MOLECULAR AND CELLULAR BIOLOGY, Mar. 2003, p. 1477–1488 Vol. 23, No. 5
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.5.1477–1488.2003

Selective Removal of the Selenocysteine tRNA[Ser]Sec Gene (*Trsp*) in Mouse Mammary Epithelium

Easwari Kumaraswamy,[1]† Bradley A. Carlson,[1] Fanta Morgan,[2] Keiko Miyoshi,[2]‡ Gertraud W. Robinson,[2] Dan Su,[3] Shulin Wang,[1] Eileen Southon,[4] Lino Tessarollo,[4] Byeong Jae Lee,[5] Vadim N. Gladyshev,[3] Lothar Hennighausen,[2] and Dolph L. Hatfield[1]*

Section on Molecular Biology of Selenium, Basic Research Laboratory, Center for Cancer Research, National Cancer Institute,[1] and Laboratory of Genetics and Physiology, National Institute of Diabetes, Digestive and Kidney Diseases,[2] National Institutes of Health, Bethesda, Maryland 20892; Department of Biochemistry, The Beadle Center, University of Nebraska—Lincoln, Lincoln, Nebraska 68588-0664[3]; Neural Development Section, Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21701[4]; and Laboratory of Molecular Genetics, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea[5]

Received 26 September 2002/Returned for modification 6 November 2002/Accepted 25 November 2002

Mice homozygous for an allele encoding the selenocysteine (Sec) tRNA[Ser]Sec gene (*Trsp*) flanked by *loxP* sites were generated. *Cre* recombinase-dependent removal of *Trsp* in these mice was lethal to embryos. To investigate the role of *Trsp* in mouse mammary epithelium, we deleted this gene by using transgenic mice carrying the *Cre* recombinase gene under control of the mouse mammary tumor virus (MMTV) long terminal repeat or the whey acidic protein promoter. While both promoters target *Cre* gene expression to mammary epithelium, MMTV-*Cre* is also expressed in spleen and skin. Sec tRNA[Ser]Sec amounts were reduced by more than 70% in mammary tissue with either transgene, while in skin and spleen, levels were reduced only with MMTV-*Cre*. The selenoprotein population was selectively affected with MMTV-*Cre* in breast and skin but not in the control tissue, kidney. Moreover, within affected tissues, expression of specific selenoproteins was regulated differently and often in a contrasting manner, with levels of Sep15 and the glutathione peroxidases GPx1 and GPx4 being substantially reduced. Expression of the tumor suppressor genes *BRCA1* and p53 was also altered in a contrasting manner in MMTV-*Cre* mice, suggesting greater susceptibility to cancer and/or increased cell apoptosis. Thus, the conditional *Trsp* knockout mouse allows tissue-specific manipulation of Sec tRNA and selenoprotein expression, suggesting that this approach will provide a useful tool for studying the role of selenoproteins in health.

Selenium is an essential micronutrient in the diet of mammals and numerous other life forms (see reference 26 for a review). Many health benefits have been attributed to this element, including a role in the prevention of cancer (10) and heart disease and other cardiovascular and muscle disorders (11), in delaying the aging process (33) and the onset of AIDS in human immunodeficiency virus-positive patients (1), in male reproduction (17), in mammalian development (5), in immune function (33), and as an antiviral agent (2). Selenium is incorporated into protein in the form of selenocysteine (Sec), and Sec has its own tRNA (designated Sec tRNA[Ser]Sec) and its own code **word**, UGA (26). Sec is indeed the 21st naturally occurring amino acid in the genetic code. Most certainly, the health benefits of selenium are due in large part to its presence in protein (26).

Sec tRNA[Ser]Sec is the only known tRNA that governs the expression of an entire class of proteins, the selenoproteins (26). This provides a unique opportunity to study the expression of selenoproteins by manipulating the levels and characteristics of Sec tRNA[Ser]Sec. For example, the levels of numerous selenoproteins were reduced in a protein- and tissue-specific manner in transgenic mice carrying mutant Sec tRNA[Ser]Sec transgenes lacking the highly modified base isopentenyladenosine in its anticodon (37). Glutathione peroxidase 1 (GPx1) and thioredoxin reductases 1 (TR1) and 3 (TR3) were the most and least affected selenoproteins, respectively, and selenoprotein expression was most and least affected in liver and testes, respectively. Increasing the level of Sec tRNA[Ser]Sec expression by severalfold (36, 37) or decreasing the level of expression by as much as one-half (5, 7) had no effect on selenoprotein expression. Removal of the Sec tRNA[Ser]Sec gene (*Trsp*), however, is embryonic lethal (5). One means of examining the role of genes that are embryonic lethal is gene silencing on a conditional basis by using *Cre*/*loxP* technology (see recent reviews in references 3 and 28). Furthermore, this approach affords us an opportunity to study the role of selenoproteins in specific tissues and organs as well as their relevance to health.

We therefore generated a conditional knockout of *Trsp* by inserting a fragment of mouse DNA encoding this gene into a vector containing the neomycin (*Neo*) and thymidine kinase (*TK*) genes. The newly prepared construct has three *loxP* sites flanking *Neo* and *Trsp*. We generated a mouse that selectively

* Corresponding author. Mailing address: National Cancer Institute, National Institutes of Health, Building 37, Room 2D09, Bethesda, MD 20892. Phone: (301) 496-2797. Fax: (301) 435-4957. E-mail: hatfield@dc37a.nci.nih.gov.

† Present address: The Wistar Institute, Philadelphia, PA 19104.

‡ Present address: Department of Biochemistry, School of Dentistry, The University of Tokoushima, Tokushima 770-8504, Japan.

replaced the *Trsp* wild-type allele with the *loxP-Neo-loxP-Trsp-loxP* construct. We also selectively removed *Neo* in recombinant embryonic stem (ES) cells by transfection with a construct encoding the *Cre* recombinase that generated a mouse encoding floxed *Trsp* (designated $Trsp^{fl}$). The $Trsp^{fl/+}$ mouse became the parental line for studying conditional knockout of *Trsp* in the *Cre-loxP* system.

Breast is an ideal tissue for studying the role of selenoproteins either in its development or in the formation of breast cancer because several model systems that target the mammary gland have been developed in mice (12, 29, 42, 43). Furthermore, this tissue is a major focus of cancer occurrence in women. In inherited breast and ovarian cancer, the *BRCA1* tumor suppressor gene frequently appears to be altered (34). Germ line mutations in *BRCA1* are associated with approximately two-thirds of all familial breast cancers (18). *BRCA1* plays an essential role in several cellular pathways, including transcriptional control, DNA repair, and transcription-coupled repair of oxidative DNA damage (13, 41). p53 is another tumor suppressor gene which normally plays a central role in maintaining the genetic integrity of the cell by preventing cells with damaged DNA from further proliferation. Mutation and deletion of p53 are the most common genetic defects seen in clinical cancer (15). *BRCA1* and p53 are known to physically interact, with *BRCA1* acting as a coactivator of p53 (45).

Transgenic mice carrying *Cre* recombinase under the control of two different regulatory elements, the promoter for the whey acidic protein gene (*WAP*) and that for the mouse mammary tumor virus (MMTV) long terminal repeat, have shown a high specificity for expression in mammary epithelium (42, 44). However, the two promoters have different temporal and tissue specificities. *WAP* is expressed in alveolar cells in midpregnancy and lactation, while MMTV is already expressed in ductal epithelial cells of newborns and also in skin and spleen (42, 43).

In the present study, we crossed $Trsp^{fl/fl}$ mice with $Trsp^{fl}$/+ *WAP-Cre* or MMTV-*Cre* mice to generate offspring carrying two floxed alleles and *Cre* recombinase. The effect of the *Cre* recombinase-driven promoters on the expression of Sec $tRNA^{[Ser]Sec}$ and, consequently, on the expression of the selenoprotein population are reported herein. These studies establish a new animal model to assess the role of selenoproteins in health in a selenoprotein-specific and organ-specific manner.

MATERIALS AND METHODS

Materials. The pPNT and the pLMJ58 plox/*Neo* vectors were from two of our laboratories (those of B. J. Lee and L. Tessarollo, respectively), the pBS246 vector was from Gibco-BRL, and the pBSKS+ vector was from Stratagene. The 129/SV mouse genomic library was obtained from Incyte Genomics, [^{75}Se]selenious acid (specific activity, 1,000 Ci/mmol) was from the Research Reactor Facility, University of Missouri, Columbia, Mo., and [^{3}H]serine (specific activity, 36 Ci/mmol) was from Amersham. Restriction endonucleases and agarose were obtained from New England Biolabs and/or Gibco-BRL. Vent DNA polymerase, *Taq* DNA polymerase, and deoxynucleoside triphosphates were from New England Biolabs, and spermidine trihydrochloride used in the digestion of genomic DNA was from Sigma.

Nylon membranes (Hybond N+) and ^{32}P-labeled nucleotides were from Amersham, hybridization solution, QuickHyb, was from Stratagene, and Dulbecco's modified Eagle's medium with high glucose and fetal calf serum were used for culturing mouse embryo fibroblasts and embryonic stem (ES) cells from Gibco-BRL and HyClone, respectively. Penicillin-streptomycin, L-Glutamine, minimal essential medium, nonessential amino acids, trypsin-EDTA, geneticin, and leukemia inhibitory factor or ESGRO were from Gibco-BRL. Gelatin and dimethyl sulfoxide were from Sigma, and ganciclovir was from Cytovene Syntex. C-20 polyclonal anti-BRCA1 antibody was from Santa Cruz, and monoclonal anti-p53 antibody was from Oncogene. All other reagents were commercial products of the highest grade available.

C57BL/6 mice used for generating a *Trsp* conditional knockout carrying *Neo-Trsp* flanked by *loxP* sites were from the stock of one of us (L. Tessarollo), transgenic FVB/N mice carrying EIIa *Cre* were obtained from Heiner Westphal (National Institutes of Health), and transgenic C57BL/6 × 129 mice carrying the *Cre* recombinase driven by the MMTV long terminal repeat promoter or the *WAP* promoter were generated previously (42). The care of animals was in accordance with the National Institutes of Health institutional guidelines under the expert direction of Grace Lidl and D. L. Sly (National Cancer Institute, National Institutes of Health, Bethesda, Md.) and Mark St. Clair (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Md.).

Construction of targeting vector and generation of cells and mice carrying target alleles. The methods for preparing the targeting vector and cells and mice encoding the target alleles are described below in Materials and Methods, while the identification of each of these components is given in the Results. The primers used in identifying the various regions inserted during vector construction and in generating the different regions cloned into the targeted vector are shown in Fig. 1B and Table 1, footnote *a*.

Targeting vector construction and generation of $Trsp^{fl}$-Neo^{fl} cells. The targeting vector was constructed with *Neo* and *Trsp* flanked by *loxP* sites (designated $Trsp^{fl}$-Neo^{fl}) and the regions upstream and downstream of the tRNA gene as shown in Fig. 1. The targeting vector was linearized with *Not*I, and ES cells were electroporated with 20 μl of DNA (1 μg/μl) with a Bio-Rad Gene Pulser set at 250 V with a capacitance of 500 μF in a 0.4-cm electrode gap electroporation cuvette (Bio-Rad). The electroporated cells were transferred to plates containing ES cell medium and incubated for 18 to 24 h, and the transfected cell population was positively and negatively selected for 8 to 9 days with 250 μg of G418 per ml and 2 μM ganciclovir, respectively.

Candidate ES cell clones were screened for homologous recombination by Southern hybridization of *Pvu*II-digested genomic DNA, and the resulting cells carrying a $Trsp^{fl}$-Neo^{fl} allele were used to generate chimeric mice.

Generation of $Trsp^{fl}$-Neo^{fl} mice. Homologous recombinant ES cell clones carrying a $Trsp^{fl}$-Neo^{fl} allele were injected into C57BL/6 blastocysts and transferred to pseudopregnant females. The resulting high-percentage chimeras (90% or greater based on coat color) were mated to wild-type C57BL/6 mice, and tail DNA samples from the F_1 offspring were analyzed for germ line transmission.

Removal of *Neo* and *Trsp*. Mice carrying a $Trsp^{fl}$ allele lacking *Neo* were generated from recombinant ES cells carrying the $Trsp^{fl}$-Neo^{fl} allele (see Fig. 1) by transfection with a vector carrying the *Cre* recombinase gene under the control of the β-actin promoter (23). Clones were obtained by G418 selection, and those cells carrying a $Trsp^{fl}$ allele without *Neo* were identified by Southern hybridization of *Pvu*II-digested genomic DNA. An ES cell clone that had lost *Neo* but retained $Trsp^{fl}$ was injected into C57BL/6 blastocysts and transferred to pseudopregnant females. Resulting chimeras were mated with wild-type C57BL/6 mice, and tail DNA from F_1 offspring were analyzed for germ line transmission by Southern hybridization and PCR.

Mice carrying $Trsp^{fl/fl}$ were mated with transgenic mice carrying EIIa *Cre* to obtain a standard knockout of *Trsp*. Tail DNA of the F_1 offspring was analyzed for the loss of the tRNA gene by PCR as given in the Results.

Conditional removal of *Trsp*. The MMTV-*Cre* transgene was identified by PCR analysis of tail DNA with primers corresponding to the MMTV long terminal repeat (5′-GGT TCT GAT CTG AGC TCT GAG TG-3′) and to the *Cre* gene (5′-CAT CAC TCG TTG CAT CGA CCG G-3′), resulting in a 280-bp PCR product. The *WAP-Cre* transgene was identified by PCR analysis of tail DNA with primers corresponding to the *WAP* promoter (5′-TAG AGC TGT GCC AGC CTC TTC C-3′) and to the *Cre* primer described above, resulting in a PCR product 240 bp in size. $Trsp^{fl/+}$-*WAP-Cre* or $Trsp^{fl/+}$-MMTV-*Cre* transgenic mice were generated by mating *WAP-Cre* and MMTV-*Cre* transgenic mice with $Trsp^{fl/fl}$ mice. The presence of $Trsp^{fl}$ in *Cre* transgenic mice was detected. The offspring were mated with $Trsp^{fl/fl}$ mice, and the resulting $Trsp^{fl/fl}$-*WAP-Cre*, $Trsp^{fl/fl}$-MMTV-*Cre*, and $Trsp^{fl/fl}$ pups were used for Sec $tRNA^{[Ser]Sec}$ and selenoprotein analysis.

Isolation and aminoacylation of tRNA, RPC-5 chromatography, and Northern hybridization. Total tRNA was isolated from tissues and aminoacylated with [^{3}H]serine under limiting tRNA conditions (24), and the resulting labeled seryl-tRNA was chromatographed twice on an RPC-5 column (27), first in buffer without Mg^{2+} and then in buffer with Mg^{2+} (14, 25, 36, 37). Seryl-$tRNA^{Ser}$ is

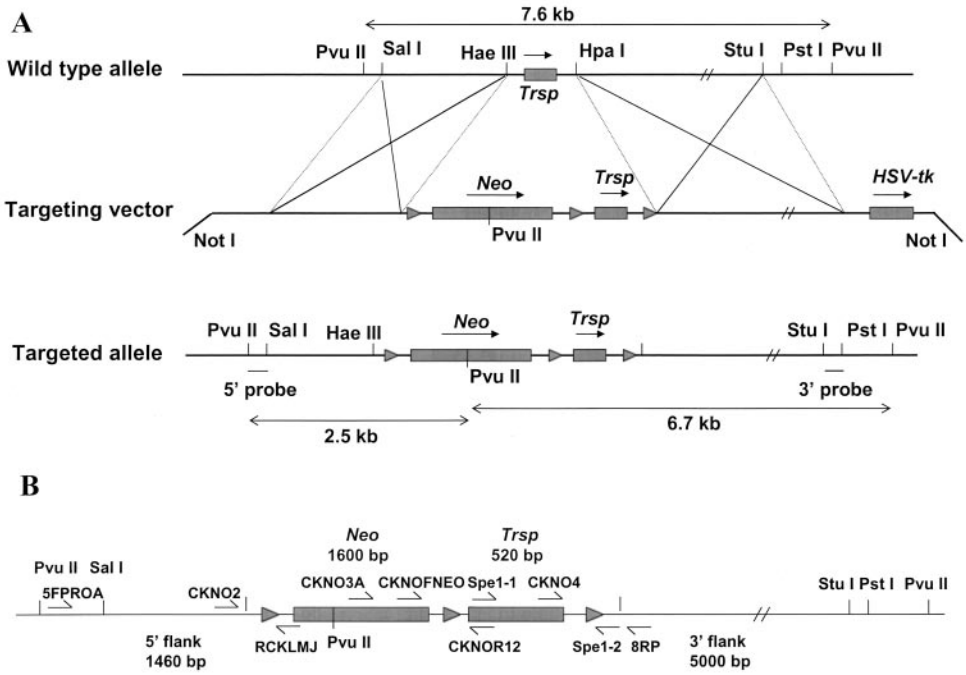

FIG. 1. Targeting vector, targeted allele, and screening strategy used in generating the conditional knockout of *Trsp*. (A) Map showing the wild-type allele (upper portion of panel A) encoding *Trsp* and the restriction sites used in generating the targeting vector (center portion of panel A) that encodes *Neo* (with its *Pvu*II restriction site, which is critical in distinguishing between the wild-type and target alleles [see below and text]), and *Trsp* flanked by *loxP* sites (designated by ▶), herpes simplex virus thymidine kinase (*HSV-tk*), and the *Not*I restriction sites used in constructing this vector, and the targeted allele (lower portion of panel A) encoding *Trsp* and *Neo* flanked by *loxP* sites (designated by ▶) and the restriction sites used in constructing this allele, in identifying homologous recombination and in distinguishing wild-type and targeted alleles (see text) are presented. Sizes of fragments generated from the wild-type allele (see top of panel A) and targeted allele (see bottom of panel A) by *Pvu*II digestion are also shown. (B) Primers used in identifying the insertion or generation of different regions during construction of the targeting vector and in determining the presence of different regions involved in generating the targeted allele and floxed *Trsp* and/or *Neo* are shown along with the sizes of each region cloned into the targeting vector. Maps in panels A and B are not drawn to scale.

more hydrophobic than seryl-tRNA$^{[Ser]Sec}$ in the absence of Mg^{2+}, and therefore elutes later on the RPC-5 column, and is less hydrophobic in the presence of Mg^{2+}, and therefore elutes earlier. The tRNA^{Ser} and tRNA$^{[Ser]Sec}$ populations can therefore be chromatographically resolved from each other and quantitated following labeling with [^{3}H]serine and chromatography as described previously (14, 25, 36, 37).

The level of the Sec tRNA$^{[Ser]Sec}$ population was also determined by Northern blot analysis by standardizing the signal obtained with a ^{32}P-5′-end-labeled oligonucleotide that was complementary to the 20 nucleotides at the 3′ end of tRNA$^{[Ser]Sec}$ (not including the CCA terminus) against that obtained with a ^{32}P-5′-end-labeled oligonucleotide that was complementary to the 20 nucleotides at the 3′ end of serine tRNA$^{Ser}_1$ (not including the CCA terminus), which was used as an internal control. This technique was a modification of that of Bosl et al. (5), where 0.01 A_{260} unit of total tRNA was electrophoresed on a 15% TBE (Tris-borate-EDTA)–urea–polyacrylamide gel for 2 h at 180 v, the tRNA was transblotted onto a nylon membrane for 1.5 h at 30 V, and the membrane was cross-linked as for Southern blot analysis (37). The membrane was then hybridized with the tRNA$^{[Ser]Sec}$ probe and washed, the signal level was determined, and the membrane was stripped of this probe and rehybridized with the serine tRNA$^{Ser}_1$ probe. 5′-End labeling of probes was carried out with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase following the vendor's procedures (Life Technologies), and hybridization assays, washing of filters, and autoradiograms were carried out as for Southern blot analysis (37).

Labeling of selenoproteins and GPx1 and TR1 assays. Lactating mice with the genotypes *Trsp$^{fl/fl}$-WAP-Cre*, *Trsp$^{fl/fl}$*-MMTV-*Cre*, and *Trsp$^{fl/fl}$* were injected intraperitoneally with 50 μCi of ^{75}Se/g and sacrificed 48 h after injection. Tissues and organs were excised and immediately placed into liquid nitrogen and stored at −80°C until ready for use. Tissues were homogenized in 40 mM Tris-HCl (pH 7.4)–1 mM EDTA–0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, sonicated for 2 min, and centrifuged at 4°C for 20 min. Supernatants were electro-

TABLE 1. Summary of PCR screening techniques used for recombination[a]

Forward primer	Reverse primer	Expected product	Product length	Sequencing primer	Identification
CKNO5FPROA	RCKLMJ	5′ flank and 1st *loxP*	1.7 kb	CKNOF11	Recombinant only
CKNO2	RCKLMJ	1st *loxP*	100 bp	CKNO2	Recombinant only
CKNOFNeo	CKNO8RP	2nd *loxP*, gene and 3rd *loxP*	800 bp	CKNO4 & CKNO8RP	Recombinant only
Spe1	CKNO8RP	Gene only and gene and 3rd *loxP*	700 bp and 800 bp		Wild type & recombinant (two products)
Spe1	Spe2	Gene and 3rd *loxP*	700 bp	Spe1	Recombinant only
CKNO3A	CKNOR12	2nd *loxP*	700 bp	CKNOR12	Recombinant only

[a] Primer designations and sequences are as follows: CKNO2, GCA ACG GCA GGT GTC GCT CTG CG; CKNO3A, GAC GGC GAT GAT CTC GTC GTG; CKNO4, GCG ACC AGC GCG CAG TTA ACC; CKNO8RP, CGT GCT CTC TCC ACT GGC TCA; CKNOF11, CCA TCA CCT AGG GAC TCA G; CKNOR12, AGT GCC TGT CTC CCT AAC T; CKNOFNEO, CGC CGC TCC CGA TTC GCA GCG; RCKLMJ, GGC TGG ACG TAA ACT CCT C; Spe1, CTA GAC TAG TGG CCG CGT GAG AAG TTT TTC; Spe2, GGC CAG TAC TAG TGA ACC TCT TC.

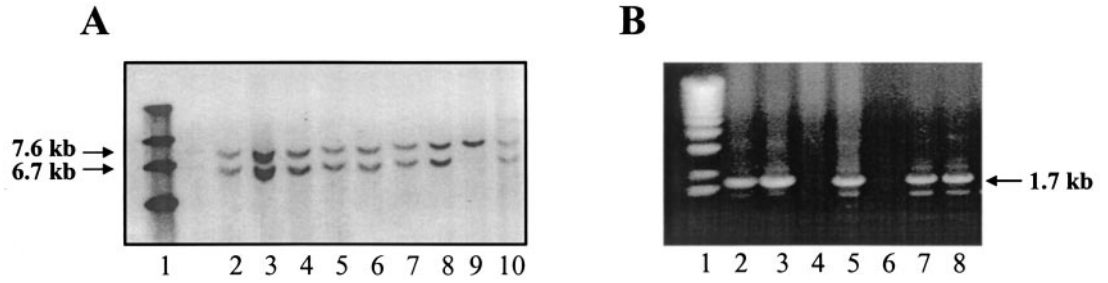

FIG. 2. Screening of ES cell clones for recombinant, targeted vector inserted DNA. In A, DNA that was isolated from 160 clones, digested with *Puv*II, electrophoresed on agarose gels, transblotted as given in Materials and Methods, and hybridized with the 3′-end probe yielded nine potential positives that were rescreened on the same blot, as shown in lanes 2 to 10. Seven of these clones (from lanes 2 to 8) yielded a 2.5-kb signal with the 5′-end probe (data not shown). In B, PCR analysis of these clones with CKNO5FPROA and RCKLMJ as primers yielded a 1.7-kb fragment (lanes 2 to 8). Subsequent sequencing of the PCR-generated fragments demonstrated the presence of Neo^{fl}-$Trsp^{fl}$ (see text). Lane 1 in panels A and B contains molecular size markers.

phoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels, separated proteins were transferred to polyvinylidene difluoride nylon membranes, and transblots were exposed to a PhosphorImager as described previously (21, 37). Gels were stained with Coomassie blue.

GPx1 activity was assayed directly in extracts, while TR1 activity was assayed after enrichment on ADP-Sepharose as described previously (37).

Tissues. The tissues used for $tRNA^{[Ser]Sec}$ and selenoprotein analyses from $Trsp^{fl/fl}$-*WAP-Cre*, $Trsp^{fl/fl}$-MMTV-*Cre*, and $Trsp^{fl/fl}$ mice were mammary (entire mammary gland), skin (ears only), spleen, and kidney. The same tissues and, in addition, liver tissues from heterozygous standard knockout ($Trsp^{+/-}$) mice and their wild-type siblings were used. Tissues were excised from labeled mice and used immediately for selenoprotein analysis (see above), while those used for tRNA analysis were immediately placed into liquid nitrogen and stored at −80°C until ready for use.

Western blot analysis. Protein samples were prepared from kidney and mammary tissues as described above, and ≈25 μg was electrophoresed on SDS–4 to 12% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-BRCA1, anti-p53, and antiactin antibodies, and the resulting band intensities were quantitated with NIH Image.

RESULTS

Targeting vector. The targeting vector was constructed from fragments encoding *Trsp* and the surrounding DNA that were isolated from a 129/Sv mouse genomic library and cloned into pPNT, which provided the backbone for the conditional knockout vector (Fig. 1A). A 520-bp *Hae*III-*Hpa*I fragment encoding *Trsp* was cloned into the *Sma*I sites of pBS246, which contained two *loxP* sites. The gene and the second *loxP* site were PCR amplified with primers Spe1 and Spe2 (see Table 1) containing *Spe*I sites at both ends and then subcloned into the *Spe*I site of pBSKS+. A 1.46-kb *Sal*I-*Not*I fragment encoding the region just upstream of the 520-bp *Trsp*-containing fragment was blunt ended and cloned into the *Eco*RI site (also blunt ended) immediately 5′ to the floxed phosphoglycerate kinase (PGK) I promoter/*Neo* (*Neo*) cassette of the pLMJ58 plox/*Neo* vector. The 4,113-bp pLMJ58 plox/*Neo* vector encodes a 1.6-kb *Eco*RI-*Xho*I pGK*Neo*bpA fragment, which was blunt ended and inserted into the blunt-ended *Bam*HI site of the plox plasmid pBS246. This fragment, which contains the 5′-flanking region of *Trsp* along with Neo^{fl}, was released from the vector as a 3.3-kb *Not*I-*Sca*I fragment and cloned into the *Not*I and *Xba*I sites upstream of $Trsp^{fl}$ in pBSKS+.

A 5.0-kb *Hpa*I-*Stu*I fragment encoding the region immediately downstream of the *Trsp*-containing fragment was prepared by initially cloning a 5.1-kb, blunt-ended *Hpa*II-*Stu*I fragment upstream of the PGK-herpes simplex virus thymidine kinase-bpA cassette (used for negative selection) into the blunt-ended *Xho*I and *Xba*I sites of pPNT, replacing the *Neo* gene. The 3′-flanking region or the long arm is upstream of the PGK- herpes simplex virus thymidine kinase-bpA cassette and was used for negative selection. The 3.8-kb fragment containing the 5′-flanking sequence and the floxed *Neo-Trsp* fragment was released from pBSKS+ with *Not*I and *Sma*I and cloned into the *Not*I and *Hpa*I sites of the pPNT vector upstream of the region containing the 3′-flanking fragment of *Trsp* to yield the targeting vector (bottom map in Fig. 1A).

Gene targeting and homologous recombination. Following electroporation of the targeting vector into ES cells and selection of recombinant cells (see Materials and Methods), candidate ES cell clones were screened for homologous recombination by isolating genomic DNA, digesting with *Pvu*II, and probing the digests by Southern hybridization. A 270-bp *Pvu*II-*Sal*I fragment located upstream of the *Neo-Trsp*-containing 520-bp fragment (designated the 5′ probe) was used to monitor insertion of the 5′-flanking region. A 250-bp *Stu*I-*Pst*I fragment located downstream of the *Neo-Trsp*-containing fragment (designated the 3′ probe) was used to monitor insertion of the 3′-flanking region.

*Pvu*II-digested, wild-type genomic DNA yielded a 7.6-kb signal with both the 5′ and 3′ probes, while recombinants yielded a 6.7-kb signal with the 3′ probe and a 2.5-kb signal with the 5′ probe, as this endonuclease cuts once inside the *Neo* gene (see Fig. 1A). One hundred sixty selected clones were screened as potential homologous recombinants. Eight were identified as homologous recombinants by screening with the 3′-end probe (Fig. 2A). Five of these eight were verified as true homologous recombinants by PCR (Fig. 2B) and sequencing with the primers shown in Fig. 1B and Table 1 (see also legend to Fig. 2B) to have three intact *loxP* sites flanking *Trsp* and *Neo*.

***$Trsp^{fl}$-Neo^{fl}* mice and Sec $tRNA^{[Ser]Sec}$ analysis.** Homologous recombinant ES cell clones were used to generate a chimeric mouse carrying a $Trsp^{fl}$-Neo^{fl} allele in its germ line as described in Materials and Methods. All F_1 offspring of parental chimeric and C57BL/6 mice yielded a 9.2-kb signal which represented the wild-type allele and either the wild-type 129/Sv 7.6-kb allele or the recombinant 6.7-kb allele as identified by Southern hybridization with the 3′ probe (data not shown). It should be noted that C57BL/6 mice encode a polymorphism in the region

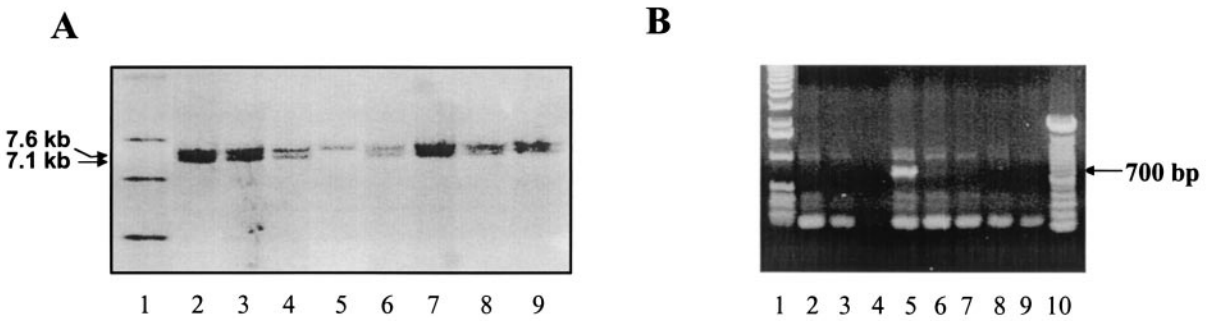

FIG. 3. Screening of *Cre*-transfected cells. In A, DNA was digested with *Pvu*II, electrophoresed on agarose gels, transblotted as given in Materials and Methods, and hybridized with the 3′ probe. Lanes 2 to 4 and 6 to 9 yielded a 7.6-kb wild-type signal and a 7.1-kb signal, indicating they have lost both *Neo* and *Trsp*. Lane 5 yielded a signal with a slight upward shift of ≈100 bp, indicating loss of *Neo*. In B, PCR analysis of the fragments in lanes 2 to 9 of panel A with CKNO2 and Spe2 as primers showed a 700-bp fragment in lane 5, confirming the loss of *Neo* and presence of *Trsp* only in the DNA of cells shown in lane 5 of panel A. Lane 1 in panel A and lanes 1 and 10 in panel B contain molecular size markers.

where the *Pvu*II site occurs in ES cells that accounts for the 9.2-kb *Pvu*II fragment in this mouse line. About 50% of the offspring were identified as heterozygous for $Trsp^{fl}$ and Neo^{fl}. Offspring from this parental mouse that carried a $Trsp^{fl}$-Neo^{fl} allele were intercrossed to yield mice homozygous for the targeted allele.

Sec tRNA[Ser]Sec levels and isoform distributions in liver, kidney, heart, lung, brain, spleen, and muscle were examined in these homozygous mice to determine whether the presence of *Neo* had any effect on the expression of *Trsp*. No differences were observed in the level of the Sec tRNA population and the distributions of the isoforms in these tissues of wild-type mice and mice carrying the homozygous targeted allele (data not shown), suggesting that the presence of *Neo* had no influence on Sec tRNA[Ser]Sec expression.

Generation of $Trsp^{fl}$ mice. *Neo* was removed from recombinant ES cells carrying the $Trsp^{fl}$-Neo^{fl} allele by transfecting these cells with a vector expressing the *Cre* recombinase under the control of the β-actin promoter. DNA was isolated from these recombinant cells and digested with *Pvu*II. A fragment about 100 bp larger than the corresponding wild-type fragment due to the presence of vector sequences was observed by Southern hybridization with the 3′ probe (Fig. 3A, lane 5). This fragment was distinguished from the allele lacking *Trsp-Neo* by being 500 bp shorter than the corresponding wild-type fragment (Fig. 3A, see lanes 2 to 4 and 6 to 9). The presence of *Trsp* and the flanking *loxP* sites (i.e., $Trsp^{fl}$) was partially confirmed by PCR with primers CKNO2 and Spe2, which yielded a 700-bp product (Fig. 3B, lane 5). Other primer combinations, Spe1 and Spe2, CKNO2 and CKNO8RP, and CKNO2 and CKNOR12 and sequencing of the PCR-generated products further confirmed the presence of $Trsp^{fl}$.

It should be noted that due to the small size difference between the wild-type and targeted alleles generated by *Pvu*II digestion, screening was also carried out by PCR with the primer combinations CKNO2 and Spe2, Spe1 and Spe2, CKNO2 and CKNO8RP, and CKNO2 and CKNOR12.

Chimeric mice carrying only $Trsp^{fl}$ were generated as described in Materials and Methods. Analysis of the DNA, as described above, from the offspring of one of the chimeric mice confirmed that about 40% of them carried $Trsp^{fl}$ without *Neo* in the germ line (data now shown).

Intercrosses between mice carrying the $Trsp^{fl}$ allele generated $Trsp^{fl/fl}$ offspring, and these mice were used in matings to generate selective deletion of *Trsp* in specific tissues and the standard *Trsp* knockout mice (see below).

Conditional knockout of *Trsp*. To induce a tissue-specific deletion of *Trsp,* we generated $Trsp^{fl/fl}$-*WAP-Cre* or $Trsp^{fl/fl}$-MMTV-*Cre* mice and used them for Sec tRNA[Ser]Sec and selenoprotein expression analyses. $Trsp^{fl/fl}$ mice were used as the control. The presence of $Trsp^{fl/fl}$ was detected by PCR analysis with the CKNO2 and CKNO8RP primers as described in Table 1, footnote *a*, and the product was a 1.1-kb fragment.

The deletion of *Trsp* from genomic DNA in mammary epithelium, skin, spleen, and kidney was determined by PCR analysis (Fig. 4). *Trsp* was virtually absent in DNA from mammary tissue of $Trsp^{fl/fl}$-*WAP-Cre* and $Trsp^{fl/fl}$-MMTV-*Cre* mice and only partially lost in DNA from skin and spleen of $Trsp^{fl/fl}$-MMTV-*Cre* mice. Interestingly, a longer film exposure of the gel in Fig. 4 revealed a partial loss of *Trsp* in DNA from the control tissue, kidney, of $Trsp^{fl/fl}$-MMTV-*Cre* mice, indicating low levels of MMTV promoter activity in the control tissue. As expected, there was no loss of *Trsp* from DNA of these tissues in $Trsp^{fl/fl}$, nontransgenic mice.

Phenotypic changes in $Trsp^{fl/fl}$-*WAP-Cre*, $Trsp^{fl/fl}$-MMTV-*Cre* and $Trsp^{fl/fl}$ mice. Primiparous females were able to nurse their litters, and no differences in pup growth were observed. Whole-mount and histological analyses of tissues harvested after lactation was fully established (day 10 of lactation) revealed normal mammary development with expanded lumina

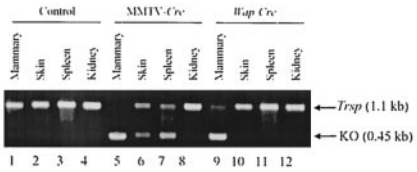

FIG. 4. Selective removal of *Trsp* in various tissues. DNA was isolated from breast, skin, spleen, and kidney, and products were generated by PCR with the CKNO2 and CKNO8RP primers with DNA from $Trsp^{fl/fl}$ (lanes 1 to 4; designated control), $Trsp^{fl/fl}$-MMTV-*Cre* (lanes 5 to 8; designated MMTV-*Cre*), and $Trsp^{fl/fl}$-*WAP-Cre* (lanes 9 to 12; designated *WAP-Cre*) mice. PCR with this DNA encoding floxed *Trsp* yielded a 1.1-kb fragment, and without floxed *Trsp* yielded a 0.45-kb fragment.

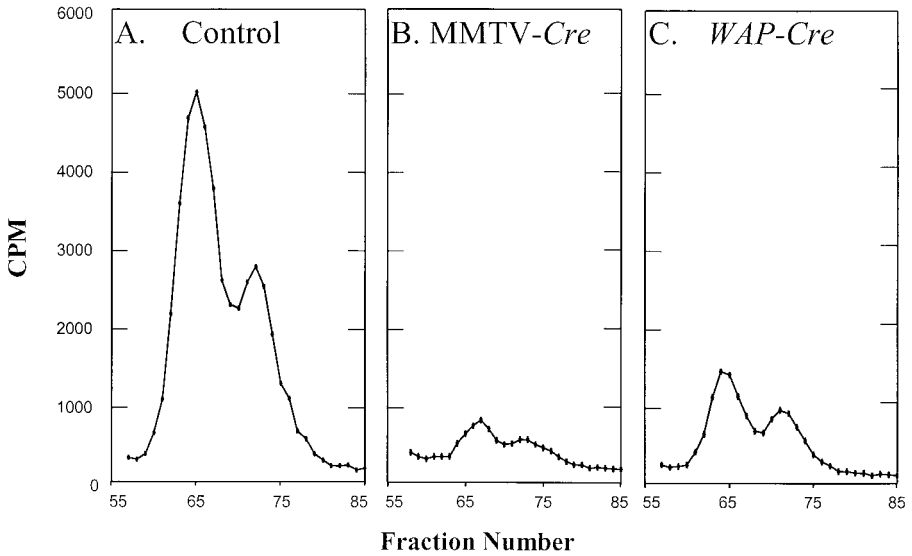

FIG. 5. Relative amounts of Sec tRNA$^{[Ser]Sec}$ in mammary tissue of conditional *Trsp* knockout mice. Total tRNA was isolated from mammary tissue of *Trsp*$^{fl/fl}$, *Trsp*$^{fl/fl}$-*WAP-Cre*, and *Trsp*$^{fl/fl}$-*MMTV-Cre* mice, the tRNA was aminoacylated with [^{3}H]serine and fractionated by RPC-5 chromatography, and the amount of [^{3}H]seryl-tRNA$^{[Ser]Sec}$ in *Trsp*$^{fl/fl}$-*WAP-Cre* and *Trsp*$^{fl/fl}$-MMTV-*Cre* mammary tissue was standardized to that found in control mammary tissue, with the total [^{3}H]seryl-tRNA^{Ser} serving as an internal control, as described in Materials and Methods.

that contained secreted milk and lipid droplets (data not shown) (42, 43).

Sec tRNA$^{[Ser]Sec}$ analysis in tissues of conditional knockout mice. tRNA was prepared from various tissues of *Trsp*$^{fl/fl}$-*WAP-Cre*, *Trsp*$^{fl/fl}$-MMTV-*Cre*, and *Trsp*$^{fl/fl}$ mice; tRNA from *Trsp*$^{fl/fl}$ mice was used as a control. The tissues selected for analysis were those reported previously to be affected by the *WAP* gene (mammary tissue [42]) or the MMTV gene (mammary tissue, spleen, and skin [42]). Kidney was used as the control tissue. Isolated tRNA from each tissue was aminoacylated with [^{3}H]serine, which labeled the serine and Sec tRNA populations. These two aminoacylated tRNA populations were separated from each other by RPC-5 chromatography, and the amounts of the two major Sec tRNA$^{[Ser]Sec}$ isoforms relative to the total seryl-tRNA were determined as described previously (14, 25, 36, 37).

The Sec tRNA$^{[Ser]Sec}$ isoforms differed from each other by a single 2′-*O*-methylribosyl moiety in the wobble position of the anticodon and were designated 5′-methylcarboxymethyluridine (mcm^{5}U) and 5′-methylcarboxymethyluridine-2′-*O*-methylribose (mcm^{5}Um). The mcm^{5}U isoform eluted earlier from the RPC-5 column than the mcm^{5}Um isoform. The levels and distributions of the Sec tRNA$^{[Ser]Sec}$ isoforms were initially examined in mammary tissue of *Trsp*$^{fl/fl}$-*WAP-Cre*, *Trsp*$^{fl/fl}$-MMTV-*Cre* and *Trsp*$^{fl/fl}$, mice as shown in Fig. 5A, B, and C, respectively. The levels of the Sec tRNA$^{[Ser]Sec}$ isoforms dropped dramatically in mammary tissue harboring the *Cre* recombinase, and the MMTV promoter-driven *Cre* was more efficient in reducing the tRNA$^{[Ser]Sec}$ population than the *WAP* promoter-driven *Cre*. The amounts of the Sec tRNA$^{[Ser]Sec}$ population relative to the seryl-tRNA population and the distributions of the isoforms were determined for each of the other tissues, skin, spleen, and kidney, in the same manner as for mammary tissue. The data for each of the four tissues examined are summarized in Table 2.

As shown in Table 2, the level of Sec tRNA$^{[Ser]Sec}$ was reduced by ≈80% in mice harboring *Cre* recombinase driven by the MMTV promoter and ≈70% in mice harboring this enzyme driven by the *WAP* promoter. The MMTV promoter-driven *Cre* also affected the Sec tRNA$^{[Ser]Sec}$ population in spleen and skin, but the decrease was less than in mammary tissue, being 54% and 39%, respectively. The distributions of mcm^{5}U and mcm^{5}Um were only slightly altered in the pres-

TABLE 2. Levels and distributions of Sec tRNA$^{[Ser]Sec}$ isoacceptors in tissues of *Trsp*$^{fl/fl}$, *Trsp*$^{fl/fl}$-MMTV-*Cre*, and *Trsp*$^{fl/fl}$-*WAP-Cre* mice[a]

Tissue	Genotype	Sec tRNA$^{[Ser]Sec}$				
		% (total)[b]	Relative amt[c]	Distribution[d] (%)		Ratio, mcm^{5}U/ mcm^{5}Um[e]
				mcm^{5}U	mcm^{5}Um	
Mammary	*Trsp*$^{fl/fl}$	2.32	1.00	65.3	34.7	1.88
	Trsp$^{fl/fl}$-MMTV-*Cre*	0.42	0.18	59.0	41.0	1.44
	Trsp$^{fl/fl}$-*WAP-Cre*	0.68	0.29	60.7	39.3	1.54
Skin	*Trsp*$^{fl/fl}$	3.34	1.00	41.9	58.1	0.72
	Trsp$^{fl/fl}$-MMTV-*Cre*	2.03	0.61	58.3	41.7	1.40
	Trsp$^{fl/fl}$-*WAP-Cre*	3.17	0.95	49.8	50.2	0.99
Spleen	*Trsp*$^{fl/fl}$	4.00	1.00	52.9	47.1	1.12
	Trsp$^{fl/fl}$-MMTV-*Cre*	1.85	0.46	50.9	49.1	1.04
	Trsp$^{fl/fl}$-*WAP-Cre*	3.84	0.96	53.8	46.2	1.16
Kidney	*Trsp*$^{fl/fl}$	4.20	1.00	36.4	63.6	0.57
	Trsp$^{fl/fl}$-MMTV-*Cre*	4.90	1.17	38.3	61.7	0.62
	Trsp$^{fl/fl}$-*WAP-Cre*	4.62	1.10	32.1	67.9	0.47

[a] Total tRNA was isolated from tissues of offspring from matings of the corresponding parents, and the tRNA was fractionated. The amounts of total Sec tRNA$^{[Ser]Sec}$ and the distributions of mcm^{5}U and mcm^{5}Um were determined as given in Materials and Methods.

[b] Percentage of tRNA$^{[Ser]Sec}$ within the seryl-tRNA population.

[c] Amount of tRNA$^{[Ser]Sec}$ relative to that in the wild type, which was assigned a value of 1.00.

[d] Percentage of mcm^{5}U and mcm^{5}Um in the seryl-tRNA population, resolved by RPC-5 chromatography (see Materials and Methods).

[e] Amount of mcm^{5}U divided by the amount of mcm^{5}Um.

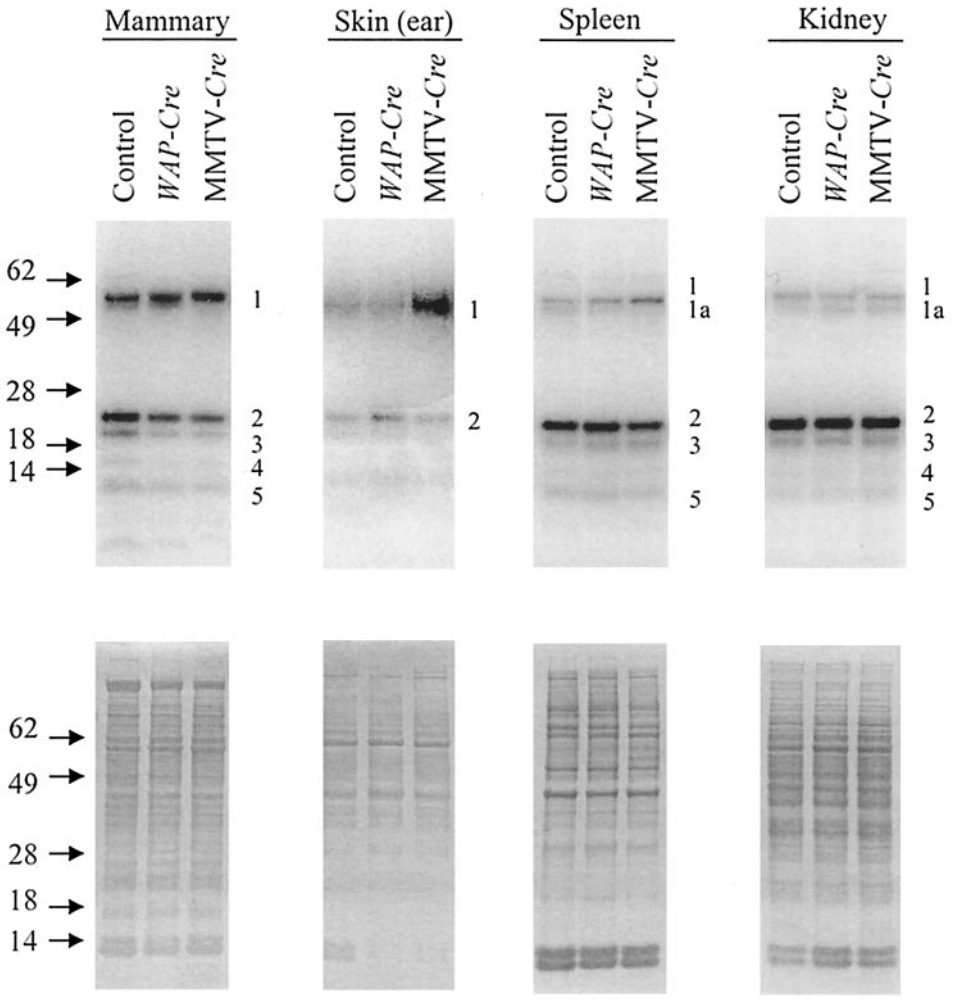

FIG. 6. Protein and selenoprotein analysis in tissues of mice carrying floxed *Trsp* and *Cre* promoter-specific transgenes. Mice were labeled with ^{75}Se; protein was extracted from tissues, electrophoresed, and stained with Coomassie blue, and ^{75}Se-labeled proteins were detected with a PhosphorImager (see Fig. 5) as given in Materials and Methods. ^{75}Se-labeled proteins from mammary, skin, spleen, and kidney tissues of *Trsp*$^{fl/fl}$ (control), *Trsp*$^{fl/fl}$-*WAP-Cre* (*WAP-Cre*), and *Trsp*$^{fl/fl}$-MMTV-*Cre* (MMTV-*Cre*) mice are shown in the upper panels, and total protein analysis is shown in the lower panels.

ence of *WAP-Cre* and MMTV-*Cre* in each tissue compared to those in the corresponding control with the exception of the values in skin. Interestingly, the distribution of the two isoforms shifted most dramatically in the presence of MMTV-*Cre*, while the distribution in the presence of *Wap-Cre* was intermediate between that of the control and MMTV-*Cre.* Similar levels and distributions of the Sec tRNA$^{[Ser]Sec}$ isoforms were observed in the kidney of *Trsp*$^{fl/fl}$-*WAP-Cre*, *Trsp*$^{fl/fl}$-MMTV-*Cre*, and *Trsp*$^{fl/fl}$ mice.

Selenoprotein synthesis in tissues of conditional knockout mice. The biosynthesis of selenoproteins in each of the four tissues from *Trsp*$^{fl/fl}$-*WAP-Cre*, *Trsp*$^{fl/fl}$-MMTV-*Cre*, and *Trsp*$^{fl/fl}$ mice was assessed by labeling the selenoprotein population with ^{75}Se. Proteins from mammary, skin, spleen, and kidney tissues of labeled mice were extracted and examined by gel electrophoresis. Coomassie blue-stained gels of total proteins from these tissues were very similar (lower panel of Fig. 6), suggesting that the presence of *Trsp*$^{fl/fl}$ or *Trsp*$^{fl/fl}$ coupled with the *Cre*-driven promoters had little or no effect on protein synthesis as a whole. However, analysis of the selenoproteins detected by ^{75}Se labeling in a representative gel showed variations in protein expression (upper panel of Fig. 6). Major selenoprotein bands are designated 1, 1a, 2, 3, 4, and 5. As reported previously, bands 1 and 1a are likely TR1 (21, 37, 39, 40), while bands 2 and 3, which have been purified and identified (21, 37), are GPx1 and GPx4, respectively. Band 4 is unknown, and band 5, which has been purified and identified (19, 21, 37), is Sep15. Bands 2 to 5 appear to be present in smaller amounts in mammary tissue carrying the *Cre*-driven promoters, whereas band 1 is increased in MMTV-*Cre* mice.

Each ^{75}Se-labeled band shown in Fig. 6 was quantitated on a PhosphorImager along with those from two other gels of protein extracts from mice that were independently labeled with ^{75}Se. The averages of these values relative to the controls are shown in Fig. 7. The most significant reductions in selenoprotein levels occurred in GPx1, GPx4, Sep15, and band 4 in mammary tissue of MMTV-*Cre* and *WAP-Cre* mice. The only other apparent significant variation occurred in the skin of

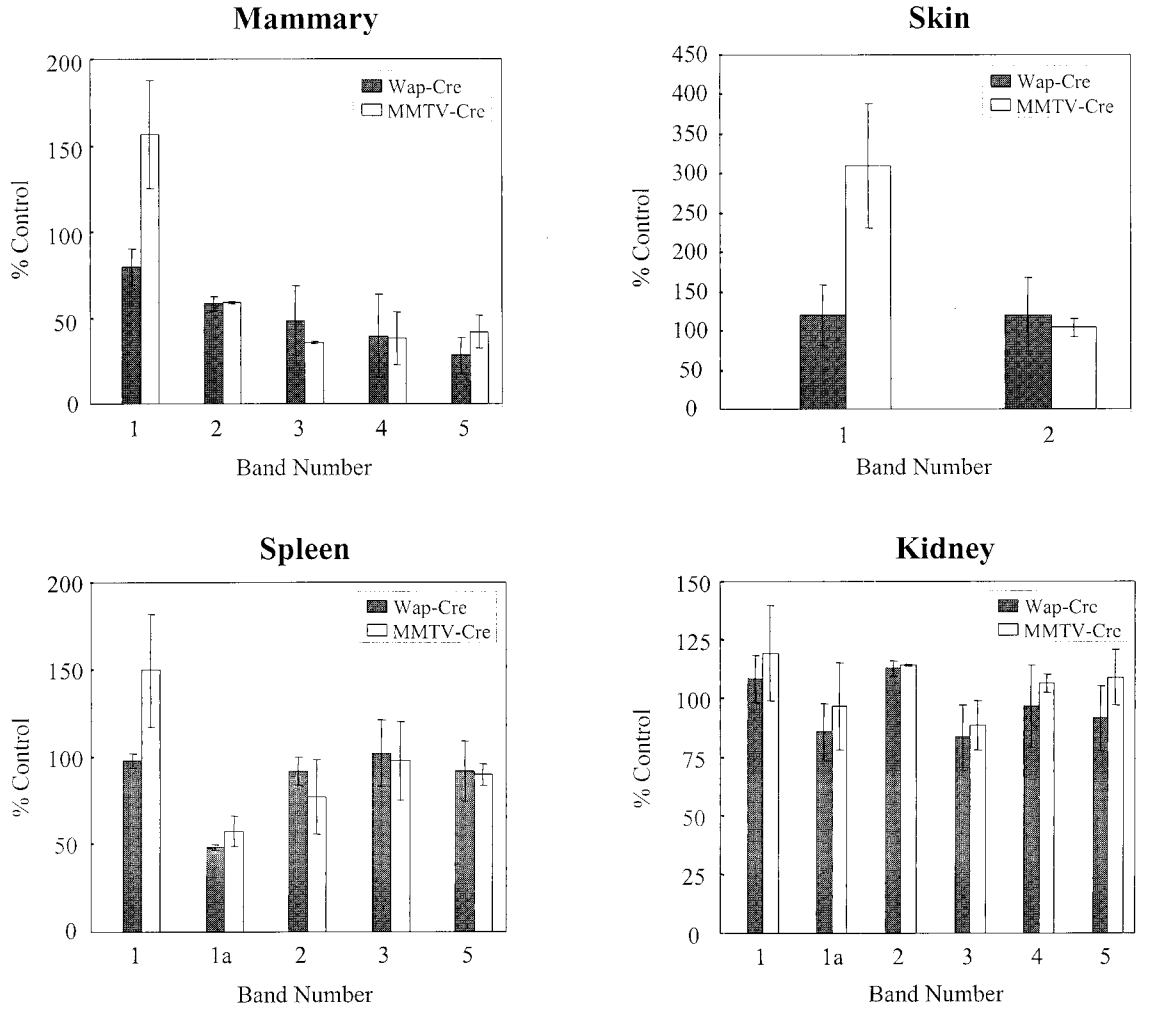

FIG. 7. Quantitation of ^{75}Se-labeled selenoproteins in tissues of mice carrying floxed *Trsp* and promoter-specific transgenes. ^{75}Se-labeled bands of selenoproteins numbered 1, 1a (in spleen and kidney only), and 2 to 5, shown in Fig. 6, and the same labeled bands from two additional experiments from different mice of the same genotype were quantitated with a PhosporImager as given in Materials and Methods. The results are reported in the figure as mean values ± standard deviation ($n = 3$). Relative amounts of each band, indicated as a bar, from mammary gland, skin, spleen, and kidney of *Trsp*$^{fl/fl}$ (control), *Trsp*$^{fl/fl}$-*WAP-Cre* (*WAP-Cre*), and *Trsp*$^{fl/fl}$-MMTV-*Cre* (MMTV-*Cre*) mice are shown.

MMTV-*Cre* mice, where TR1 was approximately 2.5 times higher than in the corresponding control tissue, and this was confirmed by direct determination of total TR activity (data not shown).

In contrast, selenoprotein expression was not affected in the kidney, as assessed both by ^{75}Se labeling (Fig. 6) and by measuring GPx1 activity (Table 3) and TR activity (data not shown). Thus, MMTV-*Cre* and *WAP-Cre* mice provide an animal model in which selenoprotein expression can be manipulated in both a selenoprotein-specific and tissue-specific manner.

As GPx1 has been shown to be highly affected during alterations of the Sec tRNA$^{[Ser]Sec}$ population (37) (Fig. 6 and 7), we used this selenoprotein as a marker to measure its activity directly to further verify the ^{75}Se labeling results and the effects of reducing the Sec tRNA$^{[Ser]Sec}$ levels in *Trsp*$^{fl/fl}$-*WAP-Cre* and *Trsp*$^{fl/fl}$-MMTV-*Cre* mice on selenoprotein synthesis (Table 3). The results of biochemical assay of GPx1 activities were similar

TABLE 3. GPx1 levels in various tissues of *Trsp*$^{fl/fl}$, *Trsp*$^{fl/fl}$-MMTV-*Cre*, and *Trsp*$^{fl/fl}$-*WAP-Cre* mice

Tissue	Genotype	Mean GPx1 activity[a] (nmol of NADPH oxidized/min) ± SD	% of control
Mammary	*Trsp*$^{fl/fl}$ (control)	47.9 ± 3.3	
	Trsp$^{fl/fl}$-MMTV-*Cre*	14.1 ± 6.6	29.4
	Trsp$^{fl/fl}$-*WAP-Cre*	18.0 ± 6.8	37.6
Skin (ear)	*Trsp*$^{fl/fl}$	70.5 ± 9.1	
	Trsp$^{fl/fl}$-MMTV-*Cre*	54.2 ± 4.8	76.9
	Trsp$^{fl/fl}$-*WAP-Cre*	79.6 ± 15.8	112.9
Spleen	*Trsp*$^{fl/fl}$	50.8 ± 10.5	
	Trsp$^{fl/fl}$-MMTV-*Cre*	34.0 ± 7.2	66.9
	Trsp$^{fl/fl}$-*WAP-Cre*	56.9 ± 9.5	112.0
Kidney	*Trsp*$^{fl/fl}$	164.9 ± 22.2	
	Trsp$^{fl/fl}$-MMTV-*Cre*	158.0 ± 18.2	95.8
	Trsp$^{fl/fl}$-*WAP-Cre*	155.3 ± 16.8	94.2

[a] Biochemical assays of GPx1 were carried out in triplicate as given previously (37).

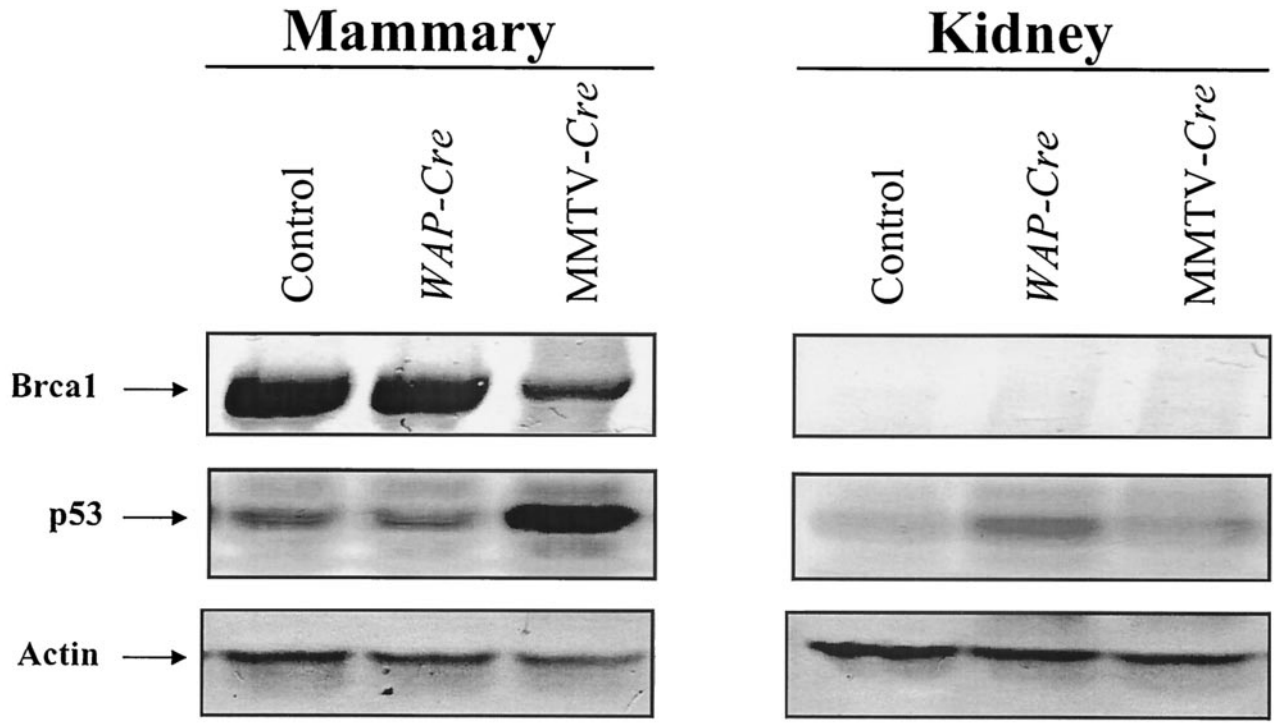

FIG. 8. Western blot analysis of protein fractions from kidney and mammary tissues of floxed *Trsp* and promoter-specific transgenes. Protein samples were separated from *Trsp*$^{fl/fl}$ (control), *Trsp*$^{fl/fl}$-*WAP-Cre* (*WAP-Cre*), and *Trsp*$^{fl/fl}$-MMTV-*Cre* (MMTV-*Cre*) mice by SDS-PAGE and blotted with antibodies against Brca1, p53, and actin (as control), and band intensities were quantitated as described in Materials and Methods. Representative Western blots are given in the figure from triplicate experiments with tissues from two different sets of animals. Brca1 was reduced by 67% ± 8%, and p53 was increased by 400% ± 20% (mean values ± standard deviation).

to those observed with ^{75}Se labeling with the exception of that in skin, which showed a slight reduction in activity.

***Brca1* and p53 cancer marker genes.** As *BRCA1* (the mouse homolog is designated *Brca1*) and p53 are significant cancer marker genes (see the introduction), we examined the levels of the corresponding proteins in mammary tissue of *Trsp*$^{fl/fl}$-MMTV-*Cre* and *Trsp*$^{fl/fl}$-*Wap-Cre* mice. Immunoblots with anti-BRCA1 antibodies showed that *Brca1* expression was decreased significantly only in mammary tissue of MMTV-*Cre* mice, as shown in Fig. 8. p53 expression was significantly upregulated in MMTV-*Cre* mice. Quantitation of these blots confirmed that *Brca1* expression was reduced by ≈3-fold and p53 expression was enhanced by ≈4-fold (see the legend to Fig. 8).

Standard *Trsp* knockout mice and Sec tRNA[Ser]Sec and selenoprotein analysis. Mice heterozygous for the loss of *Trsp* were obtained by crosses between parents carrying *Trsp*$^{fl/fl}$ and the EIIa *Cre* transgene as given in Materials and Methods. Tail DNA of the F_1 offspring was analyzed for loss of the tRNA gene by PCR with the CKNO2 and CKNO8RP primers. The mutant allele lacking *Trsp* yielded a ≈450-bp fragment, and the wild-type allele yielded a ≈900-bp fragment.

Multiple matings between mice that were heterozygous for the loss of *Trsp* yielded only wild-type or heterozygous offspring (total of 37 wild-type and 39 heterozygous were obtained from four litters). Thus, the loss of *Trsp* in our floxed mice was embryonic lethal (see also reference 5).

The levels of the Sec tRNA[Ser]Sec population in various tissues of *Trsp*$^{+/-}$ and *Trsp*$^{+/+}$ sibling mice were examined by RPC-5 chromatography and Northern blot hybridization (Table 4). The distributions of mcm^{5}U and mcm^{5}Um were examined only in kidney and liver, as shown in experiment A of the table. The distributions did not appear to vary significantly in these tissues with reduced Sec tRNA[Ser]Sec population from *Trsp*$^{+/-}$ mice compared to the corresponding control tissues from *Trsp*$^{+/+}$ mice. The levels of the Sec tRNA[Ser]Sec population were reduced only about 30% in liver, kidney, and spleen, about 20% in mammary tissue, and not at all in skin of *Trsp*$^{+/-}$ mice, as determined by Northern blot hybridization (experiment B, Table 4).

Labeling of *Trsp*$^{-/+}$ and *Trsp*$^{+/+}$ sibling mice with ^{75}Se and examination of the resulting selenoproteins in liver, kidney, spleen, mammary tissue, and skin showed no apparent changes in the selenoprotein population in these animals (data not shown). These data suggest that Sec tRNA[Ser]Sec levels are not limiting in selenoprotein biosynthesis (see also Discussion).

DISCUSSION

The present study describes the generation of mice in which the Sec tRNA[Ser]Sec gene was inactivated in specific tissues. The targeted allele encodes *Trsp*^{fl}, which can be used widely in the *loxP*/*Cre* system to remove the gene during development (4) or in a variety of tissues. Since the tRNA product of this gene governs the expression of an entire class of proteins, the selenoproteins, the ability to selectively remove *Trsp* provides

TABLE 4. Sec tRNA$^{[Ser]Sec}$ levels in *Trsp*$^{+/+}$ and *Trsp*$^{+/-}$ mice[a]

Expt	Tissue	Genotype of offspring	Sec tRNA$^{[Ser]Sec}$				
			% of total or of control	Relative amt	Distribution (%)		Ratio, mcm^{5}U/mcm^{5}Um
					mcm^{5}U	mcm^{5}Um	
A	Liver	*Trsp*$^{+/+}$	3.19	1.00	43.1	56.9	0.76
		Trsp$^{+/-}$	1.96	0.61	45.9	54.1	0.85
	Kidney	*Trsp*$^{+/+}$	4.18	1.00	44.2	55.8	0.79
		Trsp$^{+/-}$	2.89	0.69	41.0	59.0	0.69
B	Liver	*Trsp*$^{+/-}$	70.1				
	Kidney	*Trsp*$^{+/-}$	71.4				
	Spleen	*Trsp*$^{+/-}$	67.8				
	Mammary	*Trsp*$^{+/-}$	78.9				
	Skin	*Trsp*$^{+/-}$	102.7				

[a] Sec tRNA$^{[Ser]Sec}$ levels and distributions were determined by RPC-5 chromatography as described in Materials and Methods and in Table 2, footnotes *a* to *e*. The values for Sec tRNA$^{[Ser]Sec}$ levels in experiment B were determined by Northern blot analysis as described in Materials and Methods.

us with an important tool with which to perturb selenoprotein synthesis during development or in a tissue-specific manner.

Trsp was selectively removed from mammary epithelium with *Cre* recombinase that was under the control of the MMTV long terminal repeat or the *WAP* gene promoter. The two promoters target mammary epithelial cells at different times in development, with MMTV expression occurring even prior to birth, while high levels of the *WAP*-driven transgene are found only in the second half of pregnancy and lactation (42, 43). Sec tRNA$^{[Ser]Sec}$ levels were reduced by ≈70% in mammary tissue with *WAP-Cre* and ≈80% in that with MMTV-*Cre*. In turn, the selenoprotein levels were selectively reduced in mammary tissue; labeling of the selenoprotein population with ^{75}Se suggested that the amounts of GPx1, GPx4, Sep15, and an 18-kDa selenoprotein were substantially decreased, but TR1 levels were less affected. This hierarchy with respect to differential selenoprotein expression is discussed further below.

MMTV has been reported to be expressed in skin and spleen (42, 43), and indeed, the levels of the Sec tRNA$^{[Ser]Sec}$ population were substantially reduced in these tissues of *Trsp*$^{fl/fl}$-MMTV-*Cre* mice. Even though the Sec tRNA$^{[Ser]Sec}$ population was reduced less in skin than in spleen in these mice, TR1 activity was increased over twofold only in skin. This increase in activity was accompanied by a dramatic change in the distributions of the two isoforms in skin which were virtually unchanged in the spleen (Table 2). This observation suggests that the distribution of the two major Sec tRNA$^{[Ser]Sec}$ plays an important role in the expression of different selenoproteins (8, 37).

In a previous study, the amount of the wild-type Sec tRNA$^{[Ser]Sec}$ population was unaltered by the introduction of a Sec tRNA$^{[Ser]Sec}$ mutant that lacked the highly modified isopentenyladenosine base in its anticodon loop (37). The distribution of the two isoforms, however, changed dramatically in this tissue; mcm^{5}U increased and mcm^{5}Um decreased. The alteration in isoform distribution was accompanied by a substantial reduction in GPx1 in the liver, but TR1 was less affected, while TR3 was significantly enhanced. It was not clear if the alteration in selenoprotein expression was due to misreading by the isopentenyladenosine mutant Sec tRNA$^{[Ser]Sec}$ or due to a reduction in the amount of the mcm^{5}Um isoform.

During selenium deficiency in mammals, the most dramatic change in the Sec tRNA$^{[Ser]Sec}$ population involves the distribution of the two Sec isoforms: mcm^{5}U becomes more abundant and mcm^{5}Um less abundant in cells (25) and numerous tissues (8, 14). The reverse is true in the same cells and tissues when they are selenium sufficient. These observations support the possibility that GPx1 expression is linked to a Sec tRNA$^{[Ser]Sec}$ population in which mcm^{5}Um is enriched (8), while thioredoxin reductase expression is associated with enriched mcm^{5}U (37).

A hierarchy in selenoprotein expression exists in mammals, in which some selenium-containing proteins are preferentially expressed during selenium deficiency (reviewed in reference 26) or during alterations in the Sec tRNA$^{[Ser]Sec}$ population (37). The observations in the present study in which a reduction in the Sec tRNA$^{[Ser]Sec}$ population and/or an alteration in the distribution of isoforms results in an alteration of selenoprotein expression (see Table 3 and Fig. 6 and 7) provide further support that this hierarchy is directly linked to the status of the tRNA governing selenoprotein synthesis. An essential element involved in the expression of selenoproteins in mammals is a stem-loop structure in the 3′ untranslated region of their mRNAs designated the SECIS element (30). Interestingly, there are contrasting viewpoints whether the class of SECIS elements is involved in the hierarchy of selenoprotein expression (16, 31), but it would seem that the expression of certain selenoproteins may be directly linked to distributions and levels of the two Sec tRNA$^{[Ser]Sec}$ isoforms.

The effect of MMTV-*Cre*- and *WAP-Cre*-mediated recombination on Sec tRNA$^{[Ser]Sec}$ and selenoprotein levels was examined in the entire mammary gland and not specifically in the target cells, the mammary epithelium. We investigated selenoprotein levels during lactation, when approximately 90% of the mammary gland is composed of epithelium. It is therefore possible that the Sec tRNA$^{[Ser]Sec}$ could be completely removed in the epithelium of this gland and the low levels of Sec tRNA$^{[Ser]Sec}$ and altered selenoprotein synthesis observed may be contributed from other cell types in the gland, including fat, fibroblasts, and myoepithelium. Isolation of pure secretory epithelium would no doubt result in degradation of tRNA and proteins while the specific isolation of these cells was taking place. There were no phenotypic changes observed in the mammary gland with respect to generating milk or to the transfer of milk to the suckling pups, suggesting that altered selenoprotein synthesis does not play a role in these processes.

Apoptosis is a frequent phenomenon in breast cancer. It has been shown that BRCA1 may interact closely with p53 in determining whether a cell should undergo apoptosis or reversible growth arrest (32, 45). Cells with functional p53 die by apoptosis, while similar cells lacking p53 function continue to proliferate (32). Our studies showing a decrease in *Brca1* expression and an increase in p53 expression in MMTV-*Cre* mice suggest that the mice might be more susceptible to breast cancer if p53 expression was inactivated. *Brca1*, which is the mouse homolog of *BRCA1,* is crucial for the growth and development of the mouse embryo (6). It has been reported that *Brca1* homozygous deletions (−/−) are lethal early in embryonic development due to the failure of the affected cells to proliferate (22). Recent studies show that *Wap-Cre*- or MMTV-*Cre*-mediated excision of *Brca1* exon 11 in mouse mammary epithelial cells resulted in increased apoptosis (44), with a low frequency of mammary tumor formation which accelerated in the background of p53 null alleles, suggesting that genetic instability due to disruption of *Brca1* may inactivate p53 leading to tumor formation.

The relationship between selenium and p53 has been observed recently (35, 38, and references therein). Expression of p53 regulates affected selenoprotein expression in a colon cancer cell line (20), whereas one of the selenoproteins, TR1, is thought to regulate p53 activity through the redox state of thioredoxin (35). Supplementation of cells with selenium or selenomethionine activates p53 through upregulation of TR1 (38), while TR1 inhibitors repress p53 (35). However, because of contrasting patterns of expression of TR1 and GPx1 in various cancer model systems and in response to p53 expression (20), the precise relationship between p53 and selenium is not fully understood. The alteration in p53 and Brca1 levels following introduction of MMTV-*Cre* transgenes into $Trsp^{fl/fl}$ mice that was observed in the present study would appear to add another level of regulation in the relationship between selenoproteins and p53.

We also generated a standard knockout of *Trsp*. Consistent with previous findings (5), no homozygous offspring from heterozygous *Trsp* knockout parents was born, indicating that Sec $tRNA^{[Ser]Sec}$ and selenoprotein expression are essential for development. We examined $Trsp^{fl/+}$ mice in greater detail to provide a better understanding of the consequences of removing one gene copy on the Sec $tRNA^{[Ser]Sec}$ population and on selenoprotein synthesis. Despite a reduction in the Sec $tRNA^{[Ser]Sec}$ population in liver and kidney, there appeared to be little or no effect on the distribution of the two major isoforms in these tissues or on selenoprotein expression. The Sec $tRNA^{[Ser]Sec}$ population was also reduced in a number of other tissues without apparent effect on selenoprotein synthesis suggesting that the levels of this tRNA is not limiting in selenoprotein expression (5, 7, 37).

Selenium has been shown to provide enormous health benefits to mammals, such as decreasing the incidence of cancer and heart disease and delaying the aging process, as discussed in the introduction. Most certainly, the conditional knockout of *Trsp* will provide an invaluable tool with which to study the role of selenoproteins as a chemopreventive agent in a number of these diseases and under various physiological and pathological conditions. The National Cancer Institute, based on an earlier human clinical trial demonstrating a dramatic reduction in prostate cancer in males supplemented with 200 μg of selenium per day (9), recently undertook a new trial of examining the effects of selenium and/or vitamin E on prostate cancer in 32,000 men (NCI Selenium and Vitamin E Cancer Prevention Trial [SELECT], http://cancer.gov/select). This study was undertaken without knowledge of the mechanism(s) of how selenium acts at the molecular level or if selenoproteins are involved in preventing this disease. The selective reduction or loss of selenoproteins in specific tissues with *Cre*-mediated recombination undoubtedly will help elucidate the role of selenium and selenoproteins in cancer prevention.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants GM065204 and CA080946 to V.N.G. and 21C Frontier Functional Human Genome Project from the Ministry of Science and Technology of Korea to B.J.L.

REFERENCES

1. **Baum, M. K., A. Campa, M. J. Miguez-Burbano, X. Burbano, and G. Shor-Posner.** 2001. Role of selenium in HIV/AIDS, p. 247–255. *In* D. L. Hatfield (ed.), Selenium: its molecular biology and role in human health. Kluwer Academic Publishers, Norwell, Mass.
2. **Beck, M. A.** 2001. Selenium as an antiviral agent, p. 235–245. *In* D. L. Hatfield (ed.), Selenium: its molecular biology and role in human health. Kluwer Academic Publishers, Norwell, Mass.
3. **Bedell, M. A., N. A. Jenkins, and N. G. Copeland.** 1997. Mouse models of human disease. I. Techniques and resources for genetic analysis in mice. Genes Dev. **11:**1–10.
4. **Betz, U. A., C. A. Vosshenrich, K. Rajewsky, and W. Muller.** 1996. Bypass of lethality with mosaic mice generated by Cre-LoxP-mediated recombination. Curr. Biol. **6:**1307–1316.
5. **Bosl, M. R., K. Takadu, M. Oshima, S. Nishimura, and M. M. Taketo.** 1997. Early embryonic lethality caused by targeted disruption of the mouse selenocysteine tRNA gene (Trsp). Proc. Natl. Acad. Sci. USA **94:**5531–5534.
6. **Chen, Y., W. H. Lee, and H. K. Chew.** 1999. Emerging roles of BRCA1 in transcriptional regulation and DNA repair. J. Cell Physiol. **181:**385–392.
7. **Chittum, H. S., H. J. Baek, A. M. Diamond, P. Fernandez-Salguero, F. Gonzalez, T. Ohama, D. L. Hatfield, M. Kuehn, and B. J. Lee.** 1997. Selenocysteine $tRNA^{[Ser]Sec}$ levels and selenium-dependent glutathione peroxidase activity in mouse embryonic stem cells heterozygous for a targeted mutation in the $tRNA^{[Ser]Sec}$ gene. Biochemistry **36:**8634–8639.
8. **Chittum, H. S., K. E. Hill, B. A. Carlson, B. J. Lee, R. F. Burk, and D. L. Hatfield.** 1997. Replenishment of selenium deficient rats with selenium results in redistribution of the selenocysteine tRNA population in a tissue specific manner. Biochim. Biophys. Acta **1359:**25–34.
9. **Clark, L. C., G. F. Combs Jr., B. W. Turnbull, E. H. Slate, D. K. Chalker, J. Chow, L. S. Davis, R. A. Glover, G. F. Graham, E. G. Gross, A. Krongrad, J. L. Lesher, Jr., H. K. Park, B. B. Sanders, Jr., C. L. Smith, and J. R. Taylor.** 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. JAMA **276:**1957–1963.
10. **Combs, G. R., and L. Liu.** 2001. Selenium as a cancer preventative agent, p. 205–217. *In* D. L. Hatfield (ed.), Selenium: its molecular biology and role in human health. Kluwer Academic Publishers, Norwell, Mass.
11. **Coppinger, R. J., and A. M. Diamond.** 2001. Selenium deficiency and human disease, p. 219–233. *In* D. L. Hatfield (ed.), Selenium: its molecular biology and role in human health. Kluwer Academic Publishers, Norwell, Mass.
12. **Cui, Y., K. M, Miyoshi, E. Claudio, U. K. Siebenlist, F. J. Gonzalez, J. Flaws, K.-U. Wagner, and L. Hennighausen.** 2002. Loss of the peroxisome proliferation-activated receptor gamma (PPARγ) does not affect mammary development and propensity for tumor formation but leads to reduced fertility. J. Biol. Chem. **277:**17830–17835.
13. **Deng, C.-X., and S. G. Brodie.** 2000. Roles of BRCA1 and its interacting proteins. BioEssays **22:**728–737.
14. **Diamond, A. M., I. S. Choi, P. F. Crain, T. Hashizume, S. C. Pomerantz, R. Cruz, C. Steer, K. E. Hill, R. F. Burk, J. A. McCloskey, and D. L. Hatfield.** 1993. Dietary selenium affects methylation of the wobble nucleoside in the anticodon of selenocysteine $tRNA^{[Ser]Sec}$. J. Biol. Chem. **268:**14215–14223.
15. **Evan, G., and T. Littlewood.** 1998. A matter of life and cell death. Science **281:**1317–1322.
16. **Fletcher, J. E., P. R. Copeland, D. M. Driscoll, and A. Krol.** 2001. The selenocysteine incorporation machinery: interactions between the SECIS RNA and the SECIS-binding protein SBP2. RNA **7:**1442–1453.
17. **Flohe, L., R. Brigelius-Flohe, M. Maiorino, A. Roveri, J. Wissing, and F. Ursini.** 2001. Selenium and male reproduction, p. 273–281. *In* D. L. Hatfield

(ed.), Selenium: its molecular biology and role in human health. Kluwer Academic Publishers, Norwell, Mass.
18. **Futreal, P. A., Q. Liu, D. Shattuck-Eidens, C. Cochran, K. Harshman, S. Tavtigian, L. M. Bennett, A. Haugen-Strano, J. Swensen, Y. Miki, et al.** 1994. BRCA1 mutations in primary breast and ovarian carcinomas. Science **266:** 120–122.
19. **Gladyshev, V. N., K.–T. Jeang, J. C. Wootton, and D. L. Hatfield.** 1998. A new human selenium-containing protein. J. Biol. Chem. **273:**8910–8915.
20. **Gladyshev, V. N., V. M. Factor, F. Housseau, and D. L. Hatfield.** 1998. Contrasting patterns of regulation of the antioxidant selenoproteins, thioredoxin reductase, and glutathione peroxidase, in cancer cells. Biochem. Biophys. Res. Commun. **251:**488–493.
21. **Gladyshev, V. N., T. C. Stadtman, D. L. Hatfield, and K.-T. Jeang.** 1999. Levels of major selenoproteins in T cells decrease during HIV infection and low-molecular-mass selenium compounds increase. Proc. Natl. Acad. Sci. USA **96:**835–839.
22. **Gowen, L. C., B. L. Johnson, A. M. Latour, K. K. Sulik, and B. H. Koller.** 1996. Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. Nat. Genet. **12:**191–194.
23. **Gunning, P., T. H. Leavitt, G. Muscat, S. Y. Ng, and L. Kedes.** 1987. A human β-actin expression vector system directs high-level accumulation of antisense transcripts. Proc. Natl. Acad. Sci. USA **84:**4831–4835.
24. **Hatfield, D. L., C. R. Mathews, and M. Rice.** 1979. Aminoacyl-transfer RNA populations in mammalian cells: chromatographic profiles and patterns of codon recognition. Biochim. Biophys. Acta **564:**414–423.
25. **Hatfield, D. L., B. J. Lee, L. Hampton, and A. M. Diamond.** 1991. Selenium induces changes in the selenocysteine $tRNA^{[Ser]Sec}$ population in mammalian cells. Nucleic acids Res. **19:**939–943.
26. **Hatfield, D. L., and V. N. Gladyshev.** 2002. How selenium has altered our understanding of the genetic code. Mol. Cell. Biol. **22:**3565–3576.
27. **Kelmers, A. D., and D. E. Heatherly.** 1971. Columns for rapid chromatographic separation of small amounts of tracer-labeled transfer ribonucleic acids. Anal. Biochem. **44:**486–495.
28. **Kwan, K.-M.** 2002. Conditional alleles in mice: practical considerations for tissue-specific knockouts. Genesis **32:**49–62.
29. **Le Provost, F., G. Riedlinger, S. H. Yim, J. Benedict, F. J. Gonzalez, J. Flaws, and L. Hennighausen.** 2002. The aryl hydrocarbon receptor (AhR) and its nuclear translocator (Arnt) are dispensable for normal mammary gland development but are required for fertility. Genesis **32:**231–239.
30. **Low, S. C., and M. J. Berry.** 1996. Knowing when not to stop: selenocysteine incorporation in eukaryotes. Trends Biochem. Sci. **21:**203–208.
31. **Low, S. C., E. Grundner-Culemann, J. W. Harney, and M. J. Berry.** 2000. SECIS-SBP2 interactions dictate selenocysteine incorporation efficiency and selenoprotein hierarchy. EMBO J. **19:**6882–6890.
32. **MacLachlan, T. K., K. Somasundaram, M. Sgagias, Y. Shifman, R. J. Muschel, K. H. Cowan, and W. S. El-Deiry.** 2000. BRCA1 effects on the cell cycle and the DNA damage response are linked to altered gene expression. J. Biol. Chem. **275:**2777–2785.
33. **McKenzie, R. C., T. S. Rafferty, G. J. Beckett, and J. R. Arthur.** 2001. Effects of selenium on immunity and aging, p. 257–272. *In* D. L. Hatfield (ed.), Selenium: its molecular biology and role in human health. Kluwer Academic Publishers, Norwell, Mass.
34. **Miki, Y., J. Swensen, D. Shattuck-Eidens, P. A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L. M. Bennett, W. Ding, et al.** 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science **266:**66–71.
35. **Moos, P. J., K. Edes, P. Cassidy, E. Massuda, and F. A. Fitzpatrick.** 2003. Electrophilic prostaglandins and lipid aldehydes repress redox sensitive transcription factors p53 and HIF by impairing the selenoprotein thioredoxin reductase. J. Biol. Chem. **278:**745–750.
36. **Moustafa, M. E., M. A. El-Saadani, K. M. Kandeel, D. B. Mansur, B. J. Lee, D. L. Hatfield, and A. M. Diamond.** 1998. Overproduction of selenocysteine tRNA in Chinese hamster ovary cells following transfection of the mouse $tRNA^{[Ser]Sec}$ gene. RNA **4:**1436–1443.
37. **Moustafa, M., B. A. Carlson, M. A. El-Saadani, G. V. Kryukov, Q.-I. Sun, J. W. Harney, K. E. Hill, G. F. Combs, L. Feigenbaum, D. B. Mansur, R. F. Burk, M. J. Berry, A. M. Diamond, B. J. Lee, V. N. Gladyshev, and D. L. Hatfield.** 2001. Selective inhibition of selenocysteine tRNA maturation and selenoprotein synthesis in transgenic mice expressing isopentenyladenosine-deficient selenocysteine tRNA. Mol. Cell. Biol. **21:**3840–3852.
38. **Seo, Y. R., M. R. Kelley, and M. L. Smith.** Selenomethionine regulation of p53 by a ref1-dependent redox mechanism. Proc. Natl. Acad. Sci. USA **99:**14548–14553.
39. **Sun, Q.-A., Y. Wu, F. Zappacosta, K.-T. Jeang, B. J. Lee, D. L. Hatfield, and V. N. Gladyshev.** 1999. Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. J. Biol. Chem. **274:**24522–24530.
40. **Sun, Q.-A., F. Zappacosta, V. M. Factor, P. J. Wirth, D. L. Hatfield, and V. N. Gladyshev.** 2001. Heterogeneity within animal thioredoxin reductases. Evidence for alternative first exon splicing. J. Biol. Chem. **276:**3106–3114.
41. **Venkitaraman, A. R.** 2002. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell **108:**171–182.
42. **Wagner, K.-U., R. J. Wall, L. St-Onge, P. Gruss, A. Wynshaw-Boris, L. Garrett, M. Li, P. A. Furth, and L. Hennighausen.** 1997. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. **25:**4323–4330.
43. **Wagner, K.-U., K. McAllister, T. Ward, B. Davis, R. Wiseman, and L. Hennighausen.** 2001. Spatial and temporal expression of the Cre gene under the control of the MMTV-long terminal repeat in different lines of transgenic mice. Transgenic Res. **10:**545–553.
44. **Xu, X., K. U. Wagner, D. Larson, Z. Weaver, C. Li, T. Ried, L. Hennighausen, A. Wynshaw-Boris, and C. X. Deng.** 1999. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat. Genet. **22:**37–43.
45. **Zhang, H., K. Somasundaram, Y. Peng, H. Tian, H. Zhang, D. Bi, B. L. Weber, and W. S. El-Deiry.** 1998. BRCA1 physically associates with p53 and stimulates its transcriptional activity. Oncogene **16:**1713–1721.