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# Transcriptional Regulation of the Murine 1-Cys Peroxiredoxin Gene by the B Cell-Specific Activator Protein, Pax5

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**Abstract** Pax5, a member of the paired box gene family of transcription factors, is a B cell-specific activator protein (BSAP) that plays important roles in controlling the expression of lineage- and differentiation-stage specific genes during B lymphopoiesis. We identified two putative Pax5 binding sites in a 668 bp of the murine 1-cys peroxiredoxin (1-cysPrx) promoter region. These sites were located at positions −278 to −262 and −50 to −34 from the translation start site. Gel mobility shift assays showed that recombinant Pax5 protein bound specifically to the nucleotide regions −56 to −24 (MP1 probe) and −284 to −253 (MP2 probe). Furthermore, endogenous Pax5 protein from B lymphoblast cells (IM-9) formed a DNA-protein complex with MP1 and MP2 probes, indicating that Pax5 binding occurs specifically at these sequences in vivo. Transient transfection studies showed that expression of exogenous Pax5 resulted in dose-dependent increases in 1-cysPrx promoter activity, implying that Pax5 functions as a positive transcription factor for 1-cysPrx expression. Further transient cotransfection studies showed that coexpression of Pax5 and histone acetyltransferases (HATs), such as p300, cAMP-response-element-binding protein (CBP) and p300/CBP associated factor (PCAF) enhanced the Pax5-mediated 1-cysPrx promoter activity. Immunoprecipitation studies indicated that Pax5 directly binds to HATs. Chromatin immunoprecipitation assays showed that the recruitment of Pax5 to the promoter induced histone H3 and H4 hyperacetylation of chromatin. Lipopolysaccharides (LPS) stimulation of murine splenocytes resulted in coordinated expression of Pax5 and 1-cysPrx proteins. These findings suggest that Pax5 may function as a transactivator of 1-cysPrx gene expression. J. Cell. Biochem. 104: 465–476, 2008. 

**Key words:** 1-cys peroxiredoxin; B cell specific activator protein (Pax5); histone acetyltransferases (HATs); transactivation

Peroxiredoxins (Prxs) represent a superfamily of thiol-dependent antioxidant proteins that reduce a wide range of peroxides without requiring cofactors such as metals or prosthetic groups. These enzymes are ubiquitously distributed throughout all phyla and can be divided into 1- and 2-cys subgroups based on their number of conserved cysteine residue(s) for peroxidase activities [Rhee et al., 2001; Fujii and Ikeda, 2002; Hofmann et al., 2002]. Of the six mammalian Prxs, Prxs I–V are 2-cys enzymes that use thioredoxin as an electron donor. In contrast, Prx VI (also known as antioxidant protein 2 [AOP2]) is a 1-cysPrx, unlike the 2-cysPrxs has no ability to reduce peroxides with thioredoxin as a reductant. Each

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Prx is expressed in a tissue- and cell-specific manner, indicating that Prxs have independent antioxidant function. In addition to oxidant detoxification, individual Prxs are closely involved in regulating a variety of cellular processes such as cell proliferation, differentiation, and gene expression.

The 1-cysPrx protein was initially isolated from bovine ciliary bodies as a novel non-selenium glutathione peroxidase [Shichi and Demar, 1990]. Subsequently, 1-cysPrx protein and mRNA expression has been characterized in various mammalian tissues and cells [Frank et al., 1997; Akiba et al., 1998; Kim et al., 1998; Peshenko et al., 1998]. Several studies using cultured cell lines and gene-targeted mice (Prdx6−/−) have shown that 1-cysPrx can participate in cellular antioxidant defense [Manevich et al., 2001; Pak et al., 2002; Wang et al., 2003, 2004]. Analysis of the proximal promoter of the murine 1-cysPrx gene has identified binding sites for Sp1 and Pit-1a transcription factors, as well as several putative binding sites [Lee et al., 1999]. This proximal region also contains binding sites for lens epithelium-derived growth factor (LEDGF) that transactivates 1-cysPrx gene expression resulting in enhanced cell survival under oxidative stress [Fatma et al., 2001].

Pax5, a member of the paired box gene family of transcription factors, encodes the B cell-specific activator protein (BSAP) that plays important roles in controlling expression of lineage- and differentiation-stage specific genes during B lymphopoiesis [Wakatsuki et al., 1994; Busslinger and Urbanek, 1995; Morrison et al., 1998; Hagman et al., 2000]. Binding sites for Pax5 are located in the proximal promoter regions or distal enhancer positions of genes such as the immunoglobulin heavy chain locus, CD19, the B lymphoid specific tyrosine kinase blk and the surrogate light chains λ5 and $V_{preB1}$. Pax5 is considered as a bifunctional transcription factor as DNA binding can result in either activation or repression of transcription. This ability to play a dual role may be the result of degenerated consensus nucleotide sequences and/or interactions with other proteins.

The present study describes the identification of two Pax5 binding sites in the murine 1-cysPrx gene promoter and shows that Pax5 can promote 1-cysPrx gene transcription. The study also found that physical interactions of Pax5 with histone acetyltransferases (HATs) enhanced the Pax5-mediated increase in the promoter activity. In addition, the study found that Pax5 expression correlated with 1-cysPrx expression in lipopolysaccharides (LPS)-treated-murine splenocytes. These observations indicate that Pax5 is a transcription factor that regulates 1-cysPrx gene expression.

## MATERIALS AND METHODS

### Materials

Cell culture medium components were purchased from GIBCO (Grand Island, NY). Human B lymphoblastoid (HCC-1428BL) and B lymphoblast (IM-9) cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). The Taq DNA polymerase PCR system and restriction endonuclease were from Takara Bio, Inc. (Shiga, Japan). pKS-CP3-1, which contains the 5′-flanking region, exon 1, and partial intron 1 of the 129/SvJ mouse 1-cysPrx gene, was generously provided by Dr. Aron B. Fisher of the Institute for Environmental Medicine at the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania. Polyclonal antibodies to Pax5, p300, CBP and 1-cysPrx were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and LabFrontier Co. (Seoul, Korea). Mouse monoclonal antibodies to β-actin, complete protease inhibitor cocktail, and bacterial lipopolysaccharides (LPS) were obtained from Sigma–Aldrich (St. Louis, MO). All chemicals (biotechnology grade) were purchased from Amresco, Inc. (Solon, OH).

### Construction of a Murine 1-cysPrx Promoter/Luciferase Plasmid [pGL3-mPx, pGL3-mPx/MP1(Mu), and pGL3-mPx/MP2(Mu)]

The proximal 5′-flanking region of the mouse 1-cysPrx gene promoter (668 bp) was obtained through PCR using pKS-CP3-1 as a template. An Xho I site was introduced into the sense primer (5′-CTCGAGACTTGCATACACTCTG-ATCTC-3′) and a Hind III site was introduced into the antisense primer (5′-AAGCTTGGC-GGCAGTGGTGAC GCTGAG-3′). The purified PCR products were double-digested with Xho I and Hind III, ligated to the similarly digested promoterless pGL3-Basic plasmid (Promega, Madison, WI), and transformed into competent JM109 cells. DNA sequencing confirmed that the plasmids contained the 1-cysPrx promoter insert.

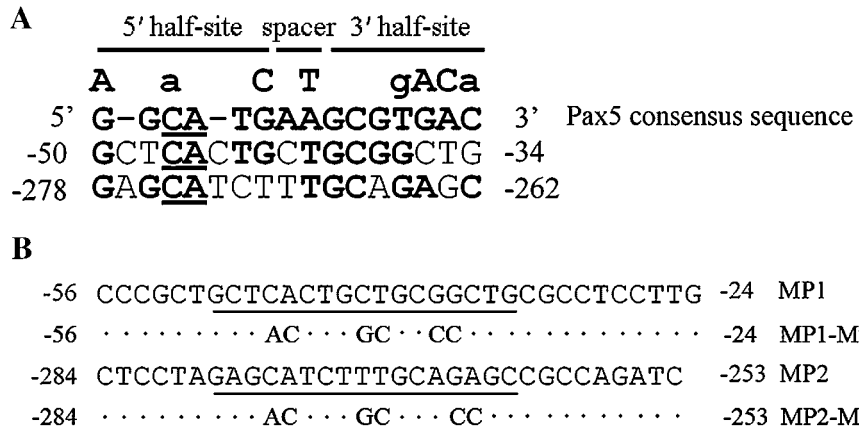


**Fig. 1.** Sequence specificity of Pax5-binding sites in the murine 1-cysPrx promoter. **A**: Alignment of the 1-cysPrx promoter region with the consensus recognition sequence for Pax5 [Busslinger and Urbanek, 1995; Hagman et al., 2000]. A: Consensus sequences are shown in bold and the nucleotide positions of the 1-cysPrx sequences are shown relative to the translation start site. **B**: Wild-type and mutant sequences of the duplex oligonucleotides used in EMSA (only upper strands are shown). For MP1-Mu and MP2-Mu, dashes indicate identity with the wild-type oligonucleotides. Pax5 binding sites within the MP1 and MP2 sequences are underlined.

Two mutant plasmids, pGL3-mPx/MP1(Mu) and pGL3-mPx/MP2(Mu), were generated by standard PCR-based site-directed mutagenesis with the template pGL3-mPx and oligonucleotides MP1-Mu or MP2-Mu, each carrying the 6 bp substitutions at the 5′ through the 3′ half-site (Fig. 1B). The final mutated PCR products were subcloned into pGL3-Basic plasmid by same method described above, and the constructs were verified by DNA sequencing in both directions.

### Plasmid Construction and Expression of Mouse Pax5 cDNA (pcDNA-Pax5 and pGEX-Pax5)

The coding region of mouse Pax5 was obtained using PCR amplification and single stranded cDNA generated from murine bone marrow total RNA. The PCR primers used were: 5′-GAATTCAAATGGATTTAGAGAAAAATTACCC-3′ as the upstream primer, and 5′-CTCGAGTCAGTGGCGGTCGTACGCAGTGG-3′ as the downstream primer, which match up to the first and last codons in the coding region (underlined). In the upstream primer, the start codon was incorporated into an EcoR I site. An Xho I site was incorporated into the downstream primer immediately after the stop codon. The PCR product (1,190 bp) was cloned into the pGEM-T Easy vector (Promega) and then transformed into competent JM109 cells. DNA sequence analysis showed that the insert had 100% identity with the coding region of murine Pax5 cDNA sequences at positions 31 and 1,208 (GenBank accession No. NM-008782). An EcoR I- Xho I-digested coding region of the Pax5 cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) for eukaryotic expression, or pGEX-2T (Amersham Biosciences, Piscataway, NJ) for the production of a glutathione S-transferase (GST)-Pax5 fusion protein, following transformation into BL21 (DE3) competent cells. Recombinant protein was purified using glutathione-Sepharose 4B beads (Amersham Biosciences).

### Immunoblot Analysis

Cells were homogenized with a lysis buffer consisting of 25 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1% Triton X-100, 10% glycerol, and a complete protease inhibitor cocktail. Insoluble material was removed by centrifugation at 10,000*g* for 30 min at 4°C, and protein concentrations were estimated using DC Protein Assays (Bio-Rad, Hercules, CA). Protein samples were subjected to SDS–PAGE and transferred to nitrocellulose (Amersham Bioscience). Membranes were probed with polyclonal antibody to 1-cysPrx or Pax5 (1:1,000 dilution), followed by horseradish-peroxidase-conjugated secondary antibody (1:5,000 dilution; Jackson ImmunoResearch Laboratory, West Grove, PA). Bands were visualized using enhanced chemiluminescence (ECL; Santa Cruz Biotechnology) and X-ray film exposure. To normalize for protein loading, following the previous probing, each membrane was washed in stripping solution (62.5 mM Tris–HCl [pH 6.8], 100 mM β-mercaptoethanol,

2% SDS) at 55°C and then incubated with a monoclonal antibody to β-actin (1:10,000).

### Nuclear Extract Preparation

Cells were harvested and nuclear proteins isolated using the Cellytic Nuclear Extraction kit (Sigma–Aldrich). Nuclear extract protein levels were measured, and extracts then stored at −70°C until use.

### Transfection and Luciferase Assay

HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and an antibiotic mixture. Cells were seeded on 48-well dishes and transiently transfected using Lipofectamine 2000 (Invitrogen) with internal control pGL3-basic, pGL3-mPx, pGL3-mPx/MP1(Mu) or pGL3-mPx/MP2(Mu) (100 ng each) reporters, and pcDNA-Pax5 (100–200 ng) in the absence or presence of CMXPL1 derivatives of p300, CBP or PCAF constructs (100–200 ng for each plasmid DNA), as described elsewhere [Seo et al., 2001]. The amount of plasmid DNA in each transfection was kept constant by the addition of the pcDNA3 empty vector. Forty-eight hours after transfection, cells were harvested and assayed for luciferase activity using a luciferase reporter assay system (Promega) according to manufacturer's instructions.

### Electrophoretic Mobility Shift Assay (EMSA)

Two sets of oligonucleotides containing the Pax5 consensus recognition sequence in the murine 1-cysPrx promoter (Fig. 1) were commercially synthesized, annealed, and then labeled with [$\alpha$-$^{32}$P] dCTP using the Klenow fragment (Roche Applied Science). The EMSA binding reaction mixture contained 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, 0.1 ng [$^{32}$P]-labeled DNA probe, and 3 μg GST-Pax5 fusion protein or 4 μg IM-9 cell nuclear extracts. The reaction mixtures were incubated at room temperature for 30 min, and the protein/DNA complexes were resolved at 4°C on nondenaturing 4% polyacrylamide gels in 0.5 × Tris-borate-EDTA running buffer. For the supershift assay, the nuclear extracts were preincubated with 1 μg of Pax5 polyclonal antibody for 30 min before addition of the labeled probe. The gels were vacuum-dried and exposed to a phosphorimager (Cyclone, Packard, Meriden, CT) for band visualization.

### Chromatin Immunoprecipitation (ChIP) Assay

ChIP was carried out as described in the protocols from Upstate Biotechnology (Lake Placid, NY). Briefly, $2 \times 10^5$ NIH3T3 cells were transfected with 3 μg pcDNA3 or pcDNA-Pax5 and harvested 48 h later. Cells were cross-linked with 1% formaldehyde and sonicated for immunoprecipitation with 5 μg anti-Pax5 antibody, anti-acetylated histone H3 or H4 antibodies (Upstate Biotechnology). After the immunoprecipitates were eluted and the cross-link reversed, DNA fragments were purified and quantitated using the following PCR conditions: 30 s at 94°C, 30 s at 60°C, 30 s at 72°C for 35 cycles. Primers utilized for the endogenous murine 1-cysPrx promoter were 5′-GCCAGACTCGCGGTCGCCCC-3′ and 5′-GGGGCTTCGTCCCCGAGAAG-3′ as P1 (detection of MP1 region), and 5′-GAGGGCTATACCCCAAGACC-3′ and 5′-GGCAAGGGAGATTCTGGAGC-3′ as P2 (detection of MP2 region). PCR products were separated using 1.5% agarose gel electrophoresis. Each experiment was repeated at least three times.

### Immunoprecipitation and Interaction Assay

In vitro [$^{35}$S] methionine-labeled Pax5 was synthesized using the pcDNA-Pax5 plasmid in the TNT Coupled Reticulocyte Lysate System (Promega), and incubated with nuclear extracts of p300- or CBP-transfected HeLa cells for 30 min at room temperature in buffer containing 20 mM Tris–HCl (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10 μg/ml BSA. Complexes were immunoprecipitated using anti-p300, anti-CBP antibodies or normal rabbit immunoglobulin (IgG), together with protein A-agarose (Amersham Bioscience). After extensive washing, immunocomplexes were dissociated, separated using SDS–PAGE, and gels were analyzed using a phosphorimager.

### Preparation of Murine Splenocytes and Activation by Bacterial LPS

Spleens were dissected from 8-week-old C57BL/6 mice in RPMI 1640 supplemented with 2% FBS and firmly pressed once with a crusher. Large cell clumps were dispersed by drawing and expelling the suspension several

times through a 6-ml syringe equipped with a 19-G needle. After removing red blood cells with ACK lysing buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2), splenocytes were resuspended and stabilized at 37°C in a humidified 5% $CO_2$ atmosphere for 3 h. For LPS treatment, cells were suspended (density = $2 \times 10^6$ cells/60 mm culture plate) in the same medium containing 5 μg/ml LPS for the indicated times.

### Statistical Analyses

Data are expressed as mean ± standard error (SE). Statistical significance was evaluated using Student's $t$ tests or one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered statistically significant when $P < 0.05$.

## RESULTS

### Identification of Two Pax5 Binding Sites in the Murine 1-cysPrx Promoter

The 668 bp region upstream of the translation start site (position −668 to −1) of the murine 1-cysPrx gene was analyzed using the MatInspector program (Genomatix Software). This analysis indicated the presence of two putative Pax5 binding sites at positions −278 to −262 and −50 to −34. Alignment of these sites with the consensus recognition sequence for the Pax5 paired domain showed that 10 of the 17 nucleotides were identical (Fig. 1A). Similar homology has been observed in other genes such as CD19, blk and mb-1 [Morrison et al., 1998].

Using EMSAs, we investigated whether a recombinant Pax5 protein would bind to two putative sites in vitro. Two sets of probes spanning nucleotides −56 to −24 (MP1) and −284 to −253 (MP2) which encompass the consensus sequence were created (Fig. 1B, rows 1 and 3). We also made 6 bp mutations within the consensus sequence. The mutated MP1 and MP2 sequences, named MP1-Mu and MP2-Mu, carried each 2 bp substitutions at the 5′, spacer and the 3′ half-site (rows 2 and 4). It has been reported that these mutations prevented Pax5 from binding to the blk promoter in vitro [Zwollo and Desiderio, 1994]. The *E. coli* expression system using pGEX-Pax5 generated a fusion protein (GST-Pax5) with an apparent molecular mass of 78 kDa on SDS–PAGE (Fig. 2A). This fusion protein mass reflects the presence of Pax5, the GST protein and additional amino

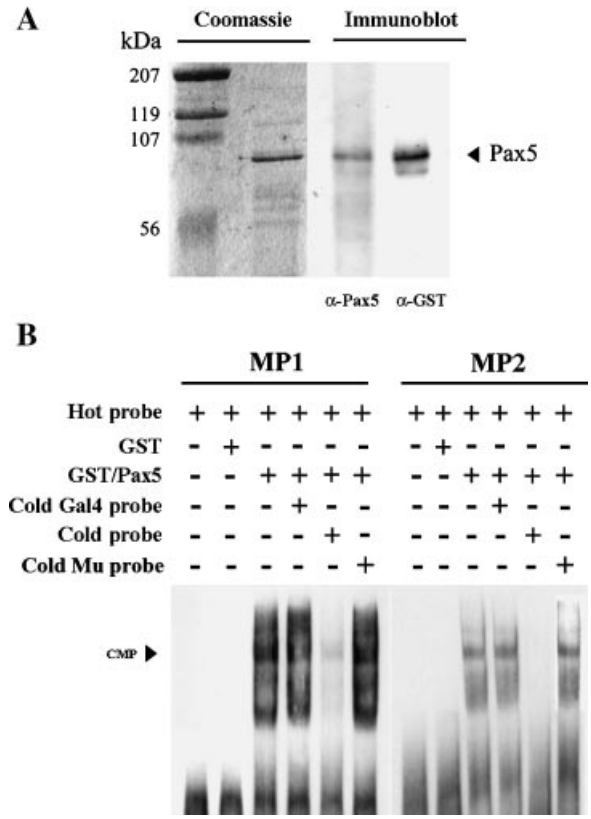


**Fig. 2.** Specific binding of purified *E. coli*-expressed GST-Pax5 to MP1 and MP2 sequences. **A**: SDS–PAGE and immunoblot analysis of the purified GST-Pax5 protein. Purified protein (1 μg) was resolved using 12% SDS–PAGE and stained with Coomassie Blue (left), or transferred to nitrocellulose membrane and then reacted with polyclonal Pax5 or GST antibodies (right). **B**: EMSA using the recombinant protein. GST-Pax5 (3 μg) was incubated with radiolabeled MP1 or MP2 probes and the resulting DNA/GST-Pax5 complex (CMP) bands (arrow head) were detected by autoradiography. Unlabeled oligonucleotides of the Gal 4 consensus sequence were added to the incubations as a non-specific competitor. A 20-fold molar excess of unlabelled MP1, MP2, MP1-Mu, or MP2-Mu were used as specific competitors.

acids from the vector. GST-Pax5 reacted with polyclonal antibodies to both Pax5 and GST (Fig. 2A, lanes 3 and 4). EMSAs showed that GST-Pax5 bound to double-stranded MP1 (33-mer) or MP2 (32-mer) probes to form GST-Pax5/DNA complexes (Fig. 2B, lanes 3 and 9). Such complexes were not observed following incubation of GST alone with the probes (Fig. 2B, lanes 2 and 8). The addition of a 20-fold molar excess of unlabeled non-specific DNA probe (Gal4 consensus sequence) did not affect complex formation (lanes 4 and 10), whereas unlabeled self-competitors reduced the amount of observed complex (lanes 5 and 11). Furthermore, unlabeled MP1-Mu and MP2-Mu duplex oligonucleotides were unable to compete for binding,

even in a 20-fold molar excess over each labeled probe (lanes 6 and 12). These results suggest that Pax5 specifically binds to sequences within the 1-cysPrx gene promoter.

Next, we investigated whether native Pax5 would bind to the MP1 and MP2 DNA sequences. MP1 and MP2 probes were incubated with B lymphoblast (IM-9) nuclear proteins, and the products were analyzed using EMSAs. Pax5 protein was only present in the B cell lines, while the expression of 1-cysPrx protein was detectable in all cell lines tested, including HeLa, NIH3T3, K-562 and B cell lines (Fig. 3A). EMSAs showed that specific bands were generated following the incubation of either probe with IM-9 cell nuclear extracts (Fig. 3B, lanes 3 and 9), but not following incubation with myeloid leukemia cell (K562) extracts (data not shown). These bands were shifted to high molecular weight positions only when Pax5-specific antibody was added, but not normal rabbit IgG (lanes 4 and 10, compared with lanes 5 and 11). Competition assays using excess unlabeled MP1 and MP2 probes resulted in a significant decrease in complex formation (lanes 2 and 8), whereas excess unlabeled mutant probes had no significant effect on complex formation (lanes 6 and 12). These results suggest that native Pax5 specifically binds to sequences within the promoter of 1-cysPrx gene in vivo.

ChIP assays were performed to investigate the binding of Pax5 to the endogenous 1-cysPrx promoter. A mouse embryonic fibroblast cell line (NIH3T3 cells) was transfected with either pcDNA-Pax5 or pcDNA3, after which chromatin was immunoprecipitated using an anti-Pax5 antibody or normal rabbit IgG. Immunoprecipitated DNA was PCR-amplified using two sets of primers. The PCR products P1 and P2, covering the MP1 and MP2 regions respectively, were synthesized with a constant level of input DNA (Fig. 3C, row 1), indicating that the primers correctly recognized the promoter region of the 1-cysPrx gene. We found that both P1 and P2 sequences were occupied by Pax5 when immunoprecipitated DNA from Pax5-transfected cells was used as a template (row 3, Pax5 of P1, and P2). No such PCR products were detected following PCR amplification from pcDNA3-transfected cells (row 3, mock of P1 and P2), or when normal rabbit IgG was used to immunoprecipitate DNA from Pax5-transfected cells (row 2).

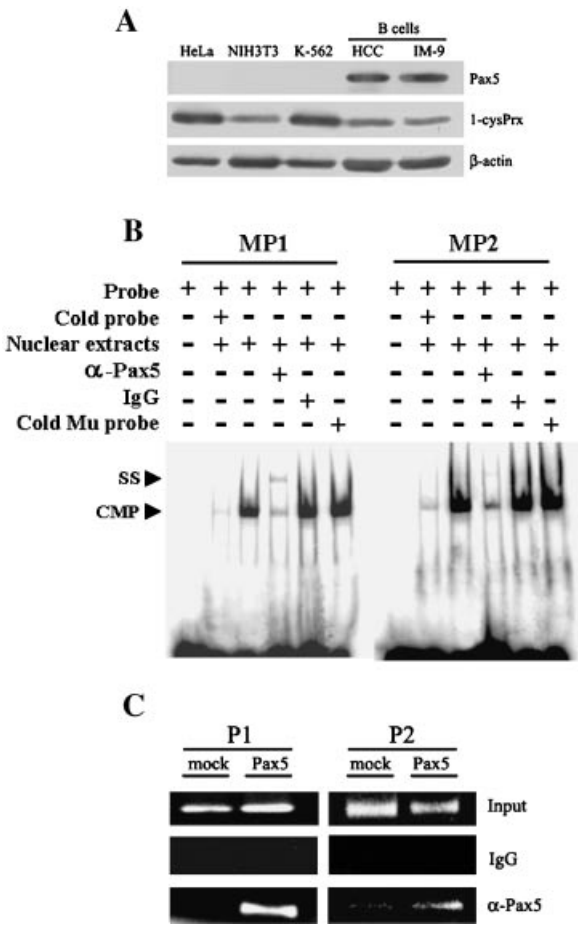


**Fig. 3.** Binding of endogenous Pax5 protein to MP1 and MP2 sequences. **A**: Immunoblot analysis of Pax5 and 1-cysPrx expression. Total soluble cell lysate protein (25 μg) was subjected to immunoblot analysis using a polyclonal Pax5 antibody. The membrane was then stripped and reprobed with a polyclonal 1-cysPrx antibody. Monoclonal β-actin antibody was used to compare total protein loading between lanes. **B**: EMSA using MP1, MP2 probes, and IM-9 cell nuclear extracts. Nuclear proteins (4 μg) from IM-9 cells were incubated with radiolabeled MP1 or MP2 probes before analysis on a non-denaturing electrophoretic gel. Bands representing the DNA/Pax5 complex (CMP, arrow head) were visualized using autoradiography. When a Pax5-specific antibody was added, the CMP band shifted to a higher molecular weight position (SS, arrow head). Specific competitors (cold MP1, MP2, MP1-Mu, and MP2-Mu) were used at a 20-fold molar excess. **C**: ChIP assays. Following transfection of NIH3T3 cells with pcDNA-Pax5 or pcDNA3, cell lysates underwent immunoprecipitation using anti-Pax5 antibody or normal IgG. The immunoprecipitated DNA (3 μg) was PCR-amplified in the presence of two sets of primers to generate P1 and P2 DNA fragments that cover the MP1 and MP2 regions of 1-cysPrx promoter, respectively.

The above data suggest that Pax5 binds to both MP1 and MP2 sequences of the 1-cysPrx promoter and may regulate 1-cysPrx expression in vitro and in vivo.

### Transactivation of the 1-cysPrx Promoter by Pax5

We investigated the effects of Pax5-binding on 1-cysPrx promoter activity. Transient cotransfection experiments were conducted using pGL3-mPx luciferase reporter and pcDNA-Pax5 constructs in HeLa cells that do not express endogenous Pax5 protein (Fig. 3A). In such a system, luciferase activity should be strictly dependent on the amount of Pax5 protein expressed. The pcDNA3 empty vector was used in all subsequent transfections as a negative control, and was added to maintain equal amounts of total transfected DNA. Transfection of the pGL3-mPx promoter-driven reporter elicited a level of basal activity above that of the promoterless basic reporter (Fig. 4, lanes 2 and 3). This activity was elevated ~7-fold in the presence of Pax5 (lane 6). Furthermore, Pax5 could dose-dependently increase promoter activity up to ~12-fold (lanes 6–8). To demonstrate that transactivation of the 1-cysPrx promoter by Pax5 is MP1- and MP2-sequence dependent, we measured luciferase activity using MP1 and MP2 mutant promoter-driven reporters. Luciferase assay showed that mutations of MP1 and MP2 region led to dramatic decreases in transcriptional activity when compared with the wild type (~60% and ~80% loss for MP1 and MP2 mutants, respectively), suggesting that both regions make a contribution to the total transactivation of the 1-cysPrx promoter (lanes 4 and 5). Similar patterns of luciferase activity were obtained when using NIH3T3 cells, another non-Pax5 expressing cell line (data not shown). These results indicate that Pax5 functions as a positive transcriptional regulator of 1-cysPrx expression.

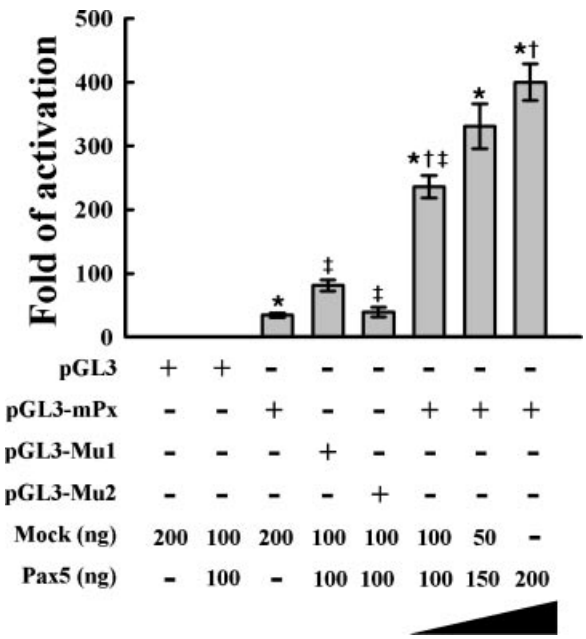


**Fig. 4.** Transactivation of 1-cysPrx-luciferase in HeLa cells. Cells were transiently transfected with the luciferase reporters pGL3, pGL3-mPx, pGL3-mPx/MP1(Mu) or pGL3-mPx/MP2(Mu) together with the indicated amounts of pcDNA-Pax5 and pcDNA3 empty vector. Forty-eight hours after transfection, cells were harvested and luciferase activity was measured. The average of the relative luciferase activity for each experiment was divided by the relative basal reporter luciferase activity, and the calculated average is shown as fold activation with a standard error. $*P < 0.05$ compared with pGL3 luciferase activity; † and ‡ difference between the groups is statistically significant ($P < 0.05$).

### Enhancement of Pax5-Mediated 1-cysPrx Promoter Activity by Histone Acetyltransferases

The histone acetyltransferases (HATs) p300/CBP and PCAF are transcription coactivators that cooperate with various transcription factors to activate target promoters. HATs act by mediating interactions between the basal transcriptional complex and DNA-binding transcription factors, as well as numerous other regulatory proteins [Goodman and Smolik, 2000]. It has been reported that Pax5 forms a trimeric complex with Daxx and CBP, suggesting that Pax5 requires HAT activity to stimulate transcription of target genes [Emelyanov et al., 2002]. We examined the effect of HATs on Pax5-mediated transactivation of 1-cysPrx expression using pGL3-mPx and HAT expression plasmids in HeLa cells. Compared with pcDNA-Pax5 transfection alone, cotransfection of pcDNA-Pax5 with plasmids for p300, CBP or PCAF expression resulted in a dose-dependent increase in 1-cysPrx reporter activity (Fig. 5A). Meanwhile, each HAT expression plasmid alone had no significant effect on the reporter activity (lanes 2, 5, and 8). CBP coexpression resulted in ~2-fold greater reporter activity than observed for either p300 or PCAF coexpression (lanes 4, 7, and 10). Similar data were obtained following coexpression of p300 or PCAF with Pax5 in NIH3T3 cells (data not shown). These results suggest that p300/CBP and PCAF play roles as transcriptional coactivators in enhancing Pax5-mediated transactivation of the 1-cysPrx promoter.

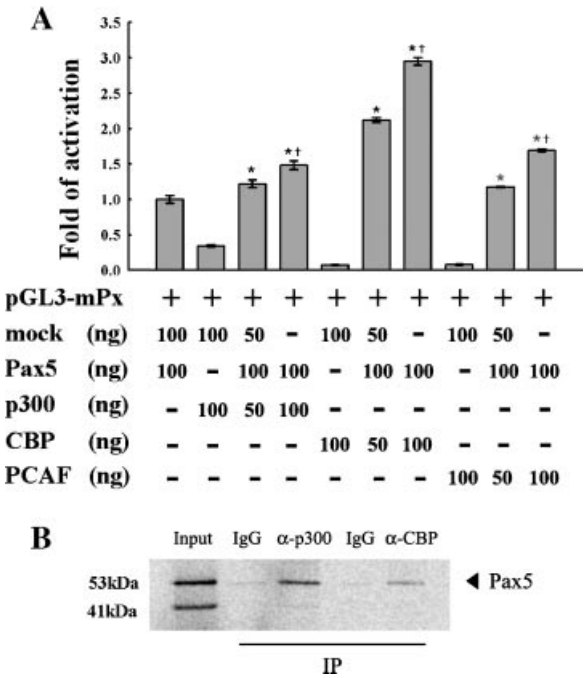


**Fig. 5.** HATs directly bind to Pax5 and enhance Pax5-mediated 1-cysPrx expression. **A**: Cotransfection of HAT expression plasmids together with pcDNA-Pax5 and pGL3-mPx in HeLa cells. Cells were transiently cotransfected with pGL3-mPx and pcDNA-Pax5 in the absence or presence of the indicated amounts of expression vectors for p300, CBP, or PCAF. Total amount of transfected DNA was kept constant by the addition of empty vector. Luciferase activity from cells cotransfected with pGL3-mPx and pcDNA-Pax5 was arbitrarily given the value of 1, and relative activities were calculated as relative fold activation. The data represent the mean ± SE from triplicate assays of three independent experiments. $*P < 0.05$ compared with pGL3-mPx/Pax5 luciferase activity. † difference between the groups is statistically significant ($P < 0.05$). **B**: Pax5 physically interacts with p300 and CBP in vivo. The [$^{35}$S]-labeled in vitro translated product of pcDNA-Pax5 was incubated with extracts from HeLa cells transfected with p300 or CBP expression plasmids. Proteins were immunoprecipitated using anti-p300 or -CBP antibodies, or normal rabbit IgG, and then resolved using 10% SDS–PAGE. Bands were visualized using a phosphorimager.

## Direct Interaction of Pax5 With p300 and CBP In Vivo

The enhancement of Pax5-mediated transcriptional activation by the p300 and CBP coactivators prompted us to examine whether Pax5 was able to interact with these proteins directly. In vitro transcribed/translated [$^{35}$S] methionine-labeled Pax5 protein was incubated with whole cell lysates from HeLa cells transfected with p300 or CBP expression plasmids, after which lysates were subjected to immunoprecipitation assays using anti-p300 or -CBP antibodies. The IgG fraction of normal rabbit serum was used as a control for immunoprecipitation specificity. Immunoprecipitation products were separated using SDS–PAGE, and gels were exposed to phosphorimager to identify labeled Pax5 protein (Fig. 5B). Expression of the Pax5 cDNA (pcDNA-Pax5) resulted in two translated products with molecular masses of ~53 kDa and ~41 kDa (lane 1). The two products are probably the results of two in-frame ATG codons positioned at nucleotides 1 and 325, as described previously [Zwollo et al., 1997]. It is likely that translation from the distal ATG codon produces a 53 kDa protein with a complete DNA-binding domain (126 amino acids), while translation from the proximal codon produces a 41 kDa protein with a partial domain (31 amino acids). We found that anti-p300 or -CBP antibodies only immunoprecipitated the 53 kDa version of Pax5 (lanes 3 and 5), suggesting that p300 and CBP bind to the Pax5 DNA-binding domain. These data suggest that Pax5 physically associates with the p300 and CBP coactivators in vivo.

## Chromatin Hyperacetylation by Targeting of Pax5 to the 1-cysPrx Promoter

Hyperacetylation of histones by coactivators generally promotes transcriptional activation, while hypoacetylation of histone by corepressors is associated with transcriptional repression [Goodman and Smolik, 2000]. We investigated the mechanism underlying Pax5-mediated transcriptional activation. ChIP assays were conducted using anti-acetylated histone H3 and H4 antibodies, and histone acetylation levels in the Pax5-binding region were measured. Using a constant level of input DNA from NIH3T3 cells transfected with either pcDNA3 or pcDNA-Pax5 (Fig. 6, row 1), we found that expression of Pax5 resulted in a substantial increase in the level of histone H3 and an even larger increase in the level of H4 acetylation in the P1 region (row 3 and 4). Normal mouse IgG was used as a negative control (row 2). Patterns of histone acetylation in the P2 region were similar to those in the P1 region (data not shown), suggesting that histone H4 might be the preferential substrate for Pax5-mediated hyperacetylation. These findings suggest that Pax5 plays an important role in transcriptional activation of the 1-cysPrx promoter via promotion of targeted histone hyperacetylation.

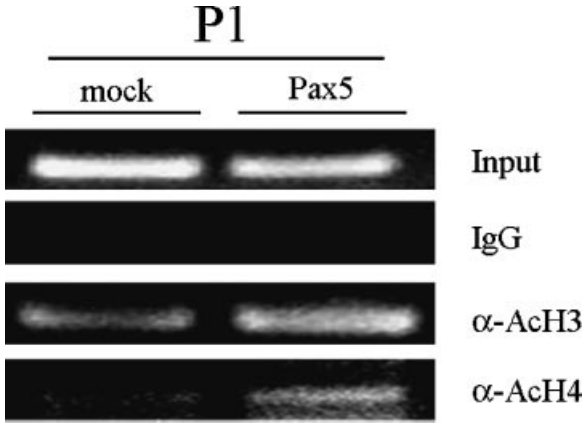


**Fig. 6.** ChIP assays using anti-acetyl histone H3 and H4 antibodies. Lysates from NIH3T3 cells transfected with pcDNA3 or pcDNA-Pax5 were immunoprecipitated with anti-acetyl histone H3, H4 antibodies, or normal mouse IgG. The immunoprecipitated 1-cysPrx promoter fragment (P1) was then detected using PCR. Input refers to amplification of total cell lysates.

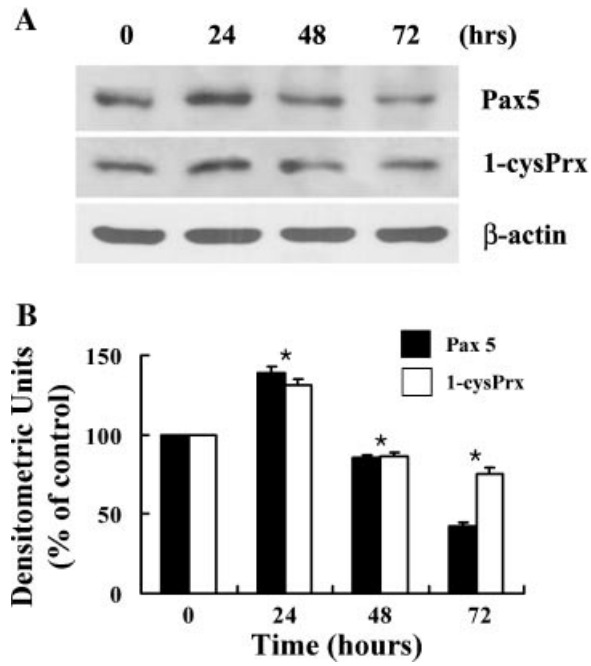


**Fig. 7.** LPS stimulates coordinated expression of Pax5 and 1-cysPrx in murine splenocytes. Cells incubated with 5 μg LPS were harvested at various time points and then analyzed for Pax5 and 1-cysPrx expression using immunoblot analysis. **A**: Representative immunoblot. **B**: Individual data were quantified as densitometric units and were normalized according to β-actin expression. Data in the graph are presented as relative percentages of the control (time 0) and are means ± SE for three independent experiments. $^*P < 0.05$ compared with the control.

### Coordinated Expression of Pax5 and 1-cysPrx Proteins in LPS-Treated Splenocytes

We investigated whether Pax5 and 1-cysPrx protein expression levels were coordinated. LPS stimulation of splenic B cells has been reported to increase Pax5 activity concurrent with an increase in B cell proliferation [Wakatsuki et al., 1994]. We performed time-course studies to examine the expression of Pax5 and 1-cysPrx in an LPS-stimulated murine splenocyte population enriched in B lymphocytes. Immunoblot assays showed that Pax5 and 1-cysPrx levels both increased by ~30% at 24 h post stimulation, and then both decreased by ~15% at 48 h post stimulation, compared with that of untreated control (Fig. 7). At 72 h post stimulation, there was ~58% decrease in Pax5 levels and ~25% decrease in 1-cysPrx levels. The expression levels of both proteins did not change significantly at any time point in untreated cells (data not shown). These data show a temporal correlation between Pax5 and 1-cysPrx expression in LPS-stimulated cells.

## DISCUSSION

The present study found that Pax5 bound to two sites in a 668 bp region of the murine 1-cysPrx promoter, and that Pax5 transactivated expression of the 1-cysPrx gene. The findings demonstrated that Pax5 targeted a gene whose expression was not limited to B cells. The study also found that the Pax5-mediated activation of the 1-cysPrx promoter was enhanced by physical interaction with HATs. To our knowledge, this is the first report to describe Pax5 and HATs-mediated transcriptional up-regulation of the 1-cysPrx gene.

Pax5, a member of the Pax family of transcription factors, is found in the pro-, pre- and mature stages of B cell development, but is silenced in terminally differentiated plasma cells [Busslinger and Urbanek, 1995]. Full-length of Pax5 contains 391 amino acids, and comprises a paired DNA-binding domain, an octapeptide, a homeobox domain, and domains involved in activation and repression [Hagman et al., 2000]. Alternative splicing results in three additional isoforms with truncated or deleted domains, which are differentially expressed during B cell maturation [Zwollo et al., 1997]. It has been suggested that the isoforms work together as positive or negative regulators of DNA-binding activity during B cell activation/proliferation, thus affecting stage-specific expression of Pax5 target genes [Lowen et al.,

2001]. In the present study, nucleotide sequence analysis and protein molecular mass data (Figs. 2A and 5B) suggested that the *E. coli*-expressed and in vitro synthesized Pax5 protein contained all functional domains.

The paired DNA-binding domain of Pax5 can be divided into two subdomains, namely the N-and C-terminal subdomains. Either the N-terminus alone or both terminals together can recognize two non-homologous half-sites of 7–8 bp separated by 2 bp. Studies have shown that even though sequence divergence in known Pax5-binding sites occurs at both sites, a specific CA dinucleotide appears to be required for high affinity binding of Pax5 to a 5′ half-site [Zwollo and Desiderio, 1994; Morrison et al., 1998; Hagman et al., 2000]. The MP1 and MP2 sites showed some homology with the consensus 5′ and 3′ half-site sequences, as do all other previously described naturally occurring Pax5 sites. The identical nucleotides comprised 4 or 5 (including a CA dinucleotide) of the 6 bp in the 5′ half-site, 1 of the 2 bp in the spacer, and 4 or 5 of 7 bp in the 3′half-site (Fig. 1A). The present study found that recombinant and native Pax5 protein bound to both MP1 and MP2 regions with similar affinity (Figs. 2B and 3B). In addition, ChIP experiments showed that exogenously expressed Pax5 bound to both MP1 and MP2 regions in the endogenous 1-cysPrx gene (Fig. 3C). The non-B cell cotransfection experiments using reporter constructs and Pax5 expression plasmids showed that this Pax5 binding led to transactivation of the 1-cysPrx promoter in a dose-dependent manner (Fig. 4).

Pax5 is known to function as both a transcriptional activator and repressor, depending on the target genes and possibly the developmental state of the B cells. This dual nature of Pax5 raises the possibility that Pax5 can interact with other transcription factors and/or regulators, thus controlling gene expression [Eberhard et al., 2000; Hagman et al., 2000]. Another interesting feature of the Pax5 interaction includes the adaptor-mediated complex formation with HATs, affecting Pax5-dependent transcription [Emelyanov et al., 2002; Barlev et al., 2003]. We also found that HATs (i.e., CBP, p300, and PCAF) enhanced Pax5-mediated 1-cysPrx promoter activation, and that this involved direct interaction between Pax5 and HAT proteins (Fig. 5). This HAT recruitment led to hyperacetylation of histone H3 and H4 on the 1-cysPrx promoter (Fig. 6), suggesting that targeted histone acetylation around the 1-cysPrx promoter mediated by Pax5 binding would cause localized chromatin remodeling and thus enhance 1-cysPrx gene transcription. The transient cotransfection assay data showed that CBP was the strongest coactivator of Pax5-mediated transactivation compared with the p300 and PCAF, yet CBP appeared to have a slightly weaker direct interaction. Further investigations regarding possible involvement of adaptor protein in Pax5-HAT complex may provide more definite information about the regulation of 1-cysPrx expression mediated by the interaction of Pax5 and HATs.

Two intronless 1-cysPrx related genes, 1-cysPrx-P1, and 1-cysPrx-P2 (also named Aop2-rs1 and Aop2-rs2, GenBank accession No. AF085220 and AF08522, respectively), have been identified in the murine genome, which they were located on a different chromosome [Phelan et al., 1998; Lee et al., 1999]. These genes shared high sequence homology with 1-cysPrx cDNA. 1-cysPrx-P1 contains an intact open reading frame (ORF) that encodes 224 amino acids, while 1-cysPrx-P2 has a disrupted ORF that might indicate an unexpressed pseudogene with no function. We noticed that the MP1 region of 1-cysPrx-P1 was almost identical to that of 1-cysPrx with the exception of a dinucleotide insertion before the 17th nucleotide in the 3′half-site. However, there was no significant homology with the MP2 region. It would be interesting to determine whether Pax5 can regulate 1-cysPrx-P1 expression.

LPS is a mitogen that activates B cells, causing proliferation and differentiation into memory cells and plasma cells. LPS treatment has been reported to increase Pax5 DNA binding activity in splenic B cells and B lymphoma cells (CH12.LX), which was coupled with an increase in proliferation [Wakatsuki et al., 1994]. Another study reported a time-dependent decrease in the levels of Pax5 mRNA, protein and DNA binding activity in LPS-activated CH12.LX cells, demonstrating the functional role of Pax5 as a repressor of B-cell differentiation [Yoo et al., 2004]. It has been also reported that LPS induced the Pax5 DNA binding activity in malignant B-1 cells during the initial activation stage (24 h), while prolonged LPS treatment (72 h) resulted in a subsequent decrease in Pax5 levels, and these events correlated with an initial augmentation

in proliferation followed by apoptosis and pathological differentiation [Zhang et al., 2001]. Increased 1-cysPrx expression was reported in actively proliferating corneal tissues during wound healing process after photorefractive keratectomy, and in proliferating bovine keratocytes as the result of growth factor treatment, suggesting 1-cysPrx involvement in cell proliferation [Tchah et al., 2005]. In the present study, expression of both Pax5 and 1-cysPrx in splenocytes increased over the 24 h after LPS treatment, during which the cells would enter an active proliferation stage. Following this increase, the expression of both proteins began to decline at 48 h, and continued to decrease over the 72 h culture period (Fig. 7), which might be explained by terminal cell differentiation. These coordinated changes in Pax5 and 1-cysPrx expression in response to LPS are consistent with the possibility that Pax5 is an important transcription factor regulating 1-cysPrx expression. Additional studies are required to examine the changes in the redox status of LPS-treated cells at time points which the expressions of Pax5 and 1-cysPrx were altered, and to evaluate 1-cysPrx expression patterns at each stage of B cell development/differentiation. Altering 1-cysPrx expression using overexpression or interference techniques will provide useful information for the physiologic function of 1-cysPrx in B lymphocytes.

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## REFERENCES

Akiba S, Dodia C, Chen X, Fisher AB. 1998. Characterization of acidic $Ca^{2+}$-independent phospholipase $A_2$ of bovine lung. Comp Biochem Physiol 120:393–404.

Barlev NA, Emelyanov AV, Castagnino P, Zegerman P, Bannister AJ, Sepulveda MA, Robert L, Kouzarides T, Bitshtein BK, Berger SL. 2003. A novel human Ada2 homologue functions with Gen5 or Brg1 to coactivate transcription. Mol Cell Biol 23:6944–6957.

Busslinger M, Urbanek P. 1995. The role of BSAP (Pax-5) in B-cell development. Curr Opin Genet Dev 5:595–601.

Eberhard D, Jimenez G, Heavey B, Busslinger M. 2000. Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. EMBO J 19:2292–2303.

Emelyanov AV, Kovac CR, Sepulveda MA, Brrshtein BK. 2002. The interaction of Pax5 (BSAP) with Daxx result in transcriptional activation in B cells. J Biol Chem 277: 11156–11164.

Fatma N, Singh DP, Shinohara T, Chyack LT Jr. 2001. Transcriptional regulation of the antioxidant protein 2 gene, a thiol-specific antioxidant, by lens epithelium-derived growth factor to protect cells from oxidative stress. J Biol Chem 276:48899–48907.

Frank S, Munz B, Werner S. 1997. The human homologue of a bovine non-selenium glutathione peroxidase is a novel keratinocyte growth factor-regulated gene. Oncogene 14:915–921.

Fujii J, Ikeda Y. 2002. Advances in our understanding of peroxiredoxin, a multifunctional, mammalian redox protein. Redox Report 7:123–130.

Goodman RH, Smolik S. 2000. CBP/p300 in cell growth, transformation, and development. Genes Dev 14:1553–1577.

Hagman J, Wheat W, Fitzsimmons D, Hodsdon W, Negri J, Dizon F. 2000. Pax-5/BSAP: Regulator of specific gene expression and differentiation in B lymphocytes. Curr Top Microbiol Immunol 245:169–194.

Hofmann B, Hecht H, Flohe L. 2002. Peroxiredoxins. Biol Chem 383:347–364.

Kim TS, Dodia C, Chen X, Hennigan BB, Jain M, Feinstein SI, Fisher AB. 1998. Cloning and expression of rat lung acidic $Ca^{2+}$-independent $PLA_2$ and its organ distribution. Am J Physiol Lung Cell Mol Physiol 274: L750–L761.

Lee TH, Yu SL, Kim SU, Kim YM, Choi I, Kang SW, Rhee SG, Yu DY. 1999. Characterization of the murine gene encoding 1-Cys peroxiredoxin and identification of highly homologous genes. Gene 234:337–344.

Lowen M, Scott G, Zwollo P. 2001. Functional analyses of two alternative isoforms of the transcriptional factor Pax-5. J Biol Chem 276:42565–42574.

Manevich Y, Sweitzer T, Pak JH, Feinstein SI, Muzykantov V, Fisher AB. 2001. 1-Cys peroxiredoxin overexpression protects cells against phospholipids peroxidation-mediated membrane damage. Proc Natl Acad Sci USA 99: 11599–11604.

Morrison AM, Nutt SL, Thevenin C, Rolink A, Busslinger M. 1998. Loss- and gain-of-function mutations reveal an important role of BSAP (Pax-5) at the start and end of B cell differentiation. Semin Immunol 10:133–142.

Pak JH, Manevich Y, Kim HS, Feinstein SI, Fisher AB. 2002. An antisense oligonucleotide to 1-cys peroxiredoxin causes lipid peroxidation and apoptosis in lung epithelial cells. J Biol Chem 277:49927–49934.

Peshenko IV, Novoselov VI, Evdokimov VA, Nikolaev Yu V, Kamzalov SS, Shuvaeva TM, Lipkin VM, Fesenko EE. 1998. Identification of a 28 kDa secretory protein from rat olfactory epithelium as a thiol-specific antioxidant. Free Radic Biol Med 25:654–659.

Phelan SA, Johnson KA, Beier DA, Paigen B. 1998. Characterization of the murine gene encoding Aop2 (Antioxidant protein 2) and identification of two highly related genes. Genomics 54:132–139.

Rhee SG, Kang SW, Chang TS, Jeong W, Kim K. 2001. Peroxiredoxin, a novel family of peroxidases. IUBMB Life 52:35–41.

Seo S, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. 2001. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the Set oncoprotein. Cell 104:119–130.

Shichi H, Demar JC. 1990. Non-selenium glutathione peroxidase without glutathione S-transferase activity from bovine ciliary body. Exp Eye Res 50:513–520.

Tchah H, Kim MJ, Kim T, Choi H, Kim JY, Kim MJ, Pak JH. 2005. Regulation of 1-Cys peroxiredoxin expression in the process of stromal wound healing after photorefractive keratectomy. Invest Ophthal Vis Sci 46:2396–2403.

Wakatsuki Y, Neurath MF, Max EE, Strober W. 1994. The B cell-specific transcription factor BSAP regulates B cell proliferation. J Exp Med 179:1099–1108.

Wang X, Phelan SA, Forsman-Semb K, Taylor EF, Petros C, Brown A, Lerner CP, Paigen B. 2003. Mice with targeted mutation of peroxiredoxin 6 develop normally but are susceptible to oxidative stress. J Biol Chem 278: 25179–25190.

Wang Y, Feinstein SI, Manevich Y, Ho Y, Fisher AB. 2004. Lung injury and mortality with hyperoxia are increased in peroxiredoxin 6 gene-targeted mice. Free Rad Biol Med 37:1736–1743.

Yoo BS, Boverhof DR, Shnaider D, Crawford RB, Zacharewski TR, Kaminski NE. 2004. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the regulation of Pax5 in lipopolysaccharide-activated B cells. Toxicol Sci 77:272–279.

Zhang M, Chong SY, Raveche ES. 2001. The role of B-cell-specific activator protein in the response of malignant B-1 cells to LPS. Exp Cell Res 264:233–243.

Zwollo P, Desiderio S. 1994. Specific recognition of the blk promoter by the B-lymphoid transcription factor B-cell-specific activator protein. J Biol Chem 269:15310–15317.

Zwollo P, Arrieta H, Ede K, Molinder K, Desiderrio S, Pollock R. 1997. The Pax-5 gene is alternatively spliced during B-cell development. J Biol Chem 272:10160–10168.