HBP1 Repression of the p47phox Gene: Cell Cycle Regulation via the NADPH Oxidase

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Received 17 June 2003/Returned for modification 8 October 2003/Accepted 30 December 2003

Several studies have linked the production of reactive oxygen species (ROS) by the NADPH oxidase to cellular growth control. In many cases, activation of the NADPH oxidase and subsequent ROS generation is required for growth factor signaling and mitogenesis in nonimmune cells. In this study, we demonstrate that the transcriptional repressor HBP1 (HMG box-containing protein 1) regulates the gene for the p47phox regulatory subunit of the NADPH oxidase. HBP1 represses growth regulatory genes (e.g., N-Myc, c-Myc, and cyclin D1) and is an inhibitor of G1 progression. The promoter of the p47phox gene contains six tandem high-affinity HBP1 DNA-binding elements at positions −1243 to −1318 bp from the transcriptional start site which were required for repression. Furthermore, HBP1 repressed the expression of the endogenous p47phox gene through sequence-specific binding. With HBP1 expression and the subsequent reduction in p47phox gene expression, intracellular superoxide production was correspondingly reduced. Using both the wild type and a dominant-negative mutant of HBP1, we demonstrated that the repression of superoxide production through the NADPH oxidase contributed to the observed cell cycle inhibition by HBP1. Together, these results indicate that HBP1 may contribute to the regulation of NADPH oxidase-dependent superoxide production through transcriptional repression of the p47phox gene. This study defines a transcriptional mechanism for regulating intracellular ROS levels and has implications in cell cycle regulation.

The production of reactive oxygen species (ROS) has various consequences, depending on the ROS concentration and the cellular environment. For example, high levels of ROS production by the NADPH oxidase complex are essential for microbial killing by phagocytic cells. In contrast, lower ROS levels that are generated by the NADPH oxidase are essential for mitogenic signaling in many cell types (e.g., see references 9 and 10). For example, both epidermal growth factor and platelet-derived growth factor require ROS for stimulated mitogenesis (reviewed in reference 26). Recent studies have highlighted a key role for ROS in modulating signaling networks through reversible cysteine oxidation and tyrosine phosphatase regulation (22, 23; reviewed in reference 37). In this paper, we provide evidence for a transcriptional mechanism for regulating intracellular ROS levels through the repression of the NADPH oxidase. Our data indicate that the transcriptional repressor and G1 inhibitor HBP1 (HMG box-containing protein 1) (e.g., see references 28, 29, and 32) represses the gene for the p47phox regulatory subunit of the NADPH oxidase. This mechanism has functional consequences for intracellular ROS homeostasis and growth regulation.

HBP1 is a transcriptional repressor and a member of the sequence-specific HMG box family of transcription factors (reviewed in reference 38). We and others originally isolated HBP1 as a binding partner of the retinoblastoma tumor suppressor and its family member p130 (13, 32). With the use of cell and animal models, it has been shown that HBP1 expression inhibits G1 progression and may regulate aspects of cellular differentiation (28, 32). Some gene targets of HBP1 include N-Myc, c-Myc, cyclin D1, myeloperoxidase, and histone H10 (14, 21, 25). Two mechanisms for transcriptional inhibition by HBP1 have been described: through direct binding to the target promoters (e.g., N-Myc [32]) and by physically inhibiting the essential transcriptional activators (e.g., cyclin D1 and Wnt signaling [25]). Since a constitutive Wnt pathway is associated with diverse epithelial cancers (reviewed in reference 24), the regulation of cyclin D1 and other Wnt target genes by HBP1 may suggest a possible tumor-suppressive role (25). With HBP1’s unexpectedly complex repression mechanisms, the identification of new gene targets is necessary for further insights into how HBP1 regulates signaling networks for tumor suppression. Through a database search (see below), we found that the promoter for the p47phox gene has a striking array of HBP1 sites and is an excellent candidate for a new target gene. The p47phox protein is a regulatory component of the NADPH oxidase complex, which is a major source of intracellular ROS.

The NADPH oxidase catalyzes the one-electron reduction of O2 to O2− with NADPH as the donor. It consists of cytoplasmic regulatory subunits (p47phox, p67phox, and p40phox) that combine with a membrane complex that includes a tissue-specific gp91phox catalytic subunit (sometimes designated NOX; reviewed in reference 3). All the various subunit names of the human NADPH oxidase components are listed below to assist with any future literature and database searches. The membrane components (p22phox and a gp91phox) are also called α and β subunits of cytochrome b558, respectively. Altogether, there are five tissue-restricted members of the gp91 catalytic subunits: gp91/CYBB/Nox2,
FIG. 1. HBP1 regulates the p47phox promoter through specific binding to the HBP1 DNA elements. (A) Schematic diagram of the p47phox promoter and associated reporter constructs. The top part depicts the array and sequence of the HBP1 sites in the p47 phox promoter at positions -1392 to -1217 from the transcriptional start site (a generous gift of Robert Clark). Letters representing the 76 bp encompassing the HBP1 sites are underlined and italicized. A schematic of the reporters used in this paper is also shown. (B) The high-affinity HBP1 sites confer transcriptional repression. HEK-293T cells were transfected with 0.5 μg of the indicated reporter and 2 μg of pRSV-β-Gal (β-gal) (to normalize for transfection efficiency). (C) Schematic diagram of wild-type HBP1 and associated mutants. MAPK, mitogen-activated protein kinase. (D) Relative activity of HBP1 and associated mutants on the native p47 phox promoter. Transfections were performed as in panel B and included 5 μg of the indicated HBP1 expression plasmid. The results are normalized for transfection efficiency and are expressed as relative transcriptional activity within each set. As shown in panel B, the activity of each reporter varies, but for comparison, the relative activity in the control (Cont.) was set to a value of 1. Each data set represents three to five experiments. (E) Relative activity of HBP1 and associated mutants on heterologous high-affinity HBP1 site reporter. Experiments were performed as described for panel D. (F) Expression of wild-type and mutant HBP1 proteins. HA-tagged proteins from experiments shown in panels D and E were detected by immunoblots (IB) with anti-HA antisera. A representative blot is shown.
FIG. 2. HBP1 occupies its high-affinity sites in the endogenous p47phox promoter. (A) ChIPs were used to test the binding of HBP1 to the endogenous p47phox gene. HEK-293T cells were transfected with pEFBOS-HBP1 or the indicated mutant plasmids. The region from position −1474 to position −1146 contains the HBP1 element and was analyzed by specific PCR. Either anti-HA or control (anti-DP1) antisera were used in the indicated lanes. Numbers representing the test and control lanes are underlined. Lanes 1 and 2 show the PCR results when no chromatin is included in the absence and presence of anti-HA, respectively. Lane 11 shows the PCR on total isolated chromatin from HEK-293T cells. Only lanes with HBP1 proteins that contain intact HMG boxes show binding to the p47phox promoter (lanes 3 and 7). At the bottom, anti-HA Western immunoblotting (IB) for HBP1 protein expression is shown. It should be noted that normal HBP1 migrates aberrantly at a molecular weight of −80,000, though the mRNA sequence specifies a 64,000-molecular-weight protein. (B) EMSA assays were performed by using a radiolabeled double-stranded probe consisting of one HBP1 high-affinity site. Ten-microgram amounts of nuclear extracts from HEK-293T cells or cells transfected with the indicated HBP1 expression plasmids were used. Cold competitors were included in the indicated lanes at 100-fold excess. The presence of specific complexes, including supershifted HA-HBP1 in the complexes, is indicated with arrows. WT, wild type.
NOX1/Mox1/gp91-2, Nox3/gp91-3, Nox4, and Nox5. The cytoplasmic components consist of p47phox/NCF1, p40phox/NCF4, p67phox/NCF2, and Rac.

The assembly of regulatory and catalytic components generates the active NADPH oxidase complex. The regulatory cytoplasmic complex is activated by phosphorylation, resulting in its translocation to the membrane to bind the catalytic subunits. The importance of a functional NADPH oxidase is underscored by the observation that mutations of the p47phox and gp91phox genes decrease overall NADPH oxidase activity and are associated with chronic granulomatosis, in which patients have increased susceptibility to bacterial and fungal infections (reviewed in reference 3). The catalytic gp91phox/NOX subunits have specific tissue distribution. Unlike the catalytic subunits, the regulatory and cytoplasmic components are largely ubiquitous. For example, the expression of the same p47phox/NCF1 gene occurs in most cell types (3).

For nonimmune cells, the regulation of NADPH oxidase activity and of intracellular ROS levels may dictate the efficacy of signaling networks involved in cancer (reviewed in references 7 and 37). Stimulation with growth factors (epidermal growth factor, platelet-derived growth factor, etc.) in nonimmune cells results in a rapid oxidative burst with the generation of ROS from the NADPH oxidase complex (reviewed in reference 26). The ROS levels in nonimmune cells are orders of magnitude lower than those in immune cells but represent ideal signaling molecules due to rapid synthesis and diffusibility. NADPH oxidase activity and ROS levels have an integral role in signaling by the G proteins Ras and Rac (9, 10). Stable transfection of NIH/3T3 fibroblasts with active Ras (H-RasV12) results in transformation and significant intracellular ROS generation, as pharmacological inhibition of the NADPH oxidase reverses transformation (9). In addition, the expression of the Nox1/Mox1 catalytic subunit of the NADPH oxidase in NIH/3T3 fibroblasts unexpectedly triggers increased superoxide production and results in oncogenic transformation (1, 30). Finally, Rac is also an obligate subunit of the cellular NADPH oxidase. The expression of active Rac1 in many cell types causes increased

![Fig. 3](http://mcb.asm.org/)

FIG. 3. Expression of HBP1 and Δ393 affects endogenous P47phox mRNA and protein expression. (A) p47 phox mRNA expression is decreased in a stable cell line that constitutively expresses HBP1. RT-PCR for the p47phox gene was performed with 2 μg of total RNA from the control C2C12 muscle cell line and a C2C12 line overexpressing HBP1 (B2 [24]). 18S RNA was used as an internal control, as indicated in Materials and Methods. The reactions were analyzed at the indicated cycle number. MW, molecular weight. (B) Transient expression of HBP1 represses endogenous p47 phox mRNA. 293T cells were cotransfected with 2 μg of F-GFP and 5 μg of HBP1 or control plasmids. The cells were selected by FACS 36 h after transfection. The mRNA was immediately isolated from the GFP-selected cells and analyzed by RT-PCR for the p47phox gene. One microgram of RNA was used with 18S RNA as an internal control with 18S competimers (Ambion; see Materials and Methods). Amplified products were analyzed at the indicated cycle numbers. (C) Transient expression of Δ393 increases p47phox mRNA. Cells were transfected with 2 μg of F-GFP or cotransfected with 5 μg of Δ393. The purification of transfected cells and RT-PCR analysis are as described for panel B. (D) Expression of Δ393 results in increased p47phox protein levels. HEK-293T cells were transfected with either 5 μg of the pmΔ393 (lanes 2 and 3) plasmid or Δ393 (lanes 4 and 5). Western blotting was performed on protein lysates using anti-human p47 phox antisera (a generous gift of Tom Leto) (top). Lane 1 contains untransfected 293T lysate. The blot was stripped and immunoblotted (IB) with anti-HA to show expression of the Δ393 and pmΔ393 proteins.
superoxide production, while the expression of a dominant-negative Rac1 blocks superoxide production (31). In addition, Jonese and Bar-Sagi have shown that superoxide production is necessary for Rac-dependent G1 progression and other cellular shape changes (10). Together, these and other observations highlight a role for ROS in cell signaling and mitogenesis.

Since variations in low intracellular ROS levels can cause major signaling changes, tight coordination of the expression of NADPH oxidase components may modulate proliferative responses. In a database search with the high-affinity HBPI site (40), we identified the p47phox gene as a potential HBPI gene target. Inspection of the fully sequenced p47phox gene (links within Unigene Hs.949; National Center for Biototechnology Information [NCBI]) revealed that the p47phox promoter contains an array of six adjacent high-affinity HBPI binding elements that are located at positions −1243 to −1318 relative to the transcriptional start site. Because of this striking arrangement, we hypothesized that HBPI could transcriptionally repress the p47phox gene. In this paper, we show that HBPI does repress the p47phox gene, with consequences for cellular superoxide production and for cell cycle progression. Taken together, our work establishes the p47phox gene as a new target of the HBPI transcriptional repressor and defines a transcriptional mechanism for regulating intracellular ROS levels.

MATERIALS AND METHODS

Database searches. The HBPI high-affinity DNA site sequence (TTCATCATTCA [40]) was used to query the Regulatory Sequence Analysis Tools by use of the Genome Scale Pattern Matching program (http://rsat.ulb.ac.be/rsat/). The five flanking sequences of the candidate genes were accessed in the Unigene database of NCBI. All sequences that were upstream of exon 1 and of the transcriptional start site were examined for the presence of the high-affinity HBPI sites. The results of this analysis indicated that one of the best candidate upstream of exon 1 and of the transcriptional start site were examined for the presence of the high-affinity HBPI sites. The results of this analysis indicated that one of the best candidate sequences of the candidate genes were accessed in the Unigene database of NCBI. All sequences that were upstream of exon 1 and of the transcriptional start site were examined for the presence of the high-affinity HBPI sites. The results of this analysis indicated that one of the best candidate sequences of the candidate genes were accessed in the Unigene database of NCBI. All sequences that were upstream of exon 1 and of the transcriptional start site were examined for the presence of the high-affinity HBPI sites. The results of this analysis indicated that one of the best candidate

Cell culture and transfection. 293T cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and glutamine (Gibco-BRL). Transfections in 100-mm dishes were performed by using the calcium phosphate method with standardized 2

Chromatin immunoprecipitation. A chromatin immunoprecipitation (ChIP) protocol was generously provided by Peggy Farnham (33–35) and was modified for HBPI studies. To cross-link protein and DNA, formaldehyde was added at a 1% (vol/vol) concentration to 293T cells in a 100-mm dish. Fixation proceeded for 10 min at room temperature with gentle shaking and was stopped with the addition of glycine to a final concentration of 0.125 M, and then the mixture was incubated at room temperature for 5 min. The cells were then washed twice with cold phosphate-buffered saline (PBS), harvested, and washed once with PBS with PMSE. The cell pellet was lysed with wash buffer (50 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 30 mM Tris [pH 8.1], 5 mM EDTA and protease inhibitors [1 mM PMSF, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml]) and incubated on ice for 10 min. The resulting lysate was sonicated to give chromatin of an average length of 600 to 1,000 bp. The lysate was then microcentrifuged at 14,000 rpm (Micro 12; Fisher Scientific) for 10 min at 4°C. The lysate was then preclaved with 50 µl of protein A beads (IP-400; Repligen) for 15 min. Immunoprecipitations were performed overnight with 1 µg of antibody in a total volume of 500 µl at 4°C. Following washes of the antibody-bound antibody was included. The reaction mixture was incubated for 30 min at room temperature and then run on a 5% polyacrylamide gel at 350 V for 2.5 h at 4°C. Chromatin immunoprecipitation

Electrophoretic mobility assays. Electrophoretic mobility shift assays (EMSA) were performed with nuclear extracts from HEK-293T cells. Nuclear extracts were isolated by lysing harvested cells in low-salt buffer (20% glycerol, 20 mM HEPES [pH 7.6], 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM EDTA, 10 mM NaCl) and then, following a low spin at 700 rpm for 15 min, lysing them in high-salt buffer (20% glycerol, 20 mM HEPES [pH 7.6], 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM EDTA, 500 mM NaCl) and spinning them at high speed for 10 min. The α- and α- phosphatase and α- and α- phosphatase sense strand probes were 5′-AT GTGTGTCCCAGGAAAGCTAGGCAT-3′ for the HBPI high-affinity promoter. The binding reaction consisted of 5% gel shift buffer [100 mM HEPES (pH 7.6), 5 mM MgCl2, 0.5 mM EDTA, 0.1% azide, 200 mM KCl, 50 mM glycerol, 1 µg of poly(dI-dC), 1 µg of salmon sperm DNA], along with 0.5 ng of probe. For supershifts, 2 µg of antibody was included. The reaction mixture was incubated for 30 min at room temperature and then run on a 5% polyacrylamide gel at 350 V for 2.5 h at 4°C. Complexes were visualized by autoradiography.

Chromatin immunoprecipitation. A chromatin immunoprecipitation (ChIP) protocol was generously provided by Peggy Farnham (33–35) and was modified for HBPI studies. To cross-link protein and DNA, formaldehyde was added at a 1% (vol/vol) concentration to 293T cells in a 100-mm dish. Fixation proceeded for 10 min at room temperature with gentle shaking and was stopped with the addition of glycine to a final concentration of 0.125 M, and then the mixture was incubated at room temperature for 5 min. The cells were then washed twice with cold phosphate-buffered saline (PBS). The amount of luciferase enzyme in each lysate was determined by using pRSV-Luciferase Assay system (Promega) according to the manufacturer's protocol. The p47phox expression vector (pREP10-p47phox) was a gift from Tom Leto.

Cell culture and transfection. 293T cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and glutamine (Gibco-BRL). Transfections in 100-mm dishes were performed by using the calcium phosphate method with standardized 2 × 10^6 RT-PCR products of 442 bp. To normalize the RT-PCR results, 18S primers and associated competimers (Ambion) were used at a 1:10 ratio. This protocol provides a linear signal to normalize experimental results. All amperometric measurements were conducted using a glassy carbon electrode (GC) for 1:10 dilution. Anti-human p47phox goat antiserum (a generous gift of Tom Leto) was used at a 1:1,000 concentration. Western blots were visualized by using enhanced chemiluminescence (ECL). Western blotting. Lysates were prepared for reporter assays (see above) or with a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 µg of leupeptin/ml. For the detection of transfected HBPI and its mutants, the HA.11 antibody (the mouse and at 1:1,010 dilution. Anti-human p47phox goat antiserum (a generous gift of Tom Leto) was used at a 1:1,000 concentration. Western blots were visualized by using enhanced chemiluminescence (ECL).
RESULTS

HBPI represses transcription of the p47phox promoter in a DNA binding-dependent manner. The HBPI high-affinity site sequence (TTCATTCATTCA) was defined in a study on chromosomal position-effect variegation (40). However, no target genes with this site have been identified. For our transcriptional studies, the high-affinity HBPI site provided a tool for identifying target promoters in queries of various databases and verifying possible target genes with the updated human genome sequences. The computer analyses indicated that the promoter for the human p47phox gene had a striking array of HBPI high-affinity sites (see Online Mendelian Inheritance in Man genome links from NCBI Unigene Hs.1583). Allowing for occasional wobble, the p47phox promoter contains six adjacent elements from position -1243 to position -1318 (see Fig. 1A).

While there are some reports of regulation in immune cells (e.g., see reference 19), transcriptional regulation of any NADPH oxidase subunits in nonimmune cells, in which it has a signaling role, has not been extensively studied. The striking number of HBPI sites prompted an investigation that used several reporter constructs to determine whether HBPI could repress the p47phox gene (Fig. 1A). To test a native p47phox promoter, the reporters p47(-1392)/luc and p47(-1217)/luc (a generous gift of Robert A. Clark [19]) were used. The (-1217)/luc p47-phox reporter gene does not contain the HBPI high-affinity sites, while the (-1392)/luc p47-phox reporter retains all HBPI sites (Fig. 1A). To directly attribute repression to the HBPI sites, four adjacent high-affinity HBPI sites were inserted upstream of a heterologous promoter (designated 4XJ) to test repression.

Additional evidence suggests that the large HBPI element is repressive. Figure 1B shows that reporters that contain the high-affinity HBPI sites [p47(-1392)/luc and 4XJ] had lower activities than control reporters [p47(-1217)/luc and pGL3, respectively] without HBPI sites. The activity of 4XJ was five-fold lower than that of the control (pGL3), and the activity of p47(-1392)/luc was 2.5-fold lower than that of p47(-1217)/luc in HEK-293T cells. Similar ratios were observed in NIH3T3 and C3A cells. There was no appreciable difference in reporter activity from position -3050 to position -2150 in the indicated cells (not shown), so we focused on the region that was downstream of position -1392.

As shown in Fig. 1C, HBPI is member of the sequence-specific HMG box family of transcription factors and is a transcriptional repressor with a central repression domain (amino acids 191 to 393) and a C-terminal HMG box DNA binding domain (amino acids 401 to 503), as well as retinoblastoma and p38 mitogen-activated protein kinase binding regulatory regions (16, 25, 29, 32, 36; M. A. McDevitt, M. Hu, J. Sun, J. Kim, B. Beraisi, V. Band, J. Blum, L. Varticovski, K. E. Paulson, and A. S. Yue, submitted for publication). Relevant mutants from our previous work are shown in Fig. 1C. To test whether HBPI expression repressed transcription of the p47phox promoter, cells were cotransfected with HA-tagged HBPI and the p47phox or the HBPI-specific 4XJ reporter gene. As shown in Fig. 1D and E, wild-type HBPI expression resulted in efficient repression of the p47(-1392)/luc reporter (Fig. 1D) or of the HBPI-specific 4XJ reporter (Fig. 1E). Wild-type HBPI expression had no effect on the p47(-1217)/luc or control pGL3 reporter, both of which lack the high-affinity HBPI sites. Thus, the data in Fig. 1 indicate that the high-affinity HBPI DNA elements of the p47phox promoter do confer transcriptional repression.

Two HBPI repression mechanisms with opposite requirements for DNA binding were previously defined (25, 32). The distinguishing feature of the two mechanisms is a requirement for an intact HMG box. N-Myc was previously defined as a sequence-specific target of HBPI. The regulation of the N-Myc gene required both the repression and the DNA binding domains of HBPI (32). In contrast, the suppression of cyclin D1 and of Wnt signaling required only the repression domain and not the DNA binding domain (25). Using selected HBPI mutants (Fig. 1C), we delineated the requirement for specific DNA binding and for the repression domain. Both the native p47phox and the 4XJ construct were used to provide complementary data for the role of HBPI. The following sections relate description of the requirements for DNA binding and the defined repression domain in the context of the p47phox promoter and of the high-affinity HBPI sites.

Using specific reagents for each mechanism, we tested the requirement for DNA binding and for the repression domains in the regulation of the p47phox promoter. Nearly identical results were obtained for the native p47phox promoter and the synthetic (4XJ) reporter (Fig. 1D and E). No effects on control reporters that lacked the high-affinity HBPI sites [( -1392)/luc and pGL3] were manifested. To dissect the requirement for DNA binding, the pmHMG mutant was used. Three residues in the HMG box were mutated in the context of the entire protein (designated pmHMG) (32). The ability of the pmHMG mutant to bind to its high-affinity site was inhibited (data not shown) (40). As shown in Fig. 1D and E, the pmHMG mutant failed to repress either the p47(-1392)/luc or the 4XJ reporter, which both possess the high-affinity HBPI
elements. Thus, repression of the p47phox promoter requires specific DNA binding through the HMG box.

Another objective of this study was to define the requirements for transcriptional repression. Using the criteria of conferring repression to a heterologous DNA binding domain, the region from amino acid 220 to amino acid 414 of HBP1 was previously defined as the repression domain (32). When co-transfected with the p47phox and 4XJ reporters, the Δ220 mutant still functioned in the repression of the p47phox promoter and through the high-affinity HBP1 sites (Fig. 1D and E, compare HBP1 with Δ220 and compare p47phox with 4XJ). Subsequently, deletion of amino acids 218 to 314 of the HBP1 protein abolished repression on both reporters with high-affinity HBP1 elements (Fig. 1D and E, compare wild-type HBP1 with Δ218–314). Thus, repression on high-affinity HBP1 sites and on the p47 promoter required specific DNA binding and a region within the repression domain. The representative anti-HA Western blots shown in Fig. 1F demonstrate that each of the mutants used in Fig. 1D and E is well expressed.

A different result was obtained for the Δ393 HBP1 mutant, which contains only the HMG box region. Rather than the inhibition of repression, the expression of Δ393 led to an approximately sixfold increase of p47 (−1392)/luc and 4XJ reporter activity (Fig. 1D and E, compare wild-type HBP1 with Δ393 on p47/1392 and 4XJ, respectively). Expression of the Δ393 mutant had no impact on the reporters that lacked the HBP1 DNA elements (Fig. 1D, p47 (−1217)/luc: Fig. 1E, pGL3), so the observed effects were specific to the HBP1 DNA sites. The ability of Δ393 to bind to the HBP1 high-affinity element was necessary, as inhibition of DNA binding by point mutations (designated pmΔ393) eliminated the increase in reporter activity (Fig. 1D and E, pmΔ393). Thus, the significant increase in the p47phox promoter activity caused by the Δ393 mutant required specific DNA binding and differed from other identified HBP1 target genes (e.g., the N-Myc gene) (32).

The p47phox promoter provided an opportunity to compare HBP1 target genes with low- and high-affinity HBP1 sites. The N-Myc promoter (a promoter with a low-affinity HBP1 site), lacking the first 220 amino acids (Δ220), still functioned in repression, but a further deletion from the HMG box abolished repression (Fig. 1C, Δ393). The results shown in Fig. 1D and E were initially puzzling, as there was no evidence for a true transcriptional activation domain in the HMG box region (13). A plausible possibility was that the Δ393 mutant might be a dominant-interfering mutant that inhibited HBP1-mediated repression on the p47phox promoter. Since overall promoter output is a balance of repression and activation, removing the repression component may provide a net activation through the activating promoter elements and factors. While the same Δ393 mutant simply showed a loss of repression on a promoter with low-affinity HBP1 sites (e.g., N-Myc [32]), the increased activity of a native p47phox and a synthetic high-affinity HBP1 heterologous promoter do suggest that Δ393 has a dominant inhibitory effect. The difference in the numbers, positions, and/or affinities of HBP1 elements in these promoters may account for this difference between promoters with high- and low-affinity HBP1 DNA elements. In the subsequent studies, we tested both wild-type HBP1 and this potential dominant-negative Δ393 mutant with regard to endogenous p47phox transcription in cell cycle progression and in superoxide production. As shown below, the Δ393 mutant has several properties that suggest a dominant inhibitory function in cellular processes that are mediated by the NADPH oxidase.

**HBP1 represses endogenous p47phox expression.** Since HBP1 expression led to reduced p47phox promoter activity, we next determined whether HBP1 could bind and repress the endogenous p47phox gene. The binding of HBP1 to the endogenous p47phox promoter was determined by a ChIP assay. The interaction with a p47phox promoter region that contains the high-affinity HBP1 sites was detected by a specific PCR assay of immunoprecipitated HBP1 DNA complexes (see Materials and Methods). A critical factor for all ChIP assays is the availability of efficient immunoprecipitating antibodies, but a reagent with this property does not exist for endogenous HBP1. Thus, HA-tagged HBP1 was used to investigate interactions with the endogenous p47phox promoter. As shown in Fig. 2A, HBP1 bound to the endogenous p47phox promoter in the region of the high-affinity HBP1 sites (lane 3). As a control, pmHBP1, which is defective in DNA binding, did not bind the endogenous p47phox gene (lane 5). Similarly, the HMG box alone (Δ393) also bound the endogenous p47phox promoter (lane 7), while the DNA binding-defective mutant pmΔ393 did not (lane 9). All transfected protein pairs (wild type and mutant) were expressed at similar levels (Fig. 2, bottom), and control immunoprecipitation (with α-DP1) yielded no signal in any pair. These results show that HBP1 can bind specifically to the endogenous p47phox promoter.

EMSA provides a complementary demonstration that HBP1 binds specifically to its high-affinity site. Zhuma et al. showed that only one species binds to the HBP1 high-affinity site in thymic nuclear extract (40). With 293T nuclear extracts, the first EMSA experiment shown in Fig. 2B demonstrates that transfected wild-type HBP1, but not an HBP1 with a point mutation in the HMG DNA-binding domain (pmHMG), resulted in increased HBP1 binding. Furthermore, the HBP1 band is specific, as determined by competition with a 50-fold excess of unlabeled HBP1 high-affinity site oligonucleotide. The second EMSA experiment shows that an additional species appears when Δ393 is transfected. This band is specific and is lost upon competition with excess unlabeled HBP1 high-affinity site oligonucleotide. In addition, the last three lanes show that the stronger band in the transfected HA-HBP1 lanes can be further shifted when anti-HA is included in the binding reaction. The supershift is not observed when anti-GAL4 is included as a negative control. Together, the EMSA and ChIP experiments indicate that HBP1 does bind to its high-affinity element in the p47 promoter.

Since HBP1 bound the endogenous p47phox promoter, the impact of HBP1 expression on endogenous p47phox mRNA expression was investigated with stable and transient expression. We had previously characterized a stable line (B2) with constitutive and higher (approximately eightfold) HBP1 levels compared to those of the parental C2C12 line (28). The relative p47phox mRNA level between the HBP1-expressing and normal lines was determined by semiquantitative RT-PCR with 18S RNA as an internal control. As shown in Fig. 3A, there was significantly less p47phox RT-PCR product after 20, 30, and 40 cycles for the HBP1-expressing B2 line than for the parental C2C12 line.
Transient HBP1 expression also reduced endogenous p47phox mRNA levels. 293T cells were cotransfected with either HBP1 or Δ393. A membrane-localized GFP (F-GFP; Promega) was used to mark transfected cells for FACs purification. GFP expression had no effect on the p47(−1392)/luc or the p47(−1217)/luc reporter (data not shown) and is an appropriate marker. The levels of p47phox mRNA were determined by semiquantitative RT-PCR in the presence and absence of HBP1 expression from purified transfected cells. As shown in Fig. 3B, after 30, 40, and 50 cycles of RT-PCR, HBP1 expression reduced p47phox gene expression compared to that of the control F-GFP-positive cells. Figure 3C shows the effect of Δ393 expression on p47phox mRNA expression. Consistent with the promoter data, expression of the Δ393 mutant also increased endogenous p47phox mRNA levels.

To determine whether HBP1-mediated changes in endogenous p47phox mRNA also resulted in increased p47 phox protein expression, immunoblots with human p47phox antisera (a gift of Tom Leto, National Institutes of Health) were performed on 293T cell extracts that expressed HBP1 or the Δ393 mutant. As shown in Fig. 3D (top), p47phox protein expression, unlike that of immune cells, was undetectable in 293T cells. It is important to note that experiments that predict reduction in p47phox protein levels with HBP1 expression will be uninformative, but increases in p47phox protein should be detectable. p47phox protein levels were increased with the expression of the Δ393 protein (lanes 4 and 5) but not when DNA binding was abolished (Δ393 pmHMG; Fig. 3D, lanes 1 and 2). The bottom panel shows the expression of the Δ393 and Δ393 pmHMG proteins in the same experiment. This result is consistent with the effects of the Δ393 HMG box mutants on the p47phox promoter (Fig. 1D) and on mRNA expression (Fig. 3C). These data further highlight the requirement of specific DNA binding for any effects on the p47phox promoter and gene. Based on p47phox reporter and endogenous gene analyses, the results presented in Figs. 1 to 3 provide evidence that HBP1 is a repressor of the p47phox gene.

**HBP1 expression alters intracellular ROS.** Since the p47phox protein is a component of the NADPH oxidase complex, we next determined whether intracellular superoxide production was affected by HBP1 expression. Superoxide production in 293T cells was detected by chemiluminescence with lucigenin, which reacts with cellular superoxide to form an unstable dioxetane that emits light upon decay (e.g., see reference 30). With GFP as a negative control and as a marker of transfection efficiency, the effect of HBP1 on superoxide production in 293T cells was measured (Fig. 4A). As predicted from the effects on the p47phox gene, HBP1 expression reduced cellular superoxide production to 40% of the control levels. Conversely, expression of the Δ393 HMG box increased superoxide production to 270% of the control levels. Consistent with the data presented in Fig. 1C, the mutation of the HMG box abolished any effects on superoxide production. As with the regulation of the p47phox promoter and the endogenous gene, the HBP1-mediated regulation of ROS production and of p47phox transcription has the same functional requirement for an intact HMG box DNA binding region.

If HBP1 expression represses superoxide production by reducing endogenous p47phox expression, exogenous p47phox expression might rescue superoxide production. As shown in Fig. 4B, exogenous p47phox can partially rescue HBP1-mediated repression of superoxide production to 70% of the control levels in a dose-dependent manner. Exogenous expression of p47phox alone caused a minor increase in superoxide production. While the data presented in Fig. 4B do suggest that p47phox regulation by HBP1 can contribute to intracellular superoxide regulation, additional factors that are regulated by HBP1 might be required to fully restore superoxide levels. Thus, the HBP1 regulation of the p47phox gene is a contributing factor to intracellular ROS status, although other yet-to-be-identified factors, possibly other NADPH oxidase subunits, may be required (see Discussion).

If HBP1 expression affects redox balance, other intracellular species (e.g., glutathione) should also be affected. Within a cell, superoxide is rapidly converted by superoxide dismutase to H2O2, which is subsequently reduced to H2O by either catalase or glutathione peroxidase. Any change in superoxide production will have profound effects on the normal intracellular redox balance of oxidized and reduced molecules. In particular, intracellular GSH, the most abundant intracellular reductant, has been shown to initially decrease in response to increased ROS levels and then to increase through the induction of both de novo biosynthetic pathways and redox recycling enzymes. Intracellular GSH levels concomitantly decline when intracellular ROS levels decline. Therefore, GSH levels are often used as a measure of redox balance, with decreased GSH being a criterion for reduced ROS levels (2, 5, 11, 15). In the cell lines that overexpress HBP1 (Fig. 3A) (28, 29), the levels of intracellular GSH were reduced relative to control levels (Fig. 4C), suggesting a decrease in intracellular ROS generation.

In addition, we have previously shown that transgenic mice expressing HBP1 have a delay in the G1 phase of the cell cycle after injury (29). Other studies have shown that increased GSH levels also correlate with the S phase in mouse liver regeneration (8). In this experiment, we asked whether the GSH levels in mouse liver were regulated by HBP1 expression. As shown in Fig. 4D, the HBP1 transgenic mice had decreased intracellular GSH levels relative to those of control mice. Because GSH levels are reduced with HBP1 expression, the results presented in Fig. 4D extend the cell-based observations of reduced ROS to a relevant animal model.

**Expression of an HBP1 dominant-interfering mutant activates G1 progression that is dependent on NADPH oxidase activity.** Work by Bar Sagi, Finkel, Lambeth, and others has linked superoxide production to increased growth and cell cycle progression through Rac-mediated mitogenesis (9, 10, 30). It has been shown that HBP1 expression can affect both cell cycle progression and superoxide production (Fig. 4) (32). Thus, we hypothesized that HBP1 and its transcriptional regulation of p47phox might contribute to cell cycle regulation. NIH3T3 fibroblasts were used for these experiments due to their exquisite sensitivity to fluctuating ROS levels (1, 30). As shown in Fig. 5A, the expression of HBP1 (in the presence of serum) reduced the number of cells in the S phase threefold compared with that of control GFP-transfected cells. Previous studies showed that mutation of the HMG box inhibits the decrease in S-phase cells (32). In Fig. 5A, the expression of the Δ393 HBP1 mutant increased the percentage of S-phase cells.
approximately threefold. Mutations of the HMG box DNA binding domains inhibited the \( \Delta 393 \) increase in the S phase and further highlight a dependence on specific DNA binding for HBP1-mediated regulation of the cell cycle.

The experiments presented in Fig. 5A further suggest that increased S phase caused by the dominant-negative \( \Delta 393 \) mutant could be dependent on intracellular ROS generation through the NADPH oxidase. Therefore, we next showed that expression of \( \Delta 393 \) did increase intracellular superoxide that was sensitive to the flavoprotein inhibitor diphenyl iodonium sulfate (DPI), which blocks NADPH oxidase activity (10). We next tested whether the S phase increase caused by \( \Delta 393 \) expression was also dependent on the observed increase in overall NADPH oxidase activity. As shown in Fig. 5C, the addition of DPI also inhibited S-phase induction by \( \Delta 393 \). Thus, data presented in Fig. 5 indicate that the induction of the S phase by \( \Delta 393 \) expression correlates with increases in intracellular ROS from the NADPH oxidase and underscores its dominant-negative phenotype for HBP1-mediated repression of the p47phox gene. All observations presented in Fig. 5 do indicate a requirement for cycling cells and highlight a role for HBP1 and ROS in regulating G1 progression. Taken together, this work defines a transcriptional mechanism for regulating intracellular ROS and highlights the role of the NADPH oxidase in cell cycle progression (see Fig. 6 and Discussion).

**FIG. 4.** HBP1 regulates endogenous superoxide levels. (A) Effects on superoxide production depend upon specific DNA binding. HEK-293T cells were cotransfected with 5 \( \mu \)g of HBP1, \( \Delta 393 \), pmHMG, or pm\( \Delta 393 \) and with F-GFP. After 36 h, the relative superoxide production level was determined by using lucigenin as a substrate. Superoxide levels from F-GFP-transfected samples were set to 100%. A total of \( 2 \times 10^6 \) cells were used for each sample. (B) The addition of exogenous p47phox partially rescues HBP1-mediated superoxide reduction. HEK-293T cells were cotransfected with 2 \( \mu \)g of F-GFP and the indicated plasmids. The p47phox protein was expressed from either 1, 3, or 5 \( \mu \)g of pREP10-p47 phox with or without 5 \( \mu \)g of HBP1. The relative superoxide levels were determined as described for panel A and in Materials and Methods. The level in the control F-GFP expressing cells was set to a value of 1. (C) GSH levels in control C2C12 cells and in HBP1-expressing C2C12 lines (B1 and B2). GSH was measured three times independently as described in Materials and Methods. GSH levels in HBP1 cells lines B1 and B2 were significantly lower than that in the corresponding control C2C12 line, as determined by Student’s t test (\( P < 0.05 \)). (D) GSH levels in the livers of nontransgenic (non-tg) and HBP1 transgenic mouse strains. GSH levels in the livers of 1Z1 and HBP4 transgenic mouse strains was significantly lower than those in the corresponding livers of nontransgenic mice, as determined by Student’s t test (\( P < 0.05 \)).
DISCUSSION

Summary of results. The model in Fig. 6 describes a mechanism by which HBP1 represses the p47phox promoter and NADPH oxidase-mediated superoxide production. In the model in Fig. 6, the superoxide species are signaling molecules that regulate signaling pathways by growth factors and cytokines. By repressing the p47phox subunit and possibly other regulatory factors, HBP1 expression reduces cell cycle progression and leads to an antiproliferative state that may attenuate any growth factor and cytokine response through receptor tyrosine kinases. This model is consistent with the observed roles of HBP1 and ROS in cell cycle progression. This NADPH-oxidase mechanism now adds to the role of HBP1 in maintaining proliferation barriers in differentiation (28, 29). It should be noted that these superoxide and ROS functions contrast with other mechanisms whereby higher ROS levels are involved in cellular damage.

The model in Fig. 6 is fully supported by the data in Fig. 1 to 5 and defines a transcriptional mechanism by which HBP1 regulates intracellular superoxide generation by the NADPH oxidase enzyme. On the basis of ChIPs, endogenous gene analysis, and experiments with HBP1 mutants and reporter constructs, we conclude that HBP1 is a repressor of the p47phox promoter in a DNA binding-dependent manner. A striking clue was the array of high-affinity HBP1 sites in the promoter for the p47phox gene. Consistent with the results of p47phox gene repression, HBP1 expression reduced intracellular ROS levels. All effects of a dominant-negative HBP1 mutant on cell cycle progression were sensitive to DPI, which has been used extensively as an NADPH oxidase inhibitor. While reexpression of the p47phox gene partially rescued the reduction in ROS levels achieved with HBP1 expression, an interesting possibility is that HBP1 may regulate other subunits of the NADPH oxidase. A Unigene database search of all NADPH oxidase subunit genes reveals that a high-affinity HBP1 site exists in the p40phox gene and prompts a future study of the possible role of HBP1 in p40phox regulation. Together, these studies provide new insight into HBP1 as a transcriptional repressor and a G1 regulator by demonstrating a solid link to...
intracellular ROS status (Fig. 6). As described below, recent work has underscored the role of ROS in regulating mitogenic and other signaling pathways.

Several HBP1 target genes have been described, yet a dominant inhibitory effect has not been reported or observed in previous promoter studies (14, 21, 25, 32) with relatively low affinity HBP1 sites. Also, while there have been reports that HBP1 may be a transcriptional activator, we have consistently observed repression or inhibition only on the promoters that are defined by our work. In three separate functional tests in the context of the p47phox gene and ROS levels, the Δ393 mutant functioned as a dominant-inhibitory protein. A possibility is that the dominant-negative Δ393 mutant competes with endogenous HBP1 for the high-affinity site on the p47phox promoter. With a unique configuration of six adjacent high-affinity sites in the p47phox promoter, HBP1 may bind cooperatively to efficiently repress the p47phox promoter.

In this study, results with both wild-type HBP1 and a dominant-inhibitory HBP1 mutant strongly suggest that HBP1 may coordinately regulate the cell cycle and ROS levels through transcriptional repression of the p47phox subunit of the NADPH oxidase. Δ393 expression increased the number of cells in the S phase in the presence of serum. This effect was not observed when Δ393 was expressed in NIH3T3 cells under serum-starved conditions (data not shown), which represents a G0 state, and this result further highlights the fact that G0 is not the principle regulatory phase in which HBP1 acts. In addition, we have shown that during the liver regeneration cell cycle, HBP1 induced a delay in the G1 phase, while no change occurred at the G0-to-G1 transition. Furthermore, HBP1 protein levels declined at the onset of the S phase and further highlighted the fact that HBP1 exerts regulation in the G1 phase (29). Together, these data from cells and animal models argue that the primary targets of Δ393 and HBP1 are also in the G1 phase and not in the G0 phase.

Implications of ROS regulation for growth control. While the role of the NADPH oxidase in immune function is well defined (6), NADPH oxidases are present in many cell types and are linked to growth factor signaling. The mechanisms that regulate the expression of the subunits of the NADPH oxidase have not been reported in nonimmune cells, in which ROS generation has an important intracellular signaling function. Superoxide generation occurs during cytokine and growth factor signaling (reviewed in reference 7). The transient oxidative burst is necessary for mitogenic and other signaling networks. Upon stimulus, the inactive NADPH oxidase regulatory subunits are phosphorylated and associate with active Rac and the NADPH oxidase catalytic subunit complex at the cell membrane. The transient oxidative burst is readily attenuated due to the diffusible nature of ROS and provides for a defined response to growth factors. This transient ROS response from the NADPH oxidase at the membrane differs significantly from the sustained mitochondrial ROS production that is linked to cellular damage (3).

Work by Bar-Sagi and others has linked Rac and ROS production to G1 progression and provided an excellent framework for designing the studies in this paper (10). Recent studies have further illuminated how transient ROS production might regulate cell signaling through the modification of tyrosine phosphatases. Tonks and coworkers have shown that several tyrosine phosphatases are reversibly oxidized and inactivated in the presence of H2O2 (22). Specifically, a key cysteine residue in the active site of the SHP-2 tyrosine phosphatase is oxidized to sulfenic acid with a concomitant blockade of

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![Diagram](http://mcb.asm.org/)

**FIG. 6.** Model of HBP1 repression and ROS regulation. The results of this paper are summarized in this schematic diagram. The repression of the endogenous p47phox gene results in decreased superoxide levels and the inhibition of proliferation. Some aspects of Ras and small G protein signaling utilize the NADPH oxidase and ROS levels as part of the signaling mechanisms. A prediction is that cells with high HBP1 expression may be refractory to growth signals that emanate from tyrosine kinase receptors and the Ras/Rac/Rho signaling networks.
activity. Since SHP-2 can dephosphorylate receptor tyrosine kinases to attenuate signaling, the inhibition of SHP-2 by ROS would lead to increased tyrosine phosphorylation of receptor tyrosine kinases and an increased mitogenic response. In other work with Rac, Bar Sagi and colleagues have reported that Rac-mediated ROS generation regulates specific tyrosine phosphatases that control the activity of the small G protein Rho in signaling cascades for actin rearrangements. All of these effects were sensitive to DPI and linked the NADPH oxidase to cellular changes through both Rac and Rho (23).

These studies show that ROS levels can dictate the efficiency of mitogenic and other signaling pathways at multiple steps. Thus, the regulation of intracellular ROS through the NADPH oxidase is an essential factor in proliferative control. By keeping NADPH oxidase activity at a low level via transcriptional repression in quiescent cells, the activity of SHP-2 and other tyrosine phosphatases will not be inhibited by the ROS-mediated oxidation of the critical cysteine in the active site. Thus, constitutive tyrosine phosphatase activity should dampen the mitogenic output of tyrosine kinase receptors. The net effects are consistent with the observed antiproliferative effects of HBPI expression. In contrast, a loss of transcriptional repression of NADPH oxidase components could lead to improper potentiation of the oxidative burst, phosphatase inactivation, and increased mitogenic signaling.

By regulating the expression of p47phox and possibly other subunits, HBPI expression may modulate the cellular response to proliferation signals. The ability of $\Delta \Delta 393$ to cause an increased number of cells in the S phase is similar to proliferative effects that have been observed with the addition of exogenous ROS or with Nox1 (1, 30) and highlights the impact of deregulated ROS levels and NADPH oxidase activity. Since its expression is ubiquitous, the action of HBPI may be part of general mechanisms that restrain expression of the p47phox gene and of NADPH oxidase activity in nonimmune cells in the absence of mitogenic signals. In contrast, the inhibition of HBPI function may deregulate ROS levels and alter cell proliferation.

A longstanding observation is that tumor cells produce high levels of ROS (reviewed in reference 26). In addition, the expression of the NADPH oxidase catalytic subunit Nox1 can cause cellular transformation (30), while HBPI expression suppresses cell growth and inhibits G1 progression (28, 29, 32). In contrast, a loss of transcriptional repression in quiescent cells, the activity of SHP-2 and other tyrosine phosphatases will not be inhibited by the ROS-mediated oxidation of the critical cysteine in the active site. Thus, constitutive tyrosine phosphatase activity should dampen the mitogenic output of tyrosine kinase receptors. The net effects are consistent with the observed antiproliferative effects of HBPI expression. In contrast, a loss of transcriptional repression of NADPH oxidase components could lead to improper potentiation of the oxidative burst, phosphatase inactivation, and increased mitogenic signaling.

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ACKNOWLEDGMENTS

We thank Robert A. Clark for the p47phox promoter constructs and Tom Leto for the p47phox antisera. We also thank Peggy Farnham for generous access to her detailed ChIP protocol.

The support of the GRASP Digestive Disease Center at New England Medical Center (grant P30 DK34928) and its Molecular Biology/Genomics Core is gratefully acknowledged. This work was supported by grants to A.S.Y. from the U.S. Army (grant BC990538) and from the NIH (grants GM44634 and CA94187) and to K.E.P. from the NIH (grant ES11518).

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