Regulation of Expression of the Phospholipid Hydroperoxide/Sperm Nucleus Glutathione Peroxidase Gene

TISSUE-SPECIFIC EXPRESSION PATTERN AND IDENTIFICATION OF FUNCTIONAL CIS- AND TRANS-REGULATORY ELEMENTS*

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A sperm nucleus glutathione peroxidase (snGPx), which is closely related to the phospholipid hydroperoxide glutathione peroxidase (phGPx), was recently discovered in late spermatids. Both GPx isozymes originate from a joint ph/snGPx gene, but their N-terminal peptides are encoded by alternative first exons. The expression of the two enzymes is differentially regulated in various cells, but little is known about the regulatory mechanisms. To explore the tissue-specific regulation of expression of the two isoenzymes, we first investigated their tissue distribution. Whereas phGPx is expressed at low levels in many organs, snGPx was only detected in testsis, kidney, and in the human embryonic kidney cell line HEK293. Subcellular fractionation studies and immunoelectron microscopy revealed a cytosolic localization. To explore the mechanistic reasons for the differential expression pattern, we first tested the activity of the putative phGPx and snGPx promoters. The 5′-flanking region of the joint ph/snGPx gene exhibits strong promoter activity. In contrast, the putative snGPx promoter, which comprises 334 bp of intronic sequences, lacks major promoter activity. However, it strongly suppresses the activity of the ph/snGPx promoter. These data suggest negative regulatory elements in the first intron of the ph/snGPx gene, and DNase protection assays revealed the existence of several protein-binding sites. The corresponding trans-regulatory proteins (SP1, ERG1, GATA1, SREBP1, USF1, and CREBP1) were identified, and in vitro binding of EGR1 and SREBP1 was shown by chromatin immunoprecipitation. These data indicate for the first time somatic expression of the snGPx and provide evidence for the existence of intronic negative cis-regulatory elements in the ph/snGPx gene. Our failure to detect an alternative snGPx promoter suggests that transcription of the ph/snGPx gene may be regulated by a joint basic promoter. The decision, which GPx isozyme is expressed in a given cell, appears to be made by alternative splicing of a joint primary transcript.

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The abbreviations used are: GPx, glutathione peroxidase; phGPx, phospholipid hydroperoxide glutathione peroxidase; snGPx, sperm nucleus glutathione peroxidase; PBS, phosphate-buffered saline; SP1, stimulating protein 1; EGR/WT, early growth factor/Wilms tumor suppressor; GATA, GATA box; SREBP, sterol regulatory element-binding protein; USF, upstream stimulating factor; CREBP, cAMP-responsive element binding protein; FF, footprint; EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; RT, reverse transcriptase; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
The various isoforms of seleno-GPx show a remarkable tissue-specific expression pattern (3). The phGPx is expressed at relatively low levels in most cells and tissues tested (8, 9) but is much more prominent in testis (10). By contrast, snGPx mRNA has only been detected in testis and its expression appears to be restricted to late stages of spermatogenesis (5). Unfortunately, the regulatory mechanisms involved in high level expression of phGPx in germinative cells and repression of snGPx expression in most somatic tissues are unknown. Moreover, the molecular processes of transcriptional activation of snGPx expression during late sperm development remain to be investigated.

The lack of experimental data on the regulation of expression of the ph/snGPx gene and the remarkable tissue-specific expression pattern of the two GPx isoforms prompted us to study the regulatory mechanisms involved in ph/snGPx expression. Here we report for the first time that snGPx does not only occur in the nucleus of late spermatids but also in the cytosol of interstitial kidney cells. Moreover, we found that the regulation of expression of the joint ph/snGPx gene appears to be a complex process that requires cis-regulatory elements in the 5′-flanking region of the ph/snGPx gene and the binding of negative trans-regulatory proteins, such as SREBP1 and EGR1, to intronic sequences (11a). The lack of major promoter activity in the 5′-flanking region of the first snGPx exon argues against the existence of alternative phGPx/snGPx promoters but strongly suggests alternative splicing of a joint ph/snGPx pre-mRNA as mechanistic reason for differential expression of the two enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemicals used were from the following sources: trisodium citrate dihydrate, magnesium sulfate heptahydrate, Triton X-100, bovine serum albumin, penicillin-streptomycin solution, and fetal bovine serum from Sigma (Deisenhofen, Germany); sodium hydroxide, sodium chloride, and Tris from Merck (Darmstadt, Germany); PWO DNA polymerase, agarose, ampicillin, avian myeloblastosis virus reverse transcriptase, and proteinase inhibitor mixture tablets (complete) from Roche Molecular Diagnostics (Mannheim, Germany); restriction endonucleases and the DNA molecular weight markers (100 bp and 1 kb) from New England Biolabs GmbH (Schwalbach, Germany); Servalyt 3–10, 6–8, and 2–4 from Serva (Heidelberg, Germany); Pan-Script DNA polymerase from PAN BIOTECH GmbH (Aidenach, Germany); Advantage 2 polymerase from Clontech (Palo Alto, CA); Bacto-tryptone and Bacto-yeast extract, Bacto-agar, and Bacto-tryptone from Difco (Detroit, MI); rainbow molecular weight markers and Hybrid-N blotting membrane from Amersham Biosciences (Freiburg, Germany); and Dulbecco's modified Eagle's medium from Invitrogen (Karlsruhe, Germany). The primers were custom synthesized by TIB MOLBIOL (Berlin, Germany).

**Cell Lines and Culture Conditions**—Human embryonic kidney cells 293 (HEK293) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C under 5% CO₂.

Animal Experiments—Mice (C57BL/6, 10–15 weeks old) were sacrificed by diethyl ether inhalation and the major organs were prepared. Peritoneal macrophages were isolated from mouse tissues or HEK293 cells using the RNA MIDI or TURBO kit (purchased fromInvitek, Karlsruhe, Germany). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C under 5% CO₂.

**Materials**—The cytotoxic proteins of kidney homogenates were mixed with 10 μl of sample buffer (1.43 g/ml urea, 10 mg/ml dithiothreitol, 10% (v/v) Triton X-100, 10 μl/ml Servalyt 3–10, 26 μl/ml Servalyt 6–8, 16 μl/ml Servalyt 2–4) and then subjected to isoelectric focusing (first dimension) in acrylamide cylinders, in which a linear pH gradient between 2 and 10 was
adjusted. SDS-PAGE (second dimension) was subsequently carried out in a 12.5% polyacrylamide gel. After SDS-PAGE, the two-dimensional chromatograms were either stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 or were transferred to a nitrocellulose membrane NC45 (Serva, Heidelberg, Germany) by a semidy blotting procedure. To stain the immunoblots the nitrocellulose membrane was first incubated for 1 h with a 1:4000 dilution of a monoclonal antibody raised against the Sec46Cys mutant of the human cytosolic phGPx. This antibody prepared in our laboratory strongly cross-reacts with the rat immunoprecipitated with 10% goat serum, 0.1% sodium deoxycholate, 1% Triton X-100, 0.25 mM EDTA, and the proteinase inhibitor mixture) and samples were incubated overnight at 4°C with 2.5 μl of a rabbit polyclonal anti-Egr1 antibody (588) or with a rabbit anti-SREBP1 antibody (H-160) obtained from Santa Cruz Biotechnology. Immune complexes were precipitated by adding 50 μl of protein A-agarose ( Santa Cruz, CA) at a 166-fold excess over the digoxigenin-labeled probe. For supershift studies, polyclonal antibodies from Santa Cruz Biotechnology were used. When either competitor or antibodies were used, they were preincubated with the nuclear extracts at 4°C for 10 or 60 min before the addition of the digoxigenin-labeled probe DNA.

**Chromatin Immunoprecipitation** — HEK293 cells were washed twice with PBS and the cellular proteins were cross-linked to DNA by adding formaldehyde to a final concentration of 1% for 15 min at room temperature. Cells were subsequently washed, removed from the culture dishes by scraping, and resuspended in 1 ml of PBS. Cells were lysed in 1.5 ml of lysis buffer (10 mM Hepes buffer, pH 7.9, containing 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT) and the proteinase inhibitor mixture. After 10 min on ice the cells were homogenized in a glass Dounce homogenizer and centrifuged at 7,000 rpm. The supernatant was added in 20 ml RIPA buffer containing 10% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, and proteinase inhibitor mixture and was sonicated on ice to fragment the genomic DNA. The mixture was then centrifuged to remove cell debris and aliquots were stored at −80°C. For chromatin immunoprecipitation aliquots were diluted in RIPA buffer (50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 0.25 mM EDTA, and the proteinase inhibitor mixture) and samples were incubated overnight at 4°C with 2.5 μl of a rabbit polyclonal anti-Egr1 antibody (588) or with a rabbit anti-SREBP1 antibody (H-160) obtained from Santa Cruz Biotechnology. Immune complexes were precipitated by adding 50 μl of protein A-agarose ( Santa Cruz, CA) at a 166-fold excess over the digoxigenin-labeled probe. For supershift studies, polyclonal antibodies from Santa Cruz Biotechnology were used. When either competitor or antibodies were used, they were preincubated with the nuclear extracts at 4°C for 10 or 60 min before the addition of the digoxigenin-labeled probe DNA.

**DNase I Protection Assay** — A genomic fragment containing intron I and PstI (Invitrogen, Groningen, The Netherlands) using purified plasmid DNA (Qiagen, Hilden, Germany) of the subcloned ph/snGPx gene as template and the following primer combinations were used (Fig. 5): 5′-flanking region of the ph/snGPx gene (construct C1, 325 bp), 5′-CCGGCCCTCATTACGGCCTGTCG-3′ and 5′-TGCGTCTCCGAGAACGACCCGCGG-3′; immediate 5′-flanking region of the first snGPx exon (construct C2, 334 bp), 5′-CGCGTCCCTATCACTGGGGCATG-3′; extended 5′-flanking region of the first snGPx exon (construct C3, 662 bp) 5′-AGCCTCCCCGGCCGCTT-GTGCGTC-3′; extended 5′-flanking region of the first snGPx exon (construct C3, 662 bp) 5′-CCGGCCCTCATTACGGCCTGTCG-3′; (same as for construct C1) and 5′-ATGCCGCCGGCCGCTT-GTGCGTC-3′. For transient transfection HEK293 cells were resuspended at a density of 0.5 × 10⁵ cells/ml of medium and plated in 60-mm culture dishes. After a culturing period of 24 h cells were transfected with a calcium phosphate transfection kit (Invitrogen). For transient transfection we used 10 μg of the β-galactosidase promoter construct and 5 μg of an internal reference plasmid containing the luciferase DNA (pGL3, Promega, Madison, WI). The transfection efficiency is a true measure of transfection. After 72 h, cells were harvested and lysed in 1.5 ml of lysis buffer (10 mM Hepes buffer, pH 7.9, containing 10% glycerol, 300 mM NaCl, 1.4 mM MgCl₂, 0.1% Triton X-100, 0.1 mM DTT), and the DNA was extracted with phenol/chloroform followed by ethanol precipitation. The DNA samples were incubated for 1 h at 60°C and the DNA was extracted with phenol/chloroform followed by ethanol precipitation. The DNA pellet was resuspended in 20 μl of RIPA buffer, twice with 1 ml of PBS, and DNA-protein complexes were eluted from the agarose by a 15-min incubation with 250 μl of elution buffer (1% SDS, 100 mM NaHCO₃). Elution was repeated, the eluates were pooled, NaCl was added to reach a final concentration of 0.3 M, and the samples were incubated at 65°C for 4 h to reverse formaldehyde-induced cross-linking. The proteins were then digested with 2 μl of proteinase K (20 mg/ml) in 40 mM Tris-HCl buffer, pH 7.5, containing 10 mM EDTA. The samples were incubated for 1 h at 60°C and the DNA was extracted with phenol/chloroform followed by ethanol precipitation. The DNA pellet was resuspended in 20 μl of sterile H₂O and 10 μl of DNA solution was used for PCR. The three PCR cycles were designed to amplify the precipitated binding regions of the transcription factors (Fig. 11) and 35 PCR cycles were run: P1 (5′-CATGTTGGGCTACTGGGAACTT-3′), P2 (5′-TCCGCGGCGAGGTACC-3′), P3 (5′-AGGCCCTGGCGCGGCGGACT-3′), P4 (5′-ATGCCGCCGGCGCTT-GTGCGTC-3′). The PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

**Immunohistochemistry** — For immunohistochemistry the animals (two rats and two mice) were perfused with ice-cold 4% (w/v) paraformaldehyde (dissolved in PBS). Kidneys were prepared, cryopreserved in 20% sucrose for 24 h at 4°C, and frozen in the gaseous phase of liquid nitrogen. Cryostat sections (10–20 μm thickness) were prepared and quenched in 50 μM NH₄Cl/CPBS for 10 min. The sections were washed twice with PBS and the cellular proteins were cross-linked to DNA by adding 30 μl of 50 mM NH₄Cl and 60 mM glycine diluted 1:20 (diluted) overnight at 4°C. After washing in 0.1% saponin/PBS, sections were incubated overnight at +4°C with a biotinylated anti-mouse IgG antiserum (Vector Laboratories, Burlingame, CA), diluted 1:250. For staining, the sections were exposed to avidin-biotin peroxidase complex reagent (Vector Laboratories) for 2 h at room temperature, and the immunoreactivity was visualized with 3,3′-diaminobenzidine as a chromogen (incubation for 8 min at room temperature with 0.07% 3,3′-diaminobenzidine and 0.001% hydrogen dissolved in 0.1 M PBS). Prior to inspection the sections were counterstained with hematoxylin. Egr1 staining was performed appropriately using a diluted premi-}

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**Electrophoretic Mobility Shift Assay (EMSA) and Supershift** — EMSA was carried out with the DIG Gel Shift Kit (Roche Molecular Diagnostics) following the users manual. In the first step single-stranded complementary oligonucleotides containing the binding sequence for transcription factors were annealed and end-labeled with digoxigenin. The labeled probes (45 fmol of double-stranded oligonucleotide) were incubated for 30 min on ice. Afterward, the probe was used as a template for PCR. The following primer combinations were used: 5′-ATGCCGCCGGCGCCGACT-3′ and 5′-ATGCCGCCGGCGGGCTGTGTCG-3′. The PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

**Immunoelectron Microscopy** — For ultrastructural studies, ph/snGPx-immunostained sections were osmicated (1% OsO₄ in 6.84% sucrose dissolved in PBS) for 5 min, dehydrated in graded ethanol, and flat embedded in Epon medium (Merck, Darmstadt, Germany) between 2 min at 10°C with 10% aqueous uranyl acetate and 60 min with lead citrate and uranyl acetate. A Zeiss EM 900 electron micro-

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**Activity Assay** — The phospholipid hydroperoxide activity was assayed measuring the decrease in absorbance at 340 nm with a coupled enzyme reaction due to a H₂O₂ dependent luciferase, containing 5 mM EDTA, 0.1% Triton X-100, 0.2 mM NADPH, 3 mM glutathione, and 1 unit of glutathione reductase in a total assay volume of 0.2 ml. The enzyme preparation (5–20 μl) was preincubated at room temperature in the assay buffer for 10 min and then the reaction was...
were prepared by incubating for 10 min soybean phospholipids in 0.2 M 235 nm using a molar extinction coefficient of 25,000 (M cm) was carried out spectrophotometrically measuring the absorbance at solid-phase extraction. Final quantification of the peroxide preparation phy (12) and the salt included in the solvent system was removed by reverse phase-high performance liquid chromatography-purified phospholipid hydroperoxide reaching a final substrate concentration of 50 \( \mu \text{M} \). The hydroperoxy phospholipids were prepared by incubating for 10 min soybean phospholipids in 0.2 M borate buffer, pH 9, containing 3 \( \text{mM} \) sodium deoxycholate and 1 mg/ml soybean lipoxygenase. After lipid extraction the conjugated dienes were started by addition of 10 \( \mu \text{l} \) of a methanolic solution of high performance liquid chromatography-purified phospholipid hydroperoxide reaching a final substrate concentration of 50 \( \mu \text{M} \). The hydroperoxy phospholipids were prepared by incubating for 10 min soybean phospholipids in 0.2 M borate buffer, pH 9, containing 3 \( \text{mM} \) sodium deoxycholate and 1 mg/ml soybean lipoxygenase. After lipid extraction the conjugated dienes were prepared by reverse phase-high performance liquid chromatography (12) and the salt included in the solvent system was removed by solid-phase extraction. Final quantification of the peroxide preparation was carried out spectrophotometrically measuring the absorbance at 235 nm using a molar extinction coefficient of 25,000 (M cm) \(^{-1}\).

RESULTS

Tissue-specific Expression of snGPx and Its Intracellular Localization—It has been previously reported that phGPx is ubiquitously expressed in small amounts in many cells and tissues (3, 8, 9) but at much higher levels in testis (10, 13). In addition, Northern blot analysis suggested a high level expression of the snGPx mRNA in testis and its absence in liver, kidney, heart, brain, spleen, and other organs (5). Within the testis the enzyme was mapped predominantly to the nuclei of late spermatids (5). Because spermatids are not suitable for mechanistic studies, the tissue-specific expression pattern of the snGPx mRNA was reinvestigated by semiquantitative RT-PCR. Here we found that the snGPx mRNA is present at a high level in testis and at lower levels in kidney (Fig. 1). In all other major organs (ileum, skin, heart, brain, liver, lung, stomach, spleen, muscle, and submandibular gland) we were unable to detect this messenger (not shown). In some experiments faint snGPx bands were observed in lung and stomach. In the snGPx-positive tissues (testis and kidney) we consistently detected an additional PCR product migrating with a higher molecular weight (Fig. 1). This PCR fragment was cloned and sequenced, and the data indicated an incomplete splicing product that still contained intron 11b (Scheme 1). However, all other introns (I2-16) had been spliced out completely. These data suggest problems of the splicing machinery during snGPx expression. It is likely that proteins binding to I1a of the joint ph/snGPx pre-mRNA may prevent proper splicing of the primary transcript.

For mechanistic studies on the regulation of gene expression, solid tissues have several limitations. Thus, we searched for a permanent cell line, in which the snGPx is expressed. For this purpose several human and murine cell lines were tested and expression of the snGPx mRNA was found in the human embryonic kidney cell line HEK293 (data not shown). Interestingly, this cell line also expresses phGPx as indicated by RT-PCR with phGPx-specific primer combinations.

To confirm the tissue-specific expression of snGPx on the protein level, immunoblots with a monoclonal anti-ph/snGPx antibody were carried out. For this purpose 20,000 \( \times g \) supernatants were prepared from murine tissues and equal protein amounts were applied to SDS-PAGE. From Fig. 2A it can be seen that immunoreactive proteins migrating in the expected 34-kDa region were present in the kidney lysis supernatant suggesting snGPx expression in this organ. In a separate experiment electrophoretic co-migration of the kidney snGPx with an snGPx standard prepared from human spermatids was observed. Moreover, an immunoreactive band of minor intensity was detected in the 20-kDa region that may be because of phGPx. By comparison, large amounts of this low molecular weight protein were found in the testis high speed supernatants. These data indicate that in testis cytosol the phGPx predominates and the snGPx appears to be absent.

The kidney snGPx exhibited a catalytic activity for reduction of hydroperoxy phosphatidylycerol of 0.057 units/mg of protein. This specific activity is similar to previously reported values (0.028 units/mg) (11). Our attempts to purify the native snGPx from murine kidney supernatants by conventional protein purification techniques were not successful. Running the supernatant over sequential Mono-Q FPLC and gel filtration resulted only in 8-fold enrichment of the immunoreactive material with substantial loss of enzymatic activity. In the most active FPLC fractions the specific activity could only be enriched by a factor of 2 (0.12 units/mg) compared with the lysis supernatant (see above).

It was previously shown that phGPx forms covalently linked polymers (10) that may migrate at a higher molecular weight region. Thus, the 34-kDa band observed in kidney cytosol may be because of a phGPx dimer instead of a snGPx monomer. To distinguish between these two protein species we carried out two-dimensional electrophoresis (Fig. 2B) and found that the immunoreactive material from the kidney lysate supernatant migrated with an apparent isoelectric point of about 7. However, a more alkaline isoelectric point has been reported for phGPx (7, 10). It should be stressed that in two-dimensional electrophoresis, we did not observe major expression of the 20-kDa phGPx. Comparison of Coomassie-stained immunoblots revealed that snGPx is not a major protein in kidney cytosol. In the region where the immunoreactive material was detected, significant protein was not observed.

The relatively large amounts of snGPx detected in kidney cytosol were somewhat surprising because the enzyme contains a nuclear import sequence and thus, should be localized preferentially in the nuclei. However, subcellular fractionation studies combined with Western blot analysis indicated the predominant cytosolic localization of the snGPx in kidney and its lack in the nucleus (Fig. 2C).

The available ph/snGPx antibodies do not distinguish between the two isoforms and thus, it was not possible to discriminate between the two GPx isoforms using immunohistochemistry. Fortunately, the Western blots indicated predominant expression of the 35-kDa snGPx in the kidney and thus, immunohistochemistry of kidney cross-sections will mainly reflect the cellular and subcellular distribution of the snGPx. In murine kidney, ph/snGPx is mainly expressed in cortical and medullary interstitial cells (Fig. 3). In contrast, the glomeruli and the epithelial cells of the tubuli and collecting ducts were free of immunoreactive material. Similar results were also obtained with rat kidneys (data not shown). These data con-
**Fig. 6.** Immunoblot analysis of snGPx expression. The 20,000 × g supernatant was prepared from the 50% homogenates of solid murine tissues and aliquots were applied for analysis. After separation the proteins were transferred to a nitrocellulose membrane by semidry blotting and immunoreactive bands were stained using the monoclonal ph/snGPx antibody. A. SDS-PAGE: 50 μg of lysate supernatant was applied to SDS-PAGE. B, two-dimensional electrophoresis: 50 μg of kidney homogenate supernatant was used for isoelectric focusing (horizontal separation) and SDS-PAGE was carried out in the second dimension (vertical separation). Protein blotting and immunostaining was performed as described above. C, subcellular localization of snGPx in murine kidney. Nuclear extract and cytosol were prepared from murine kidney as described under “Experimental Procedures” and 50 μg of total protein was applied to SDS-PAGE. Protein blotting and immunostaining were carried out as described above.

In Fig. 6 indicate: (i) the existence of at least five distinct protein-binding regions (FP1–FP5) in I1b and (ii) the presence of the corresponding trans-acting proteins in the nuclei of testis and HEK293 cells. The lack of DNase protection by albumin (data not shown) indicates the specificity of protein binding.

Next, the sequences of the different protein-binding sites were determined and examined for the presence of transcription factor binding motifs. As evident from Fig. 7A FP1 contains the consensus binding sequences for the stimulating proteins (SP1-like factors) and for the early growth response gene product/Wilms tumor suppressor gene product (EGR/WT). EMSA were subsequently carried out to test the presence of the corresponding trans-acting binding factors in testis and HEK293 cells. With testis extracts we observed a single high molecular weight shift band that was competed off by nonlabeled competitor (Fig. 7B). By contrast, two shift bands were detected when nuclear extracts of HEK293 cells were used. Addition of a consensus oligonucleotide for SP1 caused a disappearance of the upper SP1 shift band (Fig. 7C). In a similar experiment the strong upper SP1 shift band from HEK293 extracts also disappeared, whereas the lower band of minor intensity remained unaltered. The lower shift band, however, was eliminated with a consensus oligonucleotide for the EGR transcription factor family (Fig. 7B). Because HEK293 cells are embryonic kidney cells adult kidney cells were also examined for expression of the corresponding transcription factors. As shown in Fig. 7D we observed two shift bands when murine kidney nuclear extracts were added to the assays. Here again, the upper band disappeared completely when a SP1 consensus oligonucleotide was added, whereas the lower band was quenched off in the presence of EGR competitor. In a separate experiment an EGR1 antibody was added to the reaction mixture and in this sample the EGR/WT shift band was removed. However, we were not able to detect a clear supershift signal. Taken together, these data suggest that footprint 1 is caused by the binding of a member of the SP1 transcription factor family (15) and/or EGR1 (16) to appropriate binding sites in I1a. The differential quenching behavior of testis and HEK293 nuclear extracts suggest that the testis extracts may not contain sufficient amounts of EGR1.

When the nuclear extracts of HEK293 cells and murine kidney were used as source of nuclear proteins an additional
very low shift band (Fig. 7, B and C) was observed and this band does not appear when testis nuclear extracts were used. Unfortunately, it has not yet been possible to identify the corresponding trans-acting protein.

FP2 (Fig. 8A) contains a GATA1 binding motif (17) and EMSA indicated a single shift band when the nuclear extracts from testis and HEK293 cells were used as source of the binding proteins (Fig. 8B). This band was competed off by addition of unlabeled competitor (Fig. 8B) and by a GATA consensus oligonucleotide (Fig. 8C). In FP3 (Fig. 9A) a SREBP1-binding site was detected (18) and EMSA experiments indicated single shift bands when the nuclear extracts of testis and 293 cells were incubated with the corresponding DNA probe (Fig. 9B). Addition of unlabeled competitor eliminated the shift bands. A similar result was obtained when a SREBP1 consensus oligonucleotide was added (Fig. 9C). FP4 and FP5 (Fig. 10) contain binding sites for USF1 (Fig. 10A) and CREB (Fig. 10B), respectively (19, 20). After addition of the nuclear extracts of testis and 293 cells were incubated with the corresponding DNA probe we detected strong shift bands (Fig. 10, C and D). Addition of unlabeled competitor (data not shown) and consensus oligonucleotides caused a disappearance of these signals. Taken together, the EMSA experiments indicate that cis-regulatory elements for the binding SP1, EGR1, GATA1, SREBP1, USF1, and CREBP are present in the 5′-flanking region of E1b (Scheme 2) and that the corresponding trans-regulatory factors are expressed in murine testis and HEK293 embryonic kidney cells.

**In Vivo Binding of ERG1 and SREBP1 to the 5′-Flanking Region of E1b (Putative snGPx Promoter)**—The EMSA experiments indicated that the above mentioned transcription factors are present in the nuclei of testis and HEK293 cells and that they bind in vitro to the corresponding cis-regulatory sequences in the 5′-flanking region of E1b. To determine whether they also bind in vivo, chromatin immunoprecipitation was carried out. For this purpose DNA-binding proteins of HEK293 cells were covalently linked to genomic DNA by treatment of the cells with formaldehyde. The DNA-protein complexes were then sheared by sonication, and specific protein-DNA complexes were immunoprecipitated with antibodies against SREBP1 and ERG1. Covalent linkage was reversed and the precipitated double stranded DNA was amplified by PCR with binding site-specific primers. From Fig. 11B it can be seen that PCR signals were obtained when the DNA/protein adducts were immunoprecipitated with antibodies against SREBP1 and ERG1 indicating the in vivo binding of the transcription factors. In contrast, the corresponding controls, in which immunoprecipitation was performed without specific antibodies, did not show any PCR signal.
DISCUSSION

GPxs constitute a family of functionally related enzymes and the phGPx and snGPx are two members of this family. Whereas phGPx is expressed at low levels in many cells and tissues (3, 8), snGPx was only detected in late spermatids of several mammalian species (5). In these cells the enzyme was predominantly localized in the nucleus and has been implicated in chromatin structuring during sperm development and in antioxidative defense of the DNA (4–6). In this paper we report for the first time that snGPx is also expressed in selected somatic cells (interstitial kidney cells). Immunohistochemical staining, immunoelectron microscopy, and subcellular fractionation studies indicate that the enzyme is located predominantly in the cytosol.

FIG. 4. Immunoelectron microscopy of murine kidney cross-sections. Sample preparation and immunoelectron microscopy was carried out as described under “Experimental Procedures.” Panel A, distal tubule showing immunoreactive material in an interstitial cell (arrows) below the basal lamina (arrowheads) (magnification ×12,000). Panel B, immunonegative tubular epithelial cell with narrow luminal clefts (magnification ×7,000). Panel C, immunopositive endothelial cell (magnification ×20,000). Here again, the immunoprecipitate is mainly located in the cytoplasm. L, luminal side; Nc, nucleus.

FIG. 5. Functional promoter assays. Different parts of the 5′-flanking region of E1b (I1a, I1a+E1a) were ligated into a β-galactosidase-based reporter gene and HEK293 cells were transfected with different constructs. To correct the β-galactosidase activity measured for transfection efficiency cotransfections with a luciferase containing control plasmid (pGL3) were carried out. β-Galactosidase and luciferase activity were measured as described under “Experimental Procedures.” A, length (bp) of the PCR fragments of the 5′-flanking regions used for reporter gene construction. B, β-galactosidase activity of the different promoter constructs corrected for luciferase transfection. As positive control, a 325-bp fragment of the 5′-flanking region of E1a (phGPx promoter) was used. As negative control (C0) the linearized vector was religated without any insert.

FIG. 6. Footprint analysis for intron I1a of the ph/snGPx gene. DNase protection assays (footprints) were carried out as described under “Experimental Procedures.” The areas of quenched signals indicate the protein-binding sites (FP1 to FP5). Unspecific binding was tested with albumin as a probe, but we did not observe any signal quenching (data not shown). The sequences of the footprints (Figs. 7–10) were determined with the G + A ladder.

regulated in chromatin structuring during sperm development and in antioxidative defense of the DNA (4–6). In this paper we report for the first time that snGPx is also expressed in selected somatic cells (interstitial kidney cells). Immunohistochemical staining, immunoelectron microscopy, and subcellular fractionation studies indicate that the enzyme is located predominantly in the cytosol. This localization was rather surprising because snGPx contains a nuclear import peptide that has been proposed to target the protein to the nucleus. Currently, it is unclear why the enzyme is transported into the nucleus in late spermatids and why it remains in the cytosol in interstitial kidney cells. It is possible that nuclear import requires addi-
The kidney-specific function of the snGPx has not yet been investigated. Because the enzyme is mainly expressed in interstitial cells but not in the glomeruli, the tubulus epithelium, or the collecting ducts, it is unlikely to play a role in urine production. Alternatively, the renal snGPx may be involved in antioxidative defense as it has been suggested before for other GPx isoforms (21). It is well known that there is an active oxidative metabolism in renal cortex, which may be accompanied by the formation of reactive oxygen species (22, 23).

Compared with other selenoperoxidases, which are encoded by separate genes, phGPx and snGPx originate from a joint ph/snGPx gene (4, 5). Nevertheless, both isoforms are differen-
tially expressed in various cells and tissues, but virtually nothing is known about the regulatory mechanisms. Here we report that the minimal promoter of the phGPx (cytosolic isoform), which comprises the 5'-flanking region of the ph/snGPx gene and the 5'-untranslated region of its mRNA, exhibits major promoter activity. By contrast, the promoter activity of the 5'-flanking region of E1b (putative snGPx promoter) was almost negligible. These data are difficult to reconcile with the existence of alternative phGPx/snGPx promoters. They rather suggest that transcription of the ph/snGPx gene is driven by joint regulatory sequences located upstream of the 3'-ATG of the ph/snGPx gene. Interestingly, the joint ph/snGPx promoter can be strongly down-regulated by inhibitory proteins that bind to intronic regulatory sequences of I1a. Possible suppressor protein candidates include members of several transcription factor families (e.g. SP, EGR, GATA, SREBP, USF, and CREBP). Functional intronic cis-regulatory elements have been reported to be involved in the regulation of expression of several genes (24, 25), but the detailed mechanisms of action are unknown.

RT-PCR of snGPx transcripts indicated the presence of incomplete splicing products that selectively contain I1a (Fig. 1). Interestingly, we have never observed splicing problems with introns 2–5 of the ph/snGPx gene. Although the mechanistic reasons for this incomplete splicing have not been investigated in this study, the identified transcription factors may be involved. It has been reported before that transcription factors that prefer to bind to double stranded DNA are also capable of binding to single stranded RNA (26, 27). Thus, they may be of regulatory importance for the splicing process. Alternative exons are frequently flanked by suboptimal splicing sites, and an increasing body of experimental evidence has indicated that effective outsplicing of alternative exons requires the participation of splicing enhancers and/or silencers in addition to the constitutive spliceosomal machinery (28). The presence of such auxiliary proteins may help to define the exact splicing sites, and may also be important for maturation-dependent and tissue-specific alterations of the protein expression patterns. cis-Regulatory pre-mRNA sequences that bind the trans-acting splicing factors may reside in the alternative exon itself (29), in the flanking introns (30, 31), and even in an adjacent exon (32). Inclusion of an alternative exon in the final mRNA is a multiprocess that involves timely differentiated removal of the two flanking introns. In fact, in several alternatively spliced
pre-mRNA species it has been demonstrated that one flanking exon is removed before the other (33, 34). Although the detailed splicing mechanism of the ph/snGPx pre-mRNA was not the focus of this study, we have identified functional cis-regulatory sequences in the 5'-flanking intron of alternative exon E1b, which may be related to the splicing process. Additional stringent in silico search for similar cis-regulatory elements in E1b also suggested the existence of SP1/WT1-binding sites and preliminary DNase protection assays suggested their functionality (data not shown). Similarly, the 3'-flanking intron of E1b also contains binding sites for several transcription factors (GABP1, OCT1P1, and GATA3), but their functionality has not been tested. Taken together, these data suggest the presence of cis-regulatory elements in the alternative exon E1b and in the flanking introns I1a and I1b. Such a constellation is frequently found in genes containing alternative exons, and the cis-regulatory sequences may serve as binding regions for splicing enhancers and/or silencers.

In the light of these findings the functional cis-regulatory elements identified in I1a and the corresponding trans-regulatory proteins may be of dual functional importance: (i) they may act as negative regulators of transcriptional initiation of the ph/snGPx gene (Fig. 5) and (ii) they may exhibit auxiliary function for alternative splicing of the joint ph/snGPx mRNA. Work is in progress in our laboratory to provide more detailed information on either process.

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Regulation of Expression of the Phospholipid Hydroperoxide/Sperm Nucleus Glutathione Peroxidase Gene: TISSUE-SPECIFIC EXPRESSION PATTERN AND IDENTIFICATION OF FUNCTIONAL CIS- AND TRANS-REGULATORY ELEMENTS
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