Cloning, Sequencing, and Characterization of Alternatively Spliced Glutaredoxin 1 cDNA and Its Genomic Gene

CHROMOSOMAL LOCALIZATION, mRNA STABILITY, AND ORIGIN OF PSEUDOGENES*

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Alternatively spliced human glutaredoxin (Grx1as) cDNA was isolated from a neutrophil cDNA library, using a $^{32}$P-labeled human glutaredoxin (Grx1) cDNA probe under non-stringent conditions. The sequence of Grx1as cDNA indicated that the open reading frame of the gene was identical to the open reading frame of the previously reported first human glutaredoxin (Grx1) cDNA, but the 3′-untranslated region of Grx1as was not homologous to Grx1 cDNA. Northern blot and RT-PCR analyses showed Grx1as mRNA was expressed in normal human neutrophils and transformed cells including U937, HL-60, THP, and Jurkat cells. Cloning and sequencing of the genomic gene corresponding to Grx1as cDNA showed that two different glutaredoxin cDNAs (Grx1as and Grx1) were generated from the same genomic gene via alternative splicing. Origination of Grx1as and Grx1 from the same gene was confirmed by chromosomal localization of the Grx1as gene to chromosome 5q13, the same location where the Grx1 gene was localized previously. During screening of the Grx1as genomic gene, two additional glutaredoxin pseudogenes were also isolated. Surprisingly, these pseudogenes contained 3′-untranslated regions that were nearly identical to the 3′-untranslated regions of Grx1as, not Grx1, cDNA. Because 3′-untranslated regions may be important in stabilizing mRNAs, the effect of the two 3′-untranslated regions of Grx1 and Grx1as on mRNA stability was investigated using luciferase reporter vectors with the 3′-untranslated regions. Luciferase activity was 2.6-fold greater in cells transfected with the reporter vector containing the 3′-untranslated region of Grx1as cDNA compared with the 3′-untranslated region of Grx1 cDNA. These data indicate that Grx1as cDNA is an alternatively spliced human Grx1 cDNA and that the Grx1as 3′-untranslated region may have a role in stabilizing mRNA.

Glutaredoxin (Grx, thioredoxin) is a small redox protein involved in oxidoreductive processes in cells through catalyzing disulfide-thiol exchange reactions (1–3). Thiol groups in proteins may act as redox-sensitive switches and are considered to be a key element in maintaining cellular redox balance (4–8). Cellular redox imbalance induces radical oxygen species that mediate signaling events leading to proliferation, apoptosis, and differentiation (4, 5). Because of its potential importance, the redox status of thiol groups is well balanced by biological reducing molecules and proteins (6–10). Among redox proteins, glutaredoxin is a protein that regenerates S-thiolated cysteines in proteins that result from oxidative stress (11). Glutaredoxins have been isolated from prokaryotes and eukaryotes and have been proposed as redox proteins that mediate several biological reactions (3, 11–16). Recently, a second human glutaredoxin (glutaredoxin 2 or Grx2) has been cloned, and two alternatively spliced Grx2 mRNA isoforms were identified (17).

In our laboratory, we searched for mRNA isoforms of the first human glutaredoxin (glutaredoxin 1 or Grx1), because swine glutaredoxin cDNA contains a 3′-untranslated region that has no counterpart in human glutaredoxin 1 cDNAs (18, 19). A human neutrophil library was screened with $^{32}$P-labeled Grx1 cDNA using non-stringent conditions. Screening yielded a new human glutaredoxin cDNA, Grx1as. Its nucleotide sequence was identical to the open reading frame of Grx1 cDNA, but the 3′-untranslated region sequence was comparable to that of swine glutaredoxin cDNA rather than that of human Grx1 cDNA. Northern blots and RT-PCR were performed to verify Grx1as mRNA in several human cells. Genomic cloning and chromosomal localization of Grx1as were performed to determine genomic origins of Grx1 and Grx1as cDNAs. Finally, effects of 3′-untranslated regions from Grx1as and Grx1 cDNAs on mRNA stability were investigated.

EXPERIMENTAL PROCEDURES

Materials—Cloned human Grx1 cDNA (3) was maintained in Escherichia coli strain JM101. Restriction endonuclease, T4 DNA ligase, T4 polynucleotide kinase, and avian myeloblastosis virus reverse transcriptase were purchased from Promega (San Luis Obispo, CA). Taq polymerase and deoxynucleotides were obtained from PerkinElmer Life Sciences and radiolabeled nucleotides from Amersham Biosciences. A human monocyte cDNA library was purchased from Clontech (catalog no. HL1056B, Palo Alto, CA). Ascorbic acid, EDTA, 4,4′-dihydroxyethyl-1-piperazinoethanesulfonic acid, Tris-HCl, and dithiothreitol were purchased from Sigma.

Construction of Human Neutrophil cDNA and Genomic Library—A human neutrophil cDNA library was constructed as described previously (3). Briefly, first and second strand cDNA were synthesized (Stratagene, La Jolla, CA) using 5 µg of human neutrophil mRNA obtained from 10 subjects, each of whom provided ~1 × 10⁹ neutrophils isolated by apheresis (20, 21). The cDNAs were ligated to Uni-ZAP cDNA; RT-PCR, reverse transcriptase polymerase chain reaction; MV, cytomegalovirus.
vector and transfected into XL-Blue strain using Gigapack (Stratagene). Human neutrophil genomic library was constructed as described previously (18). Neutrophil genomic DNA was isolated per manufacturer's instructions (Qiagen, Valencia, CA). Isolated DNA was partially digested with Sau3A, and size 6–15 kb DNA was isolated using sucrose gradient centrifugation. The isolated DNA was ligated to λ DASHII arms predigested with BamHI (Stratagene). The ligated λ was in vitro packaged using Gigapack II Gold (Stratagene), plated, and amplified.

Isolation and Sequencing of Alternatively Spliced Human Glutaredoxin (Grx1as) cDNA—An amplified human neutrophil cDNA library was screened with random-primer 32P-labeled partial human neutrophil glutaredoxin cDNA from nucleotides 50 to 270 of the open reading frame (3). Several clones were selected from 2 × 10^6 recombinants, and clones were further purified by sequential platings. The size and integrity of cDNA was determined by PCR with primers corresponding to various regions of the human cDNA. Nucleotide sequences were determined using the dideoxy chain termination method and the ALF DNA sequencer (Amersham). During the genomic screening, we also isolated two glutaredoxin pseudogenes (GS1 and GS2).

Chromosomal Localization—The Grx1as gene was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2× sodium chloride, sodium citrate buffer (23).

Determination of Effect of 3′-Untranslated Regions on mRNA Stability—To measure the effect of short and long 3′-untranslated regions on mRNA stability, a luciferase reporter vector (pCMV-Luc) was constructed by cloning PCR-amplified luciferase gene into pcRI 3.1 (Invitrogen). The reporter vectors contained either the short or long 3′-untranslated region of human glutaredoxin: pCMV-Luc-3′UTR-Grx1 or pCMV-Luc-3′UTR-Grx1as. The vectors were constructed by insertion of each 3′-untranslated region downstream of the luciferase gene using the PsI site. Vectors were transfected with calcium phosphate into HeLa cells. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were grown to ~50% confluence in 60-mm Petri dishes. The two reporter plasmids were individually transfected into cells using calcium phosphate. After transfection, cells were incubated for an additional 48 h, harvested, and lysed. Luciferase assays of the lysates were performed according to the manufacturer’s protocol (Promega). Transfection efficiency was monitored by co-transfection with pSEAP-2 promoter vector, the alkaline phosphatase activity of which was determined according to the manufacturer’s protocol (Clontech). For expression, PCR fragments were cloned into pGEX expression vector (Amersham Biosciences). The vector was transformed into E. coli (BL21), and bacteria were cultured overnight at 37 °C in Luria Bertani medium containing ampicillin (100 μg/ml). Overnight culture was inoculated into fresh medium and cultured further with vigorous shaking. When OD600 was 0.6, protein expression was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside. After 4 h of induction, bacteria were collected by centrifugation. Recombinant

**Fig. 1.** The nucleotide sequence and deduced amino acid sequence of Grx1as glutaredoxin cDNA. Sequences 1 and 2 represent Grx1as and Grx1, respectively. The nucleotides are numbered 5′ to 3′, and amino acids are numbered from N to C termini, respectively. Two conserved regions containing a total of four cysteines are underlined. Bold nucleotides indicate that they are the same in both clones, and asterisks indicate the stop codon.

ATGGCTCAGAGTTTGTGACCTGCAAAATACGCCTGTGGAGCTGGTGACCTTACCH
ATGGCTCAGAGTTTGTGACCTGCAAAATACGCCTGTGGAGCTGGTGACCTTACCH
MELA1aG1nGluPheValAnCyslys1eGlnProGlyLysValValValPhe1leLys

CCACCTCGCCCTTACCATGAGAGGATGGCCCAAGAGATCCCTGCTCAATTGCCCATCAAACAA
ProThrCysProTyrCysArgArgAlaGlnGluLysLeuSerGlnLeuProIleLysGln

GGGCCTTTTGTTTATTGACATTGATCTACAGCCAGGAGTCAGAGCATCATACCATGCAGATG
GGGCCTTTTGTTTATTGACATTGATCTACAGCCAGGAGTCAGAGCATCATACCATGCAGATG
GlyLeuGluLeuGluPheValAspIleThrAlaThrAlaThrAsnThrAsnGluLysGluAspTyr

TTGCAACAGTCTACGGAGACAAAGGCTGGCCCTGCGATCTGATTTATAAGATATGATTATA
TTGCAACAGTCTACGGAGACAAAGGCTGGCCCTGCGATCTGATTTATAAGATATGATTATA
LeuGluGlnGluThrGluAlaArgValAlaValProValPheTyrGlyLysAspCysTyrLe

GAGCCTGGAAGTCTCCATGTCCTGAAACATGGAGCAGGACGAGGACGACAGCAGGACACAG
GAGCCTGGAAGTCTCCATGTCCTGAAACATGGAGCAGGACGAGGACGACAGCAGGACACAG
GACATGCAAGCAGAAATGGAAGATGATGCATATTTTATTATTTGATTACGACGAAAGATTAA

CTTCTTTCCTCCTCTGCTGACATTTAAATGGATGACACATCTCTATTTACATACAGGAC
CTTCTTTCCTCCTCTGCTGACATTTAAATGGATGACACATCTCTATTTACATACAGGAC

AGAAAGTTGACGGGACACTTGGTTTTCCCTTCAGAGATCTTGGTTTTCCCTACTAAAG
TATCCAGATTTCCCCATCACGTGTATTTTTTTTTTTTTACGTACTACCGAAAGATTAA

GCTGACAGAGCTTGTCGCGCCATTGCTGCATGTCCTGCTGACGACAGACAGACAGACAG
GCTGACAGAGCTTGTCGCGCCATTGCTGCATGTCCTGCTGACGACAGACAGACAGACAG

The nucleotide sequence—

**1** 5′-CCGCGCGCCATCGCGG
**2** 5′-CCGCGCGCCATCGCGG

**10428**

**Grx1as glutaredoxin cDNA. Sequences 1 and 2 represent Grx1as and Grx1, respectively. The nucleotides are numbered 5′ to 3′, and amino acids are numbered from N to C termini, respectively. Two conserved regions containing a total of four cysteines are underlined. Bold nucleotides indicate that they are the same in both clones, and asterisks indicate the stop codon.**
glutathione was expressed as glutathione S-transferase fusion protein, and the fusion protein was purified using a glutathione-Sepharose column (Amersham Biosciences).

**Dehydroascorbic Acid Reducing Activity**—Reducing activity was determined in 25 μl of 100 mM Tris-HCl buffer (pH 7.5) containing (final concentrations) 0.8 mM reduced glutathione, 500 μM dehydroascorbic acid, and appropriate amounts of enzyme. The reaction was initiated by adding dehydroascorbic acid and measured for 3 min at room temperature. The reaction was terminated by addition of 35 μl of 90% methanol in water containing 1 mM EDTA. The final mixture was centrifuged for 10 min at 14,000 × g, and the supernatant immediately analyzed by high performance liquid chromatography (24). A chemical reaction containing no enzyme was simultaneously measured under the same conditions. Where indicated chemical activity was subtracted from total reducing activity to yield enzymatic activity. Chemical activity was always measured and accounted for (3). The amount of reduced dehydroascorbic acid was determined as ascorbic acid by high performance liquid chromatography (24). A chemical activity was always measured and accounted for (3).

**Western Blot**—Western blot analysis was performed using anti-glutaredoxin serum, as described (3, 26). Protein samples were electrophoresed in 10–20% gradient SDS gels, then electroblotted onto a nitrocellulose membrane, and hybridized using the probe of the 3′-untranslated region of Grx1as. Glyceraldehyde-3-phosphate dehydrogenase controls are shown at the bottom of the figure.

**RESULTS**

**Isolation of Grx1as cDNA**—To isolate different types of human glutaredoxin cDNA, partial cDNA glutaredoxin from nucleotides 50 to 270 of the open reading frame was used as a probe (3). This region was selected because it contained four conserved cysteines including an active site cysteine. Plaques (8 × 10⁹) were screened under low stringent conditions with the 32P-labeled DNA probe. Positive plaques were selected, and the integrity of each plaque was tested by PCR, using internal primers located in the previously reported glutaredoxin cDNA (3, 27, 28) (data not shown). The nucleotide sequence of the isolated clone was determined and compared with that of Grx1 cDNA (Fig. 1). Surprisingly, the nucleotide sequences of the translation regions of both cDNAs were homologous, but the 3′-untranslated region of the newly isolated cDNA was different from that of Grx1 cDNA. To date, at least three mammalian glutaredoxin cDNAs have been reported, among which is swine glutaredoxin cDNA. Swine glutaredoxin cDNA contains a translation region exhibiting ~80% identity to the human counter part, but a region with no similarity is found in the 3′-untranslated regions of human and swine glutaredoxin cDNAs (19). The newly isolated glutaredoxin cDNA not only exhibited an identical nucleotide sequence to that of previously reported human glutaredoxin (Grx1) cDNA in the translation region, but also was quite similar to the 3′-untranslated region counterpart of swine glutaredoxin cDNA rather than Grx1 cDNA. We termed the newly isolated glutaredoxin cDNA Grx1as.

![Grx1as Is Present in Multiple Cell Types](Image)

**Expression of Grx1as mRNA**—mRNA was determined by Northern blot and RT-PCR with mRNAs from human neutrophils, U937, HL-60, TPH, and Jurkat cells. Transfers of mRNAs onto nitrocellulose membrane were performed using a standard method (22). The probe used was not a full sequence of Grx1as cDNA, because the translation region of Grx1as cDNA contained the same nucleotide sequence as Grx1 cDNA. The probe was amplified by PCR with the two primers (forward and reverse primers located at nucleotides 350–370 and 520–540, respectively in Fig. 1). The

| TABLE I | Biochemical and kinetic analyses of dehydroascorbic acid reducing activities of expressed Grx1 and Grx1as. |
|---------|-------------------------------------------------------------------------------------------------------|
|          | Recombinant Grx1 | Recombinant Grx1as |
| Optimal pH | 7.5 | 7.5 |
| Kₐ for glutathione (mM) | 2.2 | 2.2 |
| Kₐ for dehydroascorbic acid (μM) | 220 | 240 |
| Specific activity (umol/min/mg) | 3 | 3 |

![Fig. 3. Western blots of recombinant proteins expressed from Grx1 and Grx1as.](Image)
amplified DNA fragment that had no homology to Grx1 cDNA was 32P-labeled by the random primer method. The transferred nitrocellulose membrane was hybridized using stringent conditions with the 32P-labeled probe. Grx1 as cDNA was expressed in all the cells tested (Fig. 2). The data indicate that Grx1 as is expressed in cells of myelocytic and lymphocytic origins.

There are several indications that Grx1 as is a generally observed phenomenon and not an artifact from a single subject source. The library from which Grx1 as was originally isolated was prepared from neutrophils from 10 subjects. As shown in Fig. 2, Grx1 as expression was verified by Northern blot in all cells tested of myeloid origin: HL60 cells, THP cells, Jurkat cells, and PLB cells. As confirmation of these data, RT-PCR with specific primers for Grx1 as showed that the mRNA was present in all the myeloid cell types tested (data not shown). Finally, when Grx1 as specific primers were used, the mRNA was also present in a commercial myeloid library.

Characterization of Recombinant Grx1 as—The nucleotide sequences of Grx1 and Grx1 cDNAs are identical in their translation regions. Therefore, we predicted that the recombinant protein from Grx1 as cDNA would be likely to exhibit the same biochemical and kinetic properties as glutaredoxin from Grx1 cDNA. To study this, the translation region (open reading frame) of Grx1 as cDNA was amplified and ligated into pGEX fusion expression vector as described under “Experimental Procedures.” Glutathione S-transferase-fused protein was purified with specific primers for Grx1 as showed that the mRNA was present in all the myeloid cell types tested (data not shown). Finally, when Grx1 as specific primers were used, the mRNA was also present in a commercial myeloid library.

**Characterization of Recombinant Grx1 as**—The nucleotide sequences of Grx1, Grx1 as, and Grx1 cDNAs are identical in their translation regions. Therefore, we predicted that the recombinant protein from Grx1 as cDNA would be likely to exhibit the same biochemical and kinetic properties as glutaredoxin from Grx1 cDNA. To study this, the translation region (open reading frame) of Grx1 as cDNA was amplified and ligated into pGEX fusion expression vector as described under “Experimental Procedures.” Glutathione S-transferase-fused protein was purified with specific primers for Grx1 as showed that the mRNA was present in all the myeloid cell types tested (data not shown). Finally, when Grx1 as specific primers were used, the mRNA was also present in a commercial myeloid library.
using a glutathione-Sepharose column, and the fusion protein was digested with thrombin (0.1%, w/v) to yield recombinant protein. Glutaredoxin, from Grx1 cDNA, was isolated and purified as described (3).

Biochemical properties of the recombinant proteins from Grx1 and Grx1as cDNAs were characterized with respect to dehydroascorbic acid reducing activities (Table I). The following properties were virtually identical: apparent \( K_m \) for glutathione; apparent \( V_m \) for dehydroascorbic acid; optimal pH; and specific activity. Each recombinant purified protein was detected by Western blot (Fig. 3), using polyclonal antibody prepared by injecting Grx1 in rabbits (3). Taken together, these data indicate that biochemical and kinetics properties of the two proteins were indistinguishable.

**Isolation and Sequencing of the Genomic Gene of Grx1as.**—To delineate the origin of Grx1as cDNA, its genomic gene was screened in a neutrophil genomic library with the same probe used in the Northern blot. A positive plaque was selected as the candidate for the genomic gene of Grx1as cDNA. The integrity and authenticity of the clone were checked by PCR using several primers (see "Experimental Procedures"). To obtain detailed information on the nucleotide sequence of the isolated genomic gene, the clone was digested with restriction enzymes and subcloned into pGEM sequence vector. Surprisingly, the nucleotide sequence of the 5'-flanking region of Grx1as cDNA was exactly the same as that of the published genomic gene of Grx1 cDNA (17, 18). Due to this unexpected result, the nucleotides of other regions of the isolated genomic gene were further sequenced. We found that the sequenced regions of the Grx1as genomic gene were perfectly matched to the corresponding region of the Grx1 genomic gene (17, 18). Therefore, every individual subclone was amplified by PCR with specific primers used in the Northern blot in order to identify the subclone containing the unique 3'-untranslated region of Grx1as cDNA. One subclone was confirmed by PCR to contain the 3'-untranslated region, and sequenced for its verification. For clarity, a partial nucleotide sequence of this subclone is shown in Fig. 4. The subclone contained, in order as shown: an overlapping partial sequence of an intron; the 3'-untranslated region of Grx1as cDNA (as shown in Fig. 1); a unique 3'-untranslated region of Grx1as cDNA; and the 3'-untranslated region of Grx1 cDNA (also as shown in Fig. 1). The full sequence of this subclone is shown in Fig. 5 and represents the nucleotide sequence of the genomic gene of Grx1as. The sequence includes the coding region that is identical to glutaredoxin, and contains two introns and three exons. This gene organization is identical to that of the genomic gene for Grx1 (17, 18) (Fig. 6). Taken together, these data indicate that Grx1 and Grx1as cDNAs are produced from the same human glutaredoxin genomic gene via alternative splicing at ggccag/AACAGGCCC in the second intron instead of cacag/ATTCATAG (Figs. 5 and 6). Thus, Grx1as cDNA, but not Grx1, includes an additional 566 nucleotides from second intron in its 3'-untranslated region. The alternative splicing of human glutaredoxin genomic gene explains why human Grx1as and swine glutaredoxin cDNAs, but not Grx1 cDNA, are similar in their 3'-untranslated regions. To confirm this, a full-length Grx1as cDNA was cloned and completely sequenced. Sequencing the human glutaredoxin genomic gene showed that an isolated full-length cDNA of Grx1as contained the unique sequence of 3'-untranslated region of Grx1as, and 3'-untranslated region of Grx1 (Fig. 7). The 3'-untranslated region of Grx1as cDNA is approximately twice the size of the counterpart of the 3'-untranslated region of Grx1 cDNA.

**Chromosomal Localization of the Grx1as Gene.**—The sequence of the genes from Grx1 and Grx1as cDNAs indicated that each individual human glutaredoxin cDNA was probably transcribed from the same gene. This was confirmed by chromosomal localization of the isolated Grx1as gene. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidioxigenin antibodies followed by counterstaining with 4'-6-diamidino-2-phenylindole dihydrochloride. The results showed a specific labeling of chromosome 5, based on size, morphology, and banding pattern. In addition, experiments, a genomic probe (previously mapped to 5q32 and confirmed by co-hybridization with a probe from the cri du chat locus on chromosome arm 5p) was co-hybridized with the hybridized probe of the Grx1as gene. In these experiments the middle and distal long arm of chromosome 5 were specifically labeled. Measurement of ten specifically hybridized chromosomes 5 demonstrated that the glutaredoxin gene is located at a position which is 39% of the distance from the centromere to the telomere of chromosome arm 5q, an area which corresponds to the boundary between bands 5q15 and 5q21 (Fig. 8). This chromosomal position was previously mapped to the human Grx1 gene (29). The nucleotide sequence and chromosomal mapping of the human Grx1as gene indicate that Grx1as cDNA is undoubtedly transcribed from the same gene that human Grx1 cDNA comes from.

**Effect of Two 3'-Untranslated regions of Grx1 and Grx1as on Their mRNA Stability.**—During the screening of the Grx1 genomic gene, two different putative pseudogenes (GS1 and GS2) were isolated and sequenced. They seemed to display typical characteristics of pseudogenes: no intron, poly(A) tailing, and sporadic mutations. However, some regions of the 3'-untranslated regions of these genes were not homologous to the 3'-untranslated region of Grx1 cDNA. Instead, Grx1as cDNA and the two glutaredoxin pseudogenes were very similar in portions of their 3'-untranslated regions (Fig. 9). It was surprising that the pseudogenes had greater resemblance to
Grx1as than to Grx1 cDNA, because pseudogenes are commonly considered to originate from their authentic mRNA. Stability of mRNA may therefore be a contributing factor for generating pseudogenes. We investigated this possibility by measuring the effect of the 3′-untranslated regions of Grx1 and Grx1as on the stability of their mRNAs. The effect of the two 3′-untranslated regions was determined by constructing the vectors pCMV-Luc-3′-untranslated region Grx1 and pCMV-Luc-3′-untranslated region Grx1as, transfecting these vectors into cells, and measuring luciferase activity (see “Experimental Procedures”).

As shown in Table II, the decreasing order of luciferase activities was pCMV-Luc-3′-untranslated region Grx1as > pCMV-Luc-3′-untranslated region Grx1 > pCMV-Luc control. Since all three reporter vectors have the same CMV promoter, the promoter activities of the vectors and their initial mRNAs may be very similar. To verify this assumption, quantitative RT-PCR was performed with mRNAs from HeLa cells transfected with each reporter vector. Amounts of luciferase mRNA from the three transfected cells was very similar (data not shown). These data indicate that the quantity of luciferase mRNAs was similar in the cells transfected with three different reporter vectors, but mRNA containing 3′-untranslated region Grx1as might be more stable than the other two mRNAs, thereby producing more luciferase. Because the 3′-untranslated region of Grx1as mRNA may confer enhanced stability, Grx1as mRNA may be a better template than Grx1 mRNA for generating its pseudogenes.

**DISCUSSION**

On comparing the sequences of mammalian glutaredoxin cDNAs, we found that swine glutaredoxin cDNA contains a 3′-untranslated region non-homologous to human Grx1 cDNA (18, 19). The discrepancy between human and swine cDNAs suggested that human glutaredoxin cDNA might exist in more than one form. It seemed reasonable that the existence of different human glutaredoxin cDNAs and a non-homologous region in swine glutaredoxin cDNA should be addressed prior to investigating glutaredoxin regulation, because it is possible that each glutaredoxin cDNA may be regulated differently in human cells. As a way to search for different human glutaredoxin mRNA, a human neutrophil cDNA library was screened with a probe of Grx1 cDNA using non-stringent conditions. In the screening process, a different human glutaredoxin (Grx1as) cDNA was isolated. Surprisingly, the nucleotide sequence and chromosomal localization of the genomic gene of Grx1as cDNA indicated that Grx1as and Grx1 were transcriptional products derived from the same gene via alternative splicing. The isolation, sequencing, and localization of Grx1as cDNA provide an answer for the lack of identity between the 3′-untranslated regions of human glutaredoxin (Grx1) and swine glutaredoxin.

**FIG. 8.** Chromosomal localization of Grx1as gene by fluorescence in situ hybridization (FISH). Normal metaphase chromosomes from peripheral blood lymphocytes were hybridized with antidigoxygenin-labeled probe. A specific hybridization signal was detected, indicated as the top mark (G), and a cohybridization signal, indicated with the bottom mark (C) in the figure. The signal position was located in 5q15, demonstrated in the ideogram of chromosome 5.

Lucase activity was pCMV-Luc-3′-untranslated region Grx1as > pCMV-Luc control. Since all three reporter vectors have the same CMV promoter, the promoter activities of the vectors and their initial mRNAs may be very similar. To verify this assumption, quantitative RT-PCR was performed with mRNAs from HeLa cells transfected with each reporter vector. Amounts of luciferase mRNA from the three transfected cells was very similar (data not shown). These data indicate that the quantity of luciferase mRNAs was similar in the cells transfected with three different reporter vectors, but mRNA containing 3′-untranslated region Grx1as might be more stable than the other two mRNAs, thereby producing more luciferase. Because the 3′-untranslated region of Grx1as mRNA may confer enhanced stability, Grx1as mRNA may be a better template than Grx1 mRNA for generating its pseudogenes.

**FIG. 7.** Comparison of nucleotide sequences of full-length cDNAs of Grx1as and Grx1. Nucleotides are numbered 5′ to 3′, and amino acids are numbered from the N to C termini, respectively. Two conserved regions containing a total of four cysteines are underlined. The homologous regions are in bold, and the signal sequence for the poly(A) tail is double underlined.

Grx1as than to Grx1 cDNA, because pseudogenes are commonly considered to originate from their authentic mRNA. Stability of mRNA may therefore be a contributing factor for generating pseudogenes. We investigated this possibility by measuring the effect of the 3′-untranslated regions of Grx1 and Grx1as on the stability of their mRNAs. The effect of the two 3′-untranslated regions was determined by constructing the vectors pCMV-Luc-3′-untranslated region Grx1 and pCMV-Luc-3′-untranslated region Grx1as, transfecting these vectors into cells, and measuring luciferase activity (see “Experimental Procedures”). As shown in Table II, the decreasing order of luciferase activities was pCMV-Luc-3′-untranslated region Grx1as > pCMV-
splicing or from two different transcripts generated using two different promoter regions of the same gene. Primer extension and S1 mapping experiments answered this question (18). Human neutrophil mRNA exhibited only one band protected from S1 nuclease, even though neutrophils contain two different glutaredoxin mRNAs (18) (Fig. 2). These data suggest that one promoter is apparently used for expression of a pre-mRNA leading to two different human glutaredoxin mRNAs via alternative splicing. Determining how this process proceeds may advance understanding of the cellular response to oxidative stress. In our preliminary studies, a certain region of the promoter of human glutaredoxin gene was identified as a transcriptional regulatory region, which may bind to several potential transcriptional factors. Future investigation will provide detailed information regarding the regulation of human glutaredoxin via this transcriptional regulatory region.

When we began this study, it was unclear whether isoforms of human glutaredoxin existed. However, human glutaredoxin 2 (Grx2) has been isolated recently, and two mRNAs of Grx2 have been identified as alternative splicing products (17). Interestingly, both Grx1 and Grx2 have two mRNAs generated via alternative splicing, respectively. During our screening of the human glutaredoxin 1 gene, two additional genes were isolated and sequenced. Each of these genes has characteristics of a pseudogene: non-existence of introns, possession of poly(A) tail, and sporadic mutations (30). Of interest, the isolated pseudogenes (GS1 and GS2) resemble Grx1 as cDNA rather than Grx1 cDNA. Furthermore, a glutaredoxin pseudogene (GS1) has a mutation of the first conserved cysteine to phenylalanine. Further investigation of this pseudogene is now underway to determine whether the pseudogene is expressed in cells, and what biological function the pseudogene may deliver, if expressed. Although alternative splicing is a common mechanism used to generate two mRNAs in Grx1 and Grx2, respectively, the biological consequences of the 3'-untranslated regions of Grx2 are largely unknown currently.

In this report, two different human glutaredoxin 1 mRNAs were demonstrated to exist, and potential biological consequences of the two mRNAs were elucidated. Glutaredoxin pseudogenes contain a long 3'-untranslated region, similar to that of Grx1 as cDNA. According to current hypotheses, mRNAs are templates for producing pseudogenes, and pseudogenes contain a poly(A) tail, sporadic mutations, and no introns. If so, human Grx1 as cDNA with a long 3'-untranslated region was perhaps more stable than Grx1 cDNA with a short 3'-untranslated region. Greater stability would increase the likelihood of Grx1 as serving as a template for human glutaredoxin pseudogenes. This study showed that a luciferase gene with the longer 3'-untranslated region of Grx1 as had a more stable transcript.
than the shorter 3' untranslated region of Grx1, as indicated by greater luciferase activity in transfected cells. In summary, the data in this paper provided information about the expression of different human glutaredoxin 1 cDNAs and their mRNA stability, and also may provide insight about the origin and potential biological consequence of the 3'-untranslated region from Grx2.

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