 13,000 rpm for 20 min at room temperature. The supernatant was transferred to fresh tube.

The method used to make double amber sites mutant pPCZ1 was described by Ray and Nickoloff (1992). It was a PCR-based version of unique site elimination. DNA used as the template was pPCZ1am (from primers 498 and 499) which was extracted using the Wizard Minipreps kit. The PCR reaction was set up as following: pPCZ1am was diluted directly into ddH_2O. The ratio was 1:100. The final concentration was 1-

2ng/μl. Add 1μl of diluted pPCZ1am; 2μl of primers 497 and 498 respectively (300ng/μl of each); 5μl of 10X Vent buffer (New England Biolabs); 1μl of dNTP (25mM of each); 38.5μl of ddH_2O and 0.5μl of Vent DNA polymerase (2u/μl, Biolab, New England). The total volume was 50μl. The reaction was performed in GTC-2 Genetic Thermal Cycler (GL Applied Research, Inc.) at 96°C, 2min; 96°C, 30sec; 42°C, 1min for the first cycle; 96°C, 30sec, 42°C, 1min, 72°C, 2min for the next 35 cycles and after 10min at 72°C, the sample was parked at 6°C overnight. The PCR product was separated by gel electrophoresis in 0.7% agarose and excising the band and centrifuging in a pipette tip filled with glass wool in a benchtop centrifuge, 8000rpm, 10 min. The PCR-2amber primer was ethanol precipitated and phosphorylated: add 1.5pmole 1kb PCR-2amber primer, 5X Forward Reaction Buffer (GIBCO BRL), 3μls of 10mM ATP (GIBCO BRL) and 3μls of T_4 Kinase (10u/μl, GIBCO BRL). Add ddH_2O to 30μls. The sample was incubated at 37°C for one hour and shifted to 65°C for 10min to inactivate the enzyme. The following steps were the same as described in U.S.E. Mutagenesis kit (Pharmacia).

Preparation of DNA for Site-directed Mutagenesis

General methods for DNA analysis and buffers have been described (Sambrook *et al.*, 1989). The plasmid which was used as the reporter gene was pPCZ1 (Stringham *et al.*, 1992), constructed by inserting a 3500 bp *HindIII-AflII* fragment encompassing the *lacZ* gene [nucleotides 18-3518 bp of the expression vector pPD16.43, (Fire, 1990)] into the *HpaI* site [nucleotide 3565, (Russnak and Candido, 1985)] of the *hsp16-1* gene. pPCZ1 contains a complete *hsp16-48/1* gene pair extending from a *BclI* site at nucleotide 2280 to the *BamHI* site at nucleotide 4186 in the published DNA sequence (Russnak and Candido, 1985). Competent *E. coli* DH5α cells (Hanahan, 1983) were transformed with pPCZ1 and plated on LB Ampicillin media. Single colonies were used to inoculate LB Ampicillin broth which was shaken overnight at 37°C. Plasmid DNA was purified by QIAGEN plasmid mini kit (QIAGEN Inc.).

Methods Used to Prepare Oligonucleotides

The program which was applied to design the oligonucleotides was OLIGO™ software package (version 4.0; National Biosciences, Plymouth, MN, USA). The custom oligonucleotides were synthesized on a model 391 ABI oligonucleotide synthesizer and purified by oligonucleotide purification cartridge (*lacZ* primers) or SEP-pack purified (*hsp 16-48/1* primer). The primers were:

4 from *lacZ*: 497 (am cw), 22mer, M.W.: 6703.4; (5' 173-194 3');

498 (mutRVccw), 23mer, M.W.: 6950.6; (5' 1138-1116 3');

499 (amccw), 27mer, M.W.: 8338.4; (5' 281-255 3');

500 (3mutRVcw), 30mer, M.W.: 9293.0; (5' 1112-1141 3',

Kalnins *et al.*, 1983)

1 from *C. elegans hsp16-48/1* gene pair promoter region: hsDNA, 20mer, M.W.: 6056.0, from 3329 to 3348 in published sequence (Russnak and Candido, 1985).

Figure 2 shows the map of pPCZ1 and sequences of the primers.

Figure 2: Map of pPCZ1 and DNA sequences of the primers

This is the map of plasmid pPCZ1. A 3500bp lacZ fragment (shown as thin line with nuclear localization signal near its 5' end was inserted into the second exon (shown as thicker black lines, the introns and the flanking sequences are shown as open lines.) o *C. elegans* heat shock gene 16-1. The relative positions of the five primers are shown i the picture as big arrows except for primer 3mutRVcw which is shown as an ope rectangle. The small black arrows show the orientation of transcription of the tw *Caenorhabditis elegans* heat shock genes. The puc 19 backbone is shown as thick gre line.

The DNA sequences of the primers are listed below. The original nucleotides c the primers are shown above the mutated ones which are bold and underlined.

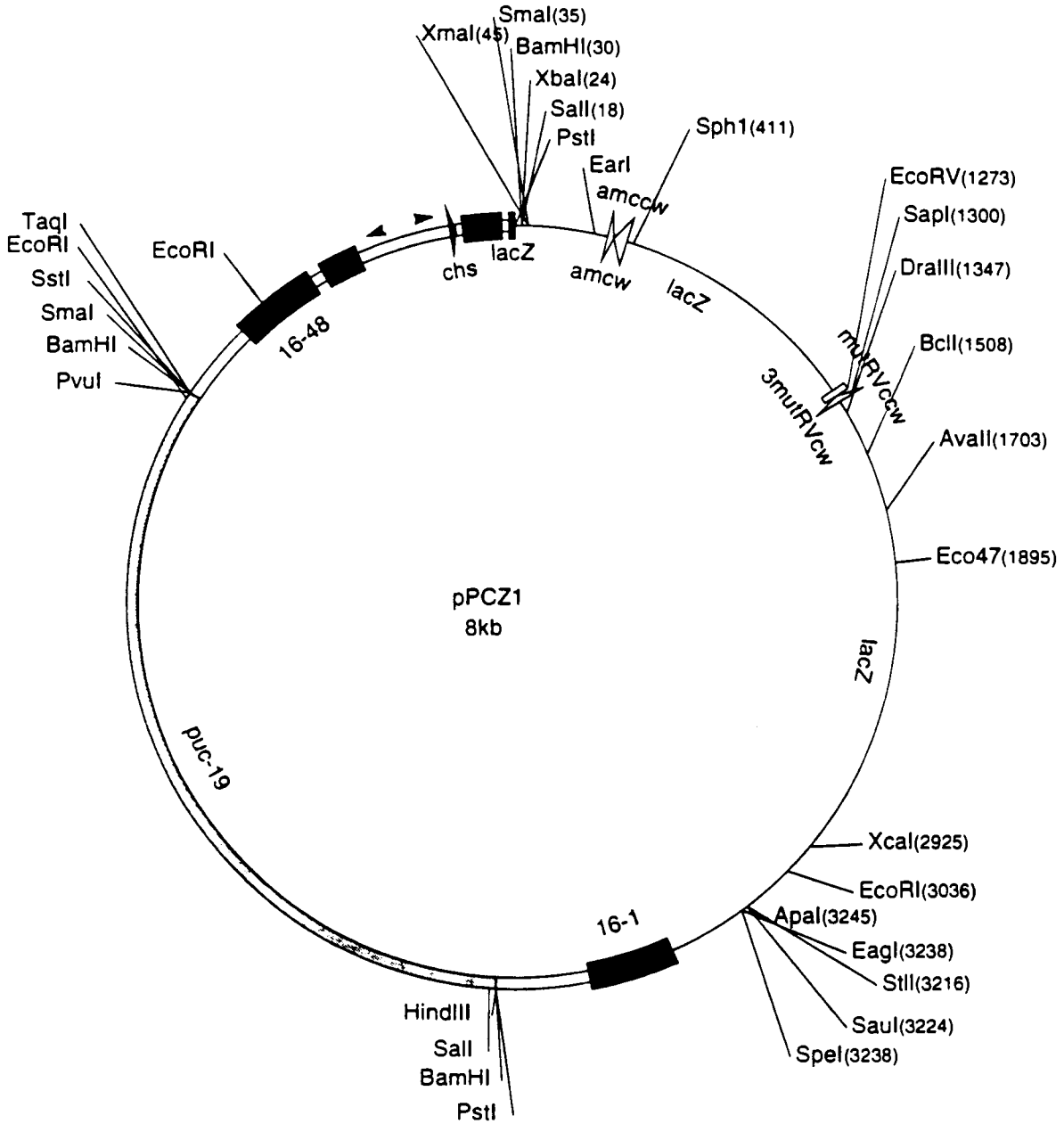
G
amcw: GGCGCTTTGCCT**A**GTTTCCGGC;

C
amccw: CCGTGCATCTGC**T**AGTTTGAGGGGACG

T CC
3mutRVcw: CGATGGTGCAGGA**C**AT**TT**TGCTGATGAAGC

G
mutRVccw: TCATCAGCAG**A**ATATCCTGCACC

chs: AAACCGAACCAAACAACATT


SmaI(35)
XmaI(45)
BamHI(30)
XbaI(24)
SalI(18)
PstI
Sph1(411)
EarI
amccw
EcoRV(1273)
SapI(1300)
DraIII(1347)
BclI(1508)
AvaII(1703)
Eco47(1895)
TaqI
EcoRI
SstI
SmaI
BamHI
PvuI
EcoRI
16-48
chs
lacZ
amcw
lacZ
3mutRVcw
mutRVccw
pPCZ1
8kb
lacZ
puc-19
16-1
XcaI(2925)
EcoRI(3036)
ApaI(3245)
EagI(3238)
StII(3216)
SauI(3224)
SpeI(3238)
HindIII
SalI
BamHI
PstI

Verification of mutagenesis by DNA sequencing

DNA sequencing was carried out using the ^{T7}Sequencing^{TM} Kit (Pharmacia Biotech). This method is base on the enzymatic dideoxy termination strategy (Sanger *et al.*, 1977). Oligonucleotides used for DNA sequencing were the same as those used for site-directed mutagenesis.

DNA templates for sequencing were prepared by Magic or Wizard miniprep kit (Promega).

Three *E. coli* strains were picked for preliminary testing for amber mutations: ***DH5α*** : *supE44ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (Hanahan, 1983); ***DH10B*** : *F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻rpsL nupG* (BRL); ***XAC-1:*** F' *lacpro* derivative of XAC (*ara Δ(lacpro) gyrA argE-am rpoB thi*) carrying Γ⁻ and Z⁻ mutations on the F'. The Z⁻is an amber mutation at coding position 17 (Kleina *et al.*, 1990).

Preparation of DNA for germline transformation

Plasmid pPCZ1 was prepared for injection by Magic or Wizard miniprep kit (Promega). The buffer used for injections was TE (10 mM Tris pH7.5, 1mM EDTA Ph8.0, Mello *et al.*, 1991). pRF4 carrying a 4kb *EcoRI* fragment of *C. elegans* genomic DNA containing the *rol-6(su 1006)* mutant collagen gene (Kramer *et al.*, 1990) was used as a dominant genetic marker for DNA transformation and purified by Nucleobond AX kit "Preparation of plasmid and cosmids (modified alkaline/SDS lysis)" (MACHEREY-NAGEL GmbH & Co. KG).

Syncytial microinjection technique

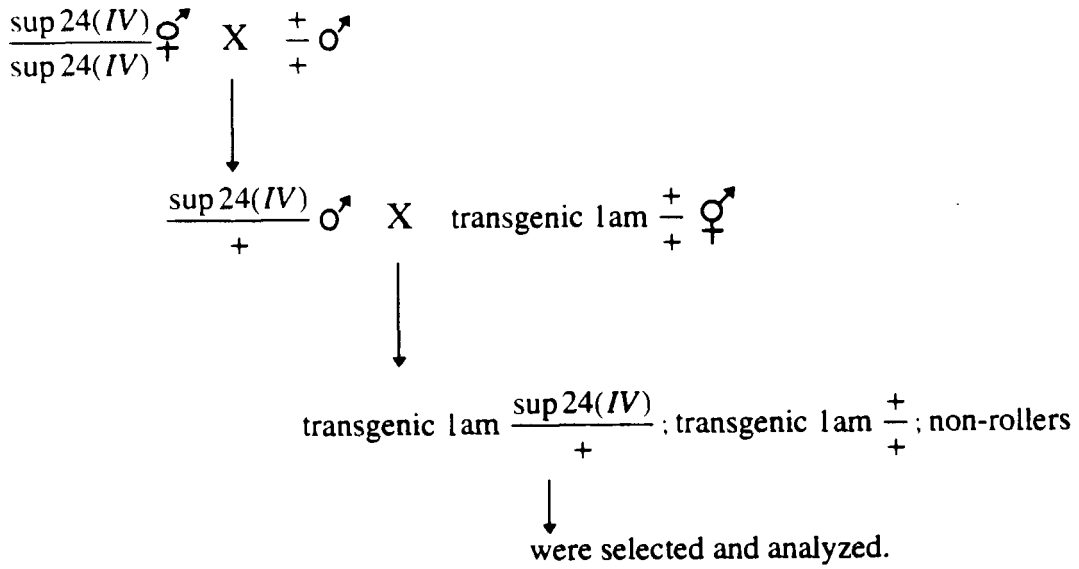
Injection needles were pulled from glass capillaries (Item No. 1B100F-6, World Precision Instruments, Inc., Sarasota, FL) using a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Co.) and were filled with *rol-6* DNA (100ng/ul) and pPCZ1 (2-20 ng/ul). Different *C. elegans* strains (N_2, *Sup29, Sup28, Sup24, Sup7*) were injected at a magnification of 400X using a IMT-2 Inverted Research Microscope (OLYMPUS) equipped with Nomarski optics and a Leitz (Wetzlar, Germany) micromanipulator according to the methods of Fire (1986) and Mello *et al.* (1991). A behavioral marker provided by an allele of *rol-6 (su1006)* (Kramer *et al*, 1990) was co-injected with pPCZ1 to monitor the production of transgenic *C. elegans* lines. This allele encodes a mutant form of collagen which alters the structure of the worm's cuticle such that the worm turns onto its right side as it attempts to move forward.

The injection solution was centrifuged for 10-30 min at top speed in the benchtop microcentrafuge before use to remove any particulate material.

Methods for introducing pPCZ1-am or pPCZ1-2am into sup-worms genetically

To introduce pPCZ1-am or pPCZ1-2am into sup-worms, 4-6 L_4 sup hermaphrodites and 8-12 L_4 N_2 males were picked up respectively and were set up for outcross on NGM mating plates seeded with *Escherichia coil* strain OP50 at 21°C (for sup-7, 23°C -24°C) (Brenner, 1974) overnight. On day 2, one hermaphrodite and one male were transferred to a fresh plate. L_4 male progeny which contained one sup gene were selected from these single hermaphrodite plates and crossed with transgenic L_4 N_2 hermaphrodites (with pPCZ1-am or pPCZ1-2am plasmid). Figure 3 shows the illustration of the genetic outcross experiments. Rolling progeny were chosen and the presence of insert was verified by PCR or *in situ* histochemical staining.

Figure 3. Illustration of the genetic outcross



Verification of germline transformation by PCR

Taq DNA polymerase (Lawyer *et al.*, 1989) (GIBCO BRL) was used in PCR reactions. Three primers, one **chs** (5' end) from *C. elegans hsp 16-48/1*(promoter region between the two genes) and another two: **amccw** and **mutRVccw** (3' end) from *lacZ* (the same as those used for site-directed mutagenesis) were used in this experiment because the vector pPCZ1 was constructed as a fusion of *C. elegans Hsp 16-1* and *E. coli lac Z* genes. The 5' end primer is 5' AAA CCG AAC CAA ACA ACA TT 3'. M.W. 6056.0, Tm 54°C.

C. elegans DNA was prepared following the method of Barstead, R. (personal communication). Two L_4 worms were picked for each reaction and transferred into 2.5 µl of worm lysis buffer (50mM KCl, 10 mM Tris HCl pH8.2, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatine, filter sterilize and store at room temperature; just before use, add 5µl of 10mg/ml proteinase K which was in H_2O and stored in aliquots at -20°C to 500µl of lysis buffer.) that was previously aliquoted into the cap of a 0.5ml microfuge tube. The tube was closed and left inverted on ice until several more worms were collected in a separate tube. Then, the lysis buffer and the worms were deposited into the bottom of each tube by a brief spin in the microfuge, and the tubes were placed at -70°C for 10 minutes. A single drop of paraffin oil is added to each tube and the tubes were then heated to 60°C for one hour, followed by 95°C for 15 minutes. At this time 22.5µl of a PCR mix [10-50 pm of each primer, 2.5µl 10X PCR reaction buffer (GIBCO BRL), 4µl of dNTP (Pharmacia) mix (1.25 mM for each dNTP), 0.125µl of Taq polymerase (Stock is at 5u/µl, GIBCO BRL), add H_2O to 22.5µl] was added to each tube and spun down through the oil droplet. PCR reaction was set up as the following: first cycle: 96°C, 2min., 96°C, 30 sec., 42°C, 1min., 2nd to 36th cycles: 96°C, 30 sec., 42°C, 1 min., 72°C, 2min., and finally the temperature was kept at 72°C for 10min.. The sample was parked at 6°C over night (GTC-2 Genetic Thermal Cycler, TS-65974-793, GL Applied Research, Inc. Grayslake, IL.). The reaction solution was separated from paraffin

oil by dropping on paraffin film. Load 1/2 of the reaction of on a 0.7-1% agarose gel containing 0.5µg/ml of ethidium bromide.

In situ histochemical staining techniques

Transgenic worms were studied for *lacZ* expression by heat shocking at 33°C for 2 hr on NG plates spread with bacteria. 33°C was chosen as a suitable heat shock temperature because it provided good expression of the *lacZ* transgene in all strains (Stringham *et al.*, 1992). Worms were subsequently allowed to recover for 15 min at 21°C before being picked on to the slide with 10 µl distilled water in the well under the microscope. It took 1-3 hrs to pick up all the samples which need to be checked. Worms were permeabilized by room temperature dry and -20°C-acetone treatment, and incubated in a histochemical stain containing X-gal (Fire *et al.*, 1990, 1992, Stringham *et al.*, 1992) overnight at 37°C. Some worms were incubated in staining solution without prior heat shock treatment.

Micro-photography

Worms were stained and photographs were taken using Kodacolor, Kodak Gold ultra ISO 400 film on an Olympus AHB S3 research photomicrographic microscope system. Recipro. was set at 3, exposure adjust was 1.00. Nuclei were scored for the presence or absence of blue stain. The identity of stained cell types was determined by the size and the shape of the nucleus and its position in the animal relative to other nuclei, as defined by Sulston (1976), Sulston and Horvitz (1977), Albertson and Thomson (1976).

RESULTS

Experimental Design

Because differential suppression efficiency was observed among individual suppressors *in vivo* in previous work (Kondo *et al.*, 1988, 1990), we chose four $tRNA_{Trp}$ amber suppressors to analyze the potential tissue-specific expression of individual members of the $tRNA_{Trp}$ gene family.

The general plan of this research was to construct a mutated reporter gene, which contains an amber codon in the coding region and is controlled by a non-tissue specific promoter which is expressed in *C. elegans*. Then the mutated reporter gene can be introduced into different sup strains by germline microinjection, or by outcrossing sup animals with transgenic strains carrying the mutated amber reporter gene. Theoretically, the expression of each sup tRNA gene can then be observed via assaying the activity of the suppressed amber reporter gene.

pPCZ1 was chosen as the reporter gene for three reasons: first of all, the heat shock promoter drives the expression of the genes in the widest range of tissues in *C elegans* (Stringham *et al.*, 1992). Expression of pPCZ1 transgenes was consistently observed in embryos from gastrulation onward, in all larval stages, and in adults. In postembryonic stages, it was expressed in most tissues, including neurons, muscle, intestine, and hypodermis. Only the germ line failed to express this fusion gene. Secondly, transcription of the reporter gene is controllable because it is totally heat-shock dependent and results in the rapid synthesis of detectable levels of β-galactosidase (Stringham *et al.*, 1992). Thirdly, the *lacZ* gene provides a simple assay for detection. Even though most organisms have some low-level endogenous β-galactosidase activity, their activity can be suppressed at neutral or slightly alkaline pH because the endogenous

enzymes are optimally active at low pH. Figure 2 shows the plasmid DNA used in this research, the sequence and relative positions of the primers.

Construction of Amber Mutations

I. Making 1 and 2 Amber Mutants

To introduce an amber site into the amino-terminus of lacZ gene in pPCZ1, USE (**u**nique site **e**limination) site-directed mutagenesis method was used (Deng and Nickoloff, 1992). The procedure employs two mutagenic oligonucleotide primers. One primer contains the desired amber mutation (amcw, means amber, clockwise) and the second contains mutations in a unique, nonessential *EcoRV* site (3mutRVcw, means clockwise, three nucleotides are mutated at the *EcoRV* site). The two primers are annealed to circular denatured single-stranded DNA, followed by direct synthesis of a new second strand containing both primers. The resulting DNA is subjected to *EcoRV* digestion and transformed into a mismatch repair defective (BMH 71-18) *E. coli* strain. The purified plasmid pPCZ1 is digested by *EcoRV* again and retransformed into *E. coli* DH5α. Wild type, linearized pPCZ1 DNA transforms bacteria 10-1000 fold less efficiently than circular, mutant *EcoRV*-resistant pPCZ1. Besides this pair of primers, a set of counterclockwise primers are also used. Three single amber pPCZ1 mutants were obtained from the counterclockwise primers, whereas none was found from the clockwise primers.

After pPCZ1-1am was made, 2-amber pPCZ1 was constructed by LP-USE.(**l**ong **p**rimer) reported by Ray and Nickoloff (1992). This method is a PCR-based modification of the USE mutagenesis procedure. The first step involves PCR using an amber primer and a second primer that eliminates the *EcoRV* site. Then the PCR products are used as long primers to finish the second mutant strand. In this experiment, pPCZ1-1am was used as the template; primer amcw was used to introduce the second amber site into the *lacZ* gene and mutRVccw (counterclockwise primer, eliminates *EcoRV* site) was the second primer for PCR. The 1kb PCR products were used as long primers for synthesizing the

new second strands. The subsequent procedures for pPCZ1-2am construction were the same as USE mutagenesis.

II. Efficiency of Different Oligonucleotides for Mutagenesis

The OLIGO software program provides general guidelines for the design of mutagenic oligonucleotide primers. Two pairs of primers were designed, amcw paired with 3mutRVcw (clockwise primers), amccw paired with RVccw (counterclockwise primers). All the mutagenic oligonucleotides except for 3mutRVcw, which had 3-base substitutions, were designed to introduce a single base change and they all had 9 to 13 perfect matches on the 5' and 3' end. Potential hairpin and dimer formation were minimized using the same program. After the first round of EcoRV digestion and successful transformation *of E. coli* BMH 71-18 mutS, a second round of *EcoRV* digestion and *E. coli* DH5α transformation was done. The transformation efficiency was 8.4×10^4 colonies/μg.

Verification of Mutagenesis by Restriction Enzyme Analysis and DNA Sequencing

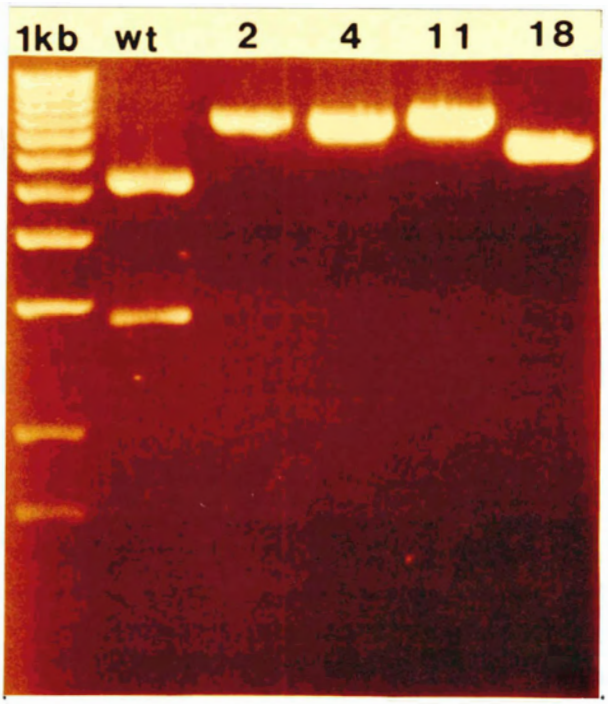
In order to identify resultant mutated plasmid clones and exclude other undesirable mutant clones, further restriction enzyme digestion was carried out. Two enzymes, *EcoRV* and *HindIII* each of which recognizes a unique site in pPCZ1, were chosen. The desired mutant pPCZ1 is linearized and should show only one band in the gel electrophoresis (0.7% agarose gel) picture whereas the non-mutated one, which still contains an *EcoRV* site, will show two bands. Figure 4 shows the gel electrophoresis picture of DNA digested by *EcoRV* and *HindIII*.

Seven *EcoRV*-digestion resistant pPCZ1 were then cut by EcoRI, and only those whose banding pattern was the same as unmutated pPCZ1 were chosen as the template for DNA sequencing. Figure 5 shows the *EcoRI*-digested pPCZ1 banding pattern of four samples. DNA sequencing was carried out by dideoxy termination strategy (Sanger *et al.*,

1977, also see MATERIALS AND METHODS). The primer amcw was used to sequence the amber site cause by amccw and vice versa. Figure 6 shows a single base substitution of an adenine for a guanine residue at the desired position. Three single amber pPCZ1 were found.

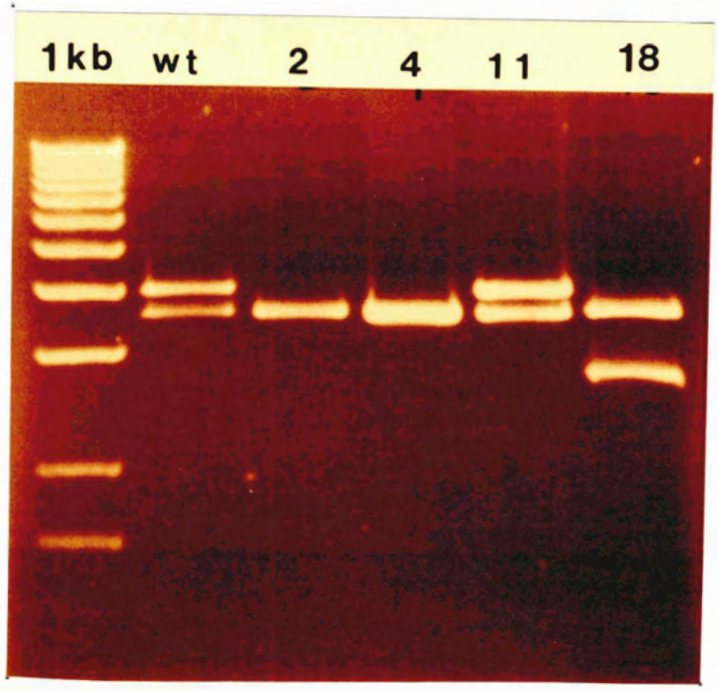
The 2-amber mutant pPCZ1 was verified by the same method except that one template DNA was sequenced twice from two primers: amcw and amccw. Five 2-amber mutant pPCZ1 were verified after 7 clones were sequenced.

Figure 4 Gel electrophoresis picture of DNA digested by *EcoRV* and *HindIII*



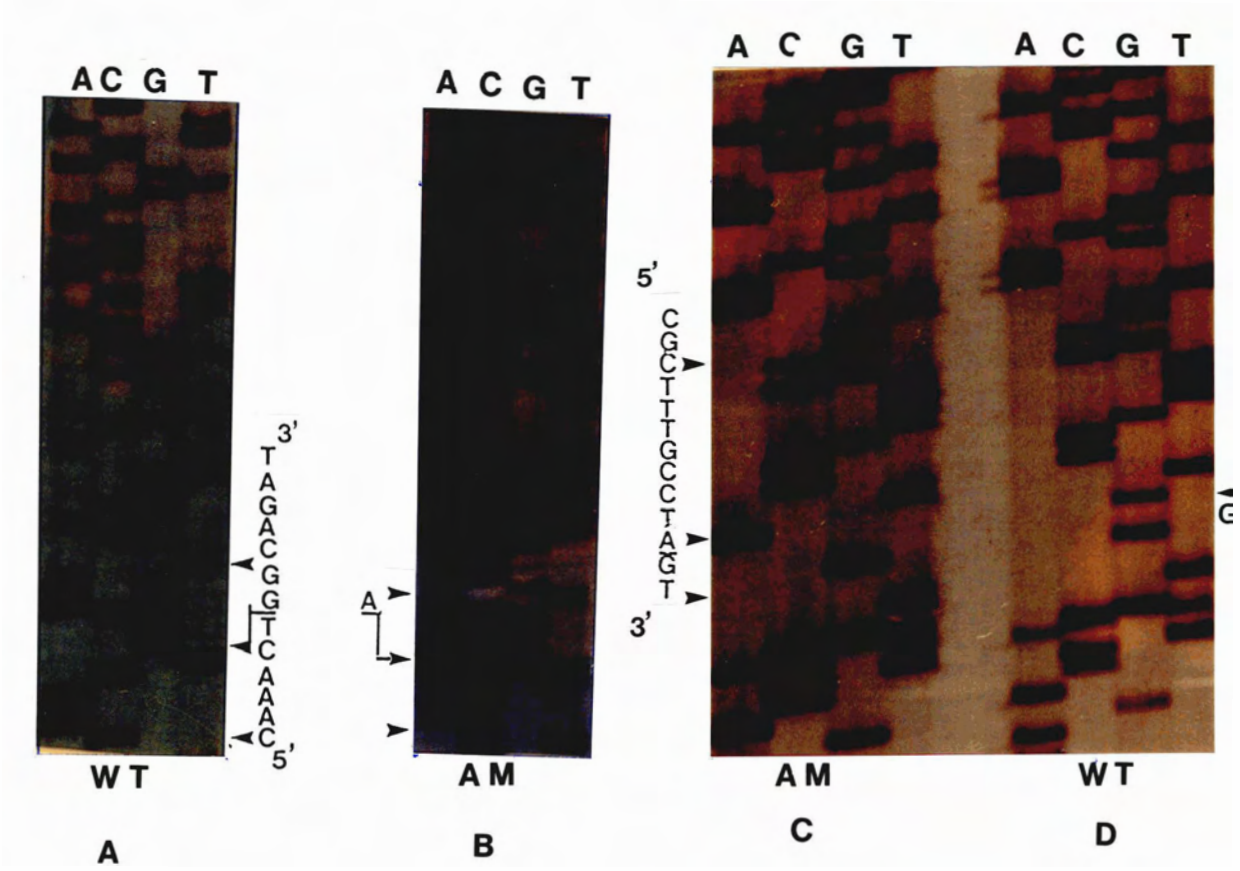
Plasmid pPCZ1 and potential mutants were digested by *EcoRV* and *HindIII*. Lane 1 shows the 1 kb marker; lane 2 shows pPCZ1 which is not mutated and was cut into two fragments: 2.8 kb and 5.2kb; lanes 3-6 show the potential *EcoRV*-resistant pPCZ1.

Figure 5 Gel electrophoresis picture of DNA digested by EcoRI



Plasmid pPCZ1 and the potential mutants were digested by *EcoRI* to limit the non-desired mutation. Lane 1 shows the 1 kb marker; lane 2 is a control which shows the non-muted pPCZ1; lanes 3-6 are the same as in Figure 4, the sample in lane 11 was picked to be sequenced.

Figure 6 DNA sequencing gel picture.



The coding sequences of *lacZ* are shown in this picture. Plates A and B show the region covered by primer amccw. Plates C and D show the sequences covered by primer amcw. The arrows at the edges specify the range of the sequences shown in letters in this picture. The middle arrows specify the position of the mutated nucleotide which are underlined. WT -- wild type, AM-- amber.

Verification of Mutagenesis by *E. coli* Transformation

To verify the amber site near the 5'-end of *lacZ* gene, three *E. coli* strains were selected as the host for transformation on the assumption that enough *lacZam* mRNA might fortuitously be expressed from mutated plasmid pPCZ1. Table II shows a brief description of each strain.

E. coli DH5α is a recombination-deficient suppressing strain, the ϕ80 lacZΔM15 might permit α-complementation with the amino-terminus of β-galactosidase. supE-encoding tRNA inserts glutamic acid at the nonsense codon UAG at amino acid 90 near the N-terminus of lacZ. This nonsense codon UAG was originally a Trp codon before the introduction of the amber site. β-galactosidase activity might be partially recovered, resulting in blue colonies on medium containing the indicator X-gal.

The second strain is *E. coli* DH10B which contains the ϕ80 lacZΔM15 marker. This marker would also allow α-complementation of the β-galactosidase, but it doesn't contain a sup gene to correct the amber mutation. The short lacZ gene product couldn't α-complement with the product from ϕ80 lacZΔM15. As a result, the colony should be white.

The third strain used is XAC-1 which carries an amber mutation in the lacZ gene. No sup gene is carried by this strain. Again, the absence of β-galactosidase activity results in white colonies on X-gal, IPTG medium. Table III summarizes the results of transformation with 1 amber pPCZ1 and 2-amber-site pPCZ1.

Table II *E. coli* Strains used to verify amber mutant pPCZ1.

Strain	Description	Reference
DH5α	supE, α-complementation;	Hanahan, 1983
DH10B	sup–, α-complementation;	BRL
XAC-1	sup–, lacZ–	Kleina *et al.*, 1990

Table III Verification of mutagenesis by *E. coli* Transformation.

Plasmid DNA	DH5α	DH10B-P_3	XAC-1
no DNA	no colony	no colony	no colony
pPCZ1	blue colonies	blue colonies	blue colonies
pPCZ1-1am-2	faint blue colonies	white colonies	white colonies
pPCZ1-1am-6	faint blue colonies	white colonies	white colonies
pPCZ1-1am-7	faint blue colonies	white colonies	white colonies
pPCZ1-2am-25	white colonies	white colonies	white colonies
pPCZ1-2am-27	white colonies	white colonies	white colonies
pPCZ1-2am-29	white colonies	white colonies	white colonies
pPCZ1-2am-31	white colonies	white colonies	white colonies
pPCZ1-2am-36	white colonies	white colonies	white colonies

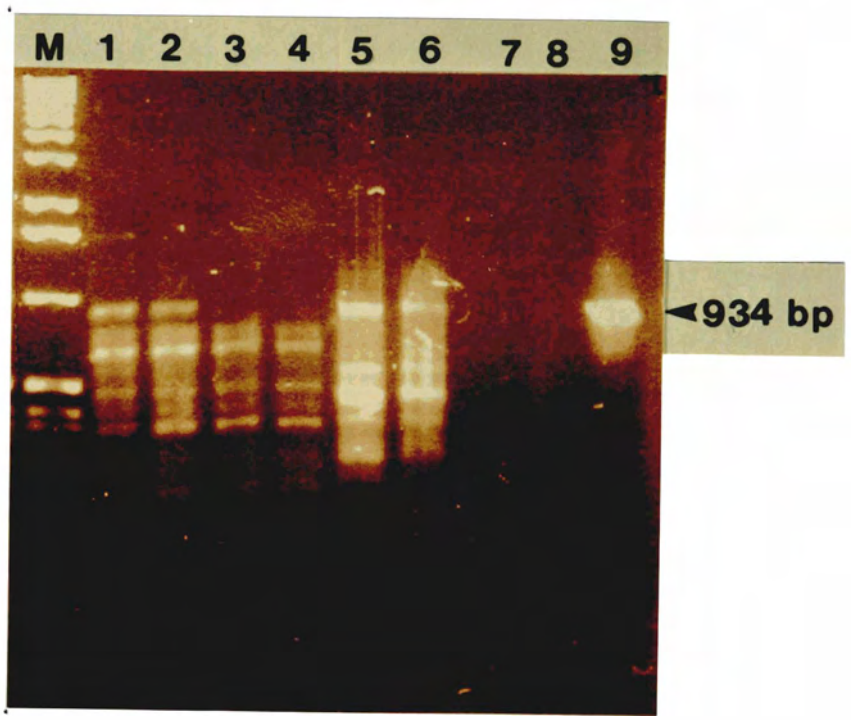
Comparison of Mutagenesis Efficiencies of U.S.E. and LP-U.S.E.

PCR-Based U.S.E. (long primer U.S.E.) is a derivative of U.S.E.. According to Ray and Nickoloff (1992), LP-U.S.E. mutagenesis efficiencies often are 100% which is much higher than that of U.S.E. (80%) (Deng and Nickoloff, 1992). In this experiment, the efficiency of desired mutagenesis by U.S.E. was very low: 3/45 for counterclockwise primers and 0 for clockwise primers. In comparison, LP-U.S.E. efficiency was 25 out of 36 isolated plasmids (no plasmid DNA was shown in the other 9 isolates) . Thus, LP-U.S.E. appears to be a more efficient method for site-directed mutagenesis.

Verification of Germline Transformation by PCR

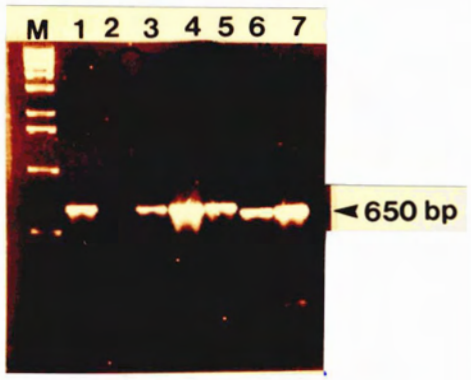
To confirm the successful germline transformation of pPCZ1-1amber in the transgenic worms, the presence of the pPCZ1-1am or pPCZ1-2am sequence was tested by PCR using amcc and mutRVccw as primers. An approximately 1kb band as expected was observed from the transgenic worms (Figure 7). pPCZ1 1am and *E.coli* OP50 were used as positive controls. In order to limit the background banding of *E.coli* OP50, another oligonucleotide (chs, *C. elegans* heat shock) derived from the *C. elegans* heat shock gene pair was synthesized (MATERIALS AND METHODS). Only pPCZ1 and its derivatives or the transgenic worms showed the unique 650bp band as predicted with primers chs and mutRVccw (Figure 8). These two experiments demonstrate that the transgenic worms possess pPCZ1-1am or pPCZ1-2am (Lane 2 shows no band in this picture because of the failure of PCR. More identical samples were used for PCR and an expected band was observed, but data is not shown.).

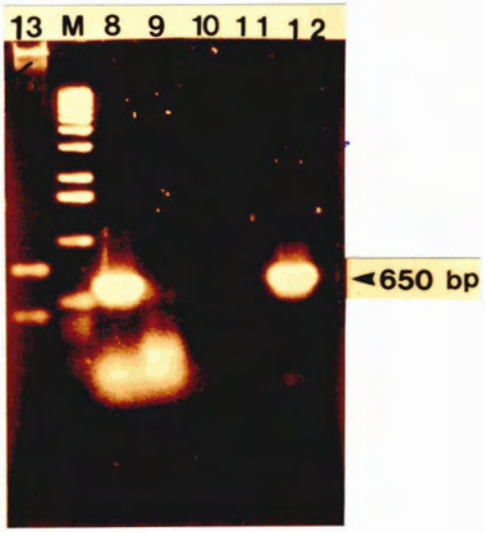
Figure 7 Verification of germline transformation by PCR from *lacZ* primers.



Lane 1 shows 1 kb marker; lanes 2 and 3 represent the DNA of transgenic worms N_2-pPCZ1-1am-A; lanes 3 and 4 show the DNA of N_2 worms; lanes 5 and 6 show the DNA of *E. coli OP50*; lanes 7 and 8 are the negative controls without worm and *E. coli*; lane 9 is a positive control with pPCZ1.

Figure 8 Verification of germline transformation by PCR from *chs* and *lacZ* primers





The primers used in this experiment were amccw and chs. The expected band should be 650 bp. Lanes M stand for 1 kb marker. Lane 1 shows the DNA from transgenic N_2-pPCZ1. Lanes 2-13 represent the results from the following samples: N_2-pPCZ1-1am-A; N_2-pPCZ1-1am-B; N_2-pPCZ1-1am-C; N_2-pPCZ1-1am-D; N_2-pPCZ1-2am-a; N_2-pPCZ1-2am-s; N_2-pPCZ1-2am-b; N_2; *E. coli OP50*; lysis buffer; miniprep pPCZ1-1am plasmid DNA.

Comparison of Suppression Efficiencies among Individual sup Strains

I. Selection and Types of Transgenic Worms

Transgenic *C. elegans* strains were constructed by microinjecting gonads of wild-type or sup young hermaphrodites with DNA of the desired construct, together with a selectable marker *rol-6*. Animals were examined by their right rolling phenotype (Kramer *et al.*, Mello *et al.*, 1991). The transformed lines obtained transmitted marker phenotype at a frequency of 42-73%. Southern analysis suggested that the injected DNA formed mixed extrachromosomal arrays, as described by Stinchcomb *et al.* (1985). Various transgenic wild types (N_2) worms and one strain of transgenic sup29/sup29 worms were obtained directly by germline microinjection technique. Transgenic heterozygote sup worms were constructed genetically. Table IV demonstrates the results of these experiments and Table V shows the transmission frequency of the marker phenotype.

Table IV Types of transgenic worms

Strains	No. of Strains
Transgenic N_2 with pPCZ1	1
Transgenic N_2 with pPCZ1-1am	4
Transgenic N_2 with pPCZ1-2am	3
Transgenic sup29/sup29 with pPCZ1-1am	1

Table V Transmission Frequency of Marker Phenotype

Strains	Frequency (% of Progenies)
N_2 with pPCZ1	67
N_2 with pPCZ1-1am-6A	73
N_2 with pPCZ1-1am-6B	48
N_2 with pPCZ1-1am-6C	70
N_2 with pPCZ1-1am-6D	46
N_2 with pPCZ1-2am-25a	42
N_2 with pPCZ1-2am-25b	56
N_2 with pPCZ1-2am-25s	67
sup29/sup29 with pPCZ1-1am-6	51

Around one third of the transgenic progeny showed partial rolling phenotype: the worms twisted only around the posterior region of the pharynx. And these animals were stained blue only in the same areas in the later experiments. About 130-285 worms were counted from each hermaphrodite.

II. Uniformity of *in situ* Histochemical Staining Techniques

Transgenic lines were assayed for expression of the *hsp16-lacZ* fusion genes from pPCZ1 and its derivatives by heat shock and staining with a solution containing X-gal (MATERIALS AND METHODS). Different heat shock temperatures were chosen for optimal conditions. 33°C gave the strongest staining in all lines compared with 30°C. The worms were treated with -20°C acetone for 2, 6, 8 min: no difference was found. The recovery period after heat shock varied from 15 min to 2h. The animals were put in 33°C incubator for 2h in the following experiments.

III. Analysis of the Different Suppression Efficiencies between *sup7/+* and *sup28/+*

To study differential suppression efficiencies among individual sup strains, heterozygote sup animals were obtained in two outcrossing steps: In the first step, sup hermaphrodites were outcrossed with N_2 males; in the second step, male progeny were picked and outcrossed with transgenic N_2 worms carrying pPCZ1-1am or pPCZ1-2am respectively. To analyze the differences between various transgenic N_2 pPCZ1-1am strains, on the differential suppression efficiencies of individual sup genes, *sup7/* males were outcrossed with 3 different transgenic lines: N_2, pPCZ1-1am-6A, B, D respectively. *sup28/* males also outcrossed with N_2, pPCZ1-1am-6A, D strains. The suppression efficiencies of *sup7/+* are consistently stronger than *sup28/+* despite different transgenic 1 amber N_2 animals were used (Figures 10 and 13.). This result suggests that the differential expression of sup strains is not caused by the differences among N_2 pPCZ1-1am strains.

Figure 9: *In situ* histochemical staining of N_2pPCZ1

LacZ expression of pPCZ1 transgenes in response to heat shock is tissue general. Staining of intestinal, body muscle, hypodermal, and pharyngeal nuclei is clearly visible. The magnification of bright field images is: A - 50X, B - 50X, C - 25X, D - 25X, E - 100X. B and E show the enlarged anterior regions of C and D respectively.

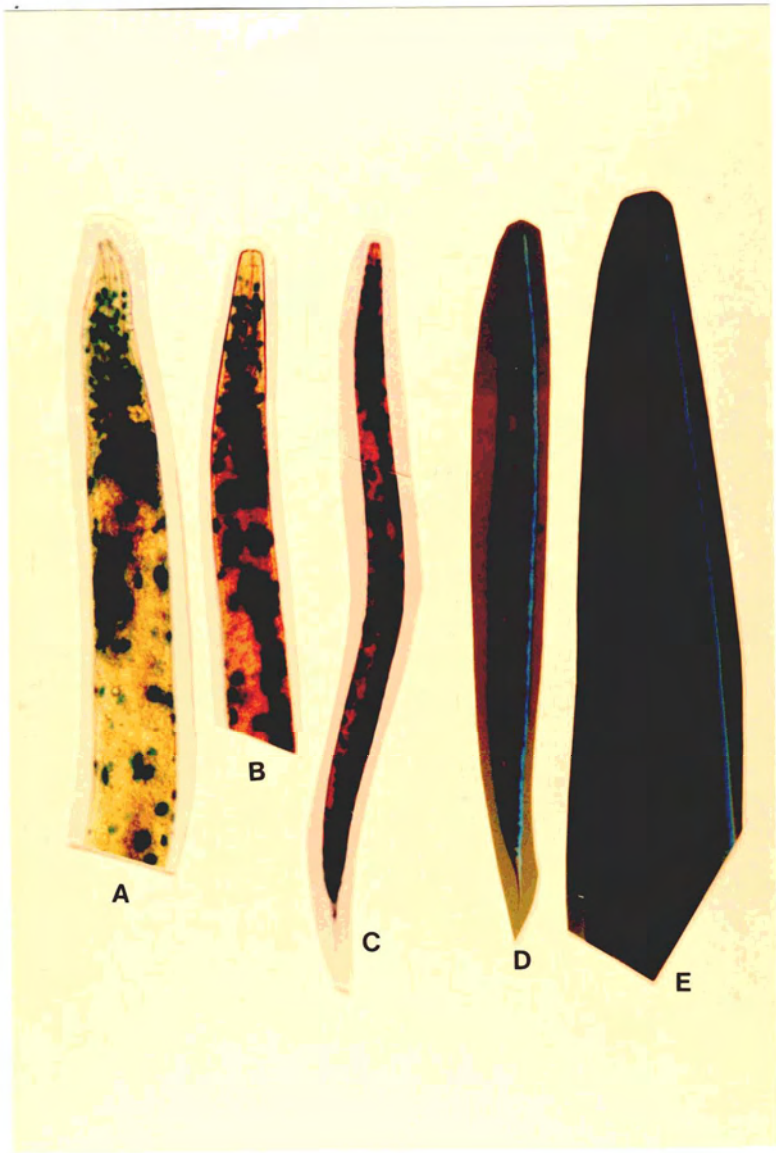

A
B
C
D
E

Figure 10: *In situ* histochemical staining of sup7/+ with pPCZ1-1am

Blue precipitate is seen in pharyngeal, intestinal, embryonic, lumbar and preanal ganglia nuclei, but fewer nuclei are stained in body muscle, hypodermis and body nerve cells than the positive control. The magnification of bright field images is: A - 100X, B - 50X, C - 50X, D - 50X, E - 25X. A and D indicate the enlarged anterior regions of B and E respectively.

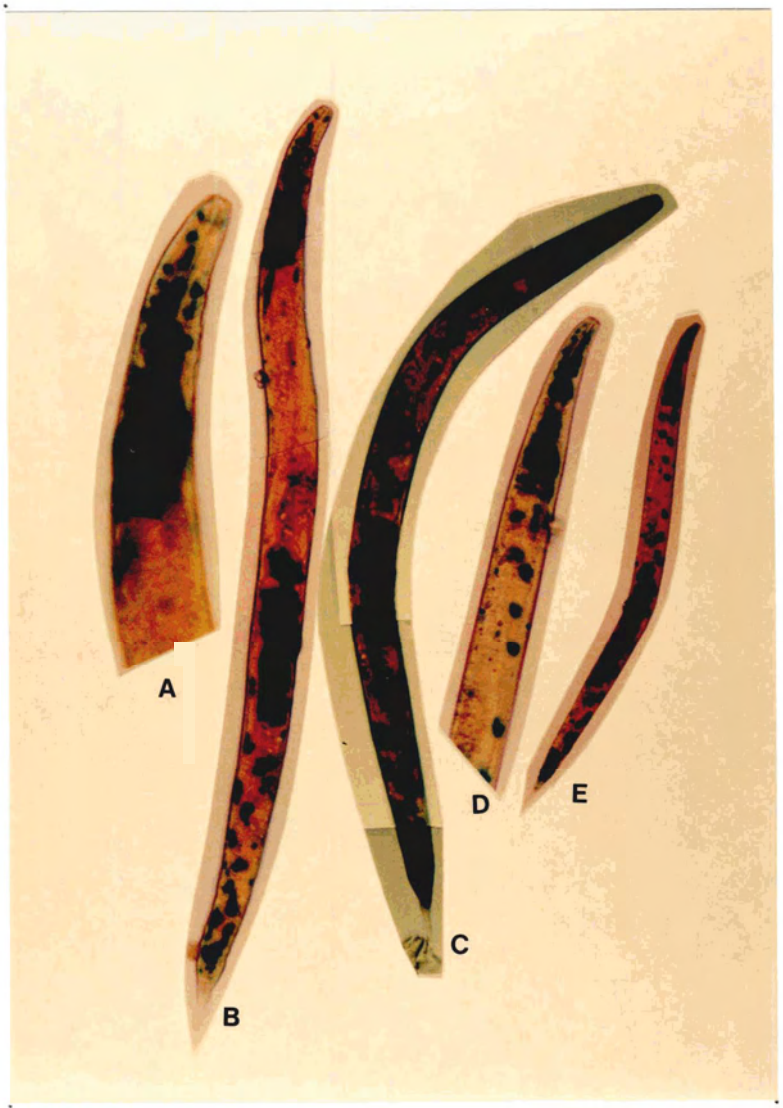

A
B
C
D
E

Figure 11: *In situ* histochemical staining of sup24/+ with pPCZ1-1am

Suppression of sup24 to pPCZ1-1am is shown as blue spots in this picture. The expression is prominent in intestinal, pharyngeal neural, hypodermal and body muscle nuclei. But the general suppression level and the suppression in pharynx are lower than sup7/+. The magnification of bright field images is: A - 40X, B - 33X, C - 40X.


A
B
C

Figure 12: *In situ* histochemical staining of sup24/+ with pPCZ1-1am in pharynx

The suppression in visible in neurons in pharynx in this picture. Staining in hypodermis cell in the same region is shown in A. The magnification of bright field images is: A - 50X, B - 100X, C - 100X, D - 100X.

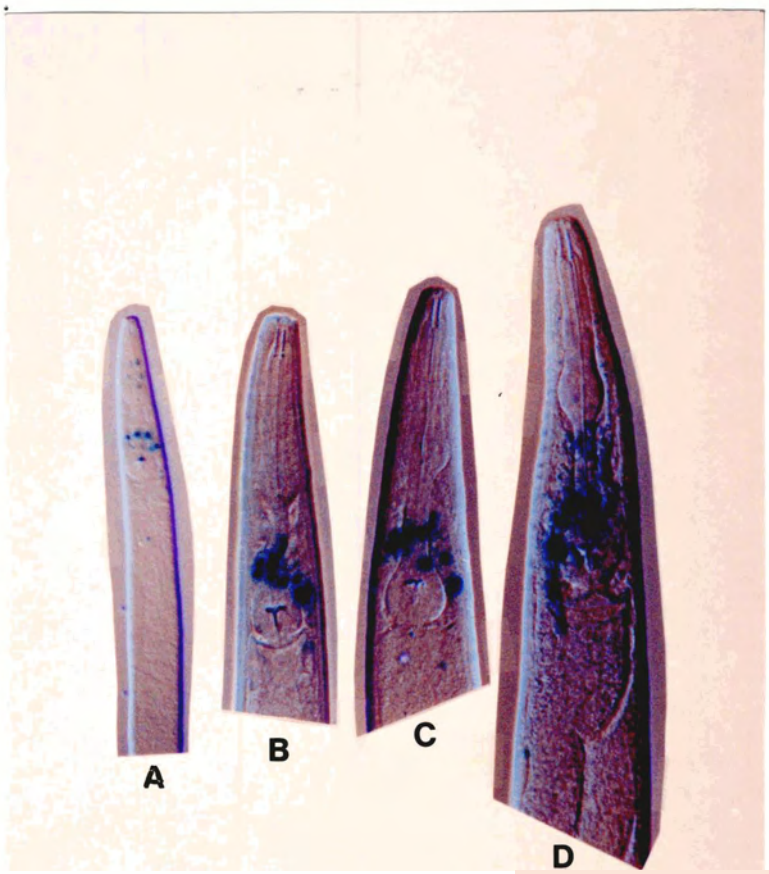

A
B
C
D

Figure 13: *In situ* histochemical staining of sup28/+ with pPCZ1-1am

Staining is prominent in hypodermal, body muscle and intestinal nuclei, but the number of cells stained in pharynx is lower than that of sup7/+ and sup24/+. Only a few hypodermal nuclei were stained. The magnification of bright field images is: A - 50X, B - 25X, C - 25X, D - 25X, E - 50X. B shows the enlarged anterior regions of B.

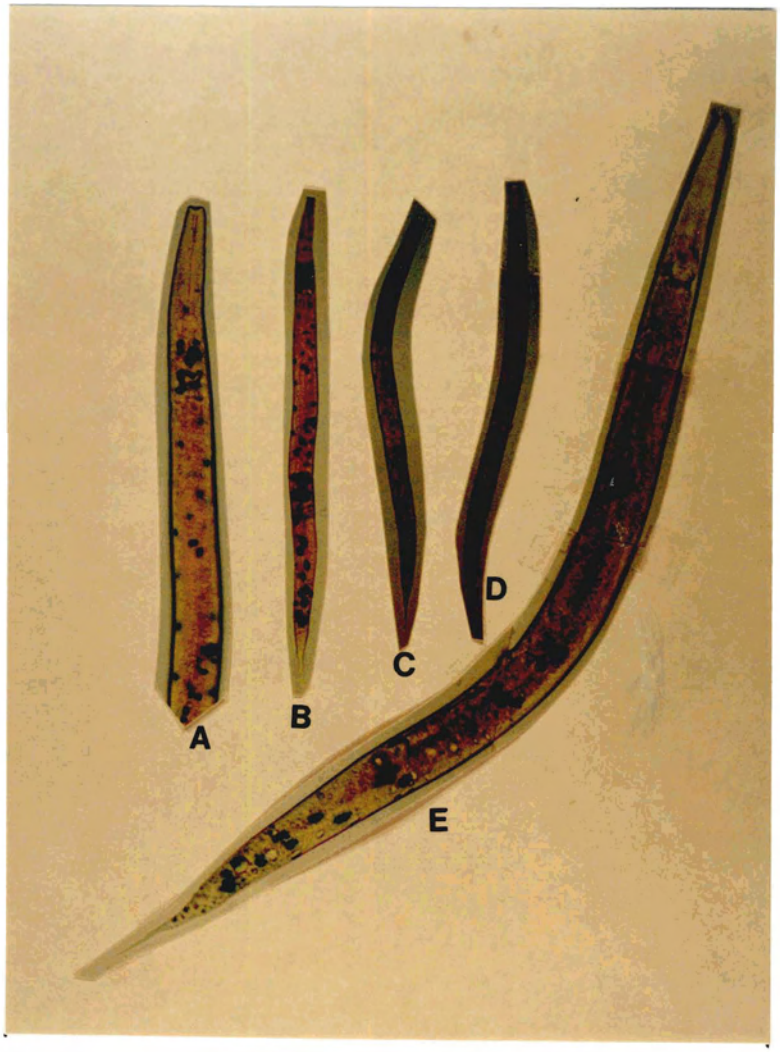

A
B
C
D
E

Figure 14: *In situ* histochemical staining of sup29/+ with pPCZ1-1am

Suppression of pPCZ1-1am by sup29/+ is shown in intestinal nuclei and a few hypodermis nuclei. No suppression is shown in neural and muscle nuclei. The magnification of bright field images is: A - 33X, B - 25X, C - 25X, D - 25X, A1 - D1 are all 50X. A1 to D1 show the enlarged anterior regions of A to D respectively.

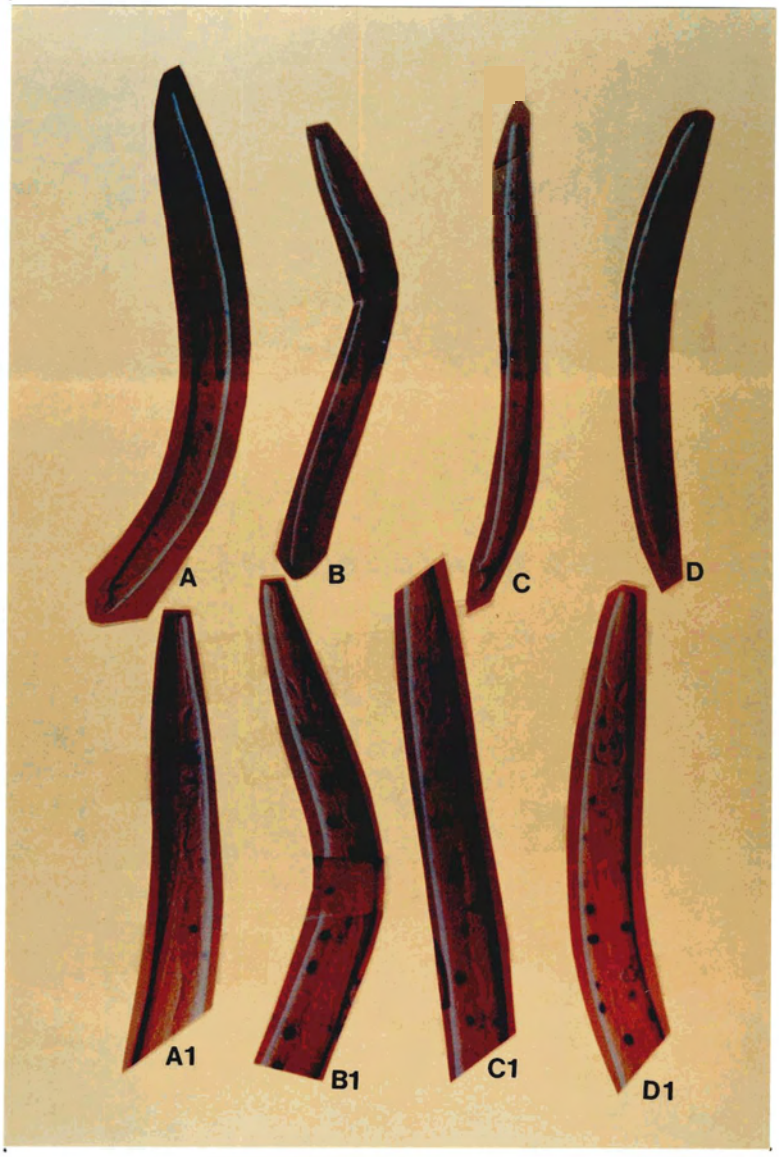

A
B
C
D
A1
B1
C1
D1

Figure 15: *In situ* histochemical staining of sup29/29 with pPCZ1-1am

The suppression is prominent in intestinal, embryonic and hypodermal nuclei. No staining is observed in neural and muscle cells. The magnification of bright field images is: A - 50X, B - 50X, C - 25X, D - 50X, E - 50X. B shows the enlarged anterior regions of C.


A
B
C
D
E

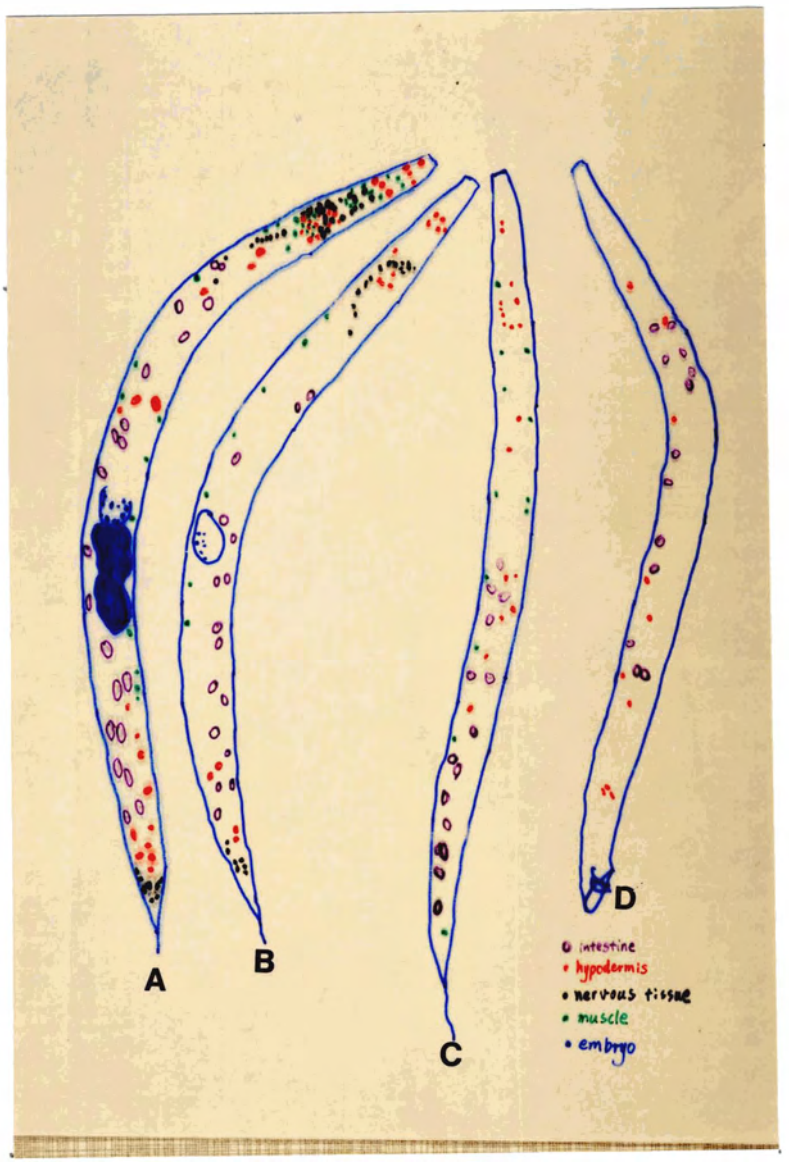
Figure 16: Summary of all types of staining

A: *sup7/+*;

B: *sup24/+*;

C: *sup28/+*;

D: *sup29/+*;


A
B
C
D
intestine
hypodermis
nervous tissue
muscle
embryo

IV. Comparison of Suppression Efficiencies in Nervous Tissue

The nervous system of *C. elegans* consists of 302 neurons and 56 glial and associated support cells in hermaphrodites, 381 neurons, 92 glial and supporting cells in males (Wood *et al.*, 1988). It is made up of two almost independent units: twenty of the cells are contained within the pharynx (Albertson and Thomson, 1976); the remaining neurons are found throughout the body of the animal. A pair of RIP (Ring/Pharynx Interneuron) cells connect the main nervous system to the pharyngeal nervous system. Most of the cell bodies of these neurons are segregated into ganglia by the arrangement of the basement membrane lining the pseudocoelom.

In the anterior region, expression of pPCZ1 in N_2 wild type transgenic animals is observed in almost all the cells in anterior ganglion, lateral and ventral ganglia and retro-vesicular ganglion (Figure 9). Suppression by *sup 7/+* gives almost the same expression pattern as that of the transgenic N_2 positive control (Figure 10). Second strongest suppression by *sup24/+* is seen in many cells except the anterior ganglion in pharynx, though not as strong as *sup7/+* (Figure 11, 12). No suppression is observed in this area in *sup28/+*, *sup29/+* and *sup29/sup29* animals (Figure 13, 14, 15).

In the ventral cord, suppression by *sup7* and *sup24* are almost the same, but not as strong as the positive control. Figure 10 and Figures 11 and 12 show that the expression is in anterior ventral cord. No suppression is detected in the ventral cord in *sup28/+*, *sup29/+* and *sup29/sup29* animals (Figure 13, 14, 15).

In preanal ganglion, lumbar and dorso-rectal ganglia, the number of stained cells of *sup7/+* are similar as the positive control (Figure9 and 10). However, suppression by *sup24* is much weaker than *sup7*: staining is less intense and fewer cells are stained. No suppression is observed in *sup28/+*, *sup29/+* and *sup29/sup29* worms. The results are consistent with the cross suppression test by Kondo (1990) except that no staining was observed in neural cells in both *sup29/+* and *sup29/sup29* animals, but barely detectable suppression was observed in *sup29/sup29*, *unc-24* worms (Table V and VI).

V. Comparison of Suppression Efficiencies in Musculature

Muscles in *C. elegans* are of two general types: single sarcomere muscles and obliquely striated muscles. All the muscles associated with the alimentary system and the sex muscles belong to the former class, whereas all the body-wall muscles belong to the latter class. Muscle cells are mononucleate, with the exception of four cells in the pharynx. The body musculature is arranged as longitudinal bands of muscle cells, with one band running in each quadrant of the body. Two rows of rhomboid-shaped muscle cells make up each band. There are a total of 95 body muscle cells in the adult: 23 in the left ventral quadrant and 24 in each of the remaining quadrants (Sulston and Horvitz, 1977).

In the pharynx, suppression in *sup7/+* animals is as strong as the positive control (Figure 9 and Figure 10), but just a few cells are stained in *sup24/+* and *sup28/+* worms (Figure 11, 12 and 13). The suppression level in *sup29/sup29* worms are a little bit higher than *sup24/+* (Figure 11); no suppression is observed by *sup29/+* (Figure 14).

In body muscle cells, good suppression is observed in *sup24/+*, *sup28/+* and *sup29/sup29* worms though it is not as strong as the positive control (Figure 11, 13 and 15). Some cells are stained in *sup7/+* and *sup29/+* worms, but not as many as in *sup24/+*. The order of suppression is *sup24* $\geq$ *sup28* $\geq$ *sup29/sup29* > *sup 7/+* $\geq$ *sup29/+*.

These results are slightly different from the *in vivo* cross suppression test: In pharynx, the order of suppression efficiency is the same as the cross suppression test except that weak suppression was observed in *sup29/sup29* animals, but not in Kondo's test. In body muscle cells, the order is different from that of Kondo's test. The difference might reflect the different sensitivity of the two methods.

Table VI Summary of cross suppression data by Kondo *et al.*

	Nervous system?			Muscle?		Hypodermis?		?
	unc-13	unc-51	unc-24	unc-15	unc-52	dpy-20	lin-1	tra-3
sup-7	++++	++++	++++	++++	++++	++++	++++	++++
sup-7/+	+++	++	++++	+	+	++	nt	+++
sup-5	+++	++++	++++	+++	+++	+++	++++	++++
sup-5/+	++	++	++++	±	±	++	++++	++++
sup-24	+	+	++	++	+++	++++	++++	++++
sup-24/+	±	±	+	nt	±	++	nt	+++
sup-33	-	±	+	+++	+++	++++	++++	++++
sup-33/+	-	-	-	±	±	++	++++	++++
sup-34	-	±	+	±	+	+++	nt	++++
sup-34/+	nt	-	-	nt	-	+	nt	nt
sup-21	-	-	±	±	+++	++++	nt	++++
sup-21/+	-	-	-	nt	±	++	++++	++++
sup-28	-	-	±	±	+++	++++	nt	++++
sup-28/+	-	-	-	nt	±	++	nt	++++
sup-29	-	-	±	-	-	±	±	++++
sup-29/+	-	-	-	-	-	-	-	++

++++, maximum suppression observed, not necessarily wild-type; ±, barely detectable suppression; -, no detectable suppression; nt, not tested.

Table VII Comparison of suppression efficiencies

	Nervous system			Musculature		Hypodermis		Intestine
	Pharynx	Body	Posterior	Pharynx	Body	Pharynx	Body	
sup7/+	++++	++	++++	++++	+	++++	++	++++
sup24/+	+++	++	++	±	++	++	+++	+++
sup28/+	-	-	-	±	++	++	+++	++
sup29/+	-	-	-	-	+	±	++	+
sup29/29	-	-	-	+	++	+	+++	++++
N_2PCZ1	++++	++++	++++	++++	++++	++++	++++	++++

The heading "Pharynx" indicates cells in the anterior region of the animal; these are predominantly pharyngeal cells. "+" symbol indicates the number of cells stained. "++++" indicates the maximum number of cells stained and "±" indicates that one to three cells were stained. "-" indicates that no cell was stained in that tissue.

VI. Comparison of Suppression Efficiencies in Hypodermis and Intestine

The external epithelium is referred to as the hypodermis. Strong suppression by *sup7/+* is shown in the pharynx as shown in Figure 10. The suppression level is as strong as the expression in the positive control. The levels of *sup24/+*, *sup28/+*, *sup29/+* and *sup29/sup29* are weaker than those of *sup7/+* (Figure 9-15). Only a few epithelial cells, marginal cells, and gland cells are stained. The total number of stained cells is much lower than in *sup7/+*. The order of the suppression efficiencies is *sup7/+* > *sup24/+* > *sup29/ sup29* > *sup28/+* > *sup29/+*.

In the body hypodermis, the suppression efficiencies by *sup24/+*, *sup28/+* and *sup29/sup29* are similar, weaker than wild type control, but stronger than *sup7/+* and *sup29/+* (Figure 9-15). The order of suppression levels is *sup24/+* ≥ *sup28/+* ≥ *sup29/sup29* > *sup7/+* ≥ *sup29/+*.

Comparing these results with Kondo's test, the order of suppression levels in both pharynx and body hypodermis cells is different from that of Kondo's test. In Kondo's test, similar suppression levels were observed in *sup7/+*, *sup24/+* and *sup28/+* animals, barely detectable suppression was detected in *sup29/sup29* worms and no detectable suppression was observed in *sup29/+* worms. In my experiment, the suppression efficiency in pharynx of *sup7/+* is much higher than that of *sup24/+*, *sup28/+*, *sup29/+* and *sup29/sup29* animals, but the order changed on body hypodermis cells (Refer to the above paragraph.).

In intestine, strong suppression was observed in *sup7/+* and *sup29/sup29* animals. It becomes weaker gradually (Table VII). The order of suppression levels is *sup7/+* ≥ *sup29/sup29* > *sup24/+* > *sup28/+* > *sup29/+*.

Discussion

The goal of this research was to study the potential tissue or stage-specific expression of the *C. elegans* $tRNA_{Trp}$ gene family *in vivo*. Previous cross-suppression tests showed that suppressor $tRNA_{Trp}$ expressed from different loci gives different suppression efficiencies (Kondo *et al.*, 1988, 1990). Since the coding regions of all members of $tRNA_{Trp}$ gene family are identical, different suppression efficiencies must be due to differential expression of these genes. In later *in vitro* transcription experiments, the *sup7* clone was found to be expressed at the highest level. Values for the others relative to *sup7* were as follows: *sup5* 90%; *sup24* 69%; *sup28* 26%; *sup29* 19% (Linning, 1992). Tissue specific tRNA expression was reported in other organisms, too. Two classes of genes that encode alanine tRNA in the silkworm, *Bombyx mori*, are distinguished by the distribution of their products among different cells. One class encodes $tRNA^{Ala}_{C}$, which is common to all cell types; the other encodes $tRNA^{Ala}_{SG}$, which is restricted to cells of the silk gland (Meza *et al.*, Sprague, *et al.*, 1977). Transcription of $tRNA^{Ala}_{C}$ gene *in vitro* is more efficient than $tRNA^{Ala}_{SG}$, which can approach the same efficiency only if more highly concentrated transcription machinery is used (Young *et al*, 1986). A similar expression pattern of silk-gland-specific $tRNA^{Ala}$ is reported in spiders (Candelas *et al.*, 1990). *In vitro* transcription of some $tRNA^{Gly}$ genes is extremely poor compared with that of others in *Bombyx mori* and indicates that $tRNA^{Gly}$ genes might be regulated as are $tRNA^{Ala}$ genes (Taneja *et al.*, 1992). Oocyte-specific and ubiquitous $tRNA^{Tyr}$ gene families are found in *X. laevis* (Stutz, *et al.*, 1989). These reports indicate that tissue-specific control of tRNA is widespread.

Although a large number of *in vitro* studies have demonstrated that the expression efficiency of tRNA genes transcribed by RNA polymerase III is different, *in vivo* studies in a multicellular organism have not been done. The nematode *C. elegans* is the only multicellular organism in which nonsense suppressors are available. Taking advantage of the powerful genetics of *C. elegans*, differential expression of individual members of *C.*

elegans $tRNA_{Trp}$ gene family was visualized by studying the suppression efficiency on an amber lacZ reporter gene which was introduced into different nonsense suppressor $tRNA^{UAG}_{Trp}$ mutant strains.

I. Analysis of Mutagenesis Efficiency

To begin this research, one or two amber sites were introduced into plasmid pPCZ1 lacZ gene which was controlled by *C. elegans* heat shock promoter (Stringham *et al.*, 1992) by USE (**u**nique **s**ite **e**limination, Deng and Nickoloff, 1992) or LP-USE (**l**ong **p**rimer) site-directed mutagenesis (Ray and Nickoloff, 1992) respectively. The two pairs of primers (**c**lock**w**ise primer pair: amcw with RVcw; **c**ounter**c**lock**w**ise primer pair: amccw with RVccw) used were designed by the OLIGO software program. The number of one amber pPCZ1 mutant from these two pairs of primers were quite different: three of 46 pPCZ1-1am were obtained from the counterclockwise pair, no desired mutation was found from the clockwise pair, though 58 isolated pPCZ1 were checked.

The self-complementarity and internal stability of oligonucleotide amcw were rechecked and two stable palindromic sequences were found at both ends. The OLIGO software package can determine a profile of an annealed oligonucleotides' internal stability by calculating a ΔG value for a sliding 5bp window. A more negative ΔG indicates greater stability. Rychlik and Rhoads (1989) have previously demonstrated the need to balance oligonucleotide 3' stability with specificity for sequencing and DNA amplification applications. Oligonucleotides having 3' pentamer ΔG values approaching –6 to -7 kcal/mol are often ideal for these applications, but if greater than -10 kacl/mol, they are unsuitable. Piechocki and Hines (1994) also suggested that a successful mutagenic oligonucleotide should have a moderately unstable 3' end to avoid extension from false-priming sites, but stable enough on the 3' and 5' ends to "clamp down" around the mutation. They indicated that the ideal primer is balanced with symmetrical stability at the 3' and 5' ends of approximately -8 kcal/mol. It is likely that the failure of the site-directed mutagenesis with amcw was due to the secondary structure of the primer and the high stability at both 3' and 5' ends. Figure 17 shows the internal stability of the primers.

Comparing the efficiency of one-amber mutants made by USE with that of two-amber mutants resulting from LP-USE, a significant difference is observed. The latter one

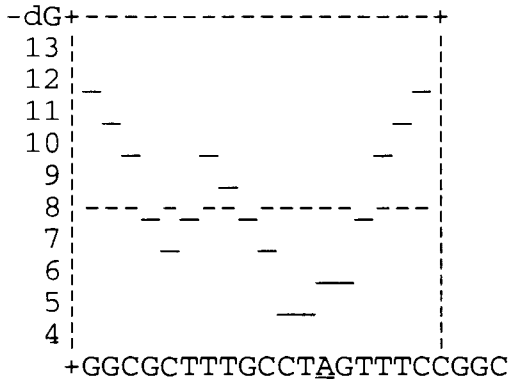
was 10 fold higher than the former one (see RESULTS). The reason might be that *EcoRV* digestion was not thorough and transformation efficiency of EvoRV-sensitive pPCZ1 was high.

Figure 17: Comparison of internal stability of primers for site-directed mutagenesis.

Internal stability of two poorly functioning primers (A and B) and two efficient primers(C and D). ΔG values were calculated for all pentamers in each primer. The last point in each inset represents the ΔG value of the sub-sequence of the last five nucleotides. The ΔG values shown for the pentamers including the position being mutagenized do not take into account the instability introduced by base mismatches.

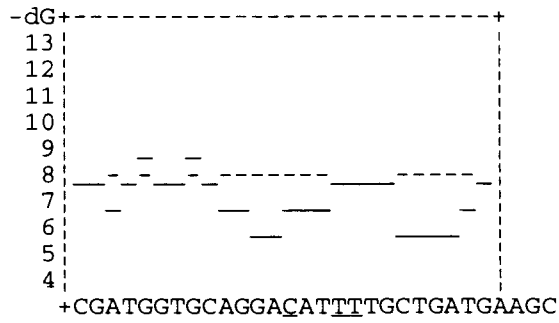
A. amcw, 22-mer,

5' GGC GCT TTG CCT AGT TTC CGG C 3'



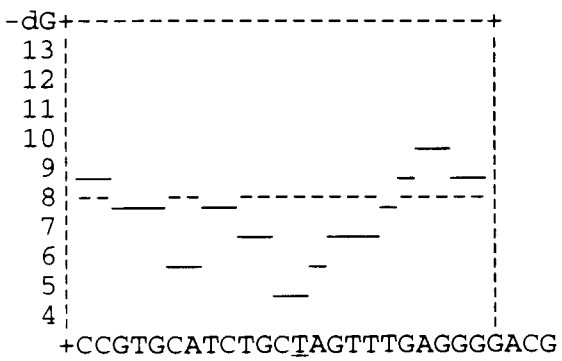
B. 3mutRVcw, 30-mer,

5' CGA TGG TGC AGG ACA TTT TGC TGA TGA AGC 3'



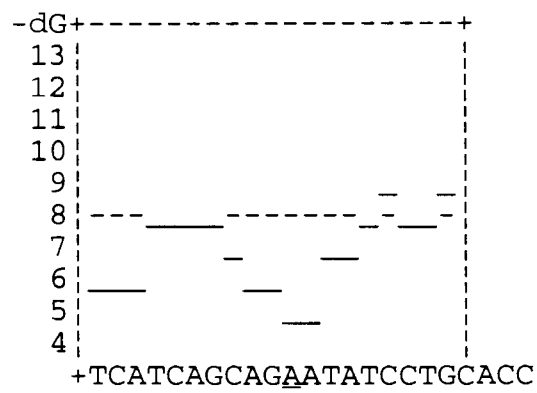
C. amccw, 27-mer,

5' CCG TGC ATC TGC TAG TTT GAG GGG ACG 3'



D. mutRVccw, 23-mer,

5' TCA TCA GCA GAA TAT CCT GCA CC 3'



II. Analysis of Suppression Patterns

The suppression efficiencies of eight suppressors to three neuronal genes (*unc-13*, *unc-51* and *unc-24*) are quite different in the previous *in vivo* cross suppression tests (Kondo *et al.*, 1988). They estimated that more than an eight fold difference exists between the strongest suppressor and the weakest suppressors in the case of unc-13. In their later experiments, they found that eight individuals of this gene family suppressed the three genes at a fixed order of suppression efficiencies: *sup7* ≥ *sup5* > *sup24*>> *sup33* ≈ *sup34* > *sup21* ≈ *sup28* ≈ *sup29* (Kondo *et al.*, 1990). They suggested that the eight genes are regulated in a similar manner in the cell-types in which these three genes are expressed. We found that the expression of *sup7/+* is very strong in the posterior region and the pharynx: almost all the 20 neurons in pharynx are stained. The number of stained cells in these two regions in *sup24/+* is lower than *sup7/+*, but higher than *sup28/+* and *sup29/+*. No suppression was observed in *sup28/+*, *sup29/+* and *sup29/sup29* worms. In cross suppression experiments, *sup7/+* suppressed *unc-51* less efficiently than *unc-13* and *unc-24*. The *unc-51* gene is required for axonal elongation and encodes a novel serine/threonine kinase. It is expressed strongly in many or most neurons including those in the nerve ring near the head, those in the ventral nerve cords, and those near the tail (Ogura *et al.*, 1994). Our experiments also show that *sup7/+* suppresses *lacZ*-1am more efficiently in the pharynx than in the ventral cord neurons.

The staining tests indicate that *sup7* is expressed constitutively (Table VII), but higher in pharynx than in body cells. These results might reflect different trp codon usage in different tissues and can not be explained by mosaic distribution of extrachromosome arrays because different transgenic *sup7/+* strains showed the similar distribution. *sup24* is expressed in a tissue general pattern, but is higher in hypodermis. *Sup28* and *sup29* are not expressed or just expressed at a very low level in nervous system, but are expressed at intermediate levels in musculature and high levels in hypodermis.

The differential suppression patterns of the four sup genes studied can't be explained by tissue-specific activity of heat shock promoter *hsp16-48*. Though this

promoter directs greater expression of β-galactosidase in muscle and hypodermis (Stringham, 1992), suppression efficiency by *sup7/+* in pharyngeal neurons is still higher than body muscle and hypodermis cells. Secondly, differential suppression is not likely caused by incomplete transmission of the extrachromosomal array or different copy numbers of the extrachromosomes for two reasons: a), the heterozygote sup strains were obtained by outcrossing the sup worms with the same transgenic N_2, pPCZ1-1am strain, but the suppression efficiencies are still different; b), two different transgenic lines were used to introduce lacZ into sup strains in both cases, *sup7/+* (by outcrossing with N_2, pPCZ1-1amA or N_2, pPCZ1-1amB respectively) suppresses *lacZ*-1am more efficiently than *sup28/+* (by outcross with N_2, pPCZ1-1amA and N_2, pPCZ1-1amB respectively). Thirdly, it is not likely caused by different levels of expression for two reasons: a), suppression levels of *dpy-20* by *sup7/+* and *sup28/+* are the same, but *sup7/+* showed strong suppression of *unc-13*, *unc-51*, and *unc-24*, whereas *sup28* showed no detectable suppression (Table VI). This result indicates the tissue-specific expression of *sup28*; b), The staining is prominent in hypodermis *sup28/+* animals, but no staining is shown in neural cells; whereas *sup7/+* showed prominent staining in both hypodermis and neural cells.

III. Dosage Effect on Suppression

The *sup29/sup29* with pPCZ1-1am strain was obtained directly by germline microinjection. Heterozygote *sup29/+* with pPCZ1-1am strain was constructed genetically (MATERIALS AND METHODS). No neuron cells are stained in either strain and the suppression in intestines of both strains is high. In the pharynx, the number of stained muscle cells and epithelial cells of heterozygote *sup29* animals is about half the number observed in homozygotes. There are more stained cells in body muscle cells and hypodermis cells in *sup29/sup29* worms than *sup29/+*. This result suggests that suppression efficiencies between one copy and two copies of *sup29* are different, however, this conclusion is tentative because the copy number of pPCZ1-1am in these two strains might be different. Table VII shows the summary of the suppression efficiencies among individual sup worms.

IV. Future Work

Recent evidence indicates that genes transcribed by RNA polymerase III may be regulated in novel ways. Additional DNA sequence elements and components of the transcription machinery could be involved in this regulation as well. To study the tissue- or developmental-stage-specific expression of tRNA genes, integrated transgenic lines will be more convincing. How the cells regulate the expression and how the regulation co-ordinates with tryptophan usage during development and tissue differentiation will be helpful to reveal the mechanism of transcriptional regulation.

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