Functional expression of rat thioredoxin reductase: selenocysteine insertion sequence element is essential for the active enzyme

Noriko FUJIWARA, Tsuneko FUJII, Junichi FUJII and Naoyuki TANIGUCHI¹

Department of Biochemistry and Molecular Biology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Mammalian thioredoxin reductase (TR) is a flavoprotein catalysing reduction of oxidized thioredoxin in an NADPH-dependent manner, and contains a selenocysteine (Sec) residue near the C-terminus. We observed that TR activity was decreased in A549 cells by the lowering of the fetal bovine serum content in the culture medium and was recovered by the addition of selenium. To study the role of Sec in TR activity, we have isolated a full-length clone of the rat TR cDNA (3.3 kb) and have expressed it in COS-1 cells in a transient-expression system. TR activities in COS-1 cells expressing rat TR were increased in accordance with supplemented sodium selenite concentrations, whereas levels of TR protein, examined by Western blotting, were not affected by sodium selenite concentrations. We introduced various deletions into the 3'-untranslated region of the TR cDNA to localize and

examine the role of a Sec insertion-sequence (SECIS) element in the functional expression of TR. TR activities were observed only in COS-1 cells transfected with the TR cDNAs containing the putative SECIS element located between 1856 and 1915 bp in the correct orientation. We also carried out radiolabelling of proteins by incubation of the cDNA-transfected cells with sodium [⁷⁵Se]selenite. ⁷⁵Se was incorporated into the expressed TR protein of the cells transfected with the SECIS element-containing cDNAs, but not into those without the SECIS element or with an inverted SECIS element. These data clearly showed a requirement of selenium for the formation of functional TR protein.

Key words: glutathione peroxidase, redox regulation, selenium.

INTRODUCTION

Selenium is an essential trace element in biological systems. Low selenium intake correlates with an increased incidence of a variety of cancers [1]. A local disease found in the Keshan district in China is well known to be caused by a deficiency of selenium in the diet [2]. Selenium is found exclusively as selenocysteine (Sec) in high-molecular-mass compounds [3]. The Sec residue has been found in seven families of mammalian gene products so far. These are glutathione peroxidases (GPXs) [4], iodothyronine hormone deiodinases [5], selenophosphate synthetase 2 [6], selenoprotein P [7], selenoprotein W [8], thioredoxin reductases (TRs) and a newly found 15-kDa protein [9]. Since the selenol group of Sec is more active than the thiol group of Cys at physiological pH, due to its low pK_a value (5.2 for the selenol group and 8.5–8.7 for the thiol group in free amino acids), proteins containing the Sec residue are more reactive and often involved in redox systems.

TR is a member of the nucleotide disulphide oxidoreductase family [10]. It catalyses the reduction of oxidized thioredoxin (TRX), with NADPH as an electron donor. TRX is a well-known electron donor to various systems, including enzymes such as ribonucleotide reductase and transcription factors such as nuclear factor-κB and activator protein 1 [11,12]. Recently, it has been shown that reduced TRX also functions as an anti-apoptotic molecule via binding to apoptosis-signalling kinase-1, which is a member of the mitogen-activated kinase superfamily [13]. Accumulated evidence suggests that a novel gene superfamily, the TRX-dependent peroxidases, also called peroxi-

redoxins, are expressed in fairly large amounts in various cells, and are involved in the detoxification of hydrogen peroxide and the protection of essential thiol groups of proteins [14]. Although bacterial TR has been well characterized and the crystallographic structure is known, mammalian TRs have only been cloned quite recently [15,16]. Structural features predicted from the primary structure of mammalian TR are totally different from the bacterial protein, and instead it has a marked similarity to glutathione reductase [17]. The similarity of their reaction mechanisms was also predicted from kinetics studies [17–20]. Mammalian TR is also a flavoprotein, like glutathione reductase, but only TR contains a Sec residue at the extended C-terminus with the sequence Gly-Cys-Sec-Gly [21,22]. Since Sec is encoded by UGA, which is generally recognized as a stop codon, a specific mechanism exists to incorporate Sec at the position encoded by the UGA [23]. It has been shown that all mRNAs encoding mammalian proteins containing Sec have a specific element called Sec insertion sequence (SECIS) in the 3'-untranslated region [3]. The distance between the UGA codon and the element in the 3'-untranslated region can be variable [24]. None of the selenoproteins can be expressed in their active form without this element [25].

Although several reports suggest that Sec is an essential residue for the catalytic reaction of mammalian TR, the functional expression of the enzyme has not yet been reported [15,19]. In this paper, we describe the expression of active rat TR in COS-1 cells for the first time, and show the requirement for incorporation of the Sec residue into the polypeptide for TR enzymic activity. We also localized the position of the SECIS

Abbreviations used: Sec, selenocysteine; TRX, thioredoxin; TR, thioredoxin reductase; SECIS, Sec insertion sequence; GPX, glutathione peroxidase; FBS, fetal bovine serum.

¹ To whom correspondence should be addressed (e-mail proftani@biochem.med.osaka-u.ac.jp).

Nucleotide sequences reported in this paper are deposited in the GenBank database with the accession number AF108213.

element in the 3'-untranslated region of the mRNA by introducing a series of deletions into the 3'-untranslated region of the rat cDNA sequences.

EXPERIMENTAL PROCEDURES

Materials

Sodium [^{75}Se]selenite was obtained from Amersham. NADPH was obtained from Oriental Yeast (Tokyo, Japan). Recombinant *Escherichia coli* TRX was purchased from Promega. Bovine insulin was obtained from Bayer. Other reagents were of the highest grade available.

Cell culture

A549 cells, a human lung adenocarcinoma cell line, and COS-1 cells, a monkey kidney simian virus 40 (SV40) transformed cell line, were maintained in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL) at 37 °C under an atmosphere of 95% air and 5% CO_2 .

Purification of TR and TRX

Purification of TR from A549 cells was carried out essentially as described previously [26], but with some modifications. A549 cells cultured in the medium supplemented with 1 μM sodium selenite were harvested by scraping with a silicon rubber scraper, washed twice with PBS and stored at -20 °C until used. The cells were homogenized in 50 mM Tris/HCl (pH 7.5)/1 mM EDTA (buffer A) and centrifuged at 100 000 g for 30 min. The supernatant was applied to a DE52 cellulose column. After washing with 50 mM NaCl in buffer A, the bound proteins were eluted with a linear gradient of NaCl (50–200 mM) in buffer A. The fractions washed with 50 mM NaCl in buffer A were used for purification of TRX. Fractions containing TR were applied to a Blue Sepharose column (Pharmacia), and eluted with a linear 0–500 mM KCl gradient in buffer A. After dialysis in buffer A, they were applied to a 2',5'-ADP-Sepharose column (Pharmacia) and washed with 200 mM potassium phosphate buffer (pH 7.5). Then the bound proteins were eluted with a linear gradient of 0–500 mM KCl in buffer A. Fractions containing TRX from DE52 were purified as described by Luthman and Holmgren [10]. The purities of TR and TRX were more than 95% as judged by SDS/PAGE.

Raising antibodies

Purified human TR or TRX protein (20 μg), described above, was emulsified in Freund's complete adjuvant, and were injected into lymph nodes of female rabbits. Another 20 or 10 μg of each protein, respectively, in the incomplete adjuvant was injected subcutaneously under the back skin at two-week intervals.

RNA preparation and Northern blotting

Total RNA was prepared from the cells as described by Chomczynski and Sacchi [27], and quantified by measuring the absorbance at 260 nm. Total RNA (20 μg) was heat-denatured at 65 °C for 15 min in the presence of 50% formamide and the running gel buffer [40 mM Mops/10 mM sodium acetate/1 mM EDTA (pH 7.0)], and then electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred on to a Zeta-Probe membrane (Bio-Rad) by capillary action, and the blotted RNAs were immobilized on the membrane by UV irradiation followed by

baking for 2 h at 80 °C under vacuum. The membranes were hybridized with a ^{32}P -labelled TR cDNA probe at 42 °C in the presence of 50% formamide. Then they were washed twice at 55 °C with 2 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS for 80 min. The Kodak XAR films were exposed for 1–3 days with an intensifying screen at -80 °C. The blots were stripped by boiling in 0.1% SDS/0.1 $\times$ SSC and reprobated serially with TRX and GPX cDNA probes using the hybridization and washing conditions given above.

Western-blot analysis

Samples (20 μg) were subjected to SDS/PAGE and then transferred on to nitrocellulose membranes (Schleicher & Schuell) under semi-dry conditions with the use of a Semiphor TE-70 semi-dry transfer unit (Hoefer). After blocking by incubation with 4% skimmed milk in Tris-buffered saline [20 mM Tris/HCl (pH 8.0)/0.15 M NaCl] for 12 h at 4 °C, and then incubated with an antibody against TR or TRX with a 1:1000 and 1:500 dilution, respectively, for 2 h at room temperature. After washing with Tris-buffered saline containing 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:4000; Organon Teknica Corp.) for 2 h. After washing, the chemiluminescence method using an ECL kit (Amersham) was employed to detect the peroxidase activity.

Activity assay

To determine the TR activity by measuring insulin reduction, the reaction was started by the addition of 10 μl of supernatant of cell homogenate to 490 μl of buffer A containing 2 μM *E. coli* TR, 500 $\mu\text{g}/\text{ml}$ insulin and 200 μM NADPH. The mixture was incubated at 37 °C for 20 min and was terminated by addition of 500 μl of 6 M guanidine hydrochloride containing 1 mM 5,5'-dithiobis(2-nitrobenzoic acid). TRX-dependent insulin reduction by NADPH was determined by subtracting the insulin reduction in the absence of TRX from that in the presence of TRX. This TRX-dependent insulin-reducing activity was given in m-units/mg of protein (where 1 unit is 1 μmol of NADPH oxidized per min), as described by Holmgren and Bjornstedt [11]. GPX activity was determined according to the method of Lawrence and Burk [28].

Cloning and sequencing the rat TR and TRX cDNAs

Two pairs of oligonucleotide primers (probe 1; TR1, GGNACNTGYGTNAAYGTNGGNTG, TR2, CCNGCRCAYTCNARNGCNACRTA: and probe 2; TR3, GARYTNACNCNGTNGCNATHCA, TR4, GGRTGDATNCCDATNGTN-SWRTC) were synthesized and used for PCR amplification of the human TR cDNA from A549 cells. The amplified DNA fragments were subcloned into T-vector (Novagene). After confirmation by sequencing, these DNA fragments were used as probes to screen a rat kidney cDNA library constructed in $\lambda\text{gt}10$ [29]. We isolated a clone covering about 2.2 kb of cDNA coding a part of the translated region and the entire 3'-untranslated region, but failed to obtain a full-length cDNA. Then we screened a rat liver cDNA library constructed in λZAPII (Stratagene) with probe 1 and finally obtained a full-length clone. A full-length rat TRX cDNA was cloned by PCR amplification of cDNA from rat liver RNA with rat-specific primers TRX1 (GCAACAGCCAAAATGGTGAAGCT) and TRX2 (CAGAGCATGATTAGGCAAACTCC). The DNA was subcloned into T-vector and confirmed by sequencing. An automated sequencer (DSQ 1000, Shimadzu) was used for the sequencing.

Construction of plasmids and transient expression

The full-length rat TR clone was excised from the λ ZAPII vector by *EcoRI* and then ligated into a pSVK3 expression vector (Pharmacia). This enabled efficient expression of cDNA in COS-1 cells in which large T-antigen is constitutively expressed to enhance expression of the gene with SV40 promoter/enhancer. Since the 5'-untranslated region contained an *NcoI* site that may have affected the efficacy of translation initiation at the *bona fide* ATG codon of TR, we destroyed the site by digestion with *NcoI* followed by mung-bean nuclease treatment and blunt-end ligation. We also performed a series of deletions by digesting the cDNA with restriction endonucleases *HindIII*, *NcoI* and *PstI*. To construct an expression plasmid with the inverted SECIS element, the *NcoI* site in the 3'-untranslated region was blunt-end ligated with a *PstI* linker after digesting with mung-bean nuclease. The *PstI* DNA fragment isolated by digestion with *PstI* was ligated back into the *PstI* site in an inverted orientation. These cDNAs were transiently transfected into COS-1 cells by electroporation (Gene Pulser; Bio-Rad).

Labelling of TR with ^{75}Se and autoradiography

COS-1 cells transfected with rat TR cDNAs with various deletions in the 3'-untranslated region were cultured in medium containing sodium [^{75}Se]selenite ($0.3 \mu\text{Ci/ml}$) for 3 days. The supernatants of the cell homogenates were applied on SDS/PAGE. The polyacrylamide gel was dried and subjected to autoradiography.

Protein assay

Protein concentrations were determined using a protein-assay kit (Bio-Rad) with BSA as a standard.

Statistical analysis

The paired Student's *t* test was used to compare the significance of the differences between data. All data are expressed as means $\pm$ S.D. of triplicate experiments.

RESULTS

Effects of selenium deficiency on TR and GPX expression in A549 cells

We have screened various cell lines to find one expressing high levels of TR, and confirmed that A549 cells expressed the highest levels. The cells were grown in a large scale, and TR and TRX were purified. Then antibodies against them were raised in rabbits and found to bind specifically to both human and rat TR and TRX (results not shown).

Since a main source for selenium in the cell-culture system is FBS supplemented in the culture medium, A549 cells were cultured for 3 weeks with various concentrations of FBS in the medium to determine the effect of selenium on TR and GPX activities. The concentration of selenium in the FBS used for cell culture was $0.48 \mu\text{M}$, as judged by atomic absorption spectroscopy. Figure 1(A) shows that TR activity was decreased significantly ($P < 0.005$) in accordance with lowering FBS concentrations, but that a significant difference for GPX activity was only observed between 10% FBS and lower concentrations. Quite a low level of TR activity was detected in the cells maintained in 0.5% FBS-supplemented medium, and the activity was enhanced by the addition of $1 \mu\text{M}$ sodium selenite. TR activity but not GPX activity was significantly higher ($P < 0.005$) in the medium with 10% FBS plus $1 \mu\text{M}$ selenium compared with the medium with 0.5% FBS plus $1 \mu\text{M}$

Figure 1 Effect of FBS deficiency and selenium supplementation on TR and GPX expression in A549 cells

A549 cells were cultured for 3 weeks in the medium supplemented with various concentrations of FBS as indicated. Sodium selenite ($1 \mu\text{M}$) was added (+). (A) TR activities (black bars) and GPX activities (white bars) of cytosolic fraction of A549 cells. Data are presented as means $\pm$ S.D. of triplicate experiments. (B) TR, and cytosolic GPX and TRX mRNAs determined by Northern blotting using specific cDNA probes. (C) TR and TRX protein levels determined by Western blotting using anti-human TR and anti-human TRX as primary antibodies.

selenium. GPX activity was sensitive to the presence of selenium, and corresponded to the level of mRNA. FBS concentrations at higher than 1% did not affect TR or TRX mRNA expression, but slightly decreased it at 0.5% FBS concentration. Addition of $1 \mu\text{M}$ sodium selenite rather suppressed TR and TRX mRNA expression, probably due to its cytotoxic effect (Figure 1B), in spite of the high TR activity. Levels of TR protein examined by

Figure 2 Constructs for TR expression in COS-1 cells

Shaded box shows the coding region. The black boxes and the arrow heads indicate a putative SECIS element and its orientation, respectively. In Δ TR4, the original *NcoI* (N) site was converted to a *PstI* (P) site by linker ligation. TGA is the codon for Sec at position 2 with respect to the C-terminus. H, *HindIII*.

Western-blot analysis, by using the antibody against human TR, corresponded to the TR activities. The levels of the TRX polypeptide were not affected by the supplementation of either FBS or selenium (Figure 1C). Thus we confirmed the regulation of TR protein production and TR enzymic activity by the presence of selenium [30,31].

Expression of the rat TR cDNA

The TR clones that we isolated from the rat kidney and liver cDNA libraries had 3'-untranslated sequences that were extended compared with the reported one [16]. Since the cDNA cloned by Zhong et al. [16] contained a poly(A) tract 550 bp downstream of the TAA stop codon and a putative SECIS element, it was assumed to be located at 1856–1915 bp [16]. This change would be caused by alternative poly(A) addition, as found in other genes. To examine a requirement of Sec for enzymic activity and the SECIS element for Sec incorporation into the TR polypeptide, we constructed several expression plasmids (Figure 2). When the expression of TR under regulation of cytomegalovirus (CMV) and SV40 promoters using pCMV and pSVK3 plasmid vectors respectively were compared, slightly higher activity was obtained by using pSVK3 in COS-1 cells (results not shown). Therefore this vector was chosen for further experiments.

We transfected the full-length TR cDNA (WT) into COS-1 cells and investigated the effect of selenium supplementation on the TR activity in conventional medium with 10% FBS. Figure 3(A) shows that the TR activity of the transfectant was increased in accordance with the added sodium selenite concentrations, and saturated at $0.5 \mu\text{M}$. The addition of $5 \mu\text{M}$ sodium selenite caused cell death (results not shown). The levels of TR protein, examined by Western-blot analysis, were not affected by the sodium selenite concentration (Figure 3B). Although COS-1 cells had low TR activity, about one-fourth of that of A549 cells, the activity was not increased by the addition of selenium (Figure 3A). The endogenous TR protein in COS-1 cells was not detected by Western blotting (Figure 3B).

Characterization of SECIS element in rat TR

The putative SECIS element was assumed to be between 1856 and 1915 bp in the 3'-untranslated region [16]. To examine a requirement of Sec for enzymic activity, and the SECIS element

Figure 3 Effect of sodium selenite concentration on TR expression in COS-1 cells

After transfection with the full-length TR cDNA in one cuvette, COS-1 cells were divided into each dish and cultured in the presence of varying concentrations of sodium selenite for 3 days. (A) TR activities in COS-1 cells transfected with the full-length TR cDNA (black bars) and untransfected control COS-1 cells (white bars). Data are presented as means $\pm$ S.D. of triplicate experiments. (B) TR protein production in COS-1 cells transfected with the full-length TR cDNA was detected by Western blotting. From left to right: lane 1, control COS-1 cells; lanes 2–7, COS-1 cells transfected with the full-length TR cDNA.

for incorporation into the TR polypeptide, we constructed expression plasmids with truncated (TR) 3'-untranslated sequences (Figure 2). Enzymic activities were observed in Δ TR1 and Δ TR2 as well as WT, with Δ TR2 showing the maximum activity. Addition of $1 \mu\text{M}$ sodium selenite markedly increased TR activity in these mutants. Δ TR3, which lacked the putative SECIS element, showed virtually no TR activity (Figure 4A). The SECIS element, which can form a potential stem-loop structure as shown in [16], was set in an inverted orientation in the same position of the cDNA, and the construct was designated Δ TR4. No TR activity was detected for this, suggesting that the SECIS element was functionally active only in the *cis* position. When co-transfection of TR cDNAs with a luciferase expression vector was performed, essentially the same fluorescent intensities by luciferase assay were obtained for all transfectants, indicating that the differences in TR activities were not due to alteration in the transfection efficiency (results not shown). Thus data from all of the truncated cDNAs together identified the location of the SECIS element as between 1856 and 1915 bp in the 3'-untranslated sequences, which covered the proposed region [16].

Requirement of the Sec residue for TR activity

Production of TR proteins in COS-1 cells expressing the full-length and truncated TR cDNAs were examined by Western blotting with a specific anti-human TR IgG. All cells transfected with the truncated cDNAs as well as the full-length one were capable of producing TR polypeptides whose levels were not affected by the addition of sodium selenite (Figure 4B). When the cDNA-transfected cells were incubated with sodium [^{75}Se]selenite for 3 days, incorporation of the radioactivity was detected at the

Figure 4 Expression of TR cDNAs with deleted 3'-untranslated sequences

(A) TR activities in COS-1 cells transfected with mutants as well as WT in the absence (white bars, —) or presence of 1 μM sodium selenite (black bars, +). Data are presented as means ± S.D. of triplicate experiments. (B) TR and TRX proteins of the same samples were detected by Western blotting. (C) Incorporation of ⁷⁵Se into the produced TR protein in COS-1 cells transfected with mutant cDNAs. After transfection with the cDNAs indicated, the cells were incubated in the medium containing sodium [⁷⁵Se]selenite (3 μCi/dish) for 3 days. Cytosolic proteins of the cells were separated by SDS/PAGE and then subjected to autoradiography.

position of TR only in WT, ΔTR1 and ΔTR2, which matched the clones exhibiting TR activities (Figure 4C). These data clearly demonstrated that the SECIS element located in the 3'-untranslated sequences in the *cis* position was essential for Sec incorporation, and that the Sec residue was a prerequisite for the catalytic activity of the TR polypeptide.

DISCUSSION

It has been demonstrated recently that mammalian TR is a selenoprotein that has a Sec residue near the C-terminus, and is quite different from the bacterial TR [22]. Several reports suggest that Sec is an essential residue for mammalian TR activity, but functional expression of TR has not been reported so far. This would be mainly due to the defect in incorporation of the Sec residue into the polypeptide in a bacterial expression system, since the machinery for Sec incorporation into the UGA codon by the function of the SECIS element in mammalian cells is different from the bacterial one.

Here, we report the functional expression of rat TR in COS-1 cells for the first time and demonstrate an essential role of the

SECIS element in the 3'-untranslated sequence in the mRNA. By using several expression constructs with deleted 3'-untranslated regions, we confirmed the prerequisite role of the SECIS element, located between 1856 and 1915 bp, for the functional expression of TR in a mammalian cell system. A preliminary trial of an application of a baculovirus/insect cell system for expressing TR produced a large amount of the TR polypeptide, but failed to produce an active TR (N. Fujiwara, T. Fujii, J. Fujii and N. Taniguchi, unpublished work). This was probably because of insufficient Sec biosynthesis or saturation of the machinery necessary for Sec incorporation into the polypeptide in response to a strong polyhedrin promoter. Several RNA-binding proteins that specifically recognize the SECIS element were detected [32], and quite recently one of the binding proteins has been cloned and identified as DNA-binding protein B [33]. The protein was bound to the SECIS element on RNA by gel-shift assay. However, it has not yet been established whether or not the protein helps Sec incorporation into the UGA codon, as demonstrated for the bacterial Sec-specific elongation factor, SELB [23].

Precise characterization of the requirement of the SECIS element for the type-I iodothyronine deiodinase and GPX genes has been reported [5,24,25,32,33]. The elements are functional on genes for other selenoproteins and even for a non-selenoprotein, rab5b, a small GTP-binding protein [25]. Conserved nucleotides in the loop and unpaired regions of the stem of the SECIS element have been found to be critical for activity [5]. It has also been shown that the distance between the UGA codon and the SECIS element is not important for Sec incorporation. There has been only one report that examined the direction of the SECIS element in the 3'-untranslated sequence [5]. Inversion of the sequences between 1856 and 1915 bp in the rat TR cDNA resulted in loss of TR activity (Figure 4A), as described previously for type-1 iodothyronine deiodinase [5]. These data clarified that the SECIS element was functional only when located in the *cis* position in the gene, and supported its unidirectional function from 3' to 5' in the mRNA. We also observed that the TR activity of ΔTR1 was comparable with the full-length cDNA, but ΔTR2 showed about 2-fold higher activity (Figure 4A). Since the produced protein level was only slightly higher in ΔTR2 transfected cells, the 3'-untranslated region deleted in ΔTR2 may have a negative regulatory element for Sec incorporation.

We have demonstrated clearly an absolute requirement of the SECIS element for the expression of TR activity through the incorporation of the Sec residue at the UGA codon. The TR proteins could be produced without the SECIS element in a transient expression system (Figure 4), and protein production was not regulated by the selenium concentration (Figure 3). This suggested that nearly the full size of the TR protein could be synthesized, even under selenium-deficient conditions or without the SECIS element, although the resultant TRs were inactive. The endogenous TR protein production in A549 cells appeared to be regulated by selenium, since TR protein level was decreased in accordance with the decrease of FBS concentration, and was increased by selenium replenishment (Figure 1C). While selenium supplementation rather decreased the levels of TR and TRX mRNA, it enhanced the levels of mRNA for GPX (Figure 1B). These data suggested that the transcriptional regulation of the TR gene by selenium was likely to differ from the GPX gene. The increase in TR protein production by selenium supplementation might be due to the enhanced stability of TR mRNA by the presence of selenium [31]. Further investigations concerning regulation at the transcriptional and translational stages by selenium will be needed. Replacement of the Sec residue by Cys in type-I iodothyronine deiodinase [5] and GPX [34] markedly

decreased their enzymic activities. Hence, it would be also interesting to know whether the substitution of Sec with Cys in TR still shows TR activity or not, because a *Caenorhabditis elegans* homologue has Cys instead of Sec at the same position [21].

We report herein that SECIS element-mediated Sec incorporation plays an important role in the production of active TR. Successful expression of active TR would enable us to analyse further details of the catalytic mechanisms of this enzyme.

We thank Dr. John M. C. Gutteridge for editing this manuscript prior to submission. This work was supported, in part, by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture, Japan, and Research Grant (9A-1) for Nervous and Mental Disorders from the Ministry of Health and Welfare, Japan.

REFERENCES

- Combs, Jr., G. F. and Clark, L. C. (1997) in *Antioxidant Nutrients and Disease Prevention* (Garewal, H., ed.), pp. 97–113. CRC Press, Boca Raton
- Keshan Disease Research Group of the Chinese Academy of Medical Sciences, Beijing (1979) *Chinese Med. J.* **92**, 477–482
- Stadtman, T. C. (1996) *Annu. Rev. Biochem.* **65**, 83–100
- Chambers, I., Framton, J., Goldfard, P., Affara, N., McBain, W. and Harrison, P. R. (1986) *EMBO J.* **5**, 1221–1227
- Berry, M. J., Banu, L., Chen, Y. Y., Mandel, S. J., Kieffer, J. D., Harney, J. W. and Larsen, P. R. (1991) *Nature (London)* **353**, 273–276
- Low, S. C., Harney, J. W. and Berry, M. J. (1995) *J. Biol. Chem.* **270**, 21659–21664
- Hill, K. E., Lloyd, R. S., Yang, J. G., Read, R. and Burk, R. F. (1991) *J. Biol. Chem.* **266**, 10050–10053
- Vendeland, S. C., Beilstein, M. A., Yeh, J. Y., Ream, W. and Whanger, P. D. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8749–8753
- Gladyshev, V. N., Jeang, K. T., Wootton, J. C. and Hatfield, D. L. (1998) *J. Biol. Chem.* **273**, 8910–8915
- Luthman, M. and Holmgren, A. (1982) *Biochemistry* **21**, 6628–6633
- Holmgren, A. and Bjornstedt, M. (1995) *Methods Enzymol.* **252**, 199–208
- Nakamura, H., Nakamura, K. and Yodoi, J. (1997) *Annu. Rev. Immunol.* **15**, 351–369
- Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y. and Kawabata, M. (1998) *EMBO J.* **17**, 2596–2606
- Chae, H. Z. and Rhee, S. G. (1994) *Biofactors* **4**, 177–180
- Gasdaska, P. Y., Gasdaska, J. R., Cochran, S. and Powis, G. (1995) *FEBS Lett.* **373**, 5–9
- Zhong, L., Arner, E. S., Ljung, J., Aslund, F. and Holmgren, A. (1998) *J. Biol. Chem.* **273**, 8581–8591
- Arcsott, L. D., Gromer, S., Schirmer, R. H., Becker, K. and Williams, Jr., C. H. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3621–3626
- Liu, S. Y. and Stadtman, T. C. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6138–6141
- Gromer, S., Arcsott, L. D., Williams, C. H., Schirmer, R. H. and Becker, K. (1998) *J. Biol. Chem.* **273**, 20096–20101
- Gromer, S., Wissing, J., Behne, D., Ashman, K., Schirmer, R. H., Flohe, L. and Becker, K. (1998) *Biochem. J.* **332**, 591–592
- Gladyshev, V. N., Jeang, K. T. and Stadtman, T. C. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6146–6151
- Tamura, T. and Stadtman, T. C. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1006–1011
- Low, S. C. and Berry, M. J. (1996) *Trends Biochem. Sci.* **21**, 203–208
- Berry, M. J., Banu, L., Harney, J. W. and Larsen, P. R. (1993) *EMBO J.* **12**, 3315–3322
- Shen, Q., Chu, F. F. and Newburger, P. E. (1993) *J. Biol. Chem.* **268**, 11463–11469
- Oblong, J. E., Gasdaska, P. Y., Sherrill, K. and Powis, G. (1993) *Biochemistry* **32**, 7271–7277
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Lawrence, R. A. and Burk, R. F. (1976) *Biochem. Biophys. Res. Commun.* **71**, 952–958
- Nishikawa, A., Ihara, Y., Hatakeyama, M., Kangawa, K. and Taniguchi, N. (1992) *J. Biol. Chem.* **267**, 18199–18204
- Hill, K. E., McCollum, G. W., Boeglin, M. E. and Burk, R. F. (1997) *Biochem. Biophys. Res. Commun.* **234**, 293–295
- Gallegos, A., Berggren, M., Gasdaska, J. R. and Powis, G. (1997) *Cancer Res.* **57**, 4965–4970
- Shen, Q., McQuilkin, P. A. and Newburger, P. E. (1995) *J. Biol. Chem.* **270**, 30448–30452
- Shen, Q., Wu, R., Leonard, J. L. and Newburger, P. E. (1998) *J. Biol. Chem.* **273**, 5443–5446
- Rocher, C., Lalanne, J. L. and Chaudiere, J. (1992) *Eur. J. Biochem.* **205**, 955–960

Received 30 November 1998/1 March 1999; accepted 23 March 1999