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The human glutaredoxin gene: determination of its organization, transcription start point, and promoter analysis

Jae B. Park, Mark Levine *

Molecular and Clinical Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, Building 10, Room 4D/52, MSC 1372, National Institutes of Health, Bethesda, MD 20892-1372, USA

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Abstract

A genomic clone for the human glutaredoxin gene was isolated and sequenced. An intron was located within the coding region and began 211 nt downstream of the initiator codon. Except for this intron, the genomic sequence shares 100% identity to the published glutaredoxin cDNA sequence. A second intron was located in the 3′ *UTR* 6 bp downstream of the terminator codon. The *tsp* of the glutaredoxin gene was determined by primer extension and confirmed by S1 mapping analysis. Analysis of the 5′-flanking region of the gene revealed that the promoter sequences TATA and CCAAT were 30 and 160 bp upstream, respectively, from the *tsp*. Other potential transcription factor binding sites included NF-E1, HNF-5, P2II and AP-1. Glutaredoxin promoter constructs inserted into a reporter plasmid for firefly luciferase were transfected into fibroblasts, and luciferase activity was 8–10-fold higher compared with controls lacking glutaredoxin promoter. These data indicate that the promoter region of the isolated glutaredoxin gene is functional. © 1997 Elsevier Science B.V.

Keywords: Thioltransferase; Dehydroascorbic acid; Ascorbic acid

1. Introduction

Glutaredoxin (GRX) (transhydrogenase or thioltransferase) (Racker, 1955), is a ubiquitous glutathione-dependent cytosolic enzyme which catalyses disulfide reduction (Bjornsted et al., 1994; Holmgren, 1978; Racker, 1955). GRX is heat-stable with an approximate molecular mass of 11 kDa (Wells et al., 1993). The protein, first isolated from yeast, has been characterized in eubacteria, archaebacteria, and eukaryotes (Wells et al., 1993; Holmgren, 1989). The defining feature of all GRXs is their active site of cys–pro–tyr(-phe)–cys. Human GRX is approximately 54% homologous to *E. coli* GRX, 46% homologous to yeast (*Saccharomyces cerevesiae*) GRX, and 90% homologous to porcine GRX (Holmgren, 1989; Wells et al., 1993). GRX is an alternative electron donor to ribonucleoside diphosphate reductase in bacteria (Holmgren, 1976), participates in deiodination of thyroxine to triiodothyronine in mammalian liver (Goswami and Rosenberg, 1985), and catalyses the exchange reaction between thiol and disulfide groups in *E. coli* (Luthman et al., 1979). Isolated pig GRX was also reported to have dehydroascorbic acid reducing activity (Wells et al., 1990).

Although the physiologic mechanism of dehydroascorbic acid reduction in cells was unclear (Winkler et al., 1994; Washko et al., 1993), GRX was recently shown to be responsible for the majority of dehydroascorbic acid reduction in normal human neutrophils (Park and Levine, 1996). However, GRX was not detected in neutrophil-like cell lines, and dehydroascorbic acid reducing activity was at least 5–10-fold less in these cells than in normal neutrophils (Park and Levine, 1996; Washko et al., 1993). These data are consistent with observations that expression of bovine GRX varies and is tissue-dependent (Rozell et al., 1993).

Although GRX expression may be regulated, the controlling mechanisms are unknown. Insights about expression are dependent on knowledge of glutaredoxin genomic DNA (*GRX*). However, no information about

* Corresponding author. Tel. +1 301 4025588, Fax +1 301 4026436, e-mail: markl@bdg8.niddk.nih.gov

Abbreviations: bp, base pair(s); cDNA, DNA complementary to RNA; GRX, glutaredoxin; *GRX*, gene (DNA) encoding glutaredoxin; kb, kilobase(s) or 1000 base pairs; kDa, kilodalton(s); nt, nucleotide(s); PCR, polymerase chain reaction; *tsp*, transcription start point(s); *UTR*, untranslated region(s).

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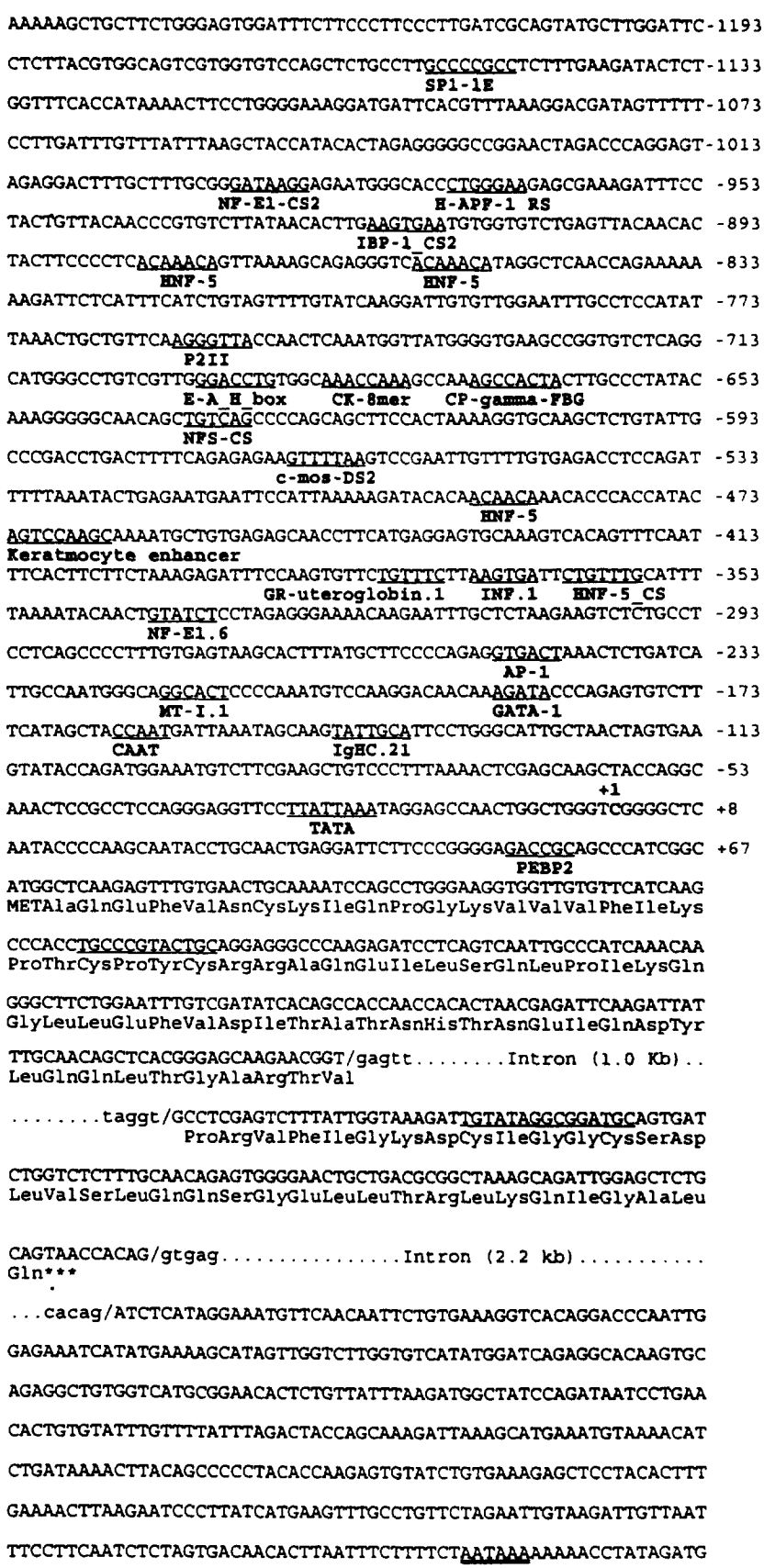
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H-APF-1 RS
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HNF-5
HNF-5
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E-A_H_box
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HNF-5
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Keratmocyte enhancer
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GR-uteroglobin.1
INF.1
HNF-5_CS
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NF-E1.6
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AP-1
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MT-I.1
GATA-1
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CAAT
IgHC.21
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+1
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PEBP2
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Gln***
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mammalian *GRX* is available, and the only data about eukaryotic *GRX* are from yeast (Bolle et al., 1992). Therefore, we present here the first isolation and sequencing of human *GRX* and initial characterization of the promoter region.

2. Experimental and discussion

2.1. Isolation and sequence of human GRX

A total of 9×10^6 recombinant λ clones were screened with labeled glutaredoxin cDNA. One clone was proven by PCR to contain the 5′-upstream region, the 3′-downstream region, and the glutaredoxin cDNA region. The approximate lengths of the 5′- and 3′-flanking regions were 3 and 4 kb, respectively. The 5′-flanking region was amplified by PCR and sequenced (Fig. 1). The PCR product contained one internal *Eco*RI site and one internal *Bam*HI site, located at nt positions 1200 and 2000, respectively (data not shown). The 5′-*UTR* of glutaredoxin cDNA shared 100% identity to *GRX* (Fig. 1). The genomic regions corresponding to the translated region of glutaredoxin cDNA were obtained by *Eco*RI digestion of the isolated genomic gene, cloned, and sequenced. An intron of approximately 1.0 kb was located 211 nt downstream from the ATG initiator codon (Fig. 1). The 3′-flanking region was also amplified by PCR and sequenced. A second intron in the 3′-*UTR* (Fig. 1) began 6 bp downstream of the terminator codon TAA. Except for the two introns, the coding region and 3′-*UTR* of glutaredoxin cDNA (Park and Levine, 1996) shared 100% identity to *GRX*.

2.2. Primer extension and S1 mapping

To investigate whether the isolated gene could be a functional gene, primer extension and S1 mapping analysis were performed. Primer extension was undertaken to identify the *GRX tsp*. A ^{32}P-labeled 18-nucleotide antisense primer corresponding to bp 1–18 of the translated region of glutaredoxin cDNA was hybridized to human neutrophil mRNA and extended with reverse transcriptase. Only one fragment size of 87 bp was detected (data not shown). To test whether the isolated genomic DNA contained this region, the 5′-end of the glutaredoxin transcript was characterized using S1 nuclease mapping. A 350-bp probe was synthesized by PCR using two primers, which corresponded to −300 to −280 bp and 87 to 69 bp of the genomic gene (Fig. 1). (Note: bp 69 to 87 of genomic DNA correspond to bp 1–18 of the translated region of glutaredoxin cDNA). The PCR product was cloned into pGEM vector. Antisense RNA ^{32}P-labeled probe was synthesized and hybridized with 10 μg of neutrophil total RNA. Hybridization of the antisense RNA probe yielded one S1-nuclease-resistant band of 87 bp, which perfectly matched the primer extension product (data not shown). Hybridization with anti-sense RNA probe could have occurred only if the appropriate mRNA was actually transcribed. These experiments suggested that the region detected by S1 nuclease mapping represented the 5′-terminus of the glutaredoxin transcript, and that the isolated genomic DNA contained the promoter region of *GRX*.

2.3. Analysis of the glutaredoxin promoter region

To learn how glutaredoxin transcription could be initiated, we sequenced the promoter region of the gene (Fig. 1). The sequence continued 1251 bp 5′ to the *tsp* (designated +1) and 87 bp 3′ to this site. Potential transcription elements in the sequence were identified. In the immediate 5′-flanking region, TATA and CCAAT sequences were located 30 and 160 bp, respectively, upstream of the *tsp*. The location and distance of these two sequences were consistent with data for other genes (Johnson and McKnight, 1989). The 5′-flanking sequence contained 15 other perfectly matched potential transcription-factor binding sites (Fig. 1).

One such site was for the transcription factor AP1, whose activity can be regulated by oxygen radicals (Abate et al., 1990). GRX and its regulation could be an important defense against oxidants because GRX catalyses dehydroascorbic acid reduction to ascorbate

Fig. 1. Nucleotide sequence of the human glutaredoxin gene. The sequence shown includes a 1.3 kb region (bp −1251 to +87) containing the 5′-flanking region of human *GRX*, the coding sequence, and the 3′-flanking region. The *tsp* is indicated by +1, putative TATA and CCAAT sequences are underlined, and potential transcription factor binding sites are marked. The deduced amino acid sequences are indicated, two conserved regions containing a total of four cysteines are underlined, and the putative poly A signal is double underlined. Nucleotides in lower case are those at the beginning and end of the two introns. The sequence was submitted to GenBank (accession No. U40574). **Methods:** An amplified human neutrophil genomic DNA library was screened with random-primed ^{32}P-labeled full-length human neutrophil glutaredoxin cDNA (Park and Levine, 1996). One positive clone contained the entire glutaredoxin gene, 3-kb upstream 5′-flanking region, and 4-kb downstream 3′-flanking region. A genomic fragment of approximately 10-kb was analyzed using PCR and restriction-endonuclease digestion. DNA containing the glutaredoxin promoter region was amplified using an 18 oligomer (5′-CACAAACTCTTGAGCCAT; primer 1) of the antisense strand complementary to nucleotide positions 1–18 of glutaredoxin cDNA and T3 primer located in the left λ arm. *GRX* was digested with *Eco*RI and six different genomic fragments were ligated to *Eco*RI-predigested TA vector (Invitrogen). The 3′-downstream DNA region was amplified by PCR with two primers: an 18 oligomer (5′-ATTGGAGCTCTGCAGTAA; primer 2) of the sense strand corresponding to 303–321 nucleotides of glutaredoxin cDNA and T7 primer in the right λ arm. Nucleotide sequences of the amplified fragments and the *Eco*RI-digested genomic fragments were determined using the dideoxy chain-termination method with modified T7 DNA polymerase (Sequenase Version 2.0, US Biochemical).

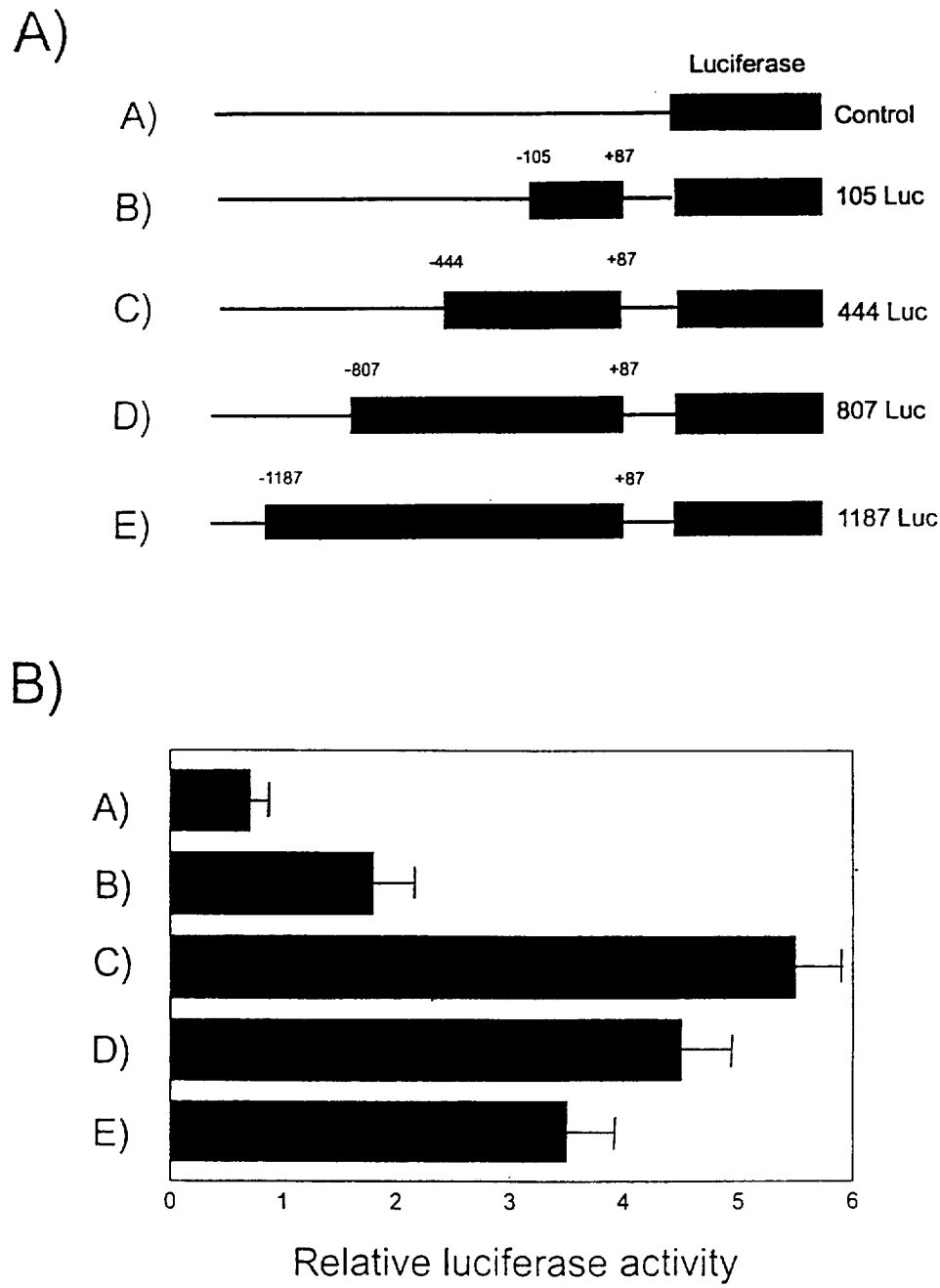

Fig. 2. Human glutaredoxin gene promoter activity. **(A)** Four different fragments of the glutaredoxin promoter (lines B–E) of increasing lengths were inserted into the reporter plasmid pGL2 (Promega) for firefly luciferase (Luc). The numbers in each fragment represent the position of the fragment in the glutaredoxin gene promoter relative to the *tsp* (+1). The control (line A) is reporter plasmid without glutaredoxin promoter. **(B)** Relative luciferase activities of the above promoter constructs transfected into normal human fibroblasts ($n=3$). Cells were transfected by the calcium phosphate method, incubated for 48 h, and lysed. Luciferase assays (Promega) were performed on the lysates.

(Park and Levine, 1996; Wells et al., 1993), dehydroascorbic acid results from ascorbate oxidation (Washko et al., 1993), and ascorbate is an ideal radical scavenger (Buettner and Moseley, 1993). AP1 activity is also induced by TPA, EGF, Ha-*ras*, *raf*, v-*mos*, NGF, TGFβ, cAMP, retinoic acid and ConA (Abate et al., 1990). A relationship might exist between oxygen radicals and these inducers, and remains to be investigated.

2.4. *Activity of the glutaredoxin gene promoter region*

To define the DNA elements directing gene expression and to identify regulatory domains within the 5′-flanking region, glutaredoxin gene promoter activity was analyzed by a transient transfection method. Four different 5′-flanking sequences of the glutaredoxin gene were constructed, containing 193–1265 nt (Fig. 2A). The

reporter plasmid pGL2 containing no promoter was used to examine the expression of firefly luciferase (Luc) under the control of the four different 5′-flanking sequences. These chimeric plasmids were transfected into human normal fibroblasts and luciferase activities were determined in cellular extracts prepared 48 h after transfection. As a control, promoterless luciferase plasmid was also transfected into fibroblasts. The construct 444-Luc exhibited maximal luciferase activity, approximately 10-fold higher than the promoterless luciferase plasmid (Fig. 2B). Transfection of construct 105-Luc resulted in approximately 65% less luciferase activity compared with construct 444-Luc. This implied that positive *cis*-regulatory elements were located between bp −444 and −105. The constructs 807-Luc and 1187-Luc showed slightly lower luciferase activity, suggesting that the region between −444 and −1187 contains mild negative regulatory elements. These data provide evidence that the promoter from the cloned glutaredoxin gene is functional. It now becomes possible to study how specific transcription factors and their binding sites regulate glutaredoxin expression in normal and tumor cells.

3. Conclusions

(1) This is the first characterization of mammalian *GRX*. We isolated and sequenced human *GRX* and described its promoter region. Human *GRX* contained one intron 211 nt downstream of the initiator codon and a second intron 6 bp downstream of the terminator codon. Except for the intron, the protein coding region of *GRX* was identical to glutaredoxin cDNA. The cloned glutaredoxin gene promoter was functional.

(2) Because GRX might protect cells from oxidant damage by maintaining high intracellular vitamin C concentrations, and the promoter region of *GRX* contains binding sites for redox-sensitive factors, regulation of GRX expression could play a central role in cellular oxidant defense. Experiments to test these possibilities are underway.

References

Abate, C., Patel, L., Rauscher, F.J., Curran, T., 1990. Redox regulation of Fos and Jun DNA-binding activity in vitro. Science 249, 1157–1161.

Bjornsted, M., Xue, J., Huang, W., Akesson, B., Holmgren, A., 1994. The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. J. Biol. Chem. 269, 29382–29384.

Bolle, P.A., Gilliquet, V., Berben, G., Dumont, J., Hilger, F., 1992. The complete sequence of K3B, a 7.9 kb fragment between PGK1 and CRY1 on chromosome III reveals the presence of seven open reading frames. Yeast 8, 205–213.

Buettner, G.R., Moseley, P.L., 1993. EPR spin trapping of free radicals produced by bleomycin and ascorbate . Free Radic. Res. Commun. (Suppl. 1) 19, 89–93.

Goswami, A., Rosenberg, I.N., 1985. Purification and characterization of a cytosolic protein enhancing glutathione-dependent microsomal iodothyronine 5′-monodeiodination. J. Biol. Chem. 260, 6012–6019.

Holmgren, A., 1976. Hydrogen donor system for *E. coli* ribonucleotide diphosphate reductase dependent upon glutathione. Proc. Natl. Acad. Sci. USA 73, 2275–2279.

Holmgren, A., 1978. A thiol-dependent transhydrogenase from yeast. J. Biol. Chem. 253, 7424–7430.

Holmgren, A., 1989. Thioredoxin and glutaredoxin systems. J. Biol. Chem. 264, 13963–13966.

Johnson, P.F., McKnight, S.L., 1989. Eukaryotic transcriptional regulatory proteins. Ann. Rev. Biochem. 58, 799–839.

Luthman, M., Eriksson, S., Holmgren, A., Thelander, L., 1979. Glutathione-dependent hydrogen donor system for calf thymus ribonucleoside-diphosphate reductase. Proc. Natl. Acad. Sci. USA 76, 2158–2162.

Park, J.B., Levine, M., 1996. Purification, cloning, and expression of dehydroascorbic acid reduction activity from human neutrophils: identification as glutaredoxin. Biochem. J. 315, 931–938.

Racker, E., 1955. Glutathione-homocystine transhydrogenase. J. Biol. Chem. 217, 867–874.

Rozell, B., Barcena, J.A., Martinez-Galistes, E., Padilla, C.A., Holmgren, A., 1993. Immunochemical characterization and tissue distribution of glutaredoxin (thioltransferase) from calf. Eur. J. Cell. Biol. 62, 314–324.

Washko, P.W., Wang, Y., Levine, M., 1993. Ascorbic acid recycling in human neutrophils. J. Biol. Chem. 268, 15531–15535.

Wells, W.W., Xu, D.P., Yang, Y., Rocque, P.A., 1990. Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. J. Biol. Chem. 265, 15361–15364.

Wells, W.W., Yang, Y., Deits, T.L., Gan, Z., 1993. Thioltransferases. Adv. Enzymol. Relat. Areas. Mol. Biol. 66, 149–201.

Winkler, B.S., Orselli, S.M., Rex, T.S., 1994. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radic. Biol. Med. 17, 333–349.