Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 64, No. 5–6, pp. 239–244, 1998
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Printed in Great Britain
PII: S0960-0760(97)00201-X
0960-0760/98 $19.00 + 0.00

Selenium Regulates Gene Expression for Estrogen Sulfotransferase and Alpha 2U-globulin in Rat Liver

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Dietary intake of the essential trace element selenium (Se) regulates expression of genes for selenoproteins and certain non-Se-containing proteins. However, these proteins do not account for all of Se's biological effects. The objective of this work was to identify additional genes whose expression is regulated by Se. Identification of these genes may reveal new functions for Se or define mechanisms for its biological effects. Weanling male Sprague–Dawley rats were fed a Torula yeast-based Se-deficient basal diet or the same diet supplemented with 0.5 mg Se/kg diet as sodium selenite for 13 weeks. Total RNA was used as template for RNA fingerprinting. Two differentially expressed cDNA fragments were identified and cloned. The first had 99% nucleotide identity with rat liver estrogen sulfotransferase (EST) isoform-6. The second had 99% nucleotide sequence identity with rat liver α 2u-globulin. The mRNA levels for both were markedly reduced in Se deficiency. Laser densitometry showed that EST mRNA in Se deficiency was 7.3% of that in Se-adequate rat liver. The level of α 2u-globulin mRNA in Se-deficient rat liver was only 12.6% of that in Se-adequate rat liver. These results indicate that dietary Se may play a role in steroid hormone metabolism in rat liver. **© 1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

The essential trace element selenium (Se) functions biochemically as a component of selenoproteins, in which Se is incorporated into the primary structure of the protein as selenocysteine, encoded by a TGA opal codon [1]. Dietary intake of Se regulates the expression of genes for selenoproteins. These include the genes for members of the glutathione peroxidase (GPX) family (EC 1.11.1.9), type I iodothyronine 5′-deiodinase (EC 3.8.1.4.), the plasma glycoprotein selenoprotein P, and selenoprotein W from muscle. For these selenoproteins, dietary Se has no effect on gene transcription, but stabilizes the steady state levels of mRNA [2, 3]. The extent of induction of selenoprotein genes by Se varies among different genes and tissues [4]. Intake of Se also affects expression of genes for non-selenoproteins, including the genes for subunits of the glutathione S-transferase family (EC 2.5.2.18) [5], transthyretin and citrate transport protein in rat liver [6].

While some biological effects of Se can be attributed to the activity of selenoenzymes or other selenoproteins, many can not. The effects of Se on hepatic heme metabolism [7], glutathione metabolism [8], cytochrome P-450 induction [9], activity of drug metabolizing enzymes [10], heavy metal toxicity [11], immune function [12], and cancer chemoprevention [13] have all been shown to be independent of GPX activity. It is likely that Se regulates expression of other genes not yet identified. Identification of such genes whose expression depends on dietary intake of Se may reveal new functions for Se, or explain some of the biological effects of Se for which the mechanisms at present are obscure. Therefore, it was the objective of this work to identify other genes whose expression in rat liver is regulated, at least in part, by dietary Se intake.

RNA fingerprinting [14] is a recently developed technique that identifies genes expressed at different levels under different conditions. Using this method we identified cDNAs for genes in rat liver whose expression varies with differences in Se intake. Differentially expressed cDNAs from the livers of rats fed Se-adequate or Se-deficient diets were cloned and

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Received 13 Feb. 1997; accepted 12 Nov. 1997.

analyzed. Our results demonstrate that expression of the genes for estrogen sulfotransferase (EST) and α 2u-globulin is markedly reduced in male rat liver by Se deficiency.

MATERIALS AND METHODS

Rats

Weanling male Sprague–Dawley rats (SASCO, Omaha, NE) were housed in pairs in stainless steel hanging wire cages in a temperature- and light-controlled room (12 h light: 12 h dark). Animals were given free access to food and tap water. All procedures related to animal use were approved by the Brigham Young University Animal Care and Use Committee.

A powdered Torula yeast-based Se-deficient diet was used as the basal diet [2, 15]. Six animals received this basal diet and six received the same diet supplemented with 0.5 mg Se/kg diet as sodium selenite for 13 weeks. There were no significant differences in weight gain between the two dietary groups. Animals were sacrificed by decapitation under ether anaesthesia. Livers were immediately removed, frozen in liquid nitrogen, and stored at −80°C.

RNA preparation

Total RNA was isolated from rat liver with TRIzol Reagent (Gibco, Gaitherburg, MD), a monophasic solution of phenol and guanidine isothiocyanate based on the method of Chomczynski and Sacchi [16]. Equal aliquots of RNA from three livers in each dietary group were combined to form a Se-adequate and a Se-deficient RNA pool. Each RNA pool was treated with RNase-free DNase I (Promega, Madison, WI) to remove contaminating DNA.

RNA fingerprinting

The Delta RNA Fingerprinting Kit (CLONTECH Laboratories, Palo Alto, CA) was used to identify differentially expressed genes. Two micrograms of total RNA were reverse transcribed with M-MLV reverse transcriptase using oligo-dT as a primer in 10 μl reactions. Single stranded cDNA products were diluted 10 and 40 times with water and used as templates in PCR reactions. One microliter of each diluted reverse transcription product was amplified in 20 μl reactions containing 1.5 mM $MgCl_2$, 50 nM dNTPs, 50 nM [α-^{33}P]dATP, 50 U/ml AmpliTaq (Perkin Elmer, Norwalk, CT) and 0.33 U/ml Deep Vent DNA Polymerase (New England Biolabs, Beverly, MA). The cycling parameters were as follows: one cycle of 94°C for 5 min, 40°C for 5 min and 68°C for 5 min; two cycles of 94°C for 2 min, 40°C for 5 min and 68°C for 5 min; 25 cycles of 94°C for 1 min, 60°C for 1 min and 68°C for 2 min; a final extension at 68°C for 7 min.

Amplified cDNAs were electrophoresed through 5% denaturing polyacrylamide sequencing gels (National Diagnostics, Atlanta, GA). Gels were dried and exposed to autoradiography film. Autoradiograms were analyzed for differences in band intensities between cDNAs amplified from Se-adequate RNA and cDNAs amplified from Se-deficient RNA. Bands of interest were cut from the gels using the autoradiograms as templates. The cDNA fragments were eluted from the gel slices by incubation in 40 μl of sterile H_2O at 100°C for 5 min. Seven μl were then used in PCR reactions to reamplify the eluted cDNA fragments using the same primers that amplified those fragments in the original reactions. Cycling parameters were as following: 94°C for 1 min, 60°C for 1 min and 68°C for 2 min for 20 cycles. Reamplified cDNA fragments were aliquoted for cloning and for use as probes in Northern blots as described below.

Cloning and sequencing of cDNA fragments

Reamplified cDNAs were cloned into the pCRII vector using the TA cloning kit (Invitrogen, San Diego, CA). Plasmid minipreps were performed using the QIAprep Spin Plasmid Kit (QIAGEN, Chatsworth, CA). Plasmid inserts were sequenced using the T7 Sequenase Quick-Denature Plasmid Sequencing Kit (Amersham Life Science, Cleveland, OH). The sequences were compared to sequences in GenBank and other data bases using the National Library of Medicine Blast programs.

Inserts of cDNA to be used as probes in Northern blots to confirm differential expression were isolated from plasmids by restriction digestion with *Eco*RI, followed by gel purification with the QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA).

Northern blot analysis

Northern blots of the original RNA were first probed with cDNA fragments reamplified from the bands cut from the sequencing gels. Callard *et al.* [17] noted that bands cut from such gels may contain several comigratory species. We used the affinity capture Northern blot method of Li *et al.* [18] to determine if our reamplified cDNAs consisted of one or more species. In each of the cases reported herein, reamplified cDNAs hybridized to only one species on Northern blots. After cloning and sequencing, these cDNA inserts were cut from the plasmid and used in a second Northern blot to confirm differential expression.

Twenty micrograms of total RNA from each pool were electrophoresed and blotted to MagnaCharge nylon membranes (MSI, Westboro, MA). The cDNAs used as probes were labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) using the DECAPrime II DNA Labeling Kit (Ambion, Austin, TX). Blots were hybridized and washed according to the instructions of the mem-

brane manufacturer, and exposed to Kodak BioMax MS film at −80°C. Following exposure, membranes were stripped and reprobed with a cDNA for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) to control for minor differences in RNA loading and transfer. Autoradiograms of Northern blots were analyzed using laser densitometry. Intensity of signals for differentially expressed cDNAs were normalized to the intensity of their corresponding signal for G3PDH.

RESULTS

Identification of the differentially expressed cDNA fragments

Figure 1 shows portions of autoradiograms of cDNA fragments amplified from Se-adequate and Se-deficient rat liver RNA pools. Figure 1(A) shows bands amplified using primers P4 and P9 from the DELTA RNA Fingerprinting Kit (sequence of primer P4: 5′-ATTAACCCTCACTAAATGCTGGTAG-3′; sequence of primer P9: 5′-ATTAACCCTCACTAAATGTGGCAGG-3′). Figure 1(B) shows results from amplification with primers P2 and P7 from the same kit (sequence of primer P2: 5′-ATTAACCCTCACTAAATGCTGGAGG-3′; sequence of primer P7: 5′-ATTAACCCTCACTAAATGCGTGGGTG-3′). The arrows indicate the cDNA bands whose intensity is greater in the lanes of bands derived from Se-adequate rat liver RNA than in the lanes of bands from Se-deficient rat liver RNA.

Northern blot analysis

Figure 2 shows the results of Northern blots run to confirm the differential expression of the newly identified cDNAs. The two bands shown in Fig. 1 (P4P9 and P2P7) were cut from the gel, eluted, reamplified and cloned into the pCRII vector. The inserts were restricted and used as the probes in Northern blots of the original RNA pools. A cDNA for G3PDH was used as a control probe. Laser densitometry of the P4P9 and G3PDH bands showed that normalized signal intensity of the mRNA in Se-deficient rat liver was only 7.3% of that found in Se-adequate rat liver. Normalized signal intensity of the P2P7 band in Se-deficient rat liver was 12.6% of that in Se-adequate rat liver.

Sequence comparison

The P4P9 fragment was 733 base pairs long and included at its ends the sequences of the two PCR primers. Excluding the primers, P4P9 consisted of a single open reading frame. Table 1 shows the results of nucleotide and deduced amino acid homology searches using the P4P9 sequence as a query in the GenBank and other data bases. As shown in the table, 705 bases of P4P9 aligned almost perfectly (only 4 mismatches) with 705 bases of a cDNA for rat liver estrogen sulfotransferase (EST) isoform 6. One of the 4 mismatches was in one of the PCR primers at the end of the P4P9 sequence. The deduced amino acid sequence of P4P9 differs from EST isoform 6 by only one, at position 144 in EST isoform 6 (EST6: aspartate vs P4P9: glycine). As shown in Table 1, nucleotide matches and deduced amino acid sequence matches were almost as good for other isoforms of EST in male rat liver. In each case some of the nucleotide mismatches were found in the PCR primers incorporated into the ends of the P4P9 fragment.

The P2P7 fragment was 490 base pairs long, including the PCR primers. Table 2 shows the results of nucleotide and deduced amino acid homology searches using the P2P7 sequence as a query in the

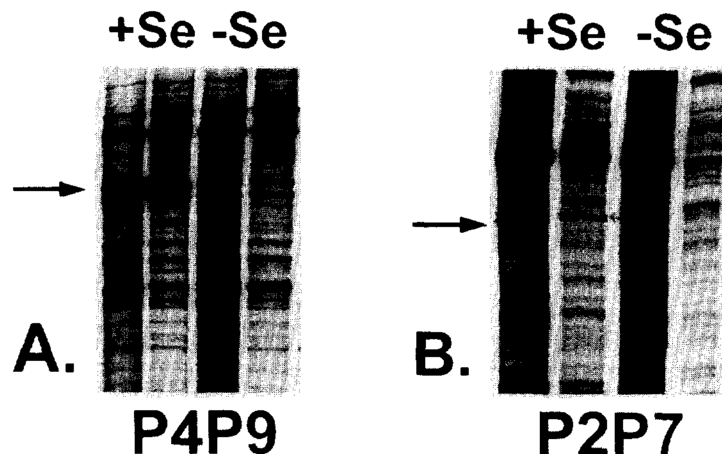

Fig. 1. Sections of the sequencing gels showing cDNA fragments amplified with two arbitrary primers in RNA fingerprinting experiments using total RNA from Se-adequate (+Se) and Se-deficient (−Se) rat livers. The arrows show cDNAs whose expression appears to be greater in +Se lanes than in −Se lanes. "P4P9" and "P2P7" refer to the different pairs of primers from the RNA Fingerprinting Kit (Clontech) used to amplify the cDNAs shown.

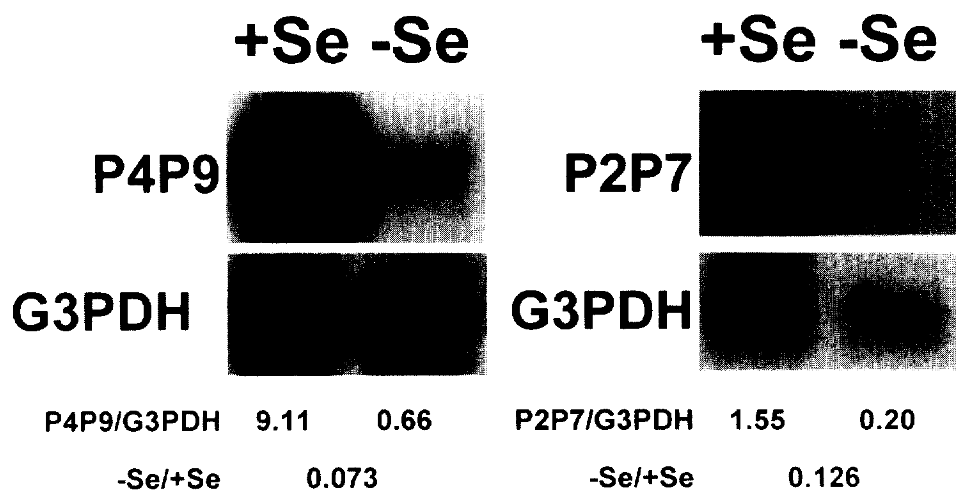

Fig. 2. Results of Northern blots probed with the bands shown in Fig. 1(A) and Fig. 1(B). Bands were eluted, PCR-amplified, radioactively labeled and used as probes in Northern blots of total rat liver RNA. After exposure, blots were stripped and reprobed with a cDNA for glyceraldehyde 3-phosphate dehydrogenase (G3PDH). Intensity of bands was quantitated by laser densitometry. Measured intensities (in arbitrary units) and the ratios of those intensities are listed.

GenBank and other data bases. A segment of 459 bases from the P2P7 sequence matched, with only 3 exceptions, a segment of equal length from a cDNA for rat α 2u-globulin (*S* type). Two of the 3 mismatches between the P2P7 and α 2u-globulin (*S* type) sequences were found in the PCR primers included at the ends of P2P7. This was the case for other close matches with different published cDNA sequences for rat liver α 2u-globulins (98–99% nucleotide and amino acid identity). Most of the nucleotide mismatches were found in the PCR primers incorporated into the ends of the P2P7 fragment. Nucleotide mismatches in the coding regions accounted for differences of one or two amino acids between the deduced amino acid sequence for P2P7 and the published sequences for rat liver α 2u-globulins.

DISCUSSION

Products of the sulfotransferase gene superfamily catalyze sulfate conjugation of drugs, steroid hormones, neurotransmitters, bile acids and xenobiotics [20]. Cytosolic estrogen sulfotransferase (EST) uses 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the donor in transferring sulfate groups to estrone and estradiol, the active forms of estrogen. Sulfoconjugation decreases the binding of these steroids to their receptor and reduces their biological activity [21]. Reiter and Wendel [10] previously showed that sulfotransferase activity was reduced by Se deficiency in mouse liver to 62% of Se-adequate control values. However, it is difficult to determine the contribution of EST to this decrease due to the overlap in specificities that exists among various sulfotransferases for substrates used in enzyme assays [22].

We have identified EST and α 2u-globulin as two proteins whose levels of mRNA are dramatically reduced in rat liver by dietary Se deficiency. For EST, the level of mRNA in Se-deficient rat liver was only 7.3% of the Se-adequate level. For rat α 2u-globulin, the Se-deficient mRNA level was 12.6% of the Se-adequate value. Previous work showed that the percentage reduction in steady state levels of mRNA in Se deficiency varies among different Se-regulated genes [4, 5]. The gene whose expression is most reduced by Se deficiency is that for cytosolic glutathione peroxidase (GPX). In Se-deficient male rat liver GPX mRNA levels are just 4–11% of Se-adequate values [4, 19]. The difference in mRNA levels

Table 1. P4P9 sequence comparisons

Species (cDNA)	% nucleotide identity	% amino acid identity	Nucleotide mismatches in primers
Rat liver EST, isoform 6	99 (701/705)	99	1
Rat liver EST, STE2	99 (703/717)	98	5
Rat liver EST, STE1	98 (693/705)	95	1
Rat liver EST	97 (698/717)	97	6
Rat liver EST, isoform 3	97 (696/717)	95	6
Mouse testis EST	89 (645/719)	87	11
Human liver EST	77 (561/724)	67	14

EST: estrogen sulfotransferase.

Table 2. P2P7 sequence comparisons

Species (cDNA)	% nucleotide identity	% amino acid identity	Nucleotide mismatches in primers
Rat α 2u-globulin (*L* type)	99 (459/464)	99	2
Rat hepatic α 2u-globulin	99 (457/461)	99	2
Rattus rattus α 2u-globulin	98 (461/471)	98	7
Rat submaxillary gland α 2u-globulin	93 (431/463)	86	2

between Se-adequate and Se-deficient male rat liver for EST and α 2u-globulin is comparable to that observed for GPX.

The mechanisms by which Se intake may regulate expression of EST remain to be explored. The expression of EST in rat liver has been shown to be regulated by androgen, progesterone and thyroid hormones [21,23]. These observations suggest an indirect means by which Se may regulate EST gene expression, as depicted in Fig. 3. Selenium plays a role in thyroid hormone metabolism by means of its incorporation into type *I* iodothyronine 5′-deiodinase [1]. This enzyme catalyzes the conversion in peripheral tissues of the prohormone thyroxin (T4) to its active form, 3,5,3′-triiodothyronine (T3). Selenium deficiency reduces mRNA levels and activity of this enzyme resulting in lower blood and tissue levels of T3 and higher levels of T4 [24,25]. Borthwick *et al.* [23] showed that i.p. injection of T4 reduced sulfotransferase activity toward estrone and estriol by half in male rat liver. Hence, the reduction we observed in male rat liver EST mRNA may be due in part to changes in thyroid hormone metabolism resulting from Se deficiency.

An alternative or additional, direct means by which Se may affect EST gene expression is suggested by previous work in our laboratory. In rat liver, Se regulation of gene expression for selenoproteins is accomplished post-transcriptionally [2–4]. However, we have also shown that binding of nuclear proteins to transcription regulatory elements is altered in Se-deficiency [26]. For example, binding of nuclear proteins to the AP-1 transcription element is reduced in Se-deficient rat liver. The existence of an AP-1 sequence (TGACTCA) in the EST 5′-flanking region may explain in part the regulation of this gene by Se. The decreased levels of mRNA for EST may be due to reduced binding of transcription regulatory elements to the AP-1 sequence secondary to Se deficiency.

Alpha 2u-globulin is one of the major proteins synthesized in the liver of male rats and accounts for more than 50% of male urinary proteins [27]. Its mRNA comprises 1–2% of total hepatic mRNA. Its function is unknown. Its expression in rat liver is controlled by multiple hormones, most likely at the level of gene transcription [28]. Androgen, glucocorticoid, insulin and growth hormone all increase α 2u-globulin synthesis while estrogen inhibits it [29,30]. Figure 4 outlines a process, consistent with the reports of others and with results obtained in this work, by which Se deficiency may reduce α 2u-globulin mRNA. As noted above, in Se-deficient rat liver expression of EST is dramatically reduced. This may result in reduced sulfation of estrogen, leading to higher levels of free, metabolically active hormone. Increased free estrogen would decrease synthesis of α 2u-globulin as previously reported by other

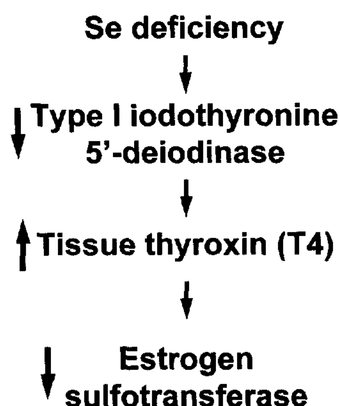

Fig. 3. Schematic of the means by which Se deficiency may reduce EST gene expression. Deficiency of Se decreases activity of iodothyronine 5′-deiodinase [1], which results in increased tissue levels of thyroxin [24,25]. Intraperitoneal injection of thyroxin was shown by others [23] to decrease liver EST.

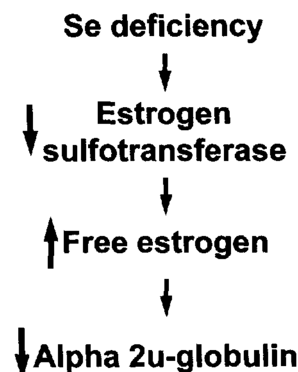

Fig. 4. Schematic of the means by which Se deficiency may reduce α 2u-globulin gene expression. Deficiency of Se decreases EST gene expression [this work] and sulfotransferase activity [10]. Decreased sulfotransferase activity would reduce estrogen sulfation, leaving a comparatively larger fraction of active hormone. Estrogen inhibits α 2u-globulin synthesis [29, 30].

investigators [29, 30]. Thus, the effect of Se deficiency on mRNA levels for α 2u-globulin could be mediated through its effects on EST expression. Also as noted above, Se deficiency reduces binding of nuclear proteins to the AP-1 transcription regulatory element. An AP-1 sequence is found in the first intron of the α 2u-globulin gene. The possibility exists that Se deficiency may exert a direct effect on α 2u-globulin gene transcription by reducing nuclear protein binding to one of its activator sequences.

In summary, these findings suggest a role for the essential trace element Se in steroid hormone metabolism. The effect of Se deficiency on mRNA levels for EST is consistent with Se's previously demonstrated role in the metabolism of thyroid hormone, a known regulator of EST expression. The results obtained for α 2u-globulin are consistent with and provide confirmation of an effect of Se deficiency on EST gene expression. Genes for both proteins include an AP-1 regulatory element to which binding is reduced in Se deficiency. Although prior experimentation offers several alternatives to explain the results obtained in this work, elucidation of the precise mechanism(s) by which Se deficiency reduces levels of mRNA for EST and α 2u-globulin awaits the results of future studies.

Acknowledgements—The authors thank Todd C. Hansen of the University of Chicago Pritzker School of Medicine for his technical assistance and encouragement.

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