This work provides the first absolute expression patterns of genes coding for all known components of both thioredoxin (Trx) and glutaredoxin (Grx) systems in mouse: Trx1, Trx2, Grx1, Grx2, TrxR1, TrxR2, thioredoxin/glutathione reductase, and glutathione reductase. We devised a novel assay that, combining the advantages of multiplex and real-time PCR, streamlines the quantitation of the actual mRNA copy numbers in whole-animal experiments. Quantitations reported establish differences among adult organs and embryonic stages, compare mRNA decay rates, explore the significance of alternative mRNA isoforms derived from TrxR1 and Grx2 genes, and examine the time-course expression upon superoxide stress promoted by paraquat. Collectively, these quantitations show: i) unique expression profiles for each transcript and mouse organ examined, yet with some general trends like the higher amounts of mRNA species coding for thioredoxins than those coding for the reductases that control their redox states and activities; ii) continuous expression during embryogenesis with outstanding up-regulations of Trx1 and TrxR1 mRNAs in specific temporal sequences; iii) drastic differences in mRNA stability, liver decay rates range from 2.8 h (thioredoxin/glutathione reductase) to ≥ 35 h (Trx1 and Trx2), and directly correlate with mRNA steady-state values; iv) testis-specific differences in the amounts (relative to total isoforms) of transcripts yielding the mitochondrial Grx2a and 67-kDa TrxR1 variants; and v) coordinated up-regulation of TrxR1 and glutathione reductase mRNAs in response to superoxide stress in an organ-specific manner. Further insights into in vivo roles of these redox systems should be gained from more focused studies of the mechanisms underlying the vast differences reported here at the transcript level.

Thioredoxin (Trx) and glutaredoxin (Grx) are ubiquitous proteins with redox-active cysteines. They were discovered as independent hydrogen donors for the essential enzyme ribonucleotide reductase. Subsequently, they were shown to be general thiol-disulfide oxidoreductases. Trx and Grx differ in their specific reductive pathways, although ultimately reducing equivalents come from NADPH. Thus, the Trx system is composed of NADPH, thioredoxin reductase (TrxR), and Trx; the Grx system of NADPH, glutathione reductase (GR), glutathione (GSH), and Grx. The properties and multiple physiological functions assigned to these systems have been reviewed, particularly in relation to redox control of cell functions and protection against oxidative damage (2, 3).

Two Trxs have been described in mammals, one predominantly cytosolic (Trx1) (4) and one mitochondrial (Trx2) (5). The cytosolic Trx1 performs many biological actions in defense against oxidative stress, regulation of gene expression, and control of growth and apoptosis, and it has also been implicated in many pathological situations including cancer (6). Trx1 and Trx2 are composed of only a thioredoxin domain (6). A second group of proteins with additional domains, like the spermatid-specific thioredoxins Sptrx1 and Sptrx2, have been also identified within the thioredoxin family (7). Grxs are regarded as reductants of disulfides via GSH controlling also the levels of GSH-mixed disulfides, particularly under conditions of oxidative stress (3). Like Trxs, Grxs are being implicated in a growing list of pivotal functions such as cellular differentiation (8), apoptosis (9), and regulation of transcription factor binding activity (10). Nowadays two different isoenzymes of mammalian Grxs have been described, the classical cytosolic enzyme (Grx1) and a novel mitochondrial protein (Grx2) with alternative splice variants (11, 12).

A single gene for GR has been described in mammals. However, mammalian GR was shown to possess a mitochondrial signal peptide, allowing it to be directed to mitochondria (13, 14). Likewise, GSH is present in the mitochondria, accounting for ~10% of the cellular GSH pool (15). Mitochondrial GR and GSH together with Grx2 may therefore play a critical role in protecting this organelle from ROS generated in the respiratory chain (11). Mammalian TrxRs are homologous to GR and have a C-terminal elongation containing a conserved catalytically active selenocysteine residue in the penultimate position (16). Mammalian TrxRs reduce not only protein disulfides such as that in oxidized Trx but also a wide variety of other disulfide and non-disulfide substrates, even H2O2 and lipid hydroperoxides (3).

Three isoenzymes of mammalian TrxRs have been described (17). TrxR1 (also called TRα and TR1) is designed as the dominant cytosolic enzyme (18, 19). TrxR1 exhibits extensive heterogeneity. In particular, a 67-kDa isofrom with an additional N-terminal sequence has been identified (20). TrxR2 (TRβ and TR3) is referred to as the mitochondrial type (19, 21, 22). TrxR2 together with Trx2 compose a mitochondrial Trx system, different from the one present in the cytosol. The third isoenzyme
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was initially described as a novel form of TrxR (or TR2) (17). Later, this new selenoprotein oxidoreductase was designated thioredoxin/glutathione reductase (TGR) for thioredoxin and glutathione reductase. TGR can reduce several components of both Trx and Grx systems. This unusual substrate specificity is achieved by a natural fusion of TrxR and Grx domains (23).

This work aimed the simultaneous analysis of the expression of gene coding proteins of mouse Trx and Grx pathways. Control of mRNA levels is crucial in regulating protein production in mammalian cells. Hence, the exhaustive investigation of the in vivo status of mRNA levels should provide valuable information for understanding the functional roles of the four redoxins (2 Trxs and 2 Grxs) and four reductases (2 TrxRs, TGR, and GR) presently known in mouse. Nowadays, reverse transcription followed by PCR is the most powerful tool for detection and quantitation of mRNAs. Herein, we report the combined use of multiplex and real-time PCR procedures for rigorous quantitation of the real number of mRNA molecules. We wanted to know how the organ context, gestational age, alternative splicing, turnover rate, and oxidizing stress conditions modulate the absolute expression patterns of investigated transcripts. Whole-animal experiments were performed for these purposes.

MATERIALS AND METHODS

Animals and Treatments—Male BALB/c mice of 7 weeks of age were from Charles River Laboratories. After a 3-day acclimation period, animals were divided into groups of 3 subjects. Mice were stressed by intraperitoneal injection with paraquat (PQ) at a dose of 30 mg kg\(^{-1}\) body weight dissolved in normal saline. For mRNA decay quantitations, mice were intraperitoneally injected with 2 mg kg\(^{-1}\) body weight of actinomycin D (AmD) dissolved in phosphate-buffered saline. Animals injected with saline solution or phosphate-buffered saline served as vehicle controls. Total RNA was extracted at the times indicated in figures. Basal expression levels were determined in non-injected mice. Animals were killed by cervical dislocation. The organs were then removed and immediately frozen in liquid nitrogen. Mice were handled according to the norms stipulated by the European Community. The investigation was approved by the Ethical Committee of Córdoba University. Mean ± S.E. values were calculated by averaging data from each experimental group.

RNA Preparations and Reverse Transcription—RNA extraction and synthesis of standard RNA and of cDNA were as detailed (24). Commercial RNAs were from Clontech, except those of ovary total RNA from Ambion. RNA sample quality was checked electrophoretically, and quantification was done spectrophotometrically. Lack of DNA contamination was checked by PCR amplification of RNA samples without prior cDNA synthesis.

Primer Design—Sequences of genes were taken from GenBank\(^{2,4}\) for primer design. Primers were made with Oligo 6.1/98 (Molecular Biology Insights) program. To obtain the highest specificity and performance, primers were required to have high Tm \((>83°C)\) and optimal 3'ΔG \((-8.4\text{kcal/mol})\) values. Two pairs of primers were designed for Grx2 and TrxR1 genes. Primers within exon 3 of Grx2 gene amplify all known types of alternative transcripts, whereas primers on exons 1 and 2 amplify only those transcripts (hereafter named Grx2a) with the mitochondrial signal sequence (12). Likewise, whereas primers within exon 9 of TrxR1 gene amplify all types of alternative splicing, amplification by primers on exon 3b and 4 was restricted to transcripts (TrxR1-III) encoding the 67-kDa isoform of mouse TrxR1 (20, 25). Primers in Table I generate specific PCR products of the desired length and nucleotide sequence.

Real-time PCR—Real-time PCR conditions were as detailed previously (26). Primers were at 0.125 μM. PCR conditions were optimized as detailed previously (26) to ensure that the amplifications were in the exponential phase and the efficiencies remained constant in the course of the PCR. Twenty-three cycles of PCR were performed. MPCR reactions were carried out in duplicate. Reaction products were quantitated as detailed previously (26), with the particularity that this time the reference was any of the investigated transcripts (typically TrxR2 mRNA). All transcripts (except Grx2a and TrxR1-III mRNAs) were amplified by MPCR in all samples. MPCR technique was used to calculate the ratio between the fluorescent signal strength (given by GeneScan electropherogram) of each PCR product and the signal strength of the reference in the same reaction. Then, by dividing the fluorescence ratios calculated in each examined sample by those given by the reference sample (typically liver from untreated mice), we obtained the fluorescence ratio variation (FV) values. Absolute measurements (mRNA molecules) were inferred from FV data, according to Equation 1,

\[
E_e = (FV \times R_e \times E_r/R_e)
\]

where \(E_e\), \(E_r\), and \(R_e\) designed the copy number of the examined (E) or reference (R) transcript, in the examined (e) or reference (r) sample, respectively. As outlined above, these values were obtained by real-time PCR. Samples for comparison of different experimental conditions were handled in parallel.

RESULTS

Absolute Quantitation of mRNA Levels—We previously designed and optimized a reverse transcription-MPCR procedure in which all target transcripts and one reference are co-amplified in a single reaction. Specific fluorescent primers are used and amplification products are analyzed with a DNA sequencer. Relative expression of the targets to the reference is measured and relative comparisons of mRNA levels among different samples are made (26, 27). Trends in gene expression can be explained by relative quantitation, but the results are strongly dependent on the normalization procedure used. Actually, we have recently raised the question about the inaccuracy of relative quantitation in complex animal studies, and we have alternatively proposed the quantitation of the real copy number of transcripts by means of real-time PCR technique (24).

Absolute real-time PCR analysis of a large number of transcripts in a large number of samples is expensive and time consuming. To solve this problem, we first devised a novel experimental approach that combines the advantages of both multiplex and real-time PCR. For the mouse genes of the Trx and Grx pathways a set of 8 primer pairs were designed (Table I). Relative data were obtained for all transcripts in all samples by MPCR, and then absolute measurements were inferred by determining by real-time PCR the actual mRNA level of one gene in all samples and those of all genes in one reference sample (see “Materials and Methods”). Table II demonstrates that the number of mRNA molecules estimated from MPCR is equivalent to that calculated by real-time PCR.

Organ Expression Profiles of Trx and Grx Transcripts—Our first goal was to quantitate the steady-state amounts of transcripts at the whole organ level (Fig. 1). Among the organs examined, the lowest Trx1 mRNA amounts were quantitated in brain, heart, and testis (average of 62 molecules/pg) and the highest in kidney (274 molecules/pg) followed by lung (159 molecules/pg). The Trx2 mRNA expression pattern did not correlate with that of Trx1. Therefore, though Trx2 mRNA appeared with maximal expression level in kidney (157 molecules/pg), high levels were quantitated also in heart and testis (average of 135 molecules/pg) and low in lung (36 molecules/pg). In the line of Trx7 transcripts, Grx1 and Grx2 mRNAs were ubiquitously synthesized and presented vast differences in abundance depending on the tissue type. Thus, Grx1 mRNA was largely overexpressed in kidney (121 molecules/pg) com-
Table I

Properties of reverse transcriptase PCR amplicons used for absolute quantitation of mRNA

Transcripts were quantitated by means of both multiplex and real-time PCRs, except for Grx2a and TrxR1-III mRNA isoforms. The standard was an engineered GAPDH cDNA with a 7 bp deletion, resulting in a PCR product of 130 bp. Exons were according to NCBI Evidence Viewer, except those for Grx2 and Grx2a, and TrxR1 and TrxR1-III that were according to Refs. 12 and 25, respectively. Standard curve equations were obtained using liver samples, except those for TGR and TrxR1-III, and that for Grx2a that were obtained using testis and brain samples, respectively. Forward (F) primers were 5' HEX-labeled for multiplexed PCRs.

| Target (GenBank) | Size (bp) | Primers | 5'-position | Exon | Standard curve equation | Real-time (correlation coefficient) | Efficiency |
|-----------------|----------|---------|-------------|------|-------------------------|--------------------------------------|------------|
| Multiplex/Real-time |          |         |             |      |                         |                                      |            |
| Trx1 (X775565) | 160      | 5'-CGT GGT GGA CTT CTC TGC TAC GTG GTG-3' (F) | 143  | 2   | y = -3.24x + 23.96 (0.99) | 1.04                                  |            |
|                  |          | 5'-CGT CGG CAT GCA TTT GAC TTC ACA GTC-3' (R) | 302  | 4   | y = -3.33x + 24.49 (0.99) | 1.00                                  |            |
| Trx2 (U85089)   | 168      | 5'-TCC TCG TCG TCG ATC CCC AGA AAG TTG-3' (F) | 348  | 3   | y = 3.49x + 25.72 (1.00) | 0.93                                  |            |
|                  |          | 5'-TGG AGA GAA GAT GGT CGC CAA GCA GCA-3' (R) | 515  | 4   | y = 3.46x + 27.90 (0.99) | 0.95                                  |            |
| Grx1 (AB013137) | 190      | 5'-CGT AGA ACC CAAT GCC ATC ACC CACT GCA AGG-3' (F) | 125  | 3   | y = -3.32x + 25.84 (0.99) | 1.00                                  |            |
|                  |          | 5'-TGG AGA CAT GAT CTC GGC CTA TG-3' (R) | 314  | 2   | y = 3.46x + 27.90 (0.99) | 0.95                                  |            |
| Grx2 (AF276918) | 123      | 5'-TGA TCT GCT CTT GCT GTT CCA TTC GGC GC-3' (F) | 142  | 3   | y = 3.36x + 27.97 (0.99) | 0.98                                  |            |
|                  |          | 5'-TGG AGA GAA GAT GGT CGC CAA GCA GCA-3' (R) | 264  | 3   | y = 3.36x + 27.97 (0.99) | 0.98                                  |            |
| TrxR1 (AB027565)| 175      | 5'-CGT AGA ACC CAAT GCC ATC GCA GCA AGG-3' (F) | 854  | 9   | y = 3.32x + 25.84 (0.99) | 1.00                                  |            |
|                  |          | 5'-TGG AGA CAT GAT CTC GGC CTA TG-3' (R) | 1028 | 10-11 | y = 3.36x + 27.97 (0.99) | 0.98                                  |            |
| TrxR2 (AB027566)| 108      | 5'-TCC CCC CCC CCC ATC AAA AAA ATC CCC CCA AC-3' (F) | 875  | 12  | y = 3.35x + 21.35 (1.00) | 1.03                                  |            |
|                  |          | 5'-GCC CCA CCG AGG ATG GTC GAA GGT GC-3' (R) | 982  | 12  | y = 3.35x + 21.35 (1.00) | 1.03                                  |            |
| TGR (AF349659)  | 116      | 5'-TGA TCG TCG TCG ATC CCC AGA AAG TTG-3' (F) | 1046 | 8-9 | y = 3.25x + 21.35 (1.00) | 1.03                                  |            |
|                  |          | 5'-TGG AGA GAA GAT GGT CGC CAA GCA GCA-3' (R) | 1161 | 9   | y = 3.32x + 25.19 (0.99) | 1.03                                  |            |
| GR (X76341)     | 100      | 5'-TTA TCA TCG TCG TCG ATC CCC AGA AAG TTG-3' (F) | 1270 | 12  | y = 3.24x + 25.19 (0.99) | 1.03                                  |            |
|                  |          | 5'-GTC TCA TCG TCG TCG ATC CCC AGA AAG TTG-3' (R) | 1369 | 12-13 | y = 3.24x + 25.19 (0.99) | 1.03                                  |            |
| Real-time        |          |         |             |      |                         |                                      |            |
| Grx2a (AF380337)| 102      | 5'-CTG GTG GCG GCG AGG AGG ATC TT-3' (F) | 73   | 1   | y = 3.28x + 27.08 (0.99) | 1.02                                  |            |
|                  |          | 5'-CTG AGA CTT CTT CCC CCA AAA GCA GCA GGT GC-3' (R) | 174  | 2   | y = 3.28x + 27.08 (0.99) | 1.02                                  |            |
| TrxR1-III (AF333036)| 168     | 5'-CTG TTT ACC GTC TCC AGA CAT GC-3' (F) | 296  | 36  | y = 3.23x + 31.50 (1.00) | 1.04                                  |            |
|                  |          | 5'-CTG ATG GTC TCC CTA GCA GCA GCA GCA ATG GC-3' (R) | 463  | 4   | y = 3.23x + 31.50 (1.00) | 1.04                                  |            |
| Standard (M32599)| 130      | 5'-GGG TGC CCA GCA TTA ATC CCC ATC GCA GCA ATG GC-3' (F) | 643  | 10-11 | y = 3.18x + 40.01 (0.99) | 1.07                                  |            |
|                  |          | 5'-AGG TCA TAG GCA CCG CCC AGA GCA GCA CAT CGC AT-3' (R) | 779  |     | y = 3.18x + 40.01 (0.99) | 1.07                                  |            |
pared with the lowest level in testis (14 molecules/pg) and with the levels in other mouse organs (average of 29 molecules/pg). In contrast, Grx2 mRNA was highly represented in testis (148 molecules/pg), compared with kidney and brain (average of 22 molecules/pg), and the rest of organs (average of 9 molecules/pg). To our knowledge, this is the first time that the organ distribution of mouse Trx and Grx mRNA has been investigated, though it was suggested a possible increased Grx2 mRNA level in mouse testis based on the relatively high frequency of expressed sequence tags (7/36) from this organ (12).

**Organ Expression Profiles of Reductase Transcripts**—The expression patterns of TrxR1, TrxR2, TGR, and GR mRNA are compared in Fig. 1B. TrxR1 transcript was more abundant in liver and kidney (around 31 molecules/pg) than in the rest of organs examined (average of 13 molecules/pg). On the contrary, the TrxR2 mRNA displayed highest levels in spleen (27 molecules/pg) followed by kidney (15 molecules/pg), intermediate levels in liver, heart, and testis (about 7.9 molecules/pg), and lowest levels in lung and brain (around 4.3 molecules/pg). Therefore, TrxR1 and TrxR2 exhibited different organ expression patterns that were distinct also from those displayed by both Trx genes. Overall, the quantitative data given here are not in good agreement with previous Northern analyses of mouse TrxR1 and TrxR2 genes (17, 19, 21). For instance, Northern blots of mouse TrxR2 mRNA showed much higher expression levels in liver than in spleen (19). On the contrary, we clearly quantitated a higher amount (by a factor of 3.9) of TrxR2 mRNA in spleen than in liver. This and other discrepancies may be explained, at least in part, by the inaccuracy of the β-actin gene as quantitative reference in organ mRNA profiling (24). GR mRNA was generally more abundant than TrxR1 and TrxR2 mRNA species. The organ expression profile was also different: kidney showed the highest amount (198 molecules/pg), followed by lung, liver, testis, and brain (about 43 molecules/pg), and then by spleen and heart (around 21 molecules/pg).

Previous studies have suggested that TGR might be testis-specific (17). Accordingly, we quantitated much higher number of TGR transcripts (760 molecules/pg) in testis than in the rest of organs (≤11 molecules/pg) (Fig. 1B). Besides this outstanding testis specificity, the refined reverse transcription-PCR procedure used in this work revealed substantial differences among other organs. Thus, TGR mRNA was overexpressed an average of 6.1 in lung and kidney (around 11 molecules/pg) compared with the lowest level in spleen and liver (about 1.8 molecules/pg). Interestingly, TGR mRNA in testis was as abundant as β-actin mRNA (24), which is regarded as a rather abundant transcript. However, in the rest of the mouse tissues, though much less abundant, TGR mRNA cannot be considered a rare transcript because its amount was roughly in the range of mRNAs coding for other reductases (particularly TrxR2).

**Alternative Splicing Forms**—Differential pre-mRNA splicing is an important mode of regulating the steady-state abundance of a specific mRNA (28). Examination of expressed sequence tags for mouse Grx2 has revealed alternative mRNAs that differ in sequences upstream of exon 2 (12). Therefore, whereas sequences corresponding to exon 2 were identical in all expressed sequence tags, alternative splicing forms lacked the sequence encoding the mitochondrial signal peptide within exon 1. Here, we quantitated this alternative splicing by designing a second pair of primers (Table I). In this pair, the forward primer was located on the mitochondrial leader sequence within exon 1 and the reverse primer on exon 2, so that the specific mRNAs encoding the mitochondrial Grx2a could be distinguished from the rest of alternative splice variants. The Grx2a mRNA isoform accounted for ~40% of all available Grx2 mRNAs in each of the mouse organs examined. The outstanding exception was the testis, where the Grx2a variant represented only 1% of total Grx2 mRNAs (data not shown).

Three alternative mRNA isoforms have been derived from the single mouse TrxR1 gene by initiating transcription at exons 1, 2, or 3, followed by alternative splicing to link the used exon to exon 4 (20). As reported previously (20, 25), TrxR1-I and TrxR1-II mRNA isoforms would contain the principal ATG start codon within exon 4 and translation of these two mRNAs would yield the first reported mouse TrxR1 protein of 54.5 kDa (19). However, the TrxR1-III mRNA isoform would utilize another ATG start codon in exon 3 (actually exon 3b), yielding the 67-kDa TrxR1 variant with the extended N-terminal domain (20). The levels of this TrxR1-III mRNA isoform were quantitated with a second pair of primers within the TrxR1 gene (Table I). In this pair, the forward sequence was located on exon 3b and the reverse sequence on exon 4. TrxR1-III mRNA was detected in all tissues examined, though at a very low level that varied in amount from 0.2 (testis) to 0.02 (rest of organs) mRNA molecules/pg (data not shown). These low quantities indicate that the TrxR1-III mRNA isoform is infrequently processed in mouse organs. The highest abundance quantitated in testis made up 1% of total TrxR1 mRNAs. This percentage fell to 0.1% in the rest of mouse tissues.

**Effect of Fetal Gestational Age on Transcript Levels**—Gene expression patterns change substantially during embryonic development (29). To investigate whether the abundance of transcripts examined here is developmentally regulated, we analyzed samples from whole mouse embryos at 3 different developmental stages (Fig. 2). Gastrulating E7 embryos are mostly reliant on anaerobic metabolism, with >90% of glucose being accounted for by lactate production (30). In E11 embryos, mitochondrial ultrastructure becomes characteristic of actively aerobically respirating cells. E17 embryos are in late gestation (total of 19 days).

A large overexpression of 11-fold in Trx1 mRNA occurred as early as E7 in the developing mouse embryo. Thereafter, the amount of Trx1 mRNA declined rapidly to the steady-state level measured in adult organs. In contrast to Trx1 mRNA elevation, there were no substantial changes in expression of Trx2, Grx1, and Grx2 during mouse gestation. The Grx2a mRNA with the mitochondrial signal displayed an elevation of 3-fold at E11. Hence, in E11 embryos, Grx2a mRNA made up 53% of total Grx2 mRNAs, compared with 35% quantitated in other stages of embryogenesis. Relative to the average value
estimated in adult organs, transcript coding for TrxR1 was elevated along murine embryogenesis, with a peak of 6-fold at E11. In contrast, no significant tendencies to elevation over adult levels were observed for transcripts coding for the other reductases, except for the near 2-fold increase of GR mRNA at E7. As in adult tissues, the TrxR1-III spliced variant was infrequently processed in mouse embryos, though it was preferentially expressed at later stages of embryogenesis. Indeed at E17, the TrxR1-III isoform made up 1.5% of total TrxR1 mRNAs, which is higher than 1% quantitated in adult testis.

**Constitutive mRNA Decay Profiles**—The steady-state abundance of every mRNA is dependent on both its rate of synthesis and rate of decay. Therefore, it follows that the constitutive stability of a particular mRNA is an important parameter to be determined. To this purpose, AmD was administered to stop transcription. The number of mRNA molecules remaining after
the transcription shut-off was then quantitated for calculation of the intrinsic half-life of every transcript.

The half-lives in liver varied widely, ranging from 2.8 h (TGR mRNA) to 35 h (Trx1 mRNA) or even longer (Trx2 mRNA) (Fig. 3). Overall, transcripts encoding Trx1 and Trx2 persisted 6- to >3-fold longer than those encoding the corresponding TrxR1 and TrxR2. Besides, mRNAs that encode the mitochondrial Trx system (Trx2 and TrxR2) turned over more slowly than those encoding the system present in the cytosol (Trx1 and TrxR1). Moreover, whereas mRNAs that encode both Trxs were more stable than those encoding both Grxs, the decay rate of the mRNA that encodes the unique GR was 2–3.5-fold above the decay rates of transcripts encoding both TrxRs. We attempt to derive a quantitative relationship between the calculated decay rates of mRNAs (Fig. 3) and their steady-state amounts (Fig. 1) by examining the association between these 2 parameters in mouse liver. A positive significant (p = 0.05) correlation (r = 0.86) was found, and this correlation was remarkably good (r = 0.94) when mitochondrial isoforms were discarded for testing data for statistical significance.

To evaluate putative organ-specific variations in mRNA stability, other 3 mouse organs were examined. AmD was less effective in lung and kidney than in liver. Hence, the decay rates of most transcripts could not be accurately quantitated in these 2 organs. The exceptions were the short-lived TrxR1 and TGR mRNAs. In these cases, half-lives in lung (5.6 and 3.0 h) and kidney (5.3 and 3.4 h) were quite similar to those in liver (6.2 and 2.8 h). In tests, no reduction in mRNA levels (including that of heme-oxygenase 1) was observed, suggesting that this mouse organ did not take up AmD at the dose and incubation time used here. AmD doses higher than 2 mg kg⁻¹ body weight (−1/2 the LD₉₀, see Ref. 31) Time courses in lung, liver, kidney, and testis were analyzed. Transcript coding for heme-oxygenase 1 controlled the effectiveness of PQ treatment (Ref. 24 and data not shown).

Lung is considered the primary target organ of PQ toxicity (a pneumotoxicant). In lung, PQ up-regulated the transcript levels of both TrxR1 and GR genes (Fig. 4). In terms of fold variations, the results indicate that induction of TrxR1 mRNA in lung may have occurred somewhat more rapidly, and did achieve a higher maximum level than that found for GR mRNA. However, in terms of absolute quantitation, the maximal variation at 4 h of PQ exposure denoted an increment of 41 TrxR1 mRNA versus 61 GR mRNA molecules/pg over their respective steady-state levels. Significant up-regulation was observed also in mouse liver, but the increments in both TrxR1 and GR mRNA levels were lower than those quantitated in lung. In contrast to what is shown in Fig. 4, PQ did not trigger any significant variation of other mRNA levels at the experimental conditions investigated in this work. Moreover, no significant response was quantitated either in kidney or testis for any of the mRNA species examined herein. This lack of effect has to be interpreted in terms of organ specificity because 3–4-fold up-regulation was readily measured in both mouse organs for heme-oxygenase 1 mRNA (data not shown).

**DISCUSSION**

Trx and Grx are major endogenous redox molecules that participate in the redox control of a great variety of biological processes in animal cells. Taking advantage of a novel assay that combines multiplex and real-time PCR, we report the first refined quantitative analysis of the actual mRNA levels of mouse Trxs and Grxs and those of TrxRs, TGR, and GR as part of the so-called Trx and Grx systems.

The absolute quantitation of the steady-state levels of transcript molecules across diverse organs in intact mice revealed a unique expression profile for each gene and organ analyzed. These particular patterns of expression presumably underlie the requirement for different proteins to contribute to the specific functions of the different mouse organs. As a whole, outstanding findings are as follows. i) Trx1 and Trx2 mRNAs are more abundant (average factor of 7.5-fold) than TrxR1 and

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**Fig. 3. Decay rates of mRNAs in mouse liver.** A, example of mRNA decay profile. Filled-in symbols represent TGR mRNA molecules/pg of total RNA in each AmD injected animal. The open symbol at 0 h represents the mean value of 5 control mice. These vehicle controls showed no significant change in any transcript level over the duration of the experiment. B, calculated half-life for the decay of each mRNA. The decays of mRNAs coding for peroxiredoxin 1, heme oxygenase 1 and β-actin were determined for comparisons.
 Transcript Amounts of Mouse Trx and Grx Systems

FIG. 4. Time courses of TrxR1 and GR mRNA inductions in mouse lung and liver in response to PQ. Data are the means of mRNA molecules/pg of total RNA ± S.E. of the samples from 3 mice in each group. Data at 0 min represent the mean values ± S.E. of 5 control mice. No time-related effect was noted in these vehicle controls. Some error bars are not visible because of small standard errors. Statistical significance was evaluated using analysis of variance followed by post-hoc multiple comparison according to the Student-Newman-Keuls method. Significant differences relative to control animals are indicated by filled-in symbols (fold variations are given for discussion).

TrxR2 mRNAs. This suggests the need for higher levels of Trxs than for the reductases that in turn control the redox state and activity of these molecules. i) Maximal fold variation among the mouse organs examined is lower for mRNA species of the Trx pathway (Trx1, Trx2, TrxR1, and TrxR2) than for mRNA species of the Grx pathway (Grx1, Grx2, and GR). This may underlie the requirement of the Trx system in wider and less specific metabolic functions than the Grx counterpart. iii) Both Trx and Grx pathways have high levels of basal expression in kidney, as illustrated by the maximal abundances of Trx1, Trx2, and TrxR1 and of Grx1 and GR transcripts. This overexpression indicates a good basal antioxidant status to sustain renal functions. iv) Transcripts encoding the atypical TGR is overrepresented in testis, where it exists in amounts as high as 760 molecules/pg of total RNA. This finding is consistent with the idea that TGR might function as the reducing system (6), given the short cell cycle times at early stages of development and the evidence that oxidative metabolism is not yet reinduced in E7 embryos (30). The switch from rather anaerobic to aerobic metabolism later in the developing mouse (at E9 to E11 stage) would explain the large up-regulation quantitated for TrxR1 mRNA in E11 embryos, given the observation that TrxR1 mRNA abundance increases in response to increased ROS production.

Alternative splicing pattern of the first exon is a common genetic mechanism for generating protein species that differ in their intracellular location and for elevated expression of a protein in a tissue-specific manner (28). A previous study (20) demonstrated a remarkable heterogeneity within TRs, which, at least in part, results from the use of alternative first exon splicing. We report here that the particular transcript encoding the minority 67-kDa form of TrxR1 identified in mouse liver preparations (20) is present in all investigated samples though at low levels. Nonetheless, the considerably highest expression quantitated in testis and late in embryogenesis supports the notion of a tissue-specific variant, whose functional properties should be studied further. Likewise, the observation that Grx2 transcripts are overrepresented in testis, where the Grx2a mRNA isoform with the mitochondrial signal is poorly expressed, deserves further attention to provide some light on how the Grx2 alternative splice forms could result in expression of functional proteins and in which biological activities they could be predominantly involved (11, 12).

The intrinsic stability of mRNAs is an important component of the gene expression program (33). Most data on mammalian mRNA stability have come from experiments with cultured cells. However, because the tissue-cultured models have unavoidable limitations, quantitations made in animal studies are critical (34). Here, we have precisely calculated the decay rates in intact animals of the mRNAs encoding the group of proteins of the Trx and Grx systems. We demonstrate drastic differences of more than 10-fold among the stabilities of these transcripts. Furthermore, our data indicate that their decay rates are directly proportional to their steady-state levels, at least when confined to mouse liver.

The mRNAs encoding both Trxs are very stable, as evidenced by half-lives ≥35 h. In contrast, the transcript encoding the novel TGR is highly unstable with a half-life of 2.8 h. The decay rate of TrxR1 mRNA is only 6.2 h, which is still relatively fast compared with those (> 10 h) of the rest of transcripts investigated in this work. It is the wide range of mRNA decay rate that contributes significantly to the regulation of gene expression in higher eukaryotes. Therefore, stable mRNAs permit a prolonged translational window for genes that are expressed at high levels. In contrast, mRNAs that code for proteins produced in response to internal or external stimuli use to have short-lives (35).

Like transcription, processing, and translation, mRNA degradation is a tightly regulated process. One important example of specific cis-acting sequences controlling the half-life of mRNAs are AU-rich elements typically found in the 3'-untranslated region (3'-UTR) of many unstable mammalian mRNAs, like the protooncogene c-fos (36). The 3'-UTR of the mRNA coding for human TrxR1 contains AU-rich elements, which down-regulate basal mRNA levels in normal unstimulated cells (37). Examination of the 3'-UTR of the corresponding mouse sequence (19) reveals the presence of 2 individual AUUUA sequences and a tandem repeat, AUUUAUUUAUUUUA, which might be crucial in determining the relatively rapid decay rate of TrxR1 mRNA, as determined here in several mouse organs. On the contrary, no such AUUUA sequences could be identified in the 3'-UTRs of transcripts coding for the other 2 isoenzymes of mouse TrxR (TrxR2 and TGR).
Further work will pursue the identification of sequences that might control the rapid turnover of Trx mRNA, as there is little doubt that unstable transcripts contain instability determinants (34). It is known that changes of only 2–4-fold in the mRNA half-life can affect its abundance by orders of magnitude (34). Unfortunately, in the present in vivo study we were unable to determine to what extent an increased TGR mRNA half-life could account for the large difference in steady-state levels quantitated between testis and liver (760 versus 1.7 transcript molecules/pg). The TGR mRNA turnovers in kidney (3.4 h) and lung (3.0 h) were close to that determined in liver (2.8 h), although they follow the same decreasing order than the corresponding basal level amount, which was highest in kidney (11 mRNA molecules/pg) and lowest in liver (1.7 mRNA molecules/pg).

TrxR1 displays significant and relatively fast (within hours) increase of protein and mRNA upon treatment of human cells with different agents, like H$_2$O$_2$ (17), lipopolysaccharide (38), and peroxynitrite (39). The in vivo up-regulation of TrxR1 mRNA after short-term exposure (15–240 min) to subtoxic PQ we present here is consistent and extend those previous findings. The stability of many short-lived mRNAs changes in response to environmental and other factors. To our knowledge no report has demonstrated an increment in the TrxR1 mRNA half-life in response to any stimulus. Nevertheless, as the human TrxR1 promoter fulfills the typical criteria of a housekeeping gene (40), the TrxR1 response to exogenous agents is currently explained by post-transcriptional regulation via its functional AU-rich elements (38, 40). According to this model, PQ might increase the TrxR1 mRNA levels in mouse by blocking its degradation through AU-rich element-interacting protein(s) responding to intracellular ROS formation. This hypothesis is, however, difficult to be conclusively assessed in vivo, because long-term toxicity of animals pretreated with PQ would limit the effective measuring time for precise quantitation of PQ-induced mRNA decay. A promoter-driven regulation can also be proposed considering the presence of potential AP-1 binding sites in the promoter region for the mouse TrxR1 gene (25). Thus, control of mouse TrxR1 mRNA levels might be directed from elements present in both 5′ and 3′-UTR regions.

Regardless of the mechanistic details that regulate the TrxR1 mRNA level in vivo, data reported here reveal the importance of the animal organ context. Therefore, whereas TrxR1 mRNA readily responded in lung and liver to the challenge posed by PQ, no significant change was quantitated in kidney. Interestingly, the model short-lived c-fos mRNA showed a distinct and opposite organ response, as much higher up-regulation was quantitated in kidney (up to 21.5-fold) than in liver (up to 2.6-fold) and lung (up to 1.6-fold) (data not shown).

The mRNA level of GR (a key component of the Grx/GSH system) responded to the oxidizing conditions posed to PQ in a similar way as the mRNA level of TrxR1 (a key component of the Trx system). The ability of both TrxR1 and GR to transcriptionally respond to PQ might indicate the presence of a coordinated antioxidant cellular response, and potentially, a common signal pathway. The highest induction in lung suggests that TrxR1 and GR constitute a first line of defense against toxic oxidants in air. More detailed investigations should provide a deeper insight into the in vivo mechanism(s) that modulate TrxR1 and GR mRNA levels in mouse and its physiological role(s).

Although TrxR1 and GR mRNAs were elevated in lung and liver of mouse upon oxidative stress promoted by PQ, the levels of Trx and Grx mRNA species remained stable. The lack of Trx1 induction in vivo was unexpected, because previous studies had reported that oxidative agents increase expression of Trx1 gene in human cultured cells (41–43). However, considerable caution is necessary when attempting to extrapolate in vitro findings to the highly intricate and complex in vivo situation. For instance, though the Trx1 mRNA increased in the lens of model mice after photochemical oxidative stress, the expression of Trx1 gene was unaltered in liver and kidney, and therefore this Trx1 up-regulation was interpreted as a tissue-specific adaptation (44). Anyhow, the ability of mouse in maintaining tight control on both TrxR1 and GR mRNA levels should certainly contribute to the effectiveness of TrxRs and Grxs under oxidative stress conditions.

In short, here we provide for the first time the absolute expression patterns of transcripts coding for all known proteins of the Trx and Grx pathways in mouse. Differences in relation to adult organs, pre-mRNA splicing, embryonic stages, mRNA turnover, and oxidative stress conditions, have been established. Further insights into the functional roles of these two redox systems should be gained from the knowledge of the mechanisms underlying the vast differences found here at the transcript level. The novel methodological approach reported here will be of relevance in these incoming experiments and for the systematic quantitation of variations in transcript levels as putative causative factor in different pathological situations.

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