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CHARACTERIZATION OF INITIATOR METHIONINE tRNA GENES FROM *CAENORHABDITIS ELEGANS*

by

Meenal Khosla

B.Sc., Simon Fraser University, 1984

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

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in the Department

of

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ABSTRACT

The subject of this thesis is the isolation and characterization of five initiator methionine tRNA (tRNA met_i) genes from Caenorhabditis elegans. First, to look at putative tRNA met_i genes in C. elegans, a Southern blot containing genomic DNA cut with several restriction endonucleases was hybridized with a Xenopus tRNA met_i gene. The Southern blot showed that C. elegans contains 8-10 tRNA met_i genes.

To isolate these genes, a C. elegans genomic library was screened with the Xenopus probe. Eighteen positives were isolated and plaque purified. Analysis of these clones revealed that the eighteen positives represented five different recombinant phage. Restriction maps of these five phage suggest that they are from different genomic locations. This result is confirmed by the fact that the tRNA genes lie on different nonoverlapping cosmid islands.

To examine the genomic organization of sequences flanking the cloned tRNA genes, EcoRI flanking phage fragments were hybridized to Southern blots of EcoRI digested genomic DNA. These blots show that a moderately repetitive DNA sequence is found within each phage and adjacent to each tRNA met_i gene. In all but one case, the localized repetitive sequences do not cross hybridize. In four of the five phage, the moderately repetitive sequence is found 5' to the tRNA gene. The repetitive sequence in Cetmet3 is found

3' to the tRNA gene.

Sequence analysis shows that all five genes contain identical coding sequences. With one exception, the flanking sequences are considerably divergent. Cetmet2 and Cetmet4 have identical flanking sequences. This could be the result of a gene duplication event.

The tRNA genes were used to program the homologous *in vitro* transcription system. All five genes produce both precursors and processed, mature products. By using flanking phage fragments as templates in the transcription system, we discovered a putative tRNA gene lying near one of the cloned tRNA met_i genes (Cetmet3).

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Introduction

The initiation of translation represents the last step of the central dogma (DNA to RNA to protein) of information transfer. Initiation of protein synthesis requires a specific methionine tRNA. In prokaryotes, as well as within the cell organelles of eukaryotes, N-formyl-methionine tRNA ($tRNA^{fMet}$) functions as the initiating species whereas in the cytoplasm of eukaryotic cells, a specific methionyl tRNA ($tRNA^{met}_i$) without formylation is used. In an attempt to understand the relationship between the structure and the specialized functions of these initiator tRNAs, the nucleotide sequence of several prokaryotic and eukaryotic initiator tRNAs have been elucidated (Gauss and Sprinzl, 1983). These studies reveal that a distinguishing feature of prokaryotic initiator tRNAs is that they lack a hydrogen bond between the 5' terminal nucleotide and the fifth nucleotide from the 3' end (Rich and RajBhandary, 1976). The possible importance of this feature is underscored by the fact that it is not shared by eukaryotic initiator tRNAs.

All eukaryotic initiators contain the sequence AUCGAAA in place of TUCG(A), a sequence present in loop IV of prokaryotic initiator tRNAs and virtually every tRNA which functions in protein synthesis. Although the conservation of this sequence suggests that it may be functionally important, so far there appears to be no simple correlation between this nucleotide sequence and initiator functions. Changing the

RNA sequence AUCG to TUCG has no effect on *in vitro* transcription and processing of a human tRNA met_i gene. Furthermore, this sequence change does not impair the aminoacylation or formylation reactions of the tRNA. (Drabkin and RajBhandary, 1985a). However, *in vivo*, the wildtype tRNA is produced in larger amounts than the mutant tRNA (Drabkin and RajBhandary, 1985b). These results suggest that maybe more subtle differences distinguish initiator tRNAs from elongating tRNAs. Studies of anticodon conformation using SI nuclease (Wrede et al, 1979) demonstrate that the anticodon loop conformation of initiator tRNAs as a class, differs from that of other tRNAs. How this structure of initiator tRNAs relates to possible functions is not known.

Although the organization of tRNA genes has been studied in several eukaryotes, these results reveal no common pattern of gene arrangement. Instead, it is evident that tRNA genes are not only found individually, but also in dispersed clusters and in tandem repeats. The variability in organization is best seen in Drosophila where tRNA genes have been localized by *in situ* hybridization of labelled tRNA to polytene chromosomes. These results indicate that a particular tRNA can be found at more than one site (Kubli and Schmidt, 1978; Hayashi et al, 1980) and that more than one type of tRNA can be localized in the same region (Yen et al, 1977; Dudler et al, 1980). The irregular arrangement of

these genes is found not only in Drosophila, but also in organisms as diverse as yeast (Cigan and Donahue, 1986; Reyes et al, 1986) and humans (Santos and Zasloff, 1981; Roy et al, 1982).

Another question that remains unsolved is the function of tRNA gene redundancy. The simplest explanation for the reiterated nature of tRNA genes is the requirement of amounts of tRNA exceeding that possible from a single template. Concurrent transcription of a number of templates would be a means of providing enough of the required gene product. In addition, reiterated genes may suggest tissue specific tRNA gene expression. The appearance of unusual isoacceptors in certain organs has been documented in B. mori (Sprague et al, 1977) and during bovine lens differentiation (Lin et al, 1980). More recently, the possibility of tissue specific expression of suppressor tRNA genes has been described in C. elegans (Hodgkin 1985).

Despite the variability found in tRNA gene number and organization, a uniform feature of these genes is their possession of intragenic promoters. The results of in vitro studies from three independent groups demonstrated that the promoter consists of two regions of essential sequence, named the A Box and B Box respectively (Hofsteller et al, 1981; Sharp et al, 1981a; Ciliberto et al, 1982a). By analyzing transcription properties of deletion clones, they showed that nucleotides 8-20 (A Box) and nucleotides 50-62 (B Box) were

required for transcription. Subsequent studies revealed that the two promoter blocks had to be separated by a distance of about 30-50 bp for optimal transcription (Ciliberto et al, 1982b). Mutational analysis of a X. laevis tRNA met_i gene showed that alteration of the sequences encoding the anticodon stem had a significant effect on promoter function (Folk and Hofstetter, 1983). Published data on mutagenesis within this region of other tRNAs show that transcription is not affected to the same extend as the tRNA met_i (Ciampi et al, 1982a; Sharp et al, 1981a). Therefore, promoter activity of the anticodon stem region may be limited to initiator tRNAs.

Cell free transcription systems from yeast (Ruet et al, 1984; Klelamp and Weil, 1986), Drosophila (Burke et al, 1983), Xenopus (Shastry et al, 1982) and HeLa cells (Lassar et al, 1983) showed that all organisms required similar factors for transcription. Fractionation of these extracts established that at least two additional factors were required along with RNA polymerase III for transcription of purified genes. These factors, TFIIIB and TFIIIC, are required for stable complex formation prior to active transcription.

Bogenhagen et al (1982) defined a 5S RNA stable complex as an interaction of transcription factors with purified genes. They showed that this association was stable, existed for many rounds of transcription, and was not disrupted by

the presence of a competing template. RNA polymerase III does not appear to be involved in stable complex formation and, therefore, recycles during rounds of transcription.

Similar experiments using tRNA genes as templates have shown that stable complex assembly on these genes involves at least two steps (Lassar et al, 1983; Schaack et al, 1983). A series of elegant competition experiments by both groups showed that TFIIIC first binds transiently to the B Box sequences forming an unstable complex. Subsequent binding of TFIIIB is then required to stabilize the complex. The stable association of TFIIIC and TFIIIB with the template is required for accurate initiation of transcription by RNA polymerase III. Experiments to purify TFIIIB and TFIIIC from crude extracts are in progress. Recently, Yoshinaga et al (1987) showed that TFIIIC activity can be separated into two different components, both of which are required for transcription.

The many advantages of working with C. elegans have been described by Brenner (1974). C. elegans is easy to maintain and has a short generation time. The adult worm has about a thousand somatic cells and because the complete cell lineage is known, it is possible to trace the origin of every somatic cell during its development (Sulston and Horvitz, 1977; Sulston et al, 1983). In addition, the small genome size of C. elegans (8×10^7 bp) makes it possible to construct a physical map of the entire genome. This map is being

generated in the following way. First, lambda clones containing nematode DNA (sent to the MRC lab by C. elegans researchers) are fingerprinted. Then, to search for overlaps, a computer compares the fingerprints of different lambda clones. Finally, aligning lambda clones in this way enables the construction of contigs-cosmid islands (A. Coulson and J. Sulston, pers comm).

With the availability of techniques such as transposon induced mutagenesis and DNA transformation, C. elegans provides an attractive system for molecular analysis. The transposon Tc1 has been used to clone genes defined only genetically but which had not been previously cloned (Moerman et al, 1986) or had no known gene products (Greenwald, 1985). There are about 30 copies of Tc1 in the Bristol (N2) strain but about 300 copies in the Bergerac (BO) strain (Emmons et al, 1983). Transposon induced mutagenesis causes a restriction fragment length polymorphism (RFLP) between the two strains of C elegans. This RFLP can then be used to isolate the gene of interest. RFLPs can also be used to determine the genetic map location of cloned DNA fragments (Rose et al, 1982). Those genes that cannot be mapped by RFLPs can be mapped to a linkage group by in situ hybridization using cosmid DNA as a probe (Albertson, 1984). More recently, a DNA transformation system has been developed for C. elegans (Fire, 1986). This procedure can be used to assay the function of specific genes in vivo.

The potential of genetic and molecular analysis in C. elegans is best illustrated by studies on the lin-12 locus. Earlier genetic studies revealed that lin-12 is a homeotic gene, responsible for determining the fate of certain cells (Greenwald et al, 1983). To determine how lin-12 specifies cell fate, the gene was cloned by Tc1 induced mutagenesis (Greenwald, 1985). Subsequent sequence analysis revealed that the putative lin-12 gene product was homologous to epidermal growth factor (Greenwald, 1985). This result led Greenwald to hypothesize that lin-12 may be involved in intercellular communication in the form of a membrane bound or secreted molecule. In addition, cosmid clones containing the lin-12 locus have been isolated (Greenwald et al, 1987). By chromosomal walking, the cosmid clones were joined and a 600 Kb contig was generated. As a result of this combined analysis, the molecular basis of some lin-12 mutations can be elucidated.

Although most research has focused on genes transcribed by polymerase II in C. elegans, some genes transcribed by polymerase III have also been characterized. Sulston and Brenner (1974) showed that C. elegans contains approximately 300 tRNA genes in the haploid genome. By hybridizing total 4S labelled RNA to blots containing EcoRI cut genomic DNA, Cortese et al (1978) obtained between 30-50 separate DNA fragments demonstrating the dispersed arrangement of tRNA genes in the genome. A $tRNA^{Pro}$ gene in C. elegans has been

studied in detail. This gene was used in initial experiments which demonstrated that the region of DNA essential for promotion of transcription is within the coding region (Ciliberto et al, 1982a; 1982b). In addition, Ciliberto and coworkers (1983) showed that a C. elegans $tRNA^{Pro}$ Box A sequence could substitute for the 5' half of the 5S internal control region and the combination constituted a functional pol III promoter.

The $tRNA^{Trp}$ family in C. elegans is the only tRNA gene family that has been extensively characterized. Southern blot analysis revealed the presence of 12 dispersed $tRNA^{Trp}$ genes (Bolton et al, 1984). Five members of this gene have been identified as amber suppressors (Wills et al, 1983; Kondo et al, 1986). Comparisons of suppressor efficiency show that these tRNA genes are expressed at different levels (Hodgkin, 1985; Kondo et al, 1986).

Kondo et al (1986) suggest that variation in gene expression could be due to the tissue specific expression of these tRNA genes. Data supporting this hypothesis was obtained when three different $tRNA^{Trp}$ suppressors (sup-7, sup-5, and sup-21) were tested against single genes such as dpy-18 and unc-13. These studies showed that the efficiency of each suppressor varied with the gene being tested. Although sup-21 was a good suppressor of dumpy mutations, it was a poor suppressor of ambers in muscle genes (Hodgkin, 1985; Kondo et al, 1986). Therefore, Hodgkin hypothesized

that sup-21 was expressed efficiently in hypodermal cells but at a low level in muscle cells.

A homologous in vitro transcription system is now available to determine template requirements of pol III genes in C. elegans. All the experiments with the $tRNA^{Pro}$ gene were done using heterologous extracts. The inability of Xenopus extracts to transcribe C. elegans 5S RNA genes (Honda et al, 1986) suggests some species specificity in transcription. Species specific transcription signals have also been identified in pol III genes from the silkworm (Sprague et al, 1977). Fractionation of the C. elegans crude extract would allow identification of nematode specific transcription factors. We undertook to investigate the organization and expression of tRNA genes in C. elegans. The subject of this thesis is the isolation and characterization of five initiator methionine tRNA genes from Caenorhabditis elegans.

MATERIALS AND METHODS

Isolation of genomic DNA

C. elegans var Bristrol (strain N2) were grown on high peptone plates containing a lawn of *E. coli* strain B as described by Rose et al (1982). For large scale DNA preparations, worms were treated with proteinase K and SDS (sodium dodecyl sulfate) as described by Emmons et al (1979) except that the worms were not frozen in liquid nitrogen but pelleted gently, left on ice for 45 minutes to allow digestion of *E. coli* and then treated. Genomic DNA was extracted three times with phenol, once with phenol/chloroform, and once with chloroform. The DNA was further purified by cesium chloride ethidium bromide centrifugation (1 gm of CsCl per ml of DNA solution containing 600ug/ml of EtBr). The EtBr was removed by repeated extractions with isopropanol saturated with 5M NaCl. Two volumes of water and 6 volumes of ethanol were added to precipitate the DNA at -20°C. The DNA was dissolved in TE (10mM Tris pH 7.5, 1 mM EDTA) and stored at 4°C (Maniatis et al, 1982).

Isolation of *C. elegans* eggs

To obtain eggs for transcription extracts, worms were grown on high peptone plates as described above. The worms were washed off the plates with 0.5M NaCl, gently pelleted and then treated with bleach and sodium hydroxide. Once the

worm cuticles had dissolved, the eggs were recovered by centrifugation, washed with cold buffer (50 mM sodium phosphate pH 6.5, 15 mM B-mercaptoethanol), and then finally resuspended in an equal volume of water. This mixture was frozen in liquid nitrogen and stored at -70°C.

Labelling of probes

DNA fragments were labelled by the nick translation method (Rigby et al, 1977) using alpha ^{32}P dATP or alpha ^{32}P dCTP. The reaction was carried out for 3 hours at 15°C and stopped with the addition of an equal volume of TE saturated phenol/chloroform. Unincorported nucleotides were removed from labelled DNA by Sephadex G-25 chromatography. DNA with specific activities of $1x10^7$ to $1x10^8$ cpm/ug were obtained. For use in hybridizations, the probe was denatured by heating in a boiling water bath for 5-7 minutes followed by rapid cooling on ice.

Southern blots

Prior to transfer, the gel was treated with three solutions (Maniatis et al, 1982). First, the gel was soaked for 10 minutes in 0.25M HCl which depurinates the DNA to allow transfer of high molecular weight DNA. The DNA was then denatured with two 15 minute washes in 0.5M NaOH/1.5M NaCl. Finally, a one hour soak in 1.0M ammonium acetate neutralized the DNA and allowed transfer to the

nitrocellulose (Schleicher and Schwell) by the method of Smith and Summers (1980). After the transfer, the filter was first air-dried and then oven baked under a vacuum at 80°C for 2 hours.

Hybridization of DNA

All filters were prehybridized in 5X SSPE (1X SSPE is 0.18M NaCl, 10mM sodium phosphate, 1mM EDTA pH 7.4) and 0.3% SDS for 2 hours. This solution was removed and hybridizations were carried out in fresh 5X SSPE, 0.3% SDS containing the denatured probe. The stringency of hybridization depended upon the homology between the probe and the DNA bound to the filter. When the Xenopus probe was used, hybridizations were done at 58°C and washes were at 58°C in 2X SSPE, 0.3% SDS. If the probe was homologous, hybridizations were carried out at 66°C and the corresponding washes were also at 66°C but in 0.2X SSPE, 0.3% SDS. Hybridizations were carried out for 12-24 hours. The filters were then exposed to Kodak XAR-5 or XK-1 film for 12-48 hours at -70°C.

Library screening

A partial EcoRI library of C. elegans DNA in the lambda phage Charon 4A (constructed by T. Snutch) was screened with a Xenopus tRNA met_i gene using the protocol of Benton and Davis (1977). The probe was a 180 bp HinfI insert from the

3.18 Kb repeat containing the intact tRNA met_i sequence of Xenopus laevis and 23 and 83 nucleotides of spacer DNA at the 5' and 3' ends respectively (Clarkson et al, 1978). 28,000 phage would have to be screened to achieve a 99% probability that a given sequence is represented in the library. This number was calculated by using Clarke and Carbon's formula N = ln(1-P) / ln(1-F) (Maniatis et al, 1982). To make sure the entire genome was represented, 56,000 phage were screened with the Xenopus probe. 15 plates, each containing approximately 4,000 plaques were screened. Positives were picked by inserting a sterile pasteur pipette into the selected plaque and immersing it into 100 ul of SM solution (SM is 0.1M NaCl, 8 mM magnesium sulfate, 0.05M Tris pH 7.5, and 0.01% gelation). Phage samples were titered and replated at 200 phage/plate for the second screen. From these plates, a well isolated positive plaque was picked and replated at 40 phage/plate for the third screen. Positives from the third screen were used as stocks of each isolated phage.

Phage DNA Isolation

Phage used for large scale DNA isolation were grown on NZYCM plates (Maniatis et al, 1982) containing a lawn of E. coli C600. 10 plates were required for each recombinant clone. Cells were allowed to grow overnight in 10 mls of NZYCM media containing 2% maltose. The next morning, cells

were centrifuged at 2500 rpm for 10 minutes and then resuspended in 4 mls of 0.01M magnesium sulfate. 100 ul aliquots of these cells were added to tubes containing 100 ul of an appropriately diluted phage stock. This mixture was incubated at 37°C for 20 minutes. After this period, 3 mls of 0.7% agarose (in NZYCM media) was added to each tube and the mixture was immediately poured on a plate. Once the agarose had hardened, the plates were incubated at 37°C until lysis was confluent. At this point, 5 mls of cold SM was added to each plate and the plates were kept at 4°C overnight. The SM was then collected and centrifuged at 4000 rpm for 10 minutes at 4°C. The phage in the supernatant were concentrated by NaCl and polyethylene glycol (PEG) precipitation. This pellet was then dissolved in TE containing cesium chloride for ultracentrifugation. DNA from CsCl purified phage was extracted with formamide as described by Davis et al (1980).

Agarose gel electrophoresis

Restriction enzymes were obtained from Bethesda Research Laboratories and Pharmacia and were used under conditions recommended by the suppliers. An excess of enzyme was used to ensure complete digestion. Phage and plasmid DNA were digested for 2 hours while genomic digests were carried out for 5-7 hours. Digestion products were separated on agarose gels (agarose dissolved in 1x TBE containing 1 ug/ml EtBr)

and run in 1X TBE buffer. TBE is 89mM Tris, 89mM Borate and 2.5mM EDTA (Maniatis et al, 1982). 0.7% agarose gels were used to separate fragments produced from genomic and phage digests while plasmid digest products were separated on gels of different agarose concentrations depending upon the size of the DNA being separated. After electrophoresis, gels were photographed on a short wave ultraviolet transilluminator.

Restriction mapping

Restriction maps of the phage were constructed from agarose gel analysis of single and double digestions of phage and plasmid DNA. In addition, single enzyme partial digests of DNA inserts labelled in vitro with alpha ^{32}P dATP at the EcoRI end were ordered as described by Smith and Birnstiel (1976). For the HindIII sites in insert A of Cetmet1 and insert A of Cetmet5, EcoRI fragments were first digested to completion with SstI. The EcoRI ends were labelled with alpha ^{32}P dATP, the fragments were separated by electrophoresis, and recovered from the gel. Each fragment was then partially digested with HindIII. Gels of different agarose concentration were used to analyze the digestion products. Large fragments were separated on 0.7% agarose gels while smaller fragments were analyzed on 1.5% gels. Lambda DNA cut with EcoRI and HindIII and pBR322 cleaved with HpaII were used as marker DNA. Gels containing labelled fragments were dried down and examined by autoradiography.

Insert A from Cetmet1 contained an internal 2 Kb SstI fragment which was not labelled. The digestion products from this fragment were visualized by photographing an ethidium bromide stained agarose gel.

Recovery of DNA from agarose gels

A slice of agarose containing the DNA was placed into dialysis tubing. The dialysis tubing contained just enough TE to cover the gel slice. The tubing was immersed in an electrophoresis tank and the DNA was electroeluted out of the gel and into the TE (Maniatis et al, 1982). The polarity was reversed for about 30 seconds to remove any DNA stuck to the dialysis tubing. This solution was then collected, centrifuged for 5 minutes in a microfuge to remove any contaminating agarose and precipitated with one-tenth volume of 3.0M sodium acetate and 2 volumes of 95% ethanol. Precipitations were usually on dry ice for 20 minutes.

Isolation of plasmid DNA

500 mls of culture was used for large scale DNA preparations and 10 mls of culture was used for small scale isolation. Plasmid containing cells were treated with lysozyme to weaken the cell wall and then lysed completely with SDS and NaOH as described by Birnboim and Doly (1979). Centrifugation removed the bulk of chromosomal DNA and the

plasmid DNA was recovered from the supernatant by ethanol precipitation. For large scale extractions, DNA was further purified by ultracentrifugation in a cesium chloride gradient in the presence of ethidium bromide. For small scale DNA isolation, the pellet was dissolved in 50 ul of TE containing 10 ug/ml RNase A (Maniatis et al, 1982).

Subcloning and ligation reactions

The EcoRI fragments from each phage were subcloned to simplify the construction of restriction maps and to use as templates for in vitro transcription reactions. pUC19 (Messing et al, 1985) DNA was first cut with the appropriate enzyme. The enzyme was inactivated by extraction with phenol/chloroform followed by ethanol precipitation. The resuspended vector DNA was subsequently used in the subcloning reaction. Insert DNA was gel purified and a five fold mass ratio of insert to vector DNA was used. The reaction was carried out in a 1.5 ml eppendorf tube which contained 100 ng of vector DNA and an appropriate amount of insert DNA in a total volume of 10 ul. To this, 6 ul of ligation mix (25mM DTT, 150 ug/ml BSA, 2.5mM ATP, 25mM magnesium chloride, and 125 mM Tris pH 7.4) and 2 units of T4 DNA ligase were added. The ligation was carried out at 15^{o}C for 2 hours. The ligation mix was then diluted with double distilled water and 100 ul of this mixture was added to the reaction tube after the 2 hour incubation. The ligation

reaction was then continued overnight at 15°C.

Half of the ligated DNA was used to transform competent E. coli JM83 cells as described by Mandel and Higa (1970). The presence of functional B galactosidase (B gal) enzyme was used to distinguish between vector containing and recombinant containing cells. JM83 cells cannot make functional B gal enzyme. The polylinker in pUC19 contains a sequence which codes for the alpha peptide of B gal (Messing et al, 1985). This peptide complements a deletion of the bacterial B gal gene. Therefore, pUC19 containing cells can produce functional B gal. However, any insertion in the vector's polylinker disrupts the alpha peptide and prevents the production of functional B gal. As a result, cells containing recombinant DNA are unable to make functional B gal enzyme. IPTG (isopropyl-thiogalactosidase) and XGAL (5-bromo-4-chloro indolyl-B-D-galactosidase) are used to detect the presence of B gal. IPTG is a synthetic inducer and XGAL when hydrolyzed by B gal yields a blue color. Transformed cells are grown on plates containing ampicillin (100 ug/ml), IPTG (160 ug/ml), and XGAL (40 ug/ml). Insert containing cells appear white and can be distinguished from blue vector containing cells.

Generation of deletion clones for sequencing reactions

Deletion clones were generated by using Exonuclease III (ExoIII) and SI nuclease as described by Henikoff (1984).

ExoIII generates unidirectional progressive deletions in the DNA because it can only digest DNA with a 5' protruding or blunt end. ExoIII cannot attack DNA with a 3' protrusion (of 4 bases) and therefore this end of the DNA is not digested. By using ExoIII and SI nuclease, an ordered set of deletion clones is easily obtained for sequencing reactions. Sequencing templates were cloned into the HincII site of pUC19. This allowed convenient selection of sites required to generate deletions in the cloned insert. The following steps were carried out to generate deletion clones:

1) 25 ug of plasmid DNA was linearized by digestion with SstI. The DNA was precipitated on dry ice for 20 minutes. The pellet was resuspended in TE and digested with BamHI.

2) The enzymes werre removed with a phenol/chloroform extraction followed by another ethanol precipitation on dry ice. This time the dried pellet was dissolved in 22 ul of distilled water.

3) To half this DNA, 36 ul of 1X ExoIII buffer (0.5M Tris pH 8.0, 100mM b-mercaptoethanol, and 50mM magnesium chloride) and 300 units of ExoIII enzyme were added. The mixture was incubated at 37°C.

4) 2 ul aliquots were removed from the above reaction at 15 second intervals and transferred into eppendorf tubes containing 2X SI buffer (2X SI buffer is 60mM potassium acetate pH 4.5, 500mM NaCl, 20mM zinc sulfate, and 10% glycerol).

5) 2 units of SI nuclease was added to each tube. Incubation was carried out at room temperature for 30 minutes.

6) To analyze the progress of digestion, half the DNA from each time point was run on an agarose gel. The rest of the DNA was extracted once with phenol/chloroform, ethanol precipitated, and resuspended in 10 ul of water.

7) To half this DNA, 2 ul of 10X ligation buffer, 3 ul of water, and Klenow enzyme were added. This mixture was incubated at 37°C for 5 minutes. A mixture of dNTPs (2.5mM each) was then added to the reaction and the incubation was continued for an additional 5 minutes.

8) Finally, in preparation for subcloning, ATP, DTT, and ligase were added and the DNA was religated overnight at 15°C.

9) Half of the ligation mix was used to transform competent E. coli JM83 cells. 4-5 colonies were picked from each appropriate time point.

10) DNA isolated from these colonies was digested, electrophoresized, blotted and probed with the Xenopus gene. Small inserts still containing the gene were selected for sequence analysis.

Sequencing reactions

All the genes were sequenced by the dideoxy method described by Sanger et al (1977). The protocol was modified

to allow sequencing of denatured supercoiled plasmid DNA (Messing et al, 1985; Hattori and Skaki, 1986). The following steps were performed for sequencing reactions.

1) Plasmid DNA isolated from deletion clones was dissolved in 50 ul of TE containing RNase A. 30 ul of a 20% PEG/2.5M NaCl solution was added to the DNA and the tube was left on ice for a few hours. The DNA was recovered by a 15 minute centrifugation in the microfuge. The pellet was then washed with 70% ethanol, dried, and resuspended in 30 ul of TE. 10 ul aliquots of DNA were used in each sequencing reaction.

2) 10 ul of 0.4N NaOH was added to the 10 ul of DNA. After denaturation, the DNA was ethanol precipitated and vacuum dried.

3) To this dried pellet, 1 ul of forward or reverse primer, 3 ul of water, and 1 ul of 5XH buffer (50mM Tris pH 7.9, 50mM magnesium chloride, and 250mM NaCl) were added. The reagents were mixed and incubated at 60^{o}C for 15 minutes followed by an incubation at room temperature for another 15 minutes.

4) To the primed plasmid, 1 ul of 100mM DTT, 2 ul of alpha ^{32}P dATP, and 5 units of Klenow were added. This combination was quickly mixed and divided into four parts and each was added to separate tubes containing 1 ul of G, A, T, and C specific dideoxy mixture (Pharmacia kit). The reaction was carried out at 42^{o}C for 20 minutes. A chase solution (1 ul of 1mM dNTPs) was added and the samples were incubated for another 20 minutes at 42^{o}C.

5) The reaction was stopped by adding 6 ul of sequencing loading dye (80% v/v deionized formamide, 50mM Tris borate pH 8.3, 1mM EDTA, 0.1% w/v xylene cyanol and 0.1% w/v bromophenol blue). The samples were heated to 85°C for 3 minutes and then quick chilled on ice. 3 ul aliquots were loaded on sequencing gels.

6) 6% polyacrylamide (29:1 acry:bis), 7M urea gels were used. Electrophoresis was in 1X TBE buffer at 1500 volts for 1 1/2 to 3 hours. The gel was then gently transferred to filter paper, dried on the paper under a vacuum, and exposed to Kodak XK-1 film for 12-24 hours at room temperature without an intensifying screen.

Preparation of transcription extracts

Transcription extracts were prepared from frozen C. elegans eggs as described by Honda et al (1986). Frozen eggs were quickly dissolved in homogenization buffer (50mM Tris pH 7.9, 0.1mM EDTA, 10mM magnesium chloride, 0.4M ammonium sulfate, 25% glycerol, 1mM phenlymethylsulfonylfluoride, and 5mM DTT) and passed through a french press twice at 12,000 psi. The resulting solution was centrifuged and the proteins remaining in the supernatant were precipitated by slow addition of ammonium sulfate. The precipitated proteins were dissolved in Buffer PC (40mM Tris pH 7.9, 1mM EDTA, and 20% glycerol) and dialyzed against buffer PC until the solution reached the same conductivity as buffer PC - 0.1M NaCl. The extract was then aliquoted and stored at -70°C.

Transcription assays

Transcription reactions contained in 25 ul: 5 ul of extract, 5 ul of DNA (containing 6 ng of tDNA and 25 ng of 5S DNA), 50mM Tris pH 7.9, 3mM magnesium chloride, 0.5mM DTT, 0.6mM ATP, 0.6mM CTP, 0.6mM UTP, 15um GTP, 10uC alpha ^{32}P GTP, 0.2 ug/ml alpha amanitin, and 0.50mM spermine. The reaction was carried out at 25°C for 60 minutes and was stopped by the addition of 5 ul of 10% SDS and 50 ul of phenol/chloroform. The aqueous layer was placed in a clean tube. An additional 25 ul of TE containing 6 ug of carrier DNA was added to the reaction tube . After another phenol/ chloroform extraction, both aqueous layers were pooled and the RNA was recovered by ethanol precipitation on dry ice for one hour.

The dried pellet was resuspended in 6 ul of sequencing loading dye. To make sure all the RNA was dissolved, the sample was heated to 85°C for 3 minutes and then loaded directly on the gel. The RNA was subjected to electrophoresis on 12% polyacrylamide gels containing 7M. urea. The electrophoresis buffer was 1X TBE. The gels were run for approximately 90 minutes at 1500 volts. Labelled RNA bands were detected by autoradiography of the dried gel.

RESULTS

Genomic organization

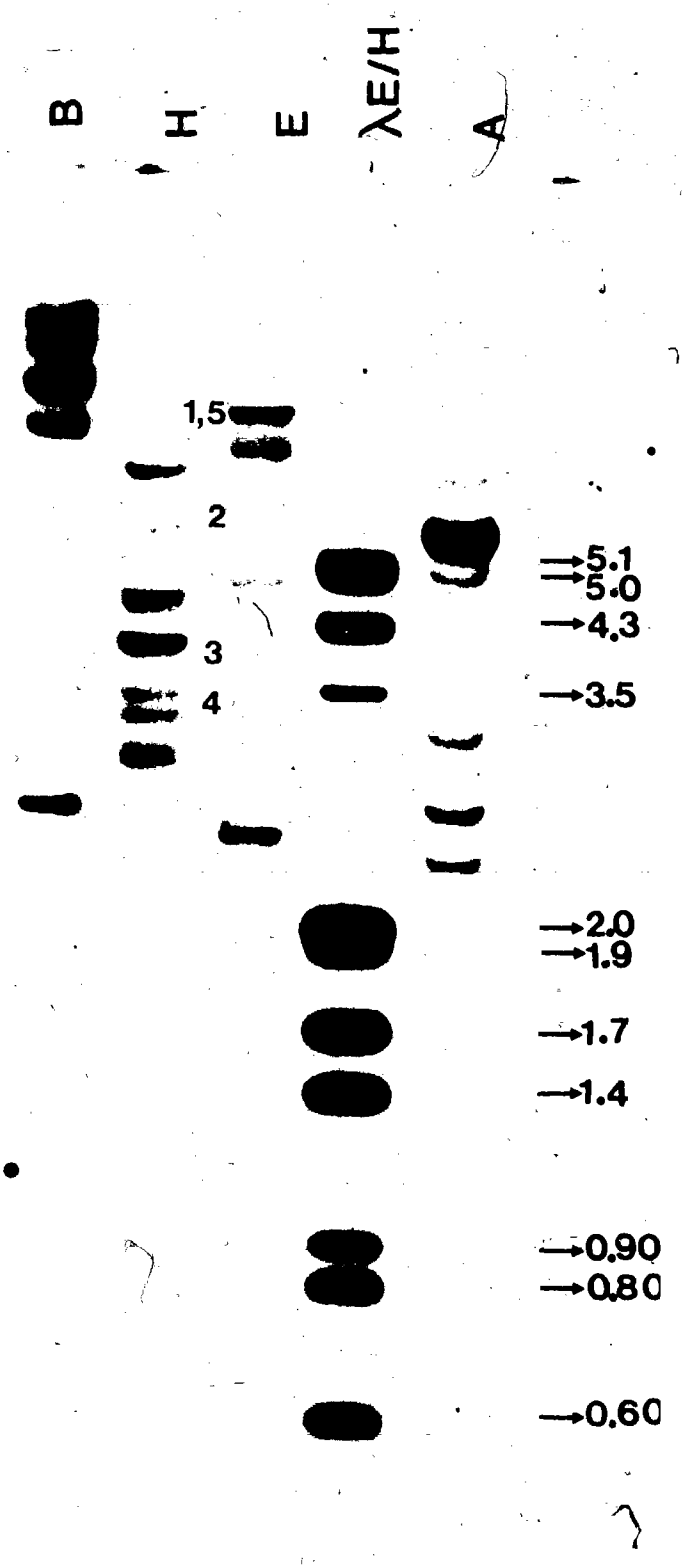
To first determine the presence of putative tRNA met_i genes in C. elegans, genomic blots were probed with a heterologous Xenopus tRNA met_i gene. C. elegans DNA cleaved with EcoRI was electrophoresed on an agarose gel, transferred to a nitrocellulose filter and hybridized with a nick translated Xenopus tRNA met_i gene. The autoradiograph (Figure 1) shows the presence of about 8-10 EcoRI initiator tRNA specific fragments ranging in size from approximately 10.2 Kb to 2.0 Kb. Blots containing genomic DNA cleaved with an enzyme recognizing a 4 bp sequence such as RsaI or AluI show 10 different bands when probed with the Xenopus gene. Neither of these restriction enzymes have sites within the tRNA met_i coding region.

The 10.2 Kb and the 2.4 Kb bands display a stronger hybridization signal implying the possible presence of two copies. Sequence comparison (see sequence data following) indicates that the homology of the probe is restricted to the coding region for all five genes isolated. The stronger hybridization signal of the two bands is not the result of greater homology to the probe.

Isolation of the tRNA met_i genes

The next step was to try to isolate these putative tRNA met_i genes for further characterization. A C. elegans

Figure 1. A. C. elegans genomic blot probed with the Xenopus tRNA met_i gene at low stringency. Lambda DNA cut with EcoRI and HindIII was used as marker DNA. The filter was exposed at -80°C for 2 1/2 days to Kodak XAR-5 film with an intensifying screen. B = BamHI, H = HindIII, E = EcoRI, A = AvaII. The numbers next to the bands in the EcoRI lane represent the different tRNA met_i genes isolated.


B
H
E
λE/H
A
1,5
2
3
4
→5.1
→5.0
→4.3
→3.5
→2.0
→1.9
→1.7
→1.4
→0.90
→0.80
→0.60

genomic library was screened with the Xenopus tRNA met_i probe using *in situ* plaque hybridization (Benton and Davis, 1977). The screening of about 56,000 phage resulted in the isolation of 18 clones which hybridized strongly to the probe. EcoRI restriction digests of the 18 positives showed only five different patterns. Therefore, the 18 positives represented only five different recombinant phage namely: Cetmet1, Cetmet2, Cetmet3, Cetmet4 and Cetmet5. Four of the eight hybridizing bands in the EcoRI lane of Figure 1 were represented in the isolated phage. As suggested previously, the 10.2 Kb band represents two different tRNA met_i genes. This result suggests that *C. elegans* contains 10 tRNA met_i genes. Of the 18 positives isolated: 4 were identical to Cetmet1, 6 were identical to Cetmet2, 4 represented Cetmet3, 2 represented Cetmet4 and 2 were the same as Cetmet5. The chromosomal inserts in Cetmet1, Cetmet3, and Cetmet5 were about 14 Kb while Cetmet2 and Cetmet4 contained inserts of approximately 12.5 Kb and 15 Kb respectively.

Characterization of phage

Restriction mapping

Since these phage clones were isolated from a partial EcoRI library, restriction with EcoRI releases more than one fragment. To make the restriction mapping a little easier, recombinant phage DNA was digested with EcoRI and the resulting fragments were inserted into the vector pUC19 at

the EcoRI site. Each insert was then cut with a variety of restriction endonucleases. Data obtained from digestion of the clones followed by fragment separation enabled the construction of the maps presented in Figure 2. The organization of restriction sites over the entire insert is different in each phage indicating that each represents a different chromosomal region. Results of Southern blotting experiments during restriction mapping of the clones narrowed down the tRNA containing region to a 1.0 Kb EcoRI HindIII fragment in Cetmet2 and Cetmet4 while the tRNA coding sequences are found in a 0.50 Kb HindIII fragment in Cetmet1, and a 2.6 Kb EcoRI XbaI insert in Cetmet5. Hybridization maps the tRNA gene in Cetmet3 to a central 1.7 Kb PstI HindIII fragment. The EcoRI HindIII fragment in Cetmet2 appears identical to the corresponding segment in Cetmet4. The rest of the two clones differ in restriction site organization. The tRNA containing region from each phage was subcloned to allow further characterization.

Sequence analysis

Table 1 summarizes the results presented below. It shows the templates used in each sequencing reaction. All the templates were cloned into the HincII site of pUC19.

Cetmet2 and Cetmet4 In both these phage, the gene had been localized to a 1.0 Kb EcoRI HindIII fragment. Using Exonuclease III and SI nuclease, progressive unidirectional deletions were made into this insert. Southern blots of

Figure 2. Restriction maps of the recombinant phage containing C. elegans tRNA met_i genes. 5' and 3' indicate the orientation of the insert in the phage. The heavy arrow in each phage shows the gene containing insert. E = EcoRI, H = HindIII, B = BamHI, S = SstI, X = XbaI, Hp = HpaII, A = AvaII.

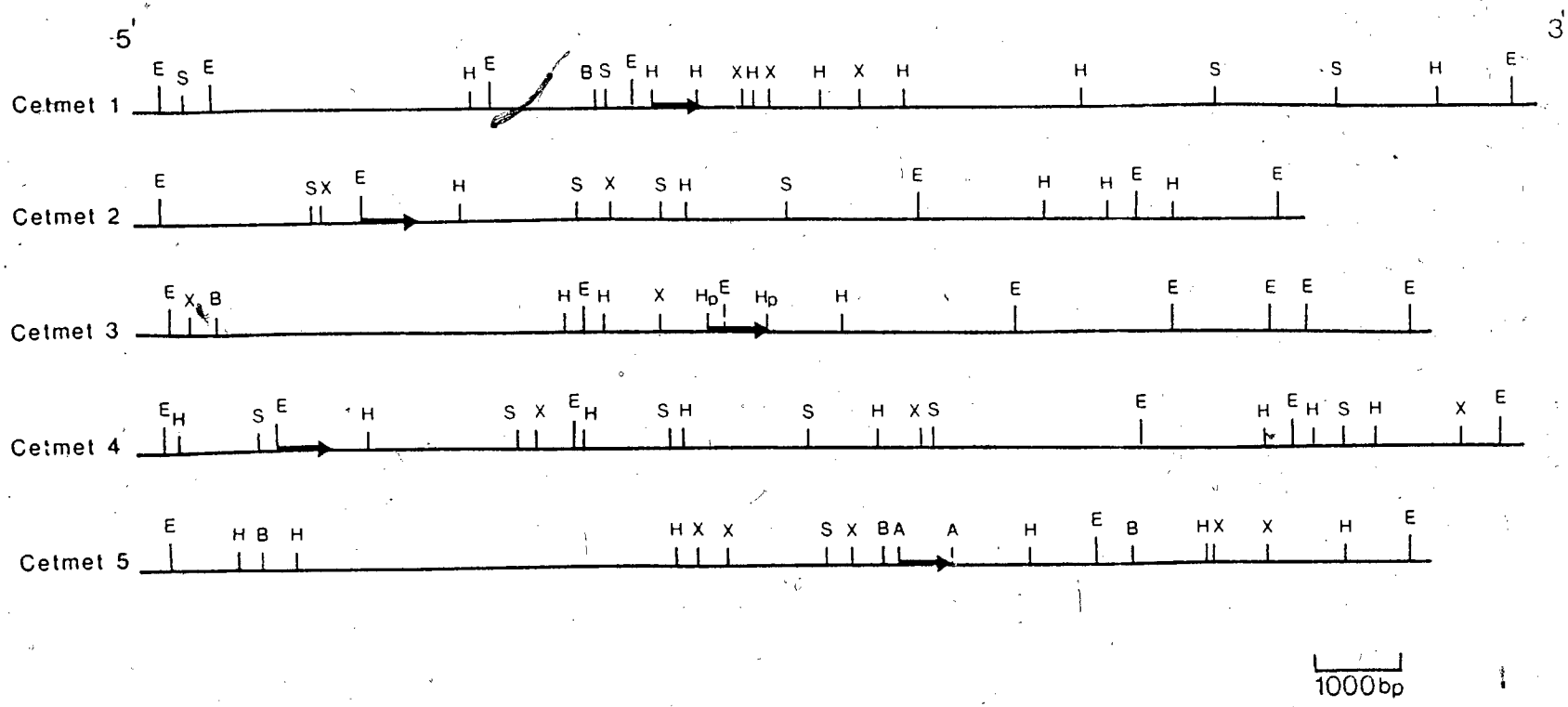

5'
3'
Cetmet 1
Cetmet 2
Cetmet 3
Cetmet 4
Cetmet 5
1000 bp

Table 1. Summary of templates used in sequencing reactions.

tRNA gene	template	Sequencing strategy
Cetmet2	1.0 Kb EcoRI HindIII insert	generated deletions with ExoIII and SI
Cetmet4	1.0 Kb EcoRI HindIII insert	generated deletions with ExoIII and SI
Cetmet3	0.55 Kb HpaII insert	generated deletions with ExoIII and SI
Cetmet5	0.56 Kb AvaII insert	generated deletions with ExoIII and SI
Cetmet1	0.30 Kb HindIII HinfI insert	subcloned and sequenced directly

these deletion clones probed with the Xenopus gene identified small gene containing inserts which were subsequently chosen for sequence analysis. By using the forward primer in one sequencing reaction and the reverse primer in another reaction, both strands of the insert were sequenced. This strategy was employed to sequence both strands of all the other tRNA genes as well. These results show that Cetmet2 and Cetmet4 have identical flanking and coding sequences (Figure 3). Both phage share at least 1 Kb of identical sequence.

Cetmet3 In this phage, digestion of the 1.7 Kb PstI HindIII fragment with HpaII generated a 550 bp HpaII gene containing insert. This insert was subcloned and used as a template to generate deletions. A 230 bp gene-containing clone was selected for sequence analysis.

Cetmet5 The tRNA coding sequence in this phage was confined to a 2.6 Kb EcoRI XbaI fragment. The gene was then further localized to a 560 bp AvaII insert. Again, ExoIII and SI were used to generate clones for sequencing reaction. By sequencing overlapping clones, the nucleotide sequence of the entire 560 bp fragment was determined.

Cetmet1 During restriction mapping, the gene was found to be contained in a 500 bp HindIII fragment. A single HinfI site cleaved this fragment into two pieces of about 200 bp and 300 bp. Hybridization analysis revealed the presence of the gene in the 300 bp insert. This insert was subcloned and

Figure 3. Nucleotide sequence of the C. elegans tRNA met_i genes. The sequence in boldtype represents the coding portion of the tRNA gene. The noncoding DNA strand is shown in each case. The A Box and the B Box promoter sequences are underlined. The transcription termination site is in boldtype in the 3' flanking sequence.

C. elegans tRNA met_i genes

5' flanking sequences

Cetmet1 TGGCCAAAATGGCAAAATTCAAAATTGCCAGTTTGTTCACAATTTTCCGC

Cetmet2 AAGCAAAAATGATCAGACTGATAAAACCATCGAAATGATATGAAAAACGC

Cetmet3 TCTTTGCGTGCCATCTCGTTGTATAGGCTGTGTGTTCTCCTATTACCAAC

Cetmet4 AAGCAAAAATGATCAGACTGATAAAACCATCGAAATGATATGAAAAACGC

Cetmet5 AACACTACACGCTCGTTTCATCGACCGCTAGTTTGTTGCTGTCAAACAAG

tRNA met_i AGCAGCGTGGCGCAGTGGAAGCGTGCTGGGCCCATAACCCAGAGGTCGGT GGATCGAAACCACTCGCTGCTA

3' flanking sequences

Cetmet1 GACTCTTTTTTTGCACTACCTCCAAATTTTAGCAATTTTTGAGTACCTGT

Cetmet2 GAACGATTTTTTTTTGAAAATATATGAATTACCTTCTAAATGTACCAAAAA

Cetmet3 GATTTTTTTCATTTTTTAAAAACAGATATTTTTAATTTTATATGATAACT

Cetmet4 GAACGATTTTTTTTTGAAAATATATGAATTACCTTCTAAATGTACCAAAAA

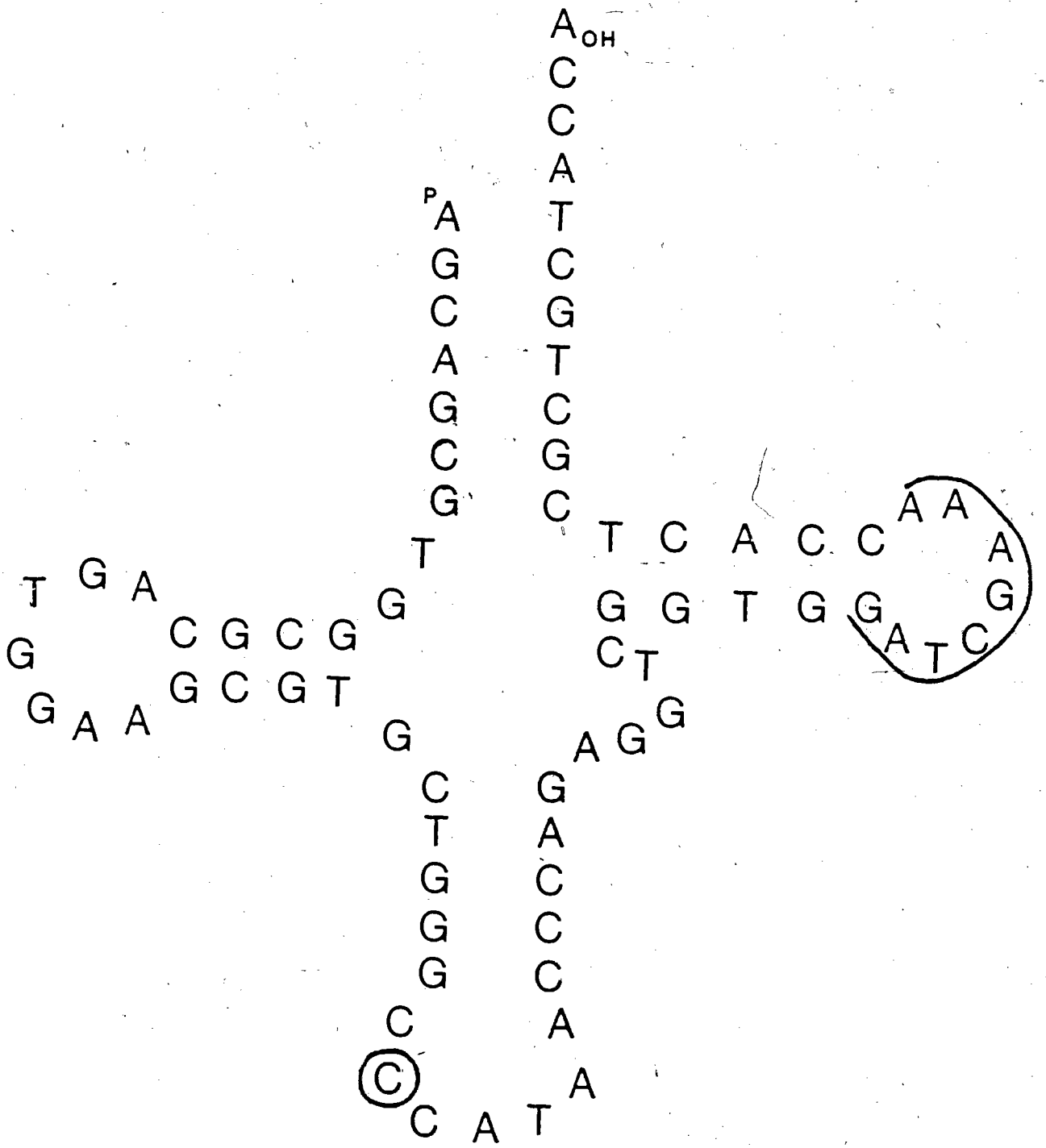
Cetmet5 AAAATTTTATTTCTATGCACAGGTAGGAGGTAGGGCTGTGCGGTTGGCTA

used as a template in sequencing reactions.

Figure 3 shows that all the tRNA met_i genes contain identical coding sequences. The structural sequence is not interrupted and does not code for the 3' terminal CCA. With the exception of Cetmet2 and Cetmet4, the sequences surrounding the tRNA genes show considerable divergence: little or no homology in the 5' and 3' flanking regions. Similarly, Kondo et al (1986) were unable to find any significant homologies in the 5' flanking sequences of five C. elegans $tRNA^{Trp}$ genes. The nematode initiator genes do contain nucleotides homologous to the A Box and the B Box promoter sequences (Ciliberto et al, 1982; Galli et al, 1981; Sharp et al, 1981). In addition, the 3' flanking region of each gene contains a block of T residues which is thought to be the transcription termination site (Korn and Brown, 1978; Bogenhagen and Brown, 1981; Koski and Clarkson, 1982).

Figure 4 displays the C. elegans tRNA met_i gene in the standard cloverleaf configuration. Like all other eukaryotic initiators, the C. elegans gene contains the characteristic ATCGAAA sequence in loop IV instead of the invariant TTCG(A) sequence found in all other tRNAs (Gillum et al, 1975; Rich and RajBhandary, 1976). In addition, the anticodon sequence CAU is preceded by a C instead of the U residue found in all other tRNAs. This is also a feature unique to eukaryotic initiators (Rich and RajBhandary, 1976; Silverman et al, 1979; Canaday et al, 1980).

Figure 4. The nucleotide sequence of the initiator methionine tRNA gene arranged as a cloverleaf. The conserved sequence is underlined.


A OH
C
C
A
P A T
G C
C G
A T
G C
C G
G C
T
T C A C C A A
G G T G G A G
C T A T C
T G A
G C G C G
G C G T
G A A
G
A G G
C G
T A
G C
G C
C A
C
C A T

Hybridization of genomic DNA to a homologous tRNA met_i gene

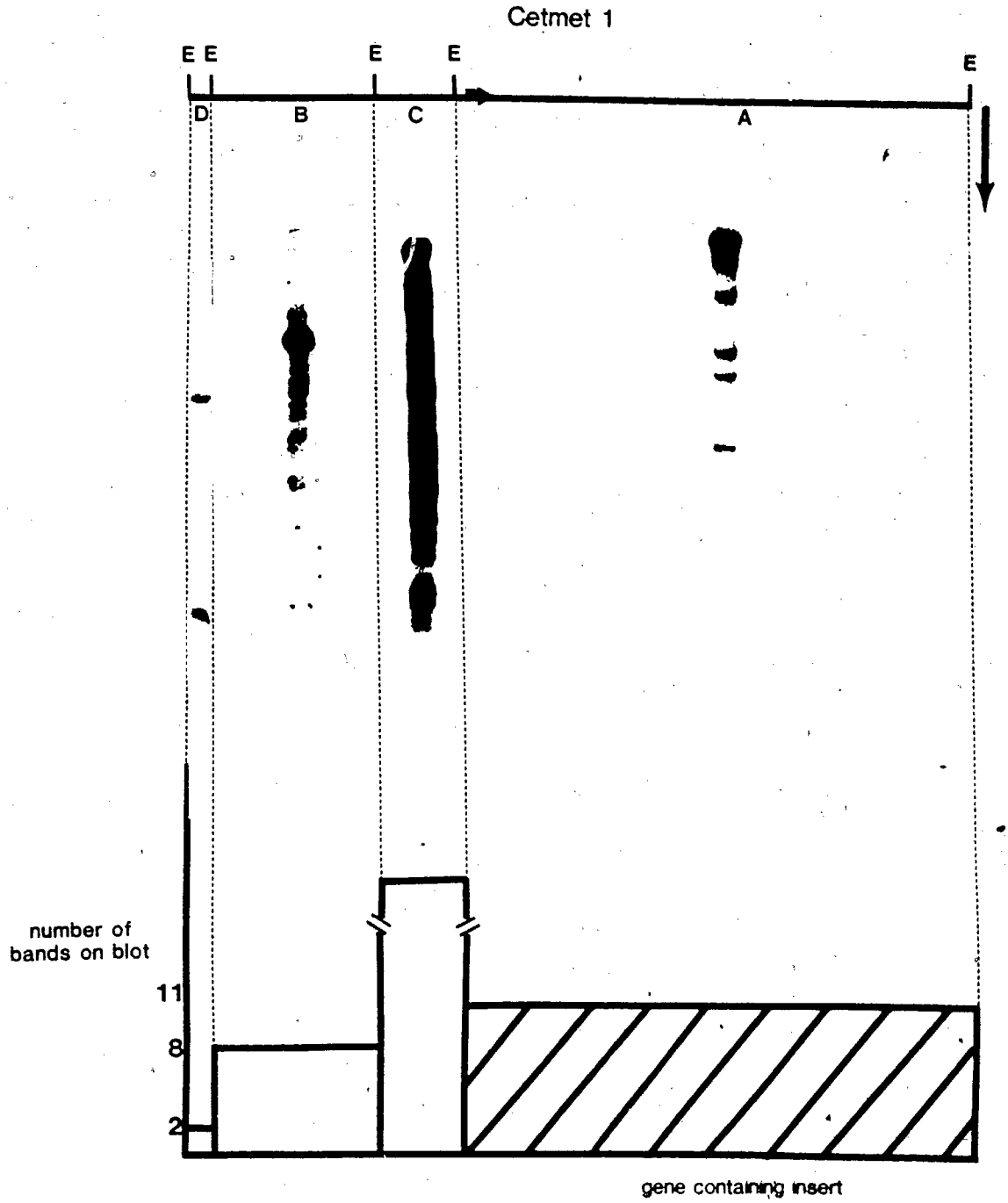
After cloning the nematode tRNA met_i genes, a 130 bp HhaI gene containing fragment from Cetmet5 was hybridized to a Southern blot containing genomic DNA cut with EcoRI. The autoradiograph produced was identical to the EcoRI lane in Figure 1. In addition, the intensity of the signal produced by the homologous tRNA met_i gene was similar to the intensity of the single band produced when a flanking phage fragment was used as a probe. This suggests that the bands on the Southern blot are the same copy number and probably represent single copy genes.

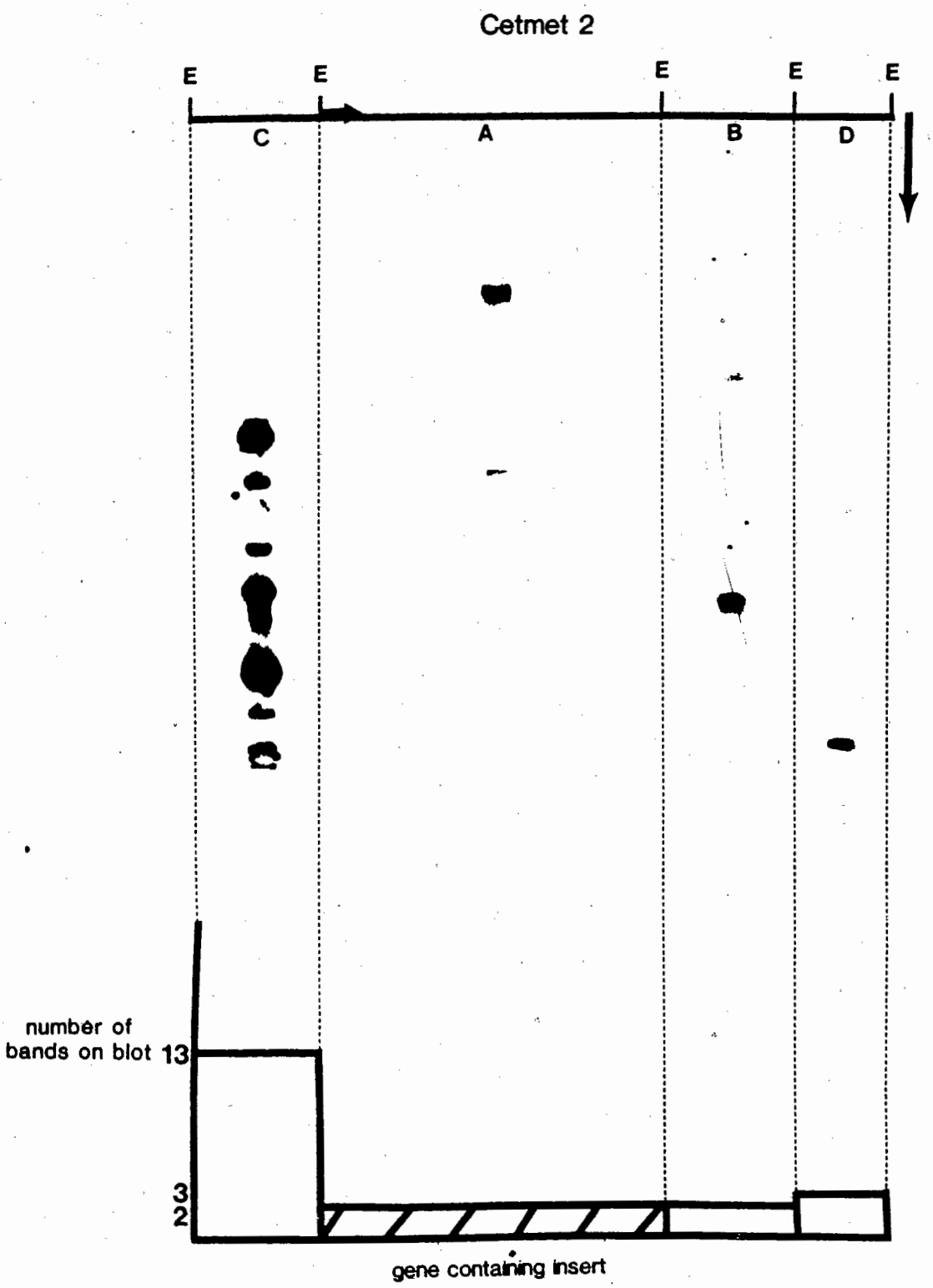
Genomic organization of sequences flanking the tRNA met_i genes

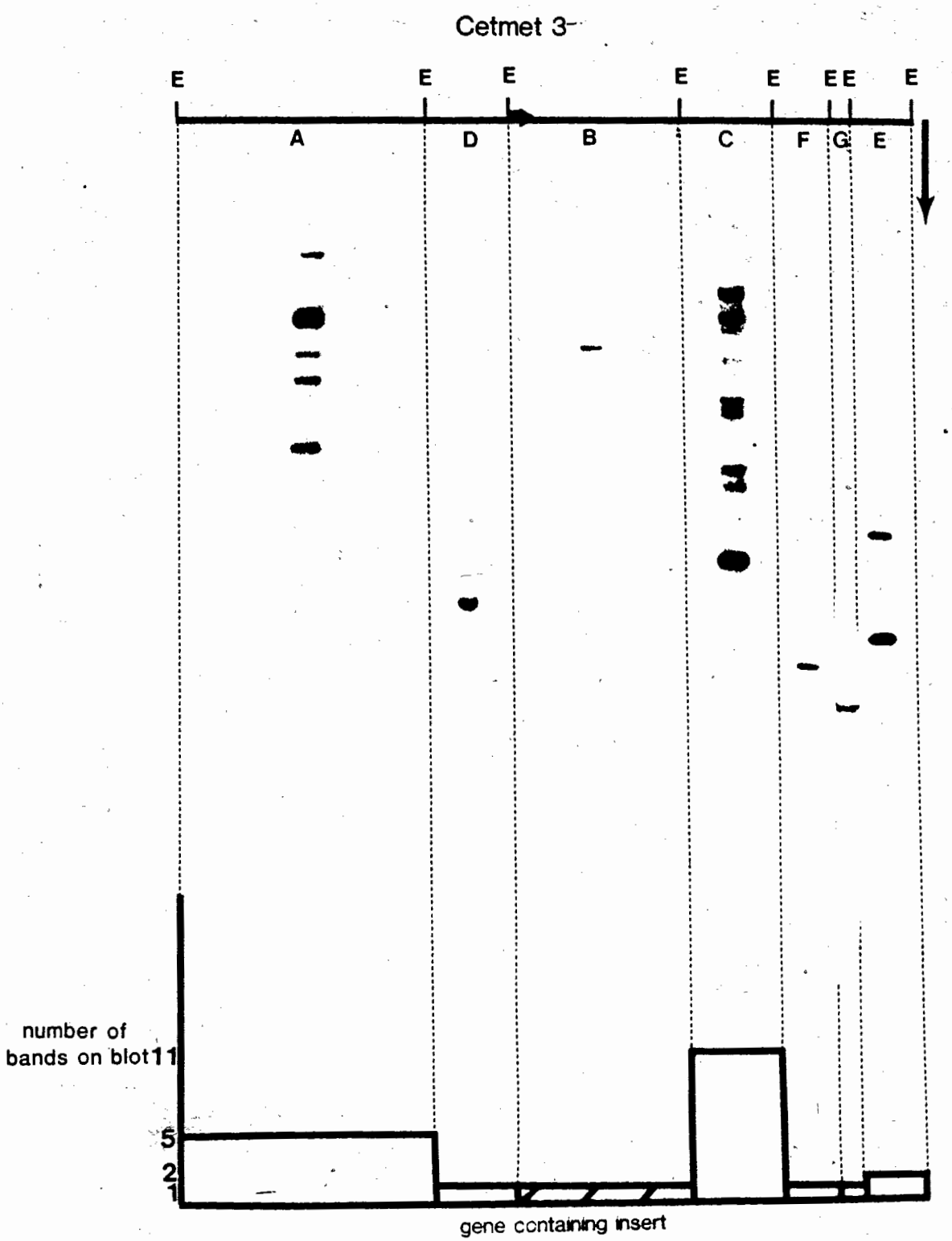
Further information regarding the genomic organization of sequences flanking the cloned tRNA met_i genes was obtained by annealing ^{32}P labelled EcoRI flanking phage fragments to Southern transfers of EcoRI digested genomic DNA (Figures 5A-5E). These results reveal the presence of a moderately repetitive DNA sequence (at least 8 copies) within each phage and adjacent to each tRNA met_i gene. In all but one case, reiterated sequences from the other phage do not cross hybridize. From this absence of homology between localized repetitive sequences, it appears that a different set is present and adjacent to each tRNA met_i gene. Finally, in four of the five phage, the repetitive sequence is found 5'

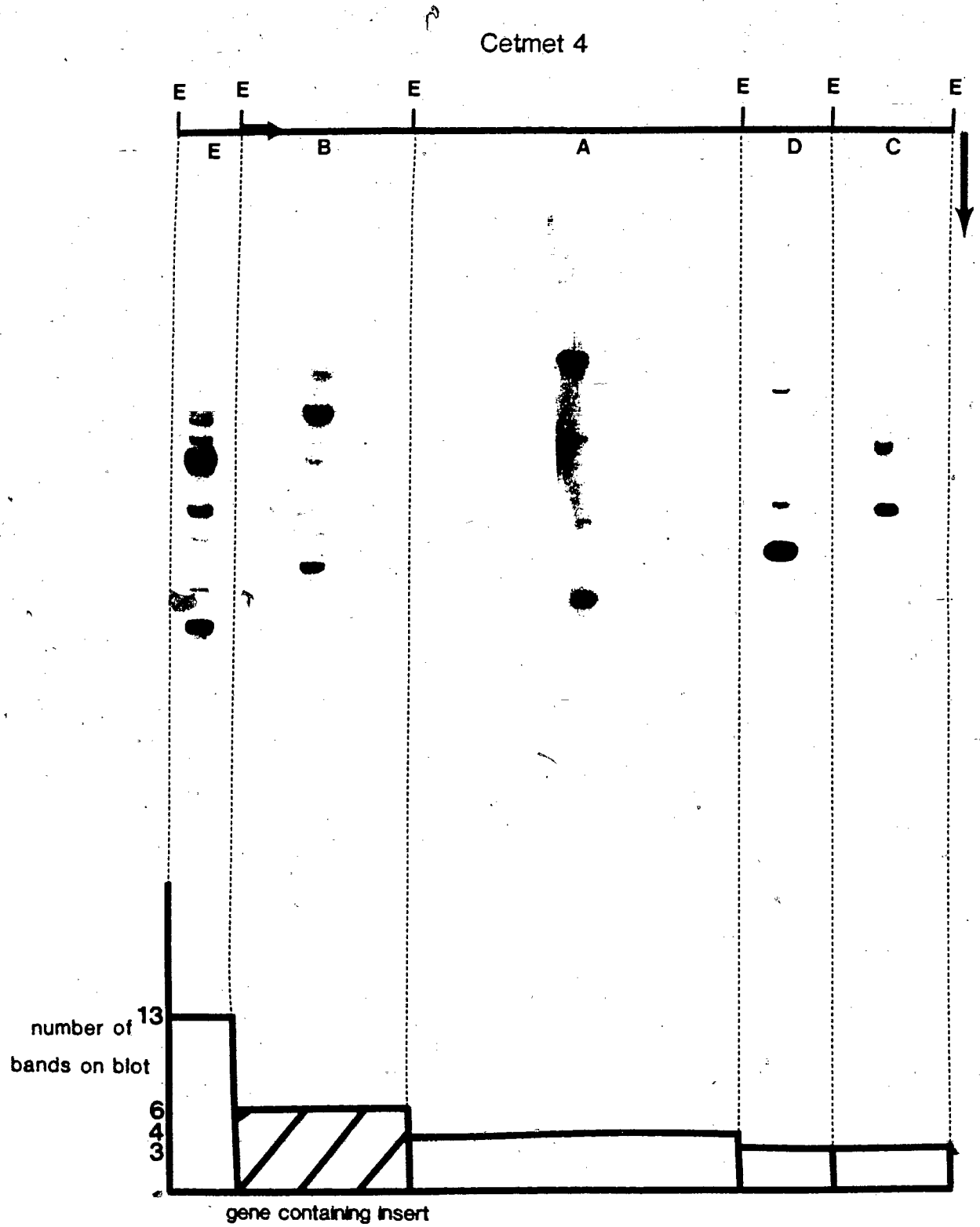
Figures 5A - 5E. The restriction map on the top shows all the EcoRI fragments in the phage. The heavy arrow represents the tRNA gene in the phage. Southern blots containing genomic DNA cut with EcoRI were probed with each EcoRI phage insert. The middle portion of the figure shows the autoradiograph produced when each EcoRI fragment was used as a probe. Hybridizations were at high stringency (wash at 66°C in 0.2X SSPE, 0.3% SDS). Filters were exposed to XAR-5 film with an intensifying screen for 48 hours. The histogram represents the estimated number of bands on the Southern blots.

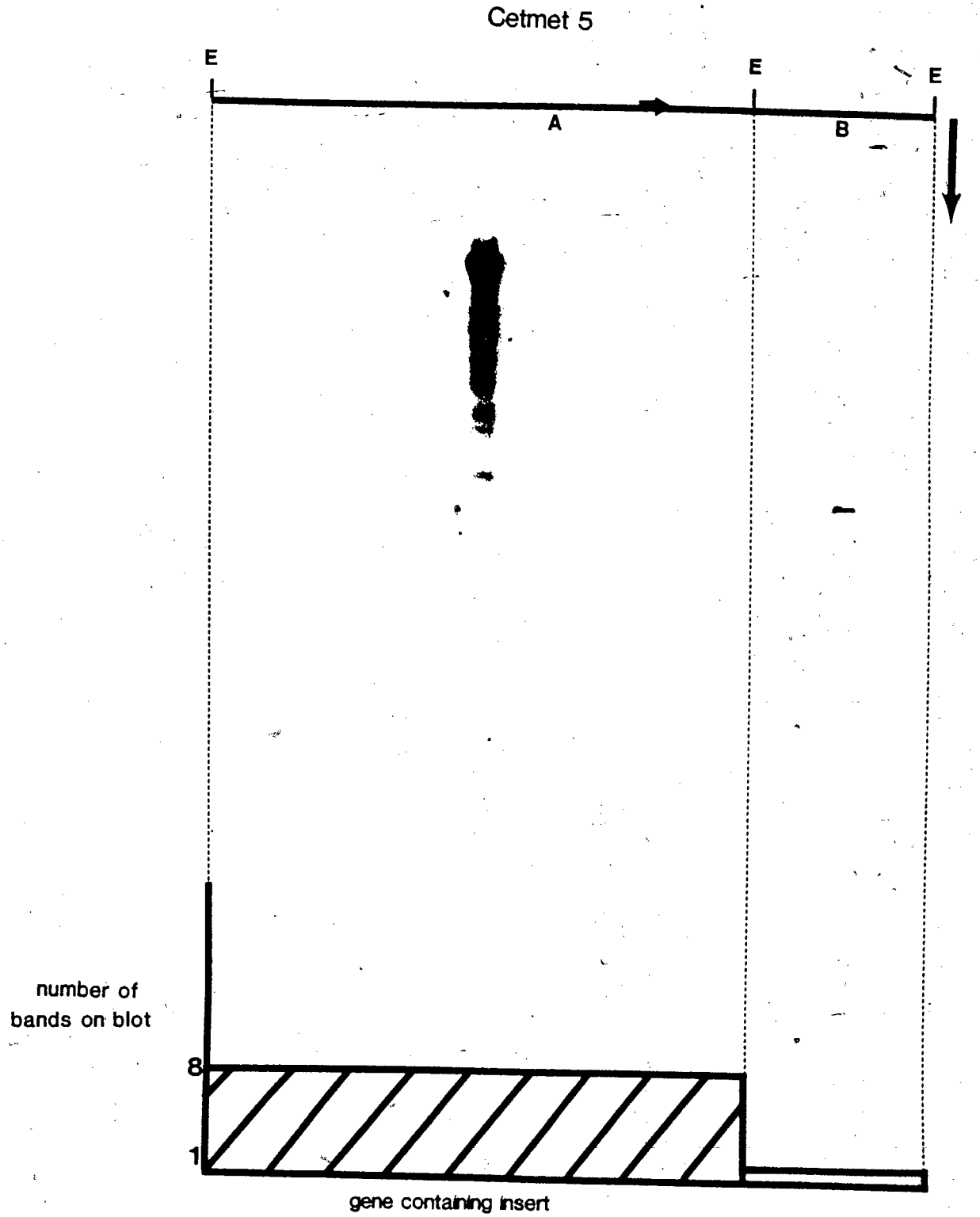
The gels for the Southern blots were run at different times. For example, the four blots shown for Cetmet1 were not from the same gel. This also applies to the blots in Figures 5B - 5E. For these figures, it is not essential to have all the blots from the same gel (for each phage). Phage blots show that in almost all cases, each EcoRI phage fragment hybridizes only to itself and does not cross hybridize to other phage fragments. Therefore, the bands on the Southern blots are not expected to line up in any particular order. The blots are only intended to show the number of bands produced by each EcoRI phage insert on a genomic Southern blot.


Cetmet 1
E E
E
E
E
D
B
C
A
number of bands on blot
11
8
2
gene containing insert


Cetmet 2
E
E
E
E
E
C
A
B
D
number of
bands on blot
13
3
2
gene containing insert


Cetmet 3
E
E
E
E
E
E
E
E
A
D
B
C
F
G
E
number of
bands on blot
11
5
2
1
gene containing insert


Cetmet 4
E
E
E
E
E
E
E
B
A
D
C
number of bands on blot
13
6
4
3
gene containing insert


Cetmet 5
E
E
E
A
B
number of
bands on blot
8
1
gene containing insert

to the tRNA gene. The repetitive sequence in Cetmet3 is found 3' to the tRNA gene.

Insert C from Cetmet1 yields no discrete bands but a smear ranging from low to high molecular weight DNA. Insert C from Cetmet2 hybridizes strongly to 8 bands and weakly to an additional 5 bands. This pattern is also produced by Insert E from Cetmet4 which also hybridizes to 13 bands. This result is not unexpected as these two fragments (Insert C from Cetmet2 and Insert E from Cetmet4) are able to cross hybridize under conditions of moderate stringency (wash at 66°C in 2X SSPE, 0.3% SDS). Insert C from Cetmet3 hybridizes to 11 bands of varying intensity. The band displaying the strongest signal is the probe hybridizing to itself. Differences in band intensity may be the result of varying degrees of homology to the probe. The repetitive sequence from Cetmet5 is found in Insert A of this phage. This fragment also happens to contain the tRNA gene. In addition to one intense band, Insert A hybridizes to seven other weaker bands. Although the reiterated sequences appear to be bounded by EcoRI restriction sites, their exact location and length in the cloned inserts is unknown.

Cosmid results

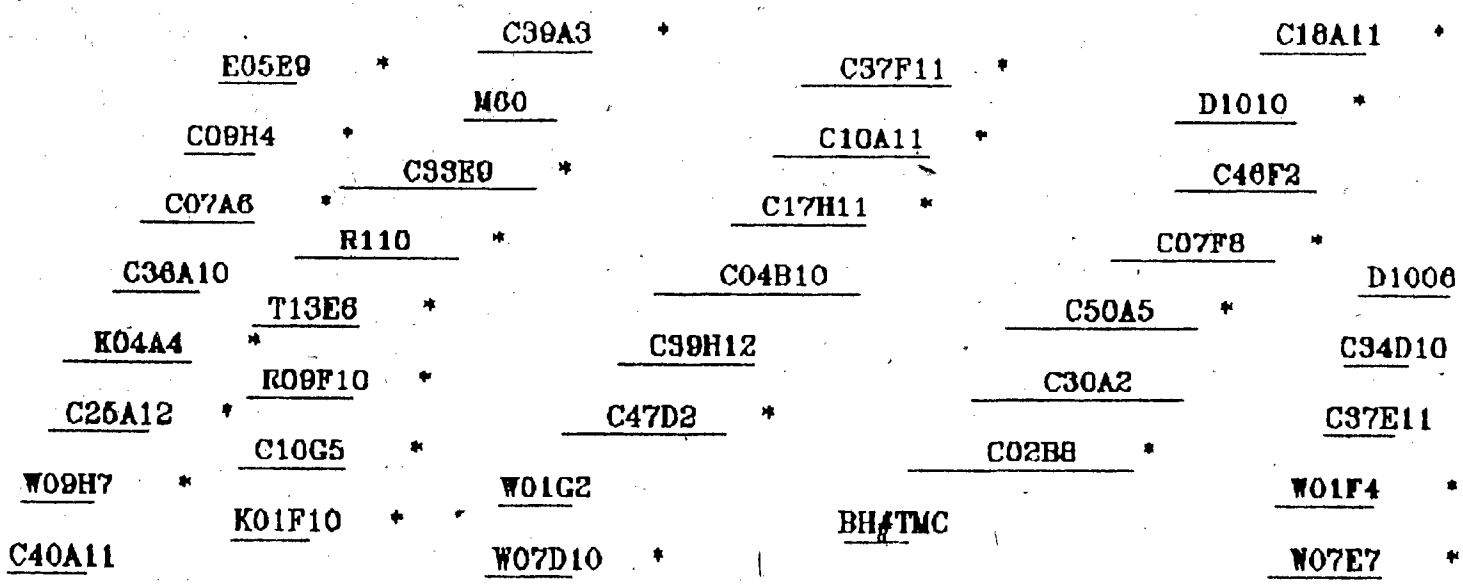
Cosmid clones have been isolated for 4 of the tRNA met_i genes (A. Coulson and J. Sulston, pers comm). The phage have been renamed such that Cetmet1 is rtm-2, Cetmet2 is rtm-4,

Cetmet3 is rtm-3, and finally Cetmet4 is rtm-1. The genes have been named **rtm** because **rt** indicates tRNA while **m** specifies methionine. All four genes lie on different, nonoverlapping cosmid islands (A. Coulson, pers comm). Absence of overlap between the cosmid clones implies that the genes are not closely linked with each other in the genome. The cosmid island containing Cetmet3 (rtm-3) is shown in Figure 6. Cetmet3 (rtm-3) has been mapped to the X chromosome by in situ hybridization and lies 39-55% from the left end (D. Albertson, pers comm).

In vitro transcriptions

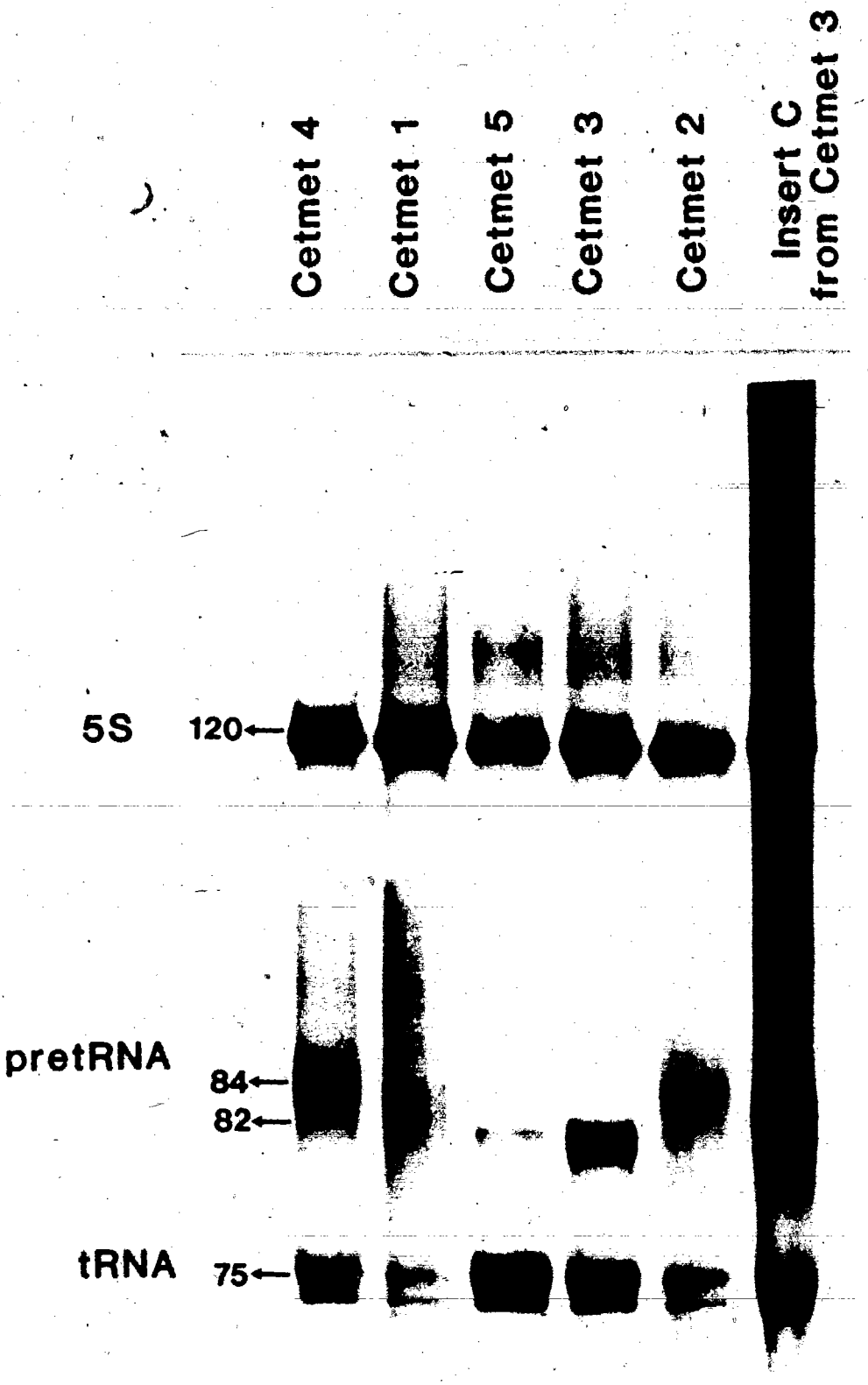
The cloned tRNA$^{met}_{i}$ genes were used as templates to program the homologous in vitro transcription system. For Cetmet2, Cetmet3, and Cetmet4, the EcoRI gene-containing insert in pUC19 was used as a template. The tRNA gene in Cetmet1 was on a large piece of DNA and, therefore, a smaller clone containing the gene was used in the transcription reaction. The HindIII gene-containing insert in pUC19 was used to direct transcription in vitro. Similarly, the 560 bp AvaII subclone from Cetmet5 was used to program the in vitro transcription system. C. elegans 5S DNA (kindly provided by D. Nelson) was used as an internal control in each reaction. All templates were supercoiled plasmids that had been purified by CsCl ultracentrifugation. Figure 7 shows that all genes direct the transcription of a precursor and mature

Figure 6. A physical map of the contig containing Cetmet3 (rtm-3). Each cosmid clone is represented by a single line. The length of each line is proportional to the number of HindIII sites in the clone. Overlapping lines indicate cosmid overlaps detected by comparing fingerprinting patterns produced by each clone. The asterisk indicates that additional similar clones have been isolated but have not been displayed in the figure.



sl.dub. C39A3 join dub. C18E11/C37E11 sl.dub.

Figure 7. *In vitro* transcription products of cloned tRNA met_i genes. 5S DNA was used as an internal control. Total RNA was analyzed on a 12% polyacrylamide gel containing 7M urea. The dried gel was exposed to Kodak XK-1 film for 24 hours at room temperature without an intensifying screen. Approximate sizes of the RNA transcripts are shown.


Cetmet 4
Cetmet 1
Cetmet 5
Cetmet 3
Cetmet 2
Insert C from Cetmet 3
5S
120
pretRNA
84
82
tRNA
75

products. The tRNA genes appear to produce precursors of different sizes. Cetmet1, Cetmet2, and Cetmet4 are similar; the precursor is approximately 84 nucleotides long. Cetmet3 and Cetmet5 produce a smaller primary transcript which is about 82 nucleotides in length. Also, two mature products (about 75 nucleotides in length are produced by each template. These may simply be the result of incomplete processing. Figure 7 also shows that using equimolar amounts of each gene template does not result in the production of equivalent amounts of product. Cetmet5 appears to be the most efficient template in directing transcription. Alternatively, differential tRNA production could result from more efficient processing or from increased stability of the Cetmet5 primary transcript.

Since the tRNA met_i coding region represented only a small portion of the entire phage insert, it was of interest to determine whether any other polymerase III genes were present. All the EcoRI flanking fragments from each phage were used to program the in vitro system. RNA coding sequences could be identified only in Insert C from Cetmet3. This clone produces a tRNA size precursor and mature products in vitro. Therefore, Insert C appears to contain a putative tRNA gene.

DISCUSSION

Gene organization

There appear to be 10 tRNA met_i genes in the haploid genome of C. elegans. The existence of only one gene in each phage insert (with the possible exception of Cetmet3) suggests that the initiator tRNA genes are arranged individually and are not clustered with other tRNA genes. The cosmid results support this hypothesis. The tRNA gene arrangement will be confirmed only when all the genes have been accurately mapped on a linkage group. So far, Cetmet3 (rtm-3) has been mapped to the X chromosome.

It is not clear why only five different recombinant phage were isolated from the screen. The growth of a recombinant phage is presumably affected by its sequence content (Maniatis et al, 1982). Therefore, phage containing unstable or poisonous sequences will be underrepresented in a library. Subsequent amplification of the library results in differential growth of recombinants and this is reflected in the sequence content of the library. Consequently, any tRNA met_i genes lying close to such sequences may also be lost from the library. Alternatively, it may be bad luck that only five different phage were isolated.

The initiator tRNA genes in C. elegans resemble those in Man and Drosophila both in number and general organization. The haploid human genome contains 12 dispersed initiator tRNA genes (Santos and Zasloff, 1981) while in Drosophila,

Southern blots indicate the presence of 8-9 scattered genes (Sharp et al, 1981b). It is not known why members of a tRNA multigene family are dispersed throughout the genome; nor is there any explanation for clustering of tRNA genes. Guthrie and Abelson (1982) hypothesize that tRNA genes are dispersed to maintain their number. If they were tandemly repeated such as 5S RNA genes, their numbers could expand or contract by unequal crossing over. They suggest that the number of identical genes in a set would tend to remain fixed if they were dispersed among different chromosomes.

To study the chromosomal regions containing tRNA met_i genes, genomic blots were probed with flanking EcoRI fragments from each phage. The results reveal the presence of a different reiterated sequence adjacent to each tRNA met_i gene. Repetitive sequences are also closely associated with three human tRNA met_i genes (Santos and Zasloff, 1980). As in the C. elegans genes, there is no homology between the reiterated sequences. Santos and Zasloff (1981) believe that the repetitive sequences surrounding the tRNA met_i genes could represent "hot spots" for recombination. They isolated two phage that had the same tRNA containing fragment but differed in the rest of the restriction site organization (similar to Cetmet2 and Cetmet4). Furthermore, the region of homology was flanked by repetitive DNA. To explain their finding, they suggest that a recombinational event (originating in the reiterated sequences) was responsible for

joining a tRNA meti gene containing fragment to two different chromosomal segments.

Similarly, it has been implied that the highly reiterated 5' flanking sequence of a D. discoideum $tRNA^{Trp}$ gene may play a role in regulating the transcription or processing of this gene (Peffley and Sogin, 1981). Olson et al (1983) use the same reasoning to propose that the consistent association between sigma elements and tRNA genes has a functional significance. However, it is unlikely that the sigma element influences tRNA gene expression since all the elements isolated do not transcribe in the same orientation as the tRNA gene. In addition, sigma cannot be required for tRNA transcription since the majority of tRNA genes do not contain such sequences. In all these cases, a role in gene regulation has been proposed, but has not yet been demonstrated.

Similarly, it is difficult to assign a regulatory role to the repetitive sequences found adjacent to the C. elegans genes. First, lack of homology between localized repetitive sequences suggests that the tRNA met_i gene is not functionally arranged with the reiterated sequence. Second, the reiterated sequence is found 5' to the tRNA coding region in only four of the five phage. In Cetmet3 a different repetitive sequence is found 3' to the gene. This sequence produces a tRNA size transcript in vitro. None of the other localized repetitive sequences produce a transcript in vitro.

Finally, Emmons et al (1981) have estimated that C. elegans contains between 130 to 1300 families of middle repetitive elements. These sequences are only a few hundred base pairs long and most are of a low copy number. These results suggest that the presence of repetitive sequences next to the tRNA genes may simply be fortuitous. Similarly, by analyzing random cloned fragments of C. elegans DNA, Rose et al (1982) found that 9 out of 27 fragments hybridized to more than one band on a Southern blot.

Sequence analysis

Although the coding region of the C. elegans tRNA met_i genes is identical, sequence heterogeneities do exist in other tRNA met_i gene families. A variant human tRNA met_i gene has been isolated by Zasloff and Santos (1981). This gene contains a T instead of a G nucleotide at position 56. Although transcriptionally active, the gene is not processed properly (Zasloff et al, 1982b) and is trapped in the nucleus (Zasloff et al, 1982c).

In addition, X. laevis contains two different tRNA met_i genes. The Met A gene contains a C residue at position 65 whereas Met B has a T nucleotide at this position (Hipskind and Clarkson, 1983). Met B transcripts have not been detected in vivo and this gene is poorly expressed in vitro. Hipskind and Clarkson (1983) showed that the single base pair change did not impair transcription. Instead, the poor

expression was due to inhibitory 5' flanking sequences.

The flanking sequences of the nematode tRNA met_i genes show no similarity. Only Cetmet2 and Cetmet4 have the same flanking sequences. These genes are found on identical 1.0 Kb DNA fragments. This arrangement could be the result of a gene duplication event. Because there is some homology between the reiterated sequences found 5' to each gene, a region greater than 1.0 Kb must have been involved in the duplication event. Similarly, the Drosophila tRNA met_i genes are found on identical 415 bp fragments (Sharp et al, 1981b). Other examples of tRNA gene duplication have been documented in Drosophila. Hosbach et al (1980) report that the three tRNA glutamate genes at chromosomal region 62A were generated by unequal crossing over between two genes that arose by duplication of an ancestral gene. In addition, single copies of a $tRNA^{Gly}$ gene are found within two direct repeats of at least 1.1 Kb at chromosomal region 56F (Hershey and Davidson, 1980). An extreme example of gene duplication is seen in the organization of two tRNA met_i genes in Xenopus laevis. In this organism, two initiator and six other tRNA genes are contained within a 3.18 Kb DNA segment that is tandemly repeated about 300 times in the haploid genome (Clarkson and Kurer, 1976).

Like other eukaryotic initiators, the C. elegans RNA sequence contains the nucleotides AUCG in the T loop region. The invariant TUCG sequence is believed to be required for

tRNA binding to the A site on the ribosome (Offengand and Henes, 1969). Because initiator tRNAs do not contain this sequence, they are restricted to the P site. However, a B. mori $tRNA^{Ala}$ containing the sequence AUCG in the T loop still functions in protein synthesis (Sprague et al, 1977). This result suggests that in addition to the invariant sequence AUCGAAA, other features must exist to distinguish initiators from other tRNAs.

Modulation of tRNA gene expression by 5' flanking sequences implies that these sequences can either inhibit or be essential for expression. Regulatory sequences have been found 5' to D. melanogaster and B. mori tRNA genes. In Drosophila, T-rich sequences are responsible for the poor in vitro transcriptional activity of lysine tRNA genes (DeFranco et al, 1981). Because the repression is position dependent, DeFranco et al (1981) suggest that the sequence may exert its effect by resembling a pol III termination signal. Also, sequences of putative stimulatory elements found 5' to Drosophila tRNA genes have been reported (Indik and Tartof, 1982; Sajjadi et al, 1987). No sequences homologous to any of these characterized sequences have been found in the 5' flanking region of the C. elegans tRNA met_i genes.

Sprague and coworkers (1983; 1984) have identified an upstream control region required for the expression of B. mori polymerase III genes. This essential region contains three block of T-rich sequence. Because the 5' and 3'

flanking regions of the nematode tRNA met_i genes are A-T rich, a computer search for the Bombyx promoter elements shows many sequences with partial homology. These sequences do not appear to be functionally significant. Data from the above studies suggests that 5' modulating sequences may influence transcription in a species specific manner. This could explain why T-rich sequences found in front of Drosophila tRNA genes are inhibitory while T-rich sequences found in front of Bombyx tRNA genes are essential for transcription.

So far, no general sequence requirements have been identified in the 5' flanking region of C. elegans pol III genes. In vitro transcription of deletion clones showed that a 13 bp sequence found 5' to the 5S RNA coding region can modulate transcription. Removal of this sequence reduces the efficiency of 5S RNA transcription in vitro (D. Nelson, pers comm). However, this sequence is not found in front of any of the nematode tRNA met_i genes. Furthermore, deletion of all but 2 bp of 5' flanking DNA from Cetmet3 does not appear to impair expression of this gene in vitro.

It is difficult to explain why the entire coding region of the tRNA met_i genes is conserved while no sequence homology is detected in the flanking DNA. This implies that strong selection pressure and/or correction mechanisms must exist to maintain sequence homology between the various members of the tRNA gene family. Weiner and Denison (1982)

propose that only gene conversion could be responsible for maintaining the homogeneity of eight yeast tyrosine tRNA genes. They argue that conversion best explains why internal sequences are homogenized while flanking sequences remain unaffected. Gene conversion is initiated when two partially homologous DNA sequences exchange single strands between themselves in the formation of a heteroduplex. Any differences between them are repaired by using either of the strands as template. Although each locus should serve as the template for repair in half of the conversion events, this does not always occur and one may be used predominately at template (Nagylaki and Petes, 1981). Conversion allows a non-reciprocal information transfer from one gene to a copy of the gene on the same (Klein and Petes, 1981) or different (Scherer and Davis, 1980) chromosome. Munz et al (1982) have demonstrated that recombination occurs between dispersed serine tRNA genes in S. pombe. They show that information transfer between three unlinked serine tRNA genes occurs by intergenic conversion events. These events appear to be restricted to the coding sequences only. Perhaps a correction mechanism exists that ensures the participation of only coding sequences in these events. In addition, some mechanism must exist to prevent the homogenization of closely related tRNAs. This mechanism would allow different isoacceptors of a tRNA to maintain their sequence heterogeneity.

tRNA-related sequences do not appear to participate in any rectification mechanisms. In fact, it has been proposed that many highly repetitive, transcribe sequences have evolved from specific tRNA genes (Rogers, 1985; Daniels and Deininger, 1985). Although the origination of these sequences is not well understood, Rogers (1985) suggests that these sequences are generated by the insertion of reverse transcribed sequences from RNA back into the genome. Because the polymerase III promoter is internal, the transposed sequences may be capable of transcription. Rogers believes that this would allow further rounds of transcription and reverse transcription and, in this way, the sequence could spread throughout the genome. One repeat sequence family homologous to a tRNA met_i gene has been found in the Galago (bushbaby) genome. There appears to be no evidence for compensating changes because members of this repetitive DNA family have accumulated numerous mutations (Daniels and Deininger, 1985).

Transcription analysis

When the cloned tRNA met_i genes are used as templates to program a homologous *in vitro* transcription system, precursors and mature products are produced. However, as mentioned earlier, primary transcripts of different sizes are transcribed from the initiator tRNA genes. The coding sequence alone cannot dictate the specific site of initiation

because all the primary transcripts of these genes should then be identical. Heterogeneity of transcription initiation sites is not unique to the nematode tRNA met_i genes. The primary transcripts of two human tRNA met_i genes differ in sequence and length of the 5' leader and 3' trailer portions (Zasloff et al, 1982a).

In addition, while studying the transcription and processing of yeast tyrosine tRNA genes in frog oocytes, Olson and Robertis (1979) noticed that each precursor had a different 5' leader sequence. This sequence varied in length between various members of the $tRNA^{Tyr}$ gene family. Variations in the size of the primary transcript could also be caused by a heterogeneous number of uridylate residues at the 3' end (Koski and Clarkson, 1982). Mature products of different length could be the result of incomplete CCA addition (Willis et al, 1986) or incomplete nucleotide modification (Cortese et al, 1982).

In vitro transcriptions also show that the genes are transcribed with different efficiencies. Although tRNA genes contain intragenic promoters, 5' flanking sequences have been shown to play a role in modulating transcription. In vitro studies have identified 5' flanking sequences which positively influence transcription and other elements which inhibit transcription. Morton et al (1980) have demonstrated that a truncated B. mori $tRNA^{Ala}$ gene containing only 14 bp of its normal 5' flanking DNA is totally inactive in

homologous transcription extracts. A similar observation has been reported by Olson and Shaw (1984). They show that expression is impaired in mutants deleted for 5' sequences within 36 bp of a yeast $tRNA^{Tyr}$ coding region. More recently, it has been established that nucleotides -38 to -34 are required for in vitro transcription of a Drosophila $tRNA^{Val4}$ gene (Sajjadi et al, 1987). In addition, their deletion analysis uncovered the inhibitory effects of nucleotides between -70 and -49. As previously mentioned, several Drosophila lysine tRNA genes contain T-rich inhibitory 5' flanking sequences (DeFranco et al, 1981). Similarly, a sequence with the ability to form Z DNA is found in front of a variant $tRNA\ met_i$ gene and is responsible for the lack of in vitro transcriptional activity (Hipskind and Clarkson, 1983).

In vivo observations also imply a role for noncoding sequences in the expression of tRNA genes. Even though the three yeast serine tRNA genes encode identical tRNAs (Broach et al, 1981) these genes are expressed at different levels as indicated by the varying efficiencies of suppression (Ono et al, 1981). Differential expression of suppressor tRNA genes has also been observed in C. elegans (Hodgkin, 1985; Kondo et al, 1986).

Alternatively, differences in transcription efficiency of the $tRNA\ met_i$ genes may be due to different ionic requirements for expression. While studying the in vitro

transcription of a Bombyx constitutive $tRNA^{Ala}$ gene and a silkgland specific $tRNA^{Ala}$ gene, Young et al (1986) discovered that expression was salt dependent. They found that the silkgland specific alanine tRNA gene was more sensitive to salt concentration changes. In addition, the silkgland specific gene required a lower salt concentration than the constitutive gene for optimum expression (Young et al, 1986). A certain ionic environment may be required for the interaction of a putative silkgland specific transcription factor with the tRNA gene. Therefore, the level of transcription of a gene can be modified by the salt concentration in a crude extract.

Proposals for further research

1) Obtain cosmid clones containing the tRNA genes and use them to search for a RFLP between the BO and N2 strains. If one exists, it can be used to map the genes to a linkage group. Alternatively, a request can be made to have the genes mapped by *in situ* hybridization.

2) The putative tRNA gene in Insert C of Cetmet3 can be identified. By testing deletion clones of Insert C *in vitro*, the polymerase III gene can be localized to a small piece of DNA. This fragment can then be sequenced to identify the gene.

3) Identify the transcription start site of the tRNA genes. This can be done by primer extension experiments.

4) Determine sequence requirements for *C. elegans* tRNA met_i gene expression *in vitro*. 5' and 3' deletion clones can be generated by using ExoIII and SI nuclease. The expression of these clones can be tested by using the homologous transcription system.

5) Determine whether pol III promoter elements are interchangeable in *C. elegans*. Hybrid genes containing promoter elements from tRNA genes and 5S RNA genes can be constructed and their expression tested *in vitro*. In addition, to find out if the 5' modulating sequence from the 5S RNA gene can also influence tRNA gene expression, this sequence can be ligated upstream from a tRNA gene. The expression of this clone can then be compared to the

expression of a wildtype tRNA gene.

6) The identity of the moderately repetitive sequence in each phage can be determined to find out how different the localized sequences are from one another.

7) A different genomic library can be screened to isolate the remaining members of the tRNA met_i gene family.

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