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The zebrafish genome contains two distinct selenocysteine tRNA[Ser]Sec genes

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Abstract The zebrafish is widely used as a model system for studying mammalian developmental genetics and more recently, as a model system for carcinogenesis. Since there is mounting evidence that selenium can prevent cancer in mammals, including humans, we characterized the selenocysteine tRNA[Ser]Sec gene and its product in zebrafish. Two genes for this tRNA were isolated and sequenced and were found to map at different loci within the zebrafish genome. The encoding sequences of both are identical and their flanking sequences are highly homologous for several hundred bases in both directions. The two genes likely arose from gene duplication which is a common phenomenon among many genes in this species. In addition, zebrafish tRNA[Ser]Sec was isolated from the total tRNA population and shown to decode UGA in a ribosomal binding assay.

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Key words: Zebrafish; Selenocysteine; tRNA; Gene; Selenium

1. Introduction

The zebrafish is becoming a prominent model organism for studying mammalian genetics and development [1] and more recently, as a model for studying oncogenes and carcinogenesis (see [1–4] and references therein). Selenium suppresses cancer in rodents, and possibly in humans, and selenoproteins are the most likely candidates responsible for the chemopreventive effect of this element (see [5,6] and references therein). As selenocysteine (Sec) tRNA[Ser]Sec is the central component in the selenoprotein biosynthesis [7], we examined this critical molecule as the initial step in determining if zebrafish may also serve as a model organism for studying selenium as a chemopreventive agent in cancer. We found that zebrafish, unlike any other known animal in which the tRNA[Ser]Sec gene has been sequenced, encodes two copies of this gene. All other higher and lower animals, including several mammals, chickens, frogs, fruit flies and worms, in which the tRNA[Ser]Sec gene has been sequenced, contain this gene only in a single copy (reviewed in [8]). Both zebrafish genes have identical encoding sequences, contain highly homologous sequences for several hundred bases upstream and downstream of the gene and map at different loci. The two genes likely result from gene duplication which is a common occurrence with many genes of the zebrafish lineage [9,10] and they may have resulted from genome duplication in an early ancestor [11].

2. Materials and methods

2.1. Materials

The zebrafish genomic library cloned in λ (EMBL 3 SP6/T7) was purchased from Clontech (Palo Alto, CA, USA) and all reagents were commercial products of the highest grade available. Adult zebrafish were quickly frozen in liquid nitrogen and stored at −80°C until ready for use.

2.2. Library screening, gene isolation and sequencing

All molecular cloning procedures including plaque lifting, Southern blotting, subcloning, restriction enzyme digestions and gel electrophoresis followed standard techniques [12] or the manufacturer's protocol unless otherwise mentioned. The zebrafish genomic library (18 plates with approximately 2×10^4 phages/plate) was screened with a 193 bp fragment encoding the human tRNA[Ser]Sec gene [13] labelled with [α-^{32}P]dCTP as probe. Following hybridization for 2 h, membranes were washed twice for 10 min each in SSC (1×SSC was used during library screening and 0.2×SSC in all subsequent hybridizations) and 0.5% SDS at room temperature and exposed to a phosphur screen for 0.5–2 h or to X-ray films for 2–10 h. Bacteriophages giving positive signals were amplified and phage DNA was isolated using Qiagen columns. Fragments encoding the Sec tRNA[Ser]Sec gene obtained by restriction endonuclease digestion were subcloned into pUC 19 for sequencing. GCG-Lite+Clustalw in the NIH Network was used for sequence alignment and analysis.

2.3. Genomic mapping

Genomic mapping of the two ZStR genes was performed using a zebrafish-hamster radiation hybrid panel (Research Genetics). PCR analyses were carried out in a final volume of 10 μl containing 1×PCR buffer (Perkin Elmer), 2 μM of each primer, 200 μM of each dNTP, 1 μl of template DNA (25 ng) and 0.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer). After an initial activation of polymerase at 95°C for 10 min, 36 cycles at 95°C for 15 s, 58°C for 15 s and 72°C for 40 s were carried out on a GeneAmp PCR System 9600 (Perkin Elmer). PCR products were separated on a 2.5% agarose gel (Gibco BRL). Analysis of the radiation hybrid panel data was performed by Dr Robert Geisler of the Max-Planck Institute.

2.4. Isolation, fractionation and codon recognition studies of tRNA[Ser]Sec

Total tRNA was isolated from 45 g of frozen adult zebrafish as described [13]. 315 A_{260} U of total tRNA were applied to a RPC-5 column in 0.45 M NaCl, 0.01 M $Mg(OAc)_2$, 0.01 M NaOAc and 0.001 M EDTA, pH 4.5, and the attached tRNA eluted in a linear 0.50 M–0.70 M NaCl gradient as described [14]. Sec tRNA[Ser]Sec was identified in fractionated tRNA by dot blotting 5 μl of every other eluted fraction onto nitrocellulose filters and hybridizing with a labelled 193 bp human DNA fragment encoding the Sec tRNA[Ser]Sec gene as probe. Fractions containing tRNA[Ser]Sec were pooled, aminoacylated with [^{3}H]serine [13], the resulting [^{3}H]seryl-tRNA[Ser]Sec was fractionated on a RPC-5 column and individual peaks of [^{3}H]seryl-tRNA were isolated, prepared for encoding studies and encoding

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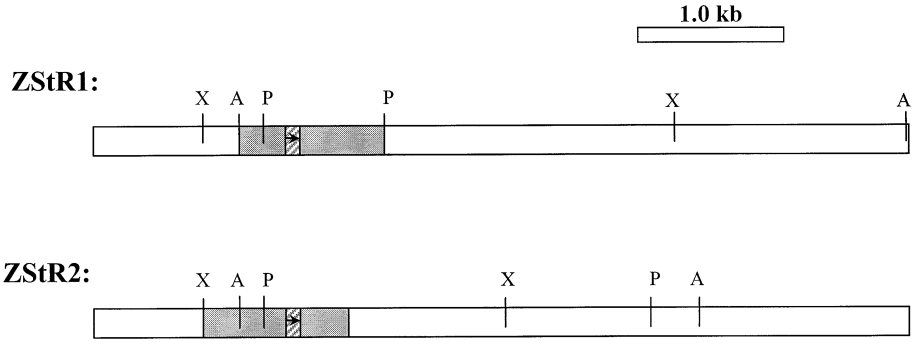

Fig. 1. Restriction map of zebrafish ZStR1 and ZStR2. The shaded regions were sequenced including the hatched areas that correspond to the encoding sequences of the tRNA[Ser]Sec genes as shown in Fig. 2. Arrows in the hatched boxes indicate the transcription orientation. X designates *Xba*I, P, *Pst*I and A, *Acc*I. The *Pst*I fragment of ZStR1 was subcloned and sequenced. The sequence of an additional 171 bp upstream of the *Pst*I site within ZStR1 was obtained by subcloning a 1 kb *Acc*I fragment of recombinant DNA that was cloned in λ near the 3′-terminus of the tRNA[Ser]Sec gene and contained an *Acc*I site inside the vector DNA. The 2.1 kb *Xba*I fragment of ZStR2 was subcloned and 1031 bp sequenced.

studies were carried out by the procedure of Nirenberg and Leder [15] as described [14].

3. Results

3.1. Restriction analysis of recombinant and genomic DNAs

The genomic library of zebrafish was screened and six positive clones were isolated. Digestion of each with endonucleases showed that they fell into two classes, designated ZStR1 and ZStR2 (see restriction map in Fig. 1). ZStR1, ZStR2 and zebrafish genomic DNA were digested with *Xba*I and with *Pst*I and the resulting fragments analyzed by Southern blotting (see Fig. 2). Genomic DNA yielded two fragments in both digests as shown in lanes 4 and 5. One fragment in each digest corresponded to those generated from ZStR1 (lanes 2 and 6), while the other corresponded to those generated from ZStR2 (lanes 3 and 7). Thus, ZStR1 and ZStR2 are located at different loci within the zebrafish genome.

3.2. Sequencing of recombinant DNAs

A total of 1031 bp in ZStR1 and 1006 bp in ZStR2 were sequenced and aligned with the corresponding tRNA[Ser]Sec gene and flanking regions in *Xenopus* as shown in Fig. 3. The encoding sequences of the two zebrafish genes are identical and differ by 5 bp from the tRNA[Ser]Sec gene in *Xenopus*. The flanking sequences of both zebrafish genes are highly homologous (97.5% in the 5′-flank and 93.6% in the 3′-flank). The upstream sequence encodes three regulatory regions, a TATA box at −30, a proximal sequence element (PSE) at −66 and an activator element (AE) at −205. These regulatory elements correspond to similar elements in *Xenopus* (see Fig. 3) that govern expression of the tRNA[Ser]Sec gene [8].

3.3. Genomic mapping of the zebrafish Sec tRNA genes

Two pairs of specific primers were used in the PCR analysis of the radiation hybrid panel for amplifying (specifically) the two ZStR genes (see Fig. 3 and figure legend). In preliminary experiments, we did not observe any cross-amplification of these two genes (data not shown). All PCR experiments were done in duplicate and the results were confirmed by using the universal primers (see Fig. 3). Retention patterns (ZStR1: 0000000000 0001000100 0000000000 0100010000 0000000100 0101100000 0000000100 1101000000 0000010000 000110 and ZStR2: 0000000100 0000001001 1100001000 0010010000 0100000000 0000010100 0110000001 1000000000 0000000000 000010) were compared with 1383 sequence-tagged site markers. Unfortunately, ZStR1 and ZStR2 did not give any significant linkage to other markers which may mean that they are located within a gap of the current map.

3.4. Identification and codon recognition properties of the gene products

Total tRNA from zebrafish was fractionated on a RPC-5

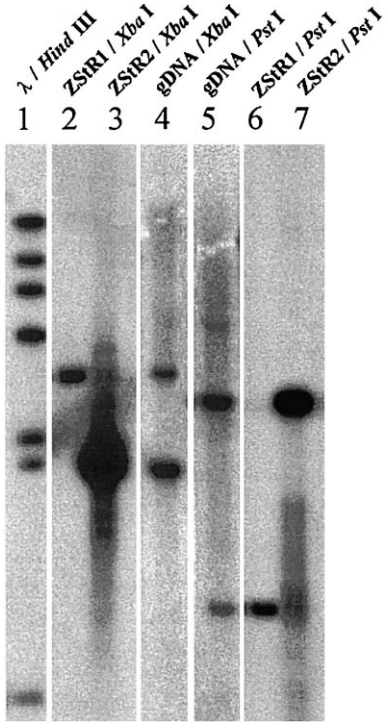

Fig. 2. Hybridization of zebrafish genomic and recombinant DNAs. Genomic DNA or recombinant DNA was digested, electrophoresed on an agarose gel, transblotted to a filter and hybridized with a 193 bp fragment of human DNA encoding the tRNA[Ser]Sec gene (see Section 2). Lanes 2 and 6 contain ZStR1, lanes 3 and 7 contain ZStR2 and lanes 4 and 5 contain genomic DNA (designated gDNA) digested with *Xba*I and *Pst*I, respectively. Lane 1 contains labelled DNA markers digested with *Hin*dIII.

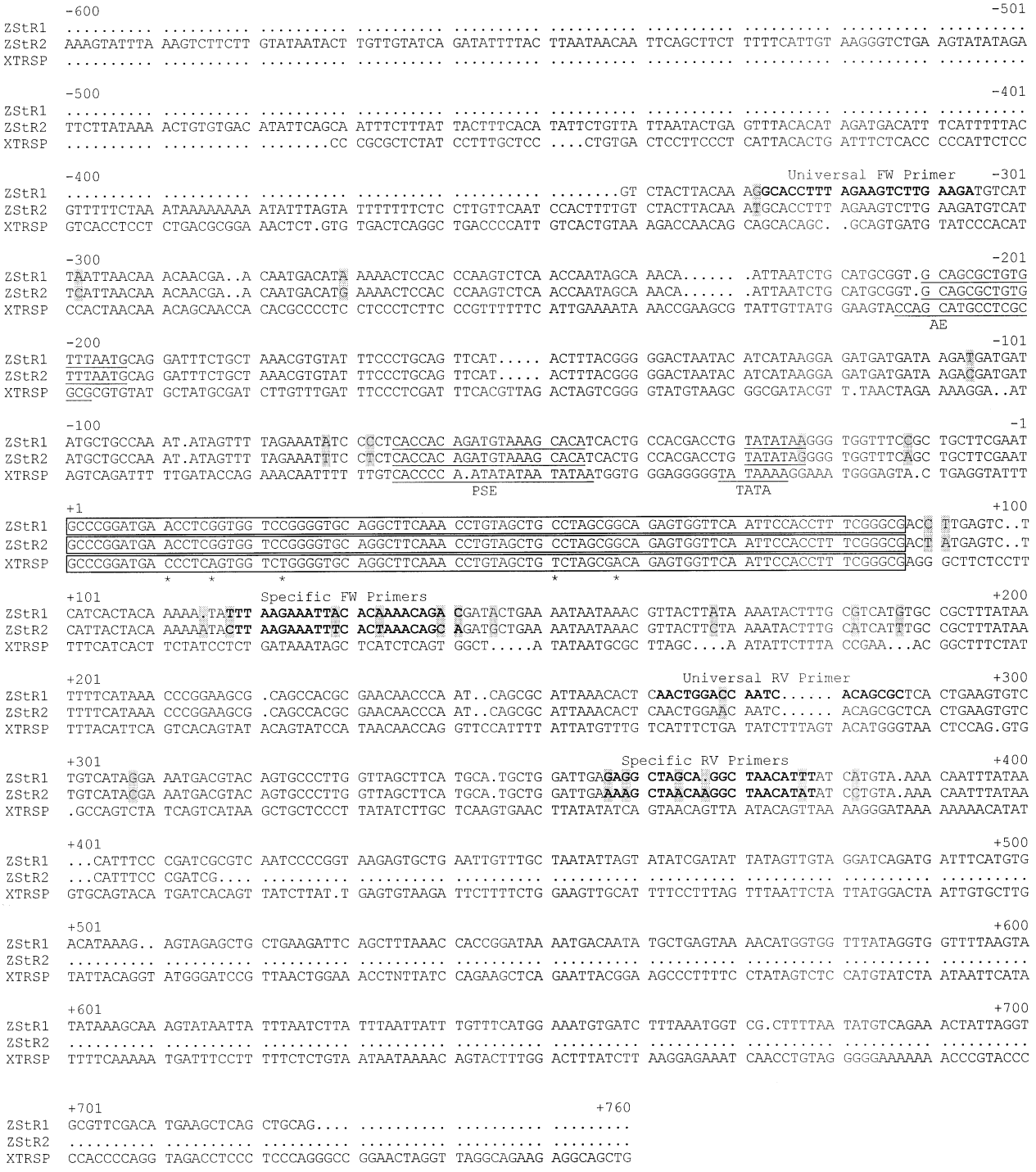

Fig. 3. Alignment of zebrafish recombinant DNA sequences and a *Xenopus* DNA sequence encoding the tRNA$^{[Ser]Sec}$ gene. The encoding sequences of the tRNA$^{[Ser]Sec}$ genes of zebrafish and *Xenopus* (boxed region) and the flanking sequences of ZStR1, ZStR2 and *Xenopus* are shown. The sequences from *Xenopus* were taken from Lee et al. [19]. Base differences within the encoding sequences are indicated with an asterisk. TATA boxes at −30 in ZStR1 and ZStR2 and at −31 in XtRSP, the PSE at −66 in ZStR1 and ZStR2 and at −64 in XStR and the AE at −205 in ZStR1 and ZStR2 and at −209 in XStR are underlined. Base differences in the flanking sequences of ZStR1 and ZStR2 are shaded. To maximize homology between sequences, dots were used to indicate gaps with the exception of the 3′- and 5′-ends of sequences where dots indicate unidentified bases. Specific and universal primers used for PCR analysis of the radiation hybrid panel are shown in bold. GenBank accession numbers for ZStR1 and ZStR2 are AF135236 and AF135237, respectively.

column and Sec tRNA$^{[Ser]Sec}$ identified in the eluted fractions by Northern hybridization (see Fig. 4A). Two peaks were observed that were pooled as shown in the figure, amino-acylated with [^{3}H]serine and refractionated over the column (Fig. 4B and C, respectively). Most of the serine tRNA^{Ser} eluted with the earlier eluting peak of tRNA$^{[Ser]Sec}$. Hence, the amount of [^{3}H]seryl-tRNA$^{[Ser]Sec}$ relative to [^{3}H]seryl-tRNA^{Ser} is much less in the earlier eluting Sec tRNA$^{[Ser]Sec}$ isoacceptor than the latter one. Both seryl-tRNA$^{[Ser]Sec}$ isoacceptors decode UGA in a ribosomal binding assay.

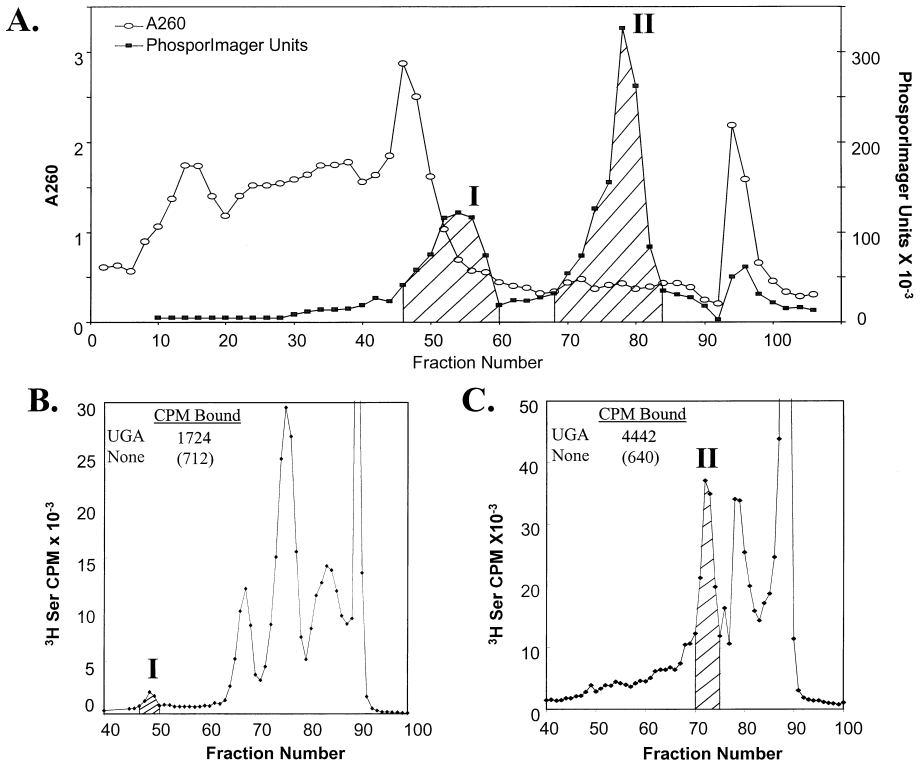

Fig. 4. Fractionation and coding responses of Sec tRNA$^{[Ser]Sec}$. In A, total tRNA was loaded onto a RPC-5 column, the column was washed and tRNA fractionated in a linear 0.50–0.70 M NaCl gradient, aliquots of every other fraction were blotted on a filter and the filter was hybridized with probe (see Section 2). Fractions were pooled as shown by the hatched areas in A, amino-acylated with [^{3}H]serine and the resulting [^{3}H]seryl-tRNAs were fractionated on the column in a linear 0.6–0.825 M NaCl gradient without Mg^{2+} (see Section 2). In B, peak I from graph A is shown and in C, peak II from graph A is shown. The hatched peak in B and that in C were pooled, prepared for encoding studies and encoding studies were carried out as given in Section 2. The total number of cpm added to assays shown in B was 4500 and that shown in C was 8430. None designates the amount of binding of [^{3}H]seryl-tRNA$^{[Ser]Sec}$ to ribosomes in the absence of trinucleoside diphosphate. Codon UGA was a gift of M.W. Nirenberg.

4. Discussion

Zebrafish contain two distinct tRNA$^{[Ser]Sec}$ genes. Both have a TATA box, a PSE and an AE in the 5′-flanking region which are the three regulatory elements [16] that govern expression of all other eukaryotic Sec tRNA$^{[Ser]Sec}$ genes examined to date [8]. Both genes are transcribed following their microinjection into *Xenopus* oocytes, but the level of transcription was quite low (data not shown) and comparable to that observed for the chicken tRNA$^{[Ser]Sec}$ gene in this transcription system [17]. The genes map at different loci within the zebrafish genome, but at present, we cannot determine whether they are localized on the same or different chromosomes.

Two Sec tRNAs$^{[Ser]Sec}$ were found in the total tRNA population. The two isoacceptors most certainly differ by base modification as the primary transcripts of both genes would have identical sequences. The isoacceptor that elutes first from the RPC-5 column may likely lack a N^6-isopentenyladenosine (i^6A) modification at position 37. This modification is characteristic for Sec tRNAs$^{[Ser]Sec}$ isolated from other animals [8] and the absence of i^6A causes tRNA$^{[Ser]Sec}$ to elute much earlier from the RPC-5 column [18] as was observed in the elution of the initial peak of zebrafish tRNA$^{[Ser]Sec}$ (see Fig. 4A).

The present studies demonstrate for the first time that the Sec tRNA$^{[Ser]Sec}$ gene occurs in two copies in the genome of an animal even though gene duplication appears to be a frequent occurrence in zebrafish [9–11]. The present study provides an initial characterization of the machinery involved in the selenoprotein biosynthesis in zebrafish and provides a foundation on which to expand the use of this organism as a model for exploring mechanisms of selenium-mediated chemoprevention in cancer.

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