The Distal-Sequence Element of the Selenocysteine tRNA Gene Is a Tissue-Dependent Enhancer Essential for Mouse Embryogenesis

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Received 23 August 2004/Returned for modification 20 September 2004/Accepted 28 January 2005

Appropriate expression of the selenocysteine tRNA (tRNA\textsuperscript{Sec}) gene is necessary for the production of an entire family of selenoprotein enzymes. This study investigates the consequence of disrupting an upstream enhancer region of the mouse tRNA\textsuperscript{Sec} gene (Trsp) known as the distal sequence element (DSE) by use of a conditional repair gene targeting strategy, in which a 3.2-kb insertion was introduced into the promoter of the gene. In the absence of DSE activity, homozygous mice failed to develop in utero beyond embryonic day 7.5 and had severely decreased levels of selenoprotein transcript. CRE-mediated removal of the selection cassette recovered DSE regulation of Trsp, restoring wild-type levels of tRNA\textsuperscript{Sec} expression and allowing the generation of viable rescued mice. Further analysis of targeted heterozygous adult mice revealed that the enhancer activity of the DSE is tissue dependent since, in contrast to liver, heart does not require the DSE for normal expression of Trsp. Similarly, in mouse cell lines we showed that the DSE functions as a cell-line-specific inducible element of tRNA\textsuperscript{Sec}. Together, our data demonstrate that the DSE is a tissue-dependent regulatory element of tRNA\textsuperscript{Sec} expression and that its activity is vital for sufficient tRNA\textsuperscript{Sec} production during mouse embryogenesis.

The tRNA molecule tRNA\textsuperscript{Sec} is the site of synthesis and carrier for the amino acid selenocysteine. With the aid of ancillary factors, tRNA\textsuperscript{Sec} decodes a UGA triplet, normally read as a stop codon (8), in the transcript of enzymes collectively known as selenoproteins. In humans, 25 individual selenoproteins are known (25), with comparable numbers existing in other metazoans (26). Among the best characterized are the glutathione peroxidases (GPX), selenoprotein P (SelP), thioredoxin reductases (TR) and iodothyronine deiodinas (DIO). Selenocysteine is found in the active site of these enzymes and is required for their catalytic activity (11). As such, the gene encoding the tRNA\textsuperscript{Sec} molecule (Trsp), a single-copy gene in mice, humans, chickens, and bovines, is central to the production and activity of the entire selenoprotein family.

The Trsp gene, like all tRNA genes, is transcribed by RNA polymerase III. In general, tRNA gene transcription requires two promoter elements situated within their transcribed region, termed A and B boxes. In contrast, transcription of the Trsp gene relies on an internal B box and three upstream elements, a TATA motif, a proximal sequence element (PSE) and a distal sequence element (DSE) (5). Interestingly, the upstream promoter arrangement of the Trsp gene is shared with many small nuclear RNA and small nuclear RNA-type gene promoters (14).

Mutational analysis of the activator elements within the DSE region of the Trsp gene of Xenopus laevis identified a motif responsible for enhanced transcription called the SpH postcotamer homology (SPH) element (34). The protein factor that recognizes the SPH element was first characterized in Xenopus (41) and was termed selenocysteine tRNA gene transcription activating factor (Staf). In mouse, a single Staf protein has been found with similar DNA-binding specificity (1), whereas in human, two Staf-like proteins have been identified, an ortholog of Xenopus Staf referred to as ZNF143 and a related protein ZNF73 (33).

Herein we describe a conditional gene repair (CR) strategy in which the DSE is physically separated from the regulation of Trsp expression. Loss of DSE regulation over Trsp terminates mouse development beyond embryonic day 7.5 (E7.5), coupled to a substantial, although differential decrease in the transcript level of certain members of the selenoprotein family. From the analysis of targeted animals and cell based assays we define the DSE as a tissue-dependent activating element of Trsp expression that is essential for adequate tRNA\textsuperscript{Sec} production during mouse embryogenesis.

MATERIALS AND METHODS

Tissue culture. Cell lines NIH 3T3, C3H/10T1/2, Swiss-3T3, and Hepa-1c1c7 were grown on six-well plates, and P815 was grown in T75 flasks in high-glucose (4.5 g/liter) Dulbecco modified Eagle medium containing 4 mM Glutaxam-I, 3.7 g of sodium bicarbonate/liter, 1 mM sodium pyruvate, penicillin-streptomycin (10 U/0.1 mg/ml), and 10% fetal bovine serum. The suspension lines MH-S, L1210, and L5178 were grown in T75 flasks and a high-glucose RPMI medium containing 2 mM l-glutamine, 2 g of sodium bicarbonate/liter, 10 mM HEPES, 1 mM sodium pyruvate, penicillin-streptomycin (10 U/0.1 mg/ml), and 10% fetal bovine serum. MH-S required the addition of 0.05 mM (final) β-mercaptoethanol to the medium. All cells were maintained in an atmosphere of 5% CO\textsubscript{2} at 37°C.

Cell line transfection and primer extension assay. NIH 3T3 and Swiss-3T3 cell lines were grown in six-well dishes to a confluency of 60%. A total of 2 ng each of Trp reporter plasmid (Fig. 1, lower panel) and Clontech pEGFP-N1 mammalian vector were transfected by using jetPEI solution (Frunakoshi). Expression of tRNA\textsuperscript{Sec} was evaluated as previously described (31) by using a primer complementary to bases 12 through 32 of the Trsp gene. A nucleotide exchange at position 9 in the Trsp transfection construct was used to distinguish the tRNA\textsuperscript{Sec}
Trsp (Fig. 1) was constructed from fragments of the 129/Sv genomic vector. The 699 to 719 mRNA product of the Clontech pd2EGFP-N1 molecule is indicated by asterisks. Primer extension generates a 6-bp extension product due to a T-to-C mutation in the trNA\textsuperscript{sec} molecule arising from the transfected constructs (lanes 2 and 3, respectively), DNA from adult wild type (WT) and enriched EGFP transcript generates a 6-bp extended product. The structure of the reporter constructs and the sequence of the wild-type or mutant SPH elements are shown.

Targeting vector construction and gene targeting. The targeting vector (Fig. 2A) was constructed from fragments of the 129/Sv genomic Trsp clone described previously (3). A pMC1/neomycin-pMC1/tdimerase kinase cassette flanked by loxP elements was inserted into the Bst1107I site of the Trsp gene, yielding an upstream SalI-Bst1107I (1.2-kbp) and downstream Bst1107I-PvuII (6-kbp) fragment, respectively. Finally, a diphtheria toxin (DT3) cassette was inserted downstream of the long-arm homology region. Targeting vector was linearized with PacI before electroporation into embryonic stem (ES) cells.

Homologous recombinant candidates were screened by PCR for 35 cycles (5 s at 94°C, 30 s at 65°C, and 30 s at 72°C). Wild-type and targeted alleles were distinguished by using CreF and CreR primers, whereas the neomycin phosphotransferase gene was detected by using the Neo5-Prime and Neo3-Prime pair (Table 1), respectively. The presence of Cre recombinase was determined by using CreF and CreR primers, whereas the neomycin phosphotransferase gene was detected by using the Neo5-Prime and Neo3-Prime pair (Table 1).

Embryo whole-mount analyses. Decidual swellings dissected at E7.5 were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at 4°C. Embryos were washed twice in PBS for 5 min each at room temperature and dissected in PBS.

\textbf{R}N\textbf{a}se protection assay. The plasmid pBS\textsuperscript{sec}RNP-probe, consisting of the cruciate 129/Sv genomic Trsp fragment in pBluescript, was linearized by using the Bst1107I-Hpal Trsp fragment in pBluescript, was linearized by using the Bst1107I-Hpal and antisense RNA with a specific activity of 4.7 \times 10^7 \text{cpm}/\mu g produced by using the Ambion T7 Maxicist kit. An 18S antisense probe, with a specific activity of 1.7 \times 10^9 \text{cpm}/\mu g, was produced from the Ambion pTRI18S plasmid. Probes were gel purified and hybridized to 2.5 \mu g of total RNA over-
night at 52°C. RNAse digestions were allowed to occur for 25 min at room temperature by using a 1:300 dilution of RNAse A/T1 mix. The protected fragments were resolved on a 10% denaturing polyacrylamide gel and detected by using a Fuji Bioimage Analyzer BAS 2000.

cDNA cloning, recombinant GPX1 expression, and antibody production. Mouse Staf cDNA (Image 3661687) was purchased from Invitrogen. The cDNA for TR1 and DIO1 were amplified from the expressed sequence tag clones A0141567 and A0156288, respectively. cDNAs for GPX1, GPX4 and SelP were amplified by using a reverse transcription-PCR (RT-PCR) with total mouse liver RNA as the source of message. The primers and cDNA sequences used in their design are shown in Table 1. The amplified cDNAs were ligated into pcR-BluntII-TOPO vectors (Invitrogen). To allow recombinant protein production of GPX1, the internal sel-
nocysteinyl codon UGA had been exchanged to a cysteine UGU codon by using the primers GPXSDFM and GPXSDFMR (Table 1). The coding region for GPX1 was subcloned into the NdeI and XhoI sites of the pET15b vector. Recombinant GPX1 was purified from soluble bacterial extracts by nickel chelate chromatography as described previously (23). The polyhistidine tag was removed by thrombin, and the protein further purified by hydroxyapatite column chromatography. Antibodies were raised in New Zealand White rabbits by MBL, Japan. For Western blotting, antisera to GPX1 were used at a 1:2,000 dilution.

RT-PCR analysis. Decidua were removed from 7.5-day-pregnant female mice, and maternal and extra-embryonic tissues were dissected from the embryo proper. Total RNA was reverse transcribed by using random hexamers, and the selenoprotein cDNA sequences were amplified by the primer pairs listed in Table 1. Real-time RT-PCR was performed to quantify GPX1, heme oxygenase 1 (HO-1), glutathione-S transferase (GST) A4, and NAD(P)H quinone oxidoreductase 1 (NQO1) mRNA levels by using an Applied Biosystems 7700 sequence detector.

RNA blotting analyses. Total RNA was size fractionated, transferred to nylon membranes, and probed by using radioactively labeled cDNA for Staf, GPX1, GPX4, TR1, SelP, and DIO1 retrieved from the pCR-BluntII-TOPO vectors described above. As a loading control the membrane was stained with methylene blue to visualize the 18S and 28S ribosomal bands.

Electrophoretic mobility shift assay. Adherent cell lines and mixed adherent or suspension cell lines were cultured on six-well plates, whereas suspension cell lines were grown in T75 flasks. Nuclei were prepared by using the Pierce NE-

RESULTS

The DSE and SPH motif make a major contributor to mouse Trsp gene expression. The contribution of the DSE to tRNA^Sec

Table 1. Primers used for mouse genotyping, cDNA cloning, and RT-PCR analyses

<table>
<thead>
<tr>
<th>Type of analysis and primer or selenoprotein name</th>
<th>Sequence (orientation)</th>
</tr>
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<tbody>
<tr>
<td>Genotype analysis of mice</td>
<td></td>
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<tr>
<td>KDFP1</td>
<td>5'-GTTCGAGATTGATGCTCTGACCTT-3' (sense)</td>
</tr>
<tr>
<td>KDRP1</td>
<td>5'-GGACTAGTGAATTGCCTCATAC-3' (sense)</td>
</tr>
<tr>
<td>SeCCDKCRFP1</td>
<td>5'-ACTCTGTCGCTAAACAGCTCA-3' (antisense)</td>
</tr>
<tr>
<td>CreF</td>
<td>5'-CTGATTACCGGTCGATGCA-3' (antisense)</td>
</tr>
<tr>
<td>CreR</td>
<td>5'-AGTTACCGGTCGACAACTGATA-3' (antisense)</td>
</tr>
<tr>
<td>Neo5-Prime</td>
<td>5'-AGTTATCCATCTAGGCTATGC-3'</td>
</tr>
<tr>
<td>Neo3-Prime</td>
<td>5'-TAGCCCAACGCTTGTCCCTGTA-3'</td>
</tr>
<tr>
<td>cDNA cloning and site-directed mutagenesis</td>
<td></td>
</tr>
<tr>
<td>GPX1</td>
<td>5'-TCACATCGAGATCAGATCTGGC-3' (sense)</td>
</tr>
<tr>
<td>GPX4</td>
<td>5'-ACACTCTGTAGTCATGCTCATG-3' (sense)</td>
</tr>
<tr>
<td>SelP</td>
<td>5'-ACATTGTAGGCAAGGATCCAGGT-3' (antisense)</td>
</tr>
<tr>
<td>DIO1</td>
<td>5'-TGAAGGCTACTGCTGCAAGATGC-3' (antisense)</td>
</tr>
<tr>
<td>TR1</td>
<td>5'-ACTCTGTGGTCTGCGTCTTCC-3' (sense)</td>
</tr>
<tr>
<td>GPX1SDMF</td>
<td>5'-CGGCGAGATGAAGCTCTGAC-3' (antisense)</td>
</tr>
<tr>
<td>GPX1SDMR</td>
<td>5'-TCACATCGAGATCAGATCTGGC-3' (sense)</td>
</tr>
<tr>
<td>RT-PCR analyses a</td>
<td></td>
</tr>
<tr>
<td>GPX1</td>
<td>5'-AGCCTTTCCTCACCATCCTATCCCGAC-3' (antisense)</td>
</tr>
<tr>
<td>GPX4</td>
<td>5'-ACACTCACTGATGCTCATGCTCATG-3' (sense)</td>
</tr>
<tr>
<td>SelP</td>
<td>5'-CCTGTAGTGAAGGATCCAGGT-3' (antisense)</td>
</tr>
<tr>
<td>DIO1</td>
<td>5'-CTCTATGCCTTCCCGTCTGATG-3' (antisense)</td>
</tr>
<tr>
<td>DIO2</td>
<td>5'-CCGTGGAAGGACAACTACATC-3' (antisense)</td>
</tr>
<tr>
<td>HPRT</td>
<td>5'-GGCAAGGACAAGGCGAGATG-3' (antisense)</td>
</tr>
<tr>
<td>a Selenoproteins.</td>
<td></td>
</tr>
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</table>

*3P-labeled target DNA (2 × 10^5 cpm). Complexes were formed in 10 mM HEPES-NaOH (pH 7.5), 5 mM MgCl_2, 1 mM dithiothreitol, 5 mM KCl, 20 mM ZnCl_2, 5% glycerol, and 0.1% NP-40 as described previously (39); dissolved in loading dye, 25 mM Tris-HCl (pH 7.5), 0.2% bromphenol blue, 0.2% xylene cyanol, and 4% glycerol; and resolved on a 5% polyacrylamide gel (acrylamide-bisacrylamide [37:5:1]) in low-ionic-strength gel buffer, 7 mM Tris-HCl (pH 7.5 at room temperature), 3 mM sodium acetate, and 1 mM EDTA.

RESULTS

The DSE and SPH motif make a major contributor to mouse Trsp gene expression. The contribution of the DSE to tRNA^Sec
expression was first evaluated in a study using mouse cell lines. A plasmid carrying the tRNA^Sec gene was introduced into NIH 3T3 mouse embryonic fibroblasts (Fig. 1) and Swiss-3T3 fibroblasts (data not shown) by transfection, and the effect of deleting the DSE region and deleting and mutating the SP1 motif on tRNA^Sec production was examined by primer extension. The tRNA^Sec transcript from the endogenous Trsp gene may be readily distinguished from the tRNA^Sec transcript arising from the transfected Trsp plasmid, since the former generates a 3-bp product and the latter generates a 6-bp extension product, respectively.

As expected, RNA isolated from nontransfected NIH 3T3 cells yielded a 3-bp extension product from endogenous tRNA^Sec molecules, whereas the primer to EGFP did not yield a product (Fig. 1, lane 1). A comparison of tRNA^Sec expression levels arising from a plasmid carrying the entire Trsp wild-type promoter (Fig. 1, lanes 2 to 4) and a basal promoter construct (Fig. 1, lanes 5 to 7), in which all sequences upstream of the promoter (Fig. 1, lanes 2 to 4) and a basal promoter construct (Fig. 1, lanes 5, 7, and 9) are transfected into NIH 3T3 cells. Similar results were obtained from studies in Swiss-3T3 fibroblasts (data not shown).

Having demonstrated the important contribution that the DSE in vivo was achieved by inserting a floxed neomycin-thymidine kinase (positive-negative selection [PNS]) cassette between the DSE and PSE, thereby physically separating the DSE from the transcribed region of the tRNASec gene by a 3.2-kbp insertion (Fig. 2A). This technique offers the advantage that specific expression of Cre recombinase can restore tRNA^Sec levels to normal by conditional excision of the PNS cassette (below).

Correct homologous recombination in mouse ES cells was demonstrated in two independent cell clones by PCR and Southern blotting analysis with a 5′ internal and 3′ external probe (Fig. 2B, lanes 2 and 3). Chimeric mice were bred onto C57BL/6, ICR, and C57BL/6/DBA mixed backgrounds to obtain heterozygous conditional rescue (Trsp^CR/CR) animals. These mice were also analyzed by Southern blotting to confirm correct transmission of the PNS cassette (Fig. 2B, lanes 5, 7, and 9).

Homozygosity for the CR locus is embryonic lethal. An initial intercross of Trsp^+/CR mice on a C57BL/6 background gave 32 progeny, 11 of which were wild type, and the remaining 21 heterozygous (Table 2). Obtaining heterozygous and wild-type progeny at a ratio of ca. 2:1, and the absence of homozygous mutants suggested homozygosity for the CR locus (Trsp^CR/CR) was embryonic lethal. This result was reproducible when we intercrossed Trsp^−/CR mice on an ICR background (results not shown).

Analysis of embryos obtained from a Trsp^+/CR intercross showed homozygous embryos could be recovered as late as E9.5 (Table 2). However, we observed that homozygous targeted embryos at E8.5 and at E9.5 had already undergone considerable resorption. The presence of Trsp^CR/CR embryos at E8.5 is significant, since Trsp-null embryos die much earlier in development at the peri-implantation stage, at approximately E4.5 (4). These results indicate a milder lethal phenotype for the Trsp^CR/CR mice relative to Trsp-null mice.

The morphology of implanted embryos at E7.5 was observed by using whole-mount analyses. All embryos that displayed a normal growth rate were wild type or heterozygous, and no discernible differences were observed between these genotypes (Fig. 3A). In contrast, Trsp^CR/CR embryos were smaller and underdeveloped (Fig. 3A). The homozygous embryos failed to compartmentalize, although the Riechart membrane appeared to have developed at near normal rates (Fig. 3B, right-hand side). Repeated efforts were made to quantify the abundance of tRNA^Sec in homozygous embryos but were unsuccessful most probably due to the low level of transcript. The failure of Trsp^CR/CR embryos to develop normally is likely due to a decrease or complete loss of certain selenoprotein species, secondary to the limited supply of tRNA^Sec. Based on previous reports, failure to supply adequate tRNA^Sec for UGA decoding could be expected to increase the susceptibility of selenoprotein transcript to degradation by nonsense mediated decay (NMD) (9).

Analysis of selenoprotein and antioxidant gene transcript levels in Trsp^+/+, Trsp^+/CR and Trsp^CR/CR embryos at E7.5. The abundance of several selenoprotein transcripts in wild-type, heterozygous, and homozygous mutant embryos at E7.5 was examined to assess the consequence of limiting amounts of tRNA^Sec (Fig. 4). We examined the mRNAs of GPX1, GPX4, TR1, SelP, and the thyroid hormone metabolizing enzymes DIO1 and DIO2. With hypoxanthine-guanine-phosphoribosyltransferase (HPRT) as a comparative control (Fig. 4A), it was found that a 20-fold greater quantity of total RNA from Trsp^CR/CR embryos was required to equal RNA levels extracted from Trsp^+/+ and Trsp^+/CR embryos. We observed that the mRNA encoding each selenoprotein did not differ in abundance between heterozygous mutant and wild-type embryos (lanes 3 and 4). In contrast, in Trsp^CR/CR embryos the message of all selenoproteins examined, with the exception of DIO2, were markedly diminished compared to wild-type and heterozygous mutants (compare lane 5 to lanes 3 and 4). This decrease is most likely caused by NMD.

It is apparent that variability exists in the relative abundance of the selenoproteins message. The levels of SelP (Fig. 4B) and DIO1 (Fig. 4C) are greatly affected in Trsp^CR/CR mice, whereas significant levels of TR1 (Fig. 4D) and GPX1 (Fig. 4E) tran-

### Table 2. Genotype analysis of progeny derived from Trsp^+/CR intercross

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>No. of progeny</th>
<th>Resorption</th>
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<tbody>
<tr>
<td>3 wk</td>
<td>+/+</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>+/CR</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CR/CR</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>E16.5</td>
<td>+/+</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>+/CR</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CR/CR</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>E15.5</td>
<td>+/+</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+/CR</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CR/CR</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>E9.5</td>
<td>+/+</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>+/CR</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>E8.5</td>
<td>+/+</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>+/CR</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>
GPX4 mRNA species could not be detected in total RNA from E7.5 embryos (Fig. 4F, lanes 3 to 5). This may be explained by the fact that GPX4 is localized to ectoderm and yolk sac, as determined by using anti-GPX4 antibodies (17), and in the present analyses the embryonic yolk sac has been removed. It is also noteworthy that transcript levels of DIO2 appear to be unaffected in homozygous mutants (Fig. 4G). These results point to selective stability of certain of the selenoprotein mRNA species under limiting tRNA^Sec conditions.

Many of the selenoprotein enzymes are known to have an antioxidant function, including TR (37), GPX and SelP (43), selenoprotein W (22), and selenoprotein R (24). As such, a decrease or loss in their activity would be expected to lead to increased oxidative stress. Recently, it has been shown that conditional removal of the Trsp gene from mouse liver resulted in the induction of a GST Mu family member (6) which, it is thought, may act to counterbalance the loss of certain of the selenoprotein activities. Therefore, we examined the expression of a number of stress response enzymes in E7.5 embryos by using real-time RT-PCR (Fig. 5). The levels of GPX1 transcript in homozygous embryos was found to be 38% that of wild type (Fig. 5A) in agreement with the earlier RT-PCR analysis (Fig. 4E). The mRNA corresponding to NQO1, the glutathione-synthesizing enzyme γ-glutamylcysteine synthase (data not shown) and GSTA4, were found not to increase in

FIG. 3. Analysis of embryos generated from a Trsp^+/CR intercross. (A) E7.5 embryos were fixed briefly in formaldehyde before removal of maternal and extra-embryonic tissues. The embryos were photographed, and genomic DNA was extracted. PCR genotyping was performed with the primer pair SeCKDCRFP1 and SeCKDCRP1, yielding a 540-bp product from the wild-type allele and SeCKDCRFP1 and KDRP1, generating a 420-bp product from the CR targeted allele. (B) High-resolution photograph of wild-type (left) and homozygous mutant E7.5 embryo (right). Bar, 100 μm.
Trsp<sup>+/−</sup>, Trsp<sup>+</sup>/CR<sup>−</sup>, and Trsp<sup>CR+/−</sup> embryos. E7.5 embryos were dissected from maternal and extra-embryonic tissues, and total RNA was extracted. RNA from three embryos of each genotype was pooled and reverse transcribed. The HPRT PCR product was used as a loading control to equalize RNA input levels in each of the RTPCR reactions. A 20-fold-greater quantity of total RNA from Trsp<sup>CR+/−</sup> embryos was required to equalize that of Trsp<sup>+/−</sup> and Trsp<sup>−/−</sup> embryos. Primer pairs specific to each of the selenoprotein species were used to amplify the RNA message of the indicated proteins. cDNA for each of the selenoproteins was used as a positive control (Ctl) in the PCRs. DNA size markers (M) were φX174 DNA digested with HaeIII. The molecular size (in base pairs) of each PCR product is presented on the left-hand side of the figure.

FIG. 4. RT-PCR analysis of selenoprotein transcript levels in Trsp<sup>+/−</sup>, Trsp<sup>+</sup>/CR<sup>−</sup>, and Trsp<sup>CR+/−</sup> embryos. E7.5 embryos were dissected from maternal and extra-embryonic tissues, and total RNA was extracted. RNA from three embryos of each genotype was pooled and reverse transcribed. The HPRT PCR product was used as a loading control to equalize RNA input levels in each of the RTPCR reactions. A 20-fold-greater quantity of total RNA from Trsp<sup>CR+/−</sup> embryos was required to equalize that of Trsp<sup>+/−</sup> and Trsp<sup>−/−</sup> embryos. Primer pairs specific to each of the selenoprotein species were used to amplify the RNA message of the indicated proteins. cDNA for each of the selenoproteins was used as a positive control (Ctl) in the PCRs. DNA size markers (M) were φX174 DNA digested with HaeIII. The molecular size (in base pairs) of each PCR product is presented on the left-hand side of the figure.

Trsp<sup>+/−</sup> and Trsp<sup>CR+/−</sup> embryos relative to wild-type Trsp<sup>+/+</sup> embryos (Fig. 5B). Interestingly, in homozygous embryos the transcript levels of the heme-metabolizing enzyme HO-1 were induced 11- and 6.3-fold, respectively, compared to heterozygous and wild-type embryos (Fig. 5C).

A diverse number of stress stimuli are known to induce HO-1 expression by increasing the cellular production of reactive oxygen species, including heme, UV light, hydrogen peroxide, and chemicals that deplete glutathione (18). Recently, the transcription factor known as Nrf2 (nuclear factor-erythroid 2 p45-related factor 2) has emerged as a key player in HO-1 induction by oxidative stress (19, 21). Therefore, we examined the expression of Nrf2 and found that, in contrast to macrophage (Fig. 5D, lane 1), Nrf2 was not expressed in E7.5 embryos (Fig. 5D, lane 2). This result suggests that a mechanism independent of Nrf2 is responsible for HO-1 induction in Trsp<sup>CR+/−</sup> mice.

Cre-mediated removal of the PNS cassette fully reestablished DSE regulation of Trsp. A key feature of the promoter targeting strategy described here is the ability to remove the PNS cassette by Cre-mediated recombination, leaving a single loxP element positioned between the DSE and PSE. Trsp<sup>−/−</sup> mice were crossed with a transgenic line expressing Cre recombinase under the direction of an AyuI promoter. It was possible to generate mice homozygous for the rescued allele Trsp<sup>R+/R</sup> and mice heterozygous for the rescued allele and the CR allele

FIG. 5. Expression of GPX1, GSTA4, HO-1, and Nrf2 in Trsp<sup>+/−</sup>, Trsp<sup>+/CR</sup>, and Trsp<sup>CR+/−</sup> embryos. The expression profiles of GPX1 (A), GSTA4 (B), and HO-1 (C) were analyzed by quantitative real-time RT-PCR with rRNA as the normalization control. (D) RT-PCR was used to examine the expression of Nrf2 and β-actin in E7.5 wild-type mice (lane 2), with peritoneal macrophage serving as the positive control (lane 1) and water as a negative control (lane 3).

Trsp<sup>CR+/+</sup>, as demonstrated both by PCR (Fig. 6A) and Southern blotting with a short-arm internal probe (Fig. 6B), a long-arm external probe (Fig. 6C), and a neomycin cassette probe (Fig. 6D). Trsp<sup>CR+/+</sup> mice were viable and fertile, suggesting that removal of the PNS cassette had restored DSE regulation of tRNA<sup>Sec</sup> expression.

By RNase protection assay (Fig. 7), we examined the levels of tRNA<sup>Sec</sup> transcript in wild-type, Trsp<sup>CR+/+</sup>, and Trsp<sup>CR−/−</sup> mice by using the 18S subunit as a comparative standard. It is apparent that disruption of the DSE activity has variable impact upon tRNA<sup>Sec</sup> expression in a tissue-dependent manner. Kidney tRNA<sup>Sec</sup> expression was the most severely affected in Trsp<sup>CR−/−</sup> mice, dropping to almost 50% that of wild-type mice. Brain and liver showed a 34 and 40% decrease in tRNA<sup>Sec</sup> relative to wild-type mice, respectively. However, in the heart tissue of Trsp<sup>CR−/−</sup> mice we found the surprising result that tRNA<sup>Sec</sup> levels did not differ substantially from wild-type mice. This indicates that in the heart DSE activity is not required for
FIG. 6. Generation of Trsp<sup>±/R</sup>, Trsp<sup>R/R</sup>, and Trsp<sup>R/CR</sup> mice. Mice carrying the Ayuul-Cre transgene were crossed with Trsp<sup>±/CR</sup> mice to generate heterozygous Trsp<sup>±/R</sup> animals. Subsequently, heterozygous rescue mice were intercrossed with Trsp<sup>±/R</sup> and Trsp<sup>±/CR</sup> mice to generate homozygous Trsp<sup>R/R</sup> and Trsp<sup>R/CR</sup> mice, respectively. (A) Genomic DNA was extracted from mouse tail samples and amplified by PCR. The primers used to detect the wild-type allele were ScCKDCRFP1 and ScCKDCRFP1, and the 5'-loxP site ScCKDCRFP1 and KDRP1, and for the neomycin phosphotransferase gene were Neo5-Prime and Neo3-Prime as described in Table 1. On the right-hand side of the panel the identity of each PCR product is indicated. After Cre-mediated recombination of the CR allele a single loxP element (50 bp, including flanking sequences) remains, positioned between the DSE and PSE. Therefore, the PCR product arising from the rescued allele is distinguishable from that of the wild-type allele due to its lower mobility. For Southern blot analysis mice that had been selected by PCR were sacrificed, and liver genomic DNA was recovered. (B and C) Genomic DNA was digested with RcaI (B) or digested simultaneously with RcaI and BamHI (a single site exits external to the loxP element) (C), and the transfer membrane was probed by using the Sspl-SwaI internal probe. (D) RcaI was used to digest the DNA, and the protected fragment was resolved by using an RNase A-RNase T1 mix, and the protected fragments were resolved on a 10% denaturing polyacrylamide gel. The migratory position of the protected fragment for tRNA<sub>Sec</sub> (91 bp) and the 18S subunit (80 bp) are indicated on the left-hand side of the figure. The relative values of the transcript signals (tRNA<sub>Sec</sub>/18S) were obtained by densitometric analyses. The values presented have been adjusted to account for a 25-fold-greater specific activity of the tRNA<sub>Sec</sub> probe relative to the 18S ribosomal probe.

FIG. 7. Quantification of tRNA<sub>Sec</sub> levels in adult Trsp<sup>±/+</sup>, Trsp<sup>±/CR</sup>, and Trsp<sup>R/CR</sup> mice by RNase protection assay. Antisense RNA to tRNA<sub>Sec</sub> (240 bp) and the 18S ribosomal subunit (130 bp) were prepared and hybridized overnight to 2.5 µg of total RNA from brains, livers, kidneys, and hearts. RNase digests were performed by using an RNase A-RNase T1 mix, and the protected fragments were resolved on a 10% denaturing polyacrylamide gel. The migratory position of the protected fragment for tRNA<sub>Sec</sub> (91 bp) and the 18S subunit (80 bp) are indicated on the left-hand side of the figure. The relative values of the transcript signals (tRNA<sub>Sec</sub>/18S) were obtained by densitometric analyses. The values presented have been adjusted to account for a 25-fold-greater specific activity of the tRNA<sub>Sec</sub> probe relative to the 18S ribosomal probe.

normal transcription of Trsp (see below). Further, it may be concluded that insertion of the floxed-PNS cassette does not affect the downstream basal promoter elements of the Trsp gene.

Removal of the PNS cassette, as can be observed in the homozygous rescued mice, restored the expression of tRNA<sub>Sec</sub> to normal in all tissues examined, showing that the single lox<sup>P</sup> element does not interfere with tRNA<sub>Sec</sub> expression. It also affirms that the effects observed in the Trsp<sup>CR+/+</sup> and Trsp<sup>CR/CR</sup> mice may be solely ascribed to a loss of DSE regulation of the Trsp gene.

Selenoprotein mRNA and GPX1 protein levels are not affected in Trsp<sup>±/CR</sup> mice. RNA blotting analyses for the expression of selenoprotein transcript (Fig. 8A) in wild-type, Trsp<sup>CR+/+</sup>, and Trsp<sup>CR−/−</sup> adult mice show that, despite significant differences in tRNA<sub>Sec</sub> expression, Trsp<sup>CR−/−</sup> mice show no change in the mRNA level of all selenoprotein species examined in brain, liver, kidney, and heart. Antibodies generated to GPX1 were used to ascertain protein levels in the liver and kidney of wild-type, Trsp<sup>CR+/+</sup>, and Trsp<sup>CR−/−</sup> mice (Fig. 8B). Again, no difference in GPX1 protein levels were found, even in kidney, where an almost 50% decrease in tRNA<sub>Sec</sub> was observed. These results reflect the earlier results from RTPCR analysis of heterozygous Trsp<sup>CR+/+</sup> embryos showing that reduced tRNA<sub>Sec</sub> levels, resulting from disruption of the DSE, does not limit selenoprotein production.

Heart does not require the DSE for normal regulation of Trsp expression. The earlier RNase protection assay results (shown in Fig. 7) revealed that disruption of the DSE differentially affected tRNA<sub>Sec</sub> expression in a tissue-specific manner, having greatest impact in liver and kidney but little recognizable affect in heart. To further investigate the impact of inserting the PNS cassette into the Trsp gene promoter, we determined tRNA<sub>Sec</sub> levels (Fig. 9A) in adult heterozygous mice relative to the 18S subunit (Fig. 9B) by means of primer extension assay. The tRNA<sub>Sec</sub> levels in Trsp<sup>CR+/+</sup> mouse livers, hearts, and muscles were 75, 106, and 83% of wild-type mice, respectively. This is in contrast to heterozygous-null mice for the Trsp gene, where in the liver and heart tRNA<sub>Sec</sub> expression was decreased to 87 and 69% of wild-type mice, respectively (4). Thus, our results indicate the DSE is a major regulator of tRNA<sub>Sec</sub> expression in liver and muscle. However, in agree-
ment with the results obtained previously, the DSE activity is not necessary for normal expression of tRNA\textsuperscript{Sec} in heart. This raises the possibility that the DSE regulates the tRNA\textsuperscript{Sec} transcription tissue specifically. Given the difficulty of examining such a possibility in mice, we decided to analyze the function of the DSE in mouse cell lines.

### tRNA\textsuperscript{Sec} levels correlate with DSE binding activity in mouse cell lines

A panel of eight mouse cell lines—adherent (NIH 3T3, C3H/10T1/2, Swiss-3T3, and Hepa1c1c7), mixed adherent-suspension (P815 and MH-S), and suspension (L1210 and L5178)—were examined for tRNA\textsuperscript{Sec} expression levels by primer extension analysis (Fig. 10A). It was observed that the tRNA\textsuperscript{Sec} molecule is differentially expressed in cell lines. Intermediate levels were detected in the embryonic fibroblast lines NIH 3T3 and C3H/10T1/2 (Fig. 10A, lanes 1 and 2); the lowest levels were detected in the embryonic fibroblast line Swiss-3T3, the hepatoma cell line Hepa-1c1c7, and the mastocytoma line P815 (Fig. 10, lanes 3, 4, and 5); and the highest levels were observed in the alveolar macrophage line MH-S and the lymphoblastic lines L1210 and L5178 (Fig. 10A, lanes 6, 7, and 8).

The promoter targeting results from mice indicated that the DSE activity is vital for setting the basal transcription level of tRNA\textsuperscript{Sec} in a tissue-specific manner. Therefore, we performed electrophoretic mobility shift assay studies on nuclear extracts from each of the cell lines with a DSE probe, covering the region from positions −254 to −179 upstream of the tRNA\textsuperscript{Sec} transcription unit of the \textit{Trsp} gene. Depending on the cell line, several complexes were formed on the DSE fragment. In the control lane (Fig. 10B, lane 1), probe alone did not form a retarded band, whereas nuclear extract from NIH 3T3 generated two retarding complexes (Fig. 10B, lane 2). Both complexes were specifically competed for by the addition of a 30-fold excess unlabeled probe, whereas the addition of unlabeled probe harboring a 9-bp mutation in the SPH element only competed for the upper band (Fig. 10B, lanes 3 and 4). This demonstrated that the lower complex contains the Staf protein. The upper retarded complex (band 1) was observed previously in nuclear extracts from lactating mammary gland in mouse; however, its identity was not explored (1).

Interestingly, nuclear extracts from the Swiss-3T3 fibroblast and Hepa-1c1c7 hepatoma lines did not contain observable amounts of probe-binding activity (Fig. 10B, lanes 7 and 8). This correlates with the low levels of tRNA\textsuperscript{Sec} seen in these cell lines. Further, the cell lines MH-S, L1210, and L5178, with the highest expression of tRNA\textsuperscript{Sec} all contained the greatest abundance of probe-binding activity (Fig. 10B, lanes 10 to 12). An exception among the lines investigated was the P815 line, for although comparatively high binding activity for the DSE probe was observed (Fig. 10B, lane 9), the tRNA\textsuperscript{Sec} levels were the lowest of all lines examined (Fig. 10A, lane 5). Intriguingly, two previously unreported complexes were also found to bind to the DSE probe in the lymphoblastic lines, shown as bands 2 and 3 (Fig. 10B, lanes 11 and 12), the identity of which is unknown. Binding studies performed by using mutated cold probes demonstrated that the additional protein complexes had the ability to bind the DSE template independently of the Staf containing complex and, conversely, that Staf was capable of interacting with the probe in the absence of the additional factors (data not shown).

It is possible that the differential probe-binding activity of each cell line arises from the variable expression of the Staf transcription factor. Therefore, RNA blotting was performed to quantify Staf transcript abundance (Fig. 10C). Comparable levels of message were observed in all cell lines, suggesting that Staf transcript abundance alone could not account for the notable differences in probe-binding activity. Similarly, in mouse tissue samples the levels of Staf message did not correlate with tRNA\textsuperscript{Sec} expression (data not shown).
DISCUSSION

In this study we have examined the transcriptional regulation of the Trsp gene through an upstream promoter region, the DSE. We demonstrate, using cell lines and by mouse gene targeting, that the activity of the DSE is tissue specific with regard to tRNASec expression. In mice, the decrease in tRNASec abundance, resulting from DSE disruption, is shown to affect selenoprotein transcript levels and inhibit embryogenesis at the pregastrulation stage.

The DSE is a tissue-specific enhancer of tRNASec transcription. Early studies on the transcriptional regulation of the Xenopus tRNASec gene identified a 15-bp SPH element within the DSE region, which when mutated resulted in a 20-fold drop in transcriptional activity (5, 34). It was found that the SPH element functions independently of other transcription elements, including an octamer or Sph1 motif, elements commonly associated with the SPH of the snRNA and snRNA-type genes (34, 38).

Our initial transfection studies in NIH 3T3 cells resulted in a comparatively modest decrease (38%) in tRNASec expression upon deletion of the DSE. This could arguably result from differences in the relative contribution of the DSE to the expression of the mouse and Xenopus Trsp genes. However, our data suggest that the role of the DSE in Trsp transcription may be more complex. In animals, heterozygous disruption of the DSE resulted in a highly variable decrease in tRNASec levels between each of the tissues examined. For example, in the kidney of heterozygous animals tRNASec abundance dropped to 50% that of wild type, whereas in heart no decrease occurred. Therefore, we hypothesize that the regulation of tRNASec transcription through the DSE is tissue specific. We found that, in support of this conclusion, the abundance of tRNASec also varied greatly between eight different cell lines, and we could show that tRNASec levels, in all but one case, corresponded to the binding activity to the DSE.

Unlike Xenopus, the tRNA^Sec gene promoters of mice and humans both contain an octamer sequence immediately downstream of the SPH motif of sequence ATGCAAAT and ATGTAAAT, respectively. The results of our experiments suggest that the octamer motif makes a relatively small contribution, if any, to the regulation of the mouse Trsp gene. Specific mutation or deletion of the SPH motif alone gave an almost identical drop in tRNASec expression to complete removal of the DSE region. The relative importance of individual motifs within the DSE and the possibility that species differences exist in tRNASec gene regulation awaits further investigation.

Gene rescue experiments by Cre-induced recombination revealed that tRNASec expression could be restored to normal by removal of the PNS cassette, leaving a single loxP element inserted between the DSE and PSE. Clearly, this shift in the position of the DSE relative to the tRNA^Sec transcription unit fails to impact on transcription of the Trsp gene. This is in agreement with earlier studies on the Xenopus tRNA^Sec gene that showed the SPH motif can be flipped or moved upstream or downstream of its normal position and still retain activity (34).

DSE regulation of tRNA^Sec transcription is essential for mouse development. The targeting method in the present study describes for the first time the use of a CR strategy in the analysis of an RNA polymerase III transcribed gene. Disruption of the DSE by CR proved successful since Trsp^CR/CR mice were underdeveloped and smaller than their wild-type and heterozygous counterparts and died during development preceding gastrulation, at approximately E7.5. This finding is in contrast to Trsp-null mutant mice, which die significantly earlier, around the peri-implantation stage, at E4.5 (4). Thus, disruption of the DSE has a milder phenotype compared to deletion of the tRNA^Sec gene.

An entire protein family of selenoenzymes relies upon the tRNA^Sec molecule for expression; therefore, the embryonic lethality of Trsp-targeted mice could be ascribed to the loss any one or a combination of these proteins. Given that the Trsp^C^CR and Trsp^+/− mice die at unrelated stages of development, it is quite probable that a different selenoenzyme(s)
accounts for the lethality. As such, two distinct blocks to development occur in the absence or limited supply of tRNA\textsubscript{Sec}.

In the case of Trsp\textsuperscript{CR/CR} mice, the loss of TR activity could contribute to the block to early embryogenesis. TR, along with its thiol-protein substrate thioredoxin, are linked to several important antioxidant and redox regulatory systems within the cell including the synthesis of deoxyribonucleotides, the regulation of several transcription factors, and the activity of protein kinases (32). Significantly, similar to Trsp\textsuperscript{−/−} mice, knockout mice for the thioredoxin gene die at the peri-implantation stage (27). The conditional repair strategy described in the present study allowed the embryo to bypass this initial block to reveal a second block to development occurring pregastrulation.

To date, the genes encoding five members of the selenoprotein family have been disrupted in mice: GPX1 (7), GPX2 (10), GPX4 (17, 46), DIO2 (40), and SelP (16). Of these, only GPX4 knockout mice were embryonic lethal, dying in utero at the pregastrulation stage. Notably, this is similar to the Trsp\textsuperscript{CR/CR} mice. Indeed, both Trsp\textsuperscript{CR/CR} and Gpx4\textsuperscript{−/−} homozygous embryos are discernible by their lack of normal structural compartmentalization (46). Interestingly, RT-PCR analysis of GPX4 mRNA in wild-type mouse embryos suggests that GPX4 message is not expressed in the embryo proper. Previous immunohistochemical studies of E7.5 embryos with antibodies to GPX4 detected protein expression in the ectoderm and yolk sac (17). These results attest to the necessity of appropriate temporal and spatial synthesis of tRNA\textsubscript{Sec} in the early embryo to allow sufficient production of essential selenoenzymes.

Trsp\textsuperscript{CR/CR} mice show a differential decrease in selenoprotein transcript levels. An investigation of selenoprotein transcript levels in E7.5 embryos showed that in mice heterozygous for the CR allele, sufficient tRNA\textsubscript{Sec} levels were present to maintain normal production of selenoenzyme message. Similarly, in the brains, livers, kidneys, and hearts of adult Trsp\textsuperscript{−/−} mice no changes in the levels of RNA message for any of the selenoprotein species studied were observed, nor was there any change in GPX1 protein levels observed in adult livers and kidneys. This is in spite of drop of 50% in tRNA\textsubscript{Sec} levels seen in the kidney. These results agree with previous reports demonstrating heterozygous levels of tRNA\textsubscript{Sec} are not limiting for selenoprotein production under normal laboratory conditions (4, 6).

By producing Trsp\textsuperscript{CR/CR} mice we had the opportunity to study the impact of decreasing tRNA\textsubscript{Sec} levels below those of heterozygous-null mice. We observed a large decrease in SelP and DIO1 mRNA abundance, an intermediate decrease in the case of GPX1 and a less-pronounced change in TR1 mRNA in Trsp\textsuperscript{CR/CR} mice relative to wild-type and heterozygous embryos. Therefore, the lower levels of tRNA\textsubscript{Sec} in Trsp\textsuperscript{CR/CR} mice leads to a differential decay in selenoprotein message. The hierarchy of selenoprotein stability observed in the current study differs somewhat from studies in which animals were deprived of selenium (15, 44), where the largest decreases were found in the GPX1 message. This may be explained by the observation that GPX1 message is actively degraded by NMD, whereas other selenoprotein members appear largely resistant to such effects (29, 42, 45). A loss or decrease in tRNA\textsubscript{Sec} may highlight alternative factors which are not observed during conditions of low dietary selenium that promote a differential decrease in selenoprotein transcript.

The results from Trsp\textsuperscript{CR/CR} mice also contrast somewhat with those obtained upon conditional removal of Trsp in mouse livers. In the latter case, the levels of GPX1 and SelP message were reduced but those of GPX4, DIO1, and TR1 were similar.
in abundance to those in heterozygous mice (6). It is possible that tissue-specific factors are involved in the preferential decay of selenoprotein mRNA that could account for the differences seen in Trsp \(^{-/-}\) liver and Trsp\(^{CCR/CCR}\) embryos. For example, it has been reported previously that although GPX4 is susceptible to NMD in mouse fibroblasts and rat hepatoma lines, in the liver and testis an uncharacterized mechanism exists to mask GPX4 mRNA from NMD (42).

Surprisingly, the abundance of mRNA coding for the DIO2 enzyme is not affected in Trsp\(^{CCR/CCR}\) mice, possibly reflecting an important role for this enzyme in embryonic development. Circulating thyroid hormone is inactivated by inner-ring deiodination through the activity of the selenoprotein DIO3. This enzyme acts as a barrier to the passage of maternal thyroid hormone, since it is highly expressed in placenta, amnion, uterius, and particularly at the site of implantation (2). The fetal expression of DIO2 may serve as a local source of active thyroid hormone.

**HO-1 mRNA levels are elevated in Trsp\(^{CCR/CCR}\) embryos.** In a recent report, conditional removal of the Trsp gene from liver was shown to induce the expression of a GST Mu family member (6). Likewise, rats that were made to be selenium deficient showed a large increase in the protein levels of GST class Alpha, Mu, and Theta and of the aldo-keto reductase 7A1 in the liver (28). It is expected that the induction of these proteins may compensate for the loss of protective antioxidant properties of the selenoproteins.

Numerous studies have documented the induction of GST enzymes, including the Mu class, in response to oxidative stress or chemical insults and shown it depends on a cis-acting DNA regulatory element known as the antioxidant response element (ARE) and its cognate transcription factor Nrf2 (12). A common feature is the ability of inducing agents to increase the intercellular oxidative stress such as would be expected to occur in the absence of selenoproteins. Apart from GST members, other detoxification and antioxidant enzymes known to occur in the absence of selenoproteins. Apart from GST members, other detoxification and antioxidant enzymes known to rely on the Nrf2 stress response pathway include NQO1, \(\gamma\)-glutamylcysteine synthase, and HO-1 (12, 13).

Therefore, we examined the expression of stress enzymes in Trsp\(^{CCR/CCR}\) embryos and were surprised to find that the levels of GSTA4, NQO1, and GCLC were unchanged relative to the age caused by reactive oxygen species. It mean that early development is particularly sensitive to damage caused by reactive oxygen species.

Intriguingly, in contrast to other antioxidant enzymes the mRNA transcript for HO-1 was found to be markedly elevated in Trsp\(^{CCR/CCR}\) animals, presumably through an Nrf2 independent mechanism. Previous reports have shown that selenium deficiency in rats increases the expression of HO-1 in liver and by using inhibitors it was possible to demonstrate that the loss of TR activity was responsible for the induction (30). HO-1 induction would be expected to enhance the antioxidant status of the cell by virtue of HO-1 ability to catabolize heme into carbon monoxide, biliverdin, and free iron. Each of these products, either directly or indirectly, has been shown to be protective in rodent models of oxidative damage and inflammation, such as ischemia-reperfusion injury and xenograft survival (36).

**Concluding remarks.** An important feature of the present targeting strategy was the ability to restore tRNA\(^{Sec}\) expression to normal by the coexpression of Cre recombinase. It is hoped that by crossing the mice described here with strains expressing Cre recombinase in various embryonic layers, it will be possible to overcome the lethality at pregastrulation. In this way it may be possible to gain further insight into the role of the selenoenzymes during embryogenesis and development.

**ACKNOWLEDGMENTS**

We thank Hozumi Motohashi, Ken Itoh, Tania O'Connor, and Pawel Jalouszynski for helpful discussion and advice.

This study was supported by grants from ERATO-JST; the Ministry of Education, Science, Sports, and Culture; the Ministry of Health, Labor, and Welfare; and the Atherosclerosis Foundation.

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