Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene

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Heme oxygenase-1 (HO-1) protects cells from various insults including oxidative stress. Transcriptional activators, including the Nrf2/Maf heterodimer, have been the focus of studies on the inducible expression of *ho-1*. Here we show that a heme-binding factor, Bach1, is a critical physiological repressor of *ho-1*. Bach1 bound to the multiple Maf recognition elements (MAREs) of *ho-1* enhancers with MafK *in vitro* and repressed their activity *in vivo*, while heme abrogated this repressor function of Bach1 by inhibiting its binding to the *ho-1* enhancers. Gene targeting experiments in mice revealed that, in the absence of Bach1, *ho-1* became expressed constitutively at high levels in various tissues under normal physiological conditions. By analyzing *bach1/nrf2* compound-deficient mice, we documented antagonistic activities of Bach1 and Nrf2 in several tissues. Chromatin immunoprecipitation revealed that small Maf proteins participate in both repression and activation of *ho-1*. Thus, regulation of *ho-1* involves a direct sensing of heme levels by Bach1 (by analogy to *lac* repressor sensitivity to lactose), generating a simple feedback loop whereby the substrate effects repressor–activator antagonism.
Keywords: BTB domain/heme/Maf/transcription repression

Introduction

Coupling of metabolic activity and gene expression is fundamental to maintain homeostasis. Heme is an essential molecule that plays a central role as the prosthetic group of many heme proteins in reactions involving molecular oxygen, electron transfer and diatomic gases. In addition, heme is known to participate in gene regulation as a ligand for transcription factors in prokaryotes (Monson *et al.*, 1992; Qi *et al.*, 1999) and yeast (Zhang and Guarente, 1995; Zhang *et al.*, 1998). Heme has been proposed to fulfill similar regulatory roles in higher eukaryotes as well (Sassa and Nagai, 1996). Consistent with this hypothesis, we recently found that mammalian transcriptional repressor Bach1 (Oyake *et al.*, 1996) is a heme protein the DNA binding activity of which is negatively regulated by heme binding *in vitro* (Ogawa *et al.*, 2001). The physiological significance of this heme–Bach1 interaction is addressed here within the context of heme metabolism and repressor function in conditional gene induction.

Although heme is integral to life, it is toxic because of its ability to catalyze the formation of reactive oxygen species, which can cause oxidative damage to cellular macromolecules. In higher eukaryotes, the toxic activity of heme is counteracted by the inducible heme oxygenase (HO)-1 system (Maines, 1997). As in the classic view of metabolic control, expression of *ho-1* is induced by the substrate heme (Tenhunen *et al.*, 1970; Shibahara *et al.*, 1978; Alam *et al.*, 1989). In addition, expression of HO-1 is robustly induced in mammalian cells by various pro-inflammatory stimulants such as cytokines, heavy metals, heat shock and oxidants that induce inflammatory damage (Shibahara *et al.*, 1985, 1987; Alam *et al.*, 1989; Keyse and Tyrrell, 1989; Taketani *et al.*, 1989). Heme oxygenases are the rate-limiting enzymes in heme degradation, generating ferrous iron, carbon monoxide and biliverdin, which is rapidly reduced to bilirubin. Carbon monoxide and biliverdin, as well as bilirubin, have antioxidant and anti-inflammatory activities *in vivo*. Thus, HO-1 is an antioxidant defense enzyme that converts toxic heme into antioxidants and is essential for higher eukaryotes in order to cope with various aspects of cellular stress and to regulate cellular iron metabolism (Poss and Tonegawa, 1997). In clinical conditions, HO-1 expression has been associated with increased resistance to tissue injury (Hancock *et al.*, 1998; Soares *et al.*, 1998; Yachie *et al.*, 1999; Odaka *et al.*, 2000; Ducker *et al.*, 2001), thus leading to a gene therapy approach employing HO-1 (Otterbein *et al.*, 1999).

The molecular mechanism that confers inducible expression of *ho-1* in response to numerous and diverse conditions has remained elusive. One important clue came recently from a detailed analysis of the transcriptional regulatory mechanisms controlling the mouse and human *ho-1* genes. The induction of *ho-1* is regulated principally by two upstream enhancers, E1 and E2 (Alam *et al.*, 1989, 1995, 2000a; Alam, 1994). Both enhancer regions contain multiple stress responsive elements (StRE; Inamdar *et al.*, 1996) that also conform to the sequence of the Maf

recognition element (MARE; Kataoka *et al.*, 1994). Previous studies have implicated heterodimers of NF-E2-related factor 2 (Nrf2) and one or another of the small Maf proteins (i.e. MafK, MafF and MafG) in induction of *ho-1* through these MAREs (Itoh *et al.*, 1997; Alam *et al.*, 2000a,b; Ishii *et al.*, 2000; Kataoka *et al.*, 2001).

The above model, centered on Nrf2 activity, suggests that the *ho-1* locus is situated in a chromatin environment that is permissive for activation. Since the MARE can be bound by various heterodimeric basic leucine zipper (bZip) factors including NF-E2, as well as several other NF-E2-related factors (Nrf1, Nrf2 and Nrf3), Bach, Maf and AP-1 families (Andrews *et al.*, 1993b; Igarashi *et al.*, 1994; Kataoka *et al.*, 1994, 1995; Moi *et al.*, 1994; Oyake *et al.*, 1996; Toki *et al.*, 1997; Kobayashi *et al.*, 1999), random interactions of activators with the *ho-1* enhancers would be expected to cause spurious expression. This raises a paradox as to how cells reduce transcriptional noise from the *ho-1* locus in the absence of metabolic or environmental stimulation. This problem could be reconciled by the activity of repressors that prevent non-specific activation.

DNA binding activity of Bach1 is negatively regulated by heme binding *in vitro* (Ogawa *et al.*, 2001). The Bach1–heme interaction is mediated by evolutionarily conserved heme regulatory motifs (HRM), including the cysteine–proline (CP) dipeptide sequence in Bach1. While Bach1 forms heterodimers with the small Maf proteins (as does Nrf2), the resulting Bach1 heterodimers repress MARE-dependent transcription (Oyake *et al.*, 1996; Igarashi *et al.*, 1998). To explore the possibility that Bach1 normally negatively regulates *ho-1* gene expression, we attempted to analyze the interaction of Bach1 with the *ho-1* enhancers *in vitro*. As a genetic test, we examined expression of *ho-1* in mice with targeted mutations in the *bach1* and *nrf2* loci. We show that loss of *bach1* function is sufficient to uncouple *ho-1* from stress-responsive control, increasing the availability and activity of its enhancers. We propose a mechanism for the *ho-1* control in which Bach1 directly senses and responds to the levels of heme, the substrate of HO-1, thereby generating a simple feedback loop by the substrate, as originally shown in the *lac* operon.

Results

Heme-regulated repression of ho-1 enhancers by Bach1

To explore the possibility that Bach1 regulates *ho-1* enhancers, we investigated its effect on HO-1 reporter plasmids in transfection assays (Figure 1). The reporter plasmid pHO15luc contains the upstream 15 kb DNA of *ho-1* (Figure 1A) and was previously shown to recapitulate inducible expression of *ho-1* in response to heme and cadmium (Alam, 1994; Alam *et al.*, 1995, 2000a). When a Bach1 expression plasmid was co-transfected with pHO15luc in NIH 3T3 cells, Bach1 repressed its expression (Figure 1B). To verify that Bach1 regulated the reporter gene through the E1 and E2 enhancers, reporter activities of the plasmids lacking either or both of these enhancers were determined (Figure 1A and B). While deletions of E1 or E2 did not affect basal level expression, both deletions resulted in less sensitivity to Bach1-
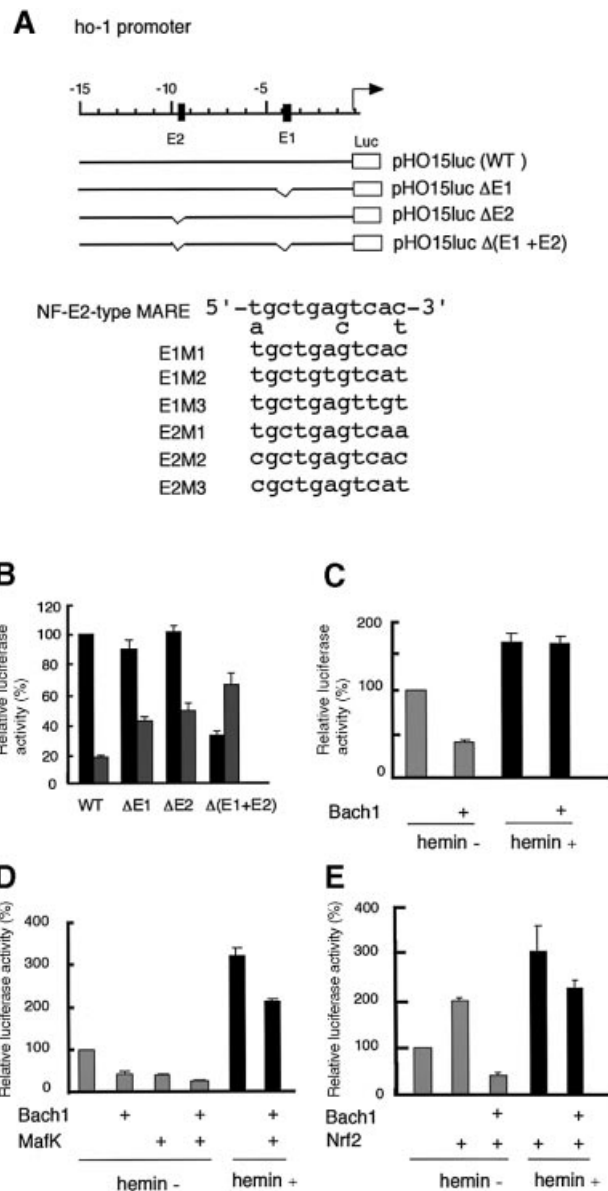

Fig. 1. Repression of HO-1 enhancers by Bach1 is alleviated by heme. (**A**) Reporter plasmids carrying upstream 15 kb DNA with or without deletions of E1 and/or E2. Comparison of NF-E2 binding site (Andrews *et al.*, 1993a) and MARE-like elements within the E1 (E1M1-3) and E2 enhancers (E2M1-3) is shown below. (**B**) NIH 3T3 cells were transfected with the wild-type reporter or enhancer-less reporters (0.5 µg) together with (shaded) or without (filled) a Bach1-expression plasmid (0.1 µg). (**C**) 293 cells were transfected with the wild-type reporter with or without Bach1-expression plasmid. Where indicated, 10 µM hemin was added to the medium. (**D** and **E**) 293 cells were transfected with the wild-type reporter along with 0.1 µg each of expression plasmids for Bach1, MafK (**D**) and Nrf2 (**E**), as indicated, in the absence or presence of added hemin.

mediated repression. Deletion of both E1 and E2 significantly reduced the basal activity and abolished Bach1-mediated repression. Thus, Bach1 represses expression of the *ho-1* reporter plasmid through its binding to E1 and E2.

Because both E1 and E2 enhancers are involved in the inducible expression of *ho-1* (Alam *et al.*, 2000b; Gong *et al.*, 2001), Bach1 may function as a repressor of *ho-1*. As reported previously (Alam *et al.*, 2000b), the activity of

pHO15luc was induced by adding hemin to the culture medium after transfection into human kidney-derived 293 cells (Figure 1C), probably by endogenous Nrf2 activation. When hemin was added, overexpressed Bach1 failed to repress the reporter expression. This inhibitory effect of hemin is consistent with our previous observations using a synthetic model promoter carrying MAREs from a β-globin enhancer (Ogawa *et al.*, 2001). Since Bach1 binds to DNA as a heterodimer with a small Maf protein, overexpressed Bach1 may repress the *ho-1* enhancers with an endogenous small Maf protein. To test this hypothesis, we carried out similar transfection assays in the presence of a MafK expression plasmid (Figure 1D). Both Bach1 and MafK repressed the *ho-1* reporter when individually co-transfected. When both were expressed, they showed a co-operative repression. Repression by Bach1/MafK was significantly alleviated when hemin was added to the culture medium. Thus, these results suggest that the Bach1/MafK heterodimer is subject to regulation by heme *in vivo*.

We next examined the regulatory relationship between Nrf2 and Bach1 by co-transfection assays (Figure 1E). Nrf2 activated the *ho-1* reporter in the absence of exogenous Bach1. When co-expressed, however, Bach1 antagonized Nrf2 activation and repressed the reporter activity. Interestingly, the dominant-repressive effect of Bach1 over Nrf2 was lost upon hemin addition. These data show that Bach1 represses the *ho-1* gene enhancers in concert with a small Maf protein, and that Bach1 antagonizes the activity of activator Nrf2. By increasing the heme levels, the repressor activity of Bach1 is lost, shifting the balance of this antagonism toward gene activation.

Binding of Bach1 to the ho-1 enhancers

Because both E1 and E2 contain multiple MAREs (Figure 1A), Bach1 may repress the enhancers by binding to these elements. To identify the DNA sequences bound by Bach1 and MafK, we carried out a footprint analysis using a 562 bp DNA fragment from the E2 enhancer that contained three MAREs (E2M1, E2M2 and E2M3; Figure 1A). As shown in Figure 2A, MafK protected E2M3 (compare lanes 1 and 2 or 7), whereas Bach1 did not protect any region (data not shown). When both Bach1 and MafK were present, E2M1 and E2M2 were also protected in addition to E2M3 (Figure 2A, lanes 3–6). One of the features of the HO-1 enhancers is the clustering of multiple MAREs, which is very rare among known MARE-containing enhancers. Since Bach1 and MafK bind to DNA by generating a multivalent DNA-binding complex through Bach1 BTB (bric a brac, tramtrack, broad complex) domain-mediated protein–protein interactions (Igarashi *et al.*, 1998; Yoshida *et al.*, 1999), we further investigated the effects of Bach1/MafK interactions on the entire E2 enhancer DNA fragment. To this end, we used the same 562 bp DNA fragment from the E2 enhancer as a probe in electrophoretic gel mobility-shift assays (EMSAs) on agarose gels (Figure 2B). Bach1 failed to bind to the probe (Figure 2B, lane 10). MafK alone generated a marginally lower mobility band (lane 8, MK/MK). In the presence of both Bach1 and MafK, a discrete low mobility complex was observed (lanes 5–7, B1/MK). As shown previously, formation of such a complex was dependent on the presence of the BTB domain of Bach1: in the presence of both MafK and Bach1ΔBTB, lacking the BTB domain, we observed only much faster migrating complexes (Figure 2B, lanes 2–4, B1ΔBTB/MK). The diffuse bands generated by Bach1ΔBTB/MafK may reflect unstable binding to the multiple MAREs. Consistent with this interpretation, E2M2 and E2M1 were protected in the footprint assays more efficiently with the wild-type Bach1 than with Bach1ΔBTB (Figure 2A, compare lanes 3–6 with 8–11).

To further verify the binding of Bach1 and MafK to individual MAREs in E2 and E1, we carried out a conventional EMSA using synthetic oligonucleotide DNA probes. As shown in Figure 2C, recombinant Bach1ΔBTB or MafK failed to bind to these sites efficiently on their own. However, when both were present, we observed strong binding to five of the MAREs in E1 and E2, as well as to the chicken β-globin enhancer MARE. One of the putative MAREs in E1 (E1M3) was not bound by MafK or Bach1/MafK. These results suggest that the clustered, multiple MAREs within the *ho-1* enhancers may provide preferentially selective targets for the multivalent DNA-binding complex of Bach1/small Mafs among many more enhancers within the nucleus that have single, isolated MARE sequences.

Heme alleviates binding of Bach1 to ho-1 enhancers

We showed previously that the binding of Bach1 to an oligonucleotide probe was inhibited by heme (Ogawa *et al.*, 2001). The binding of Bach1/MafK to the clustered MAREs within the E2 enhancer was also significantly inhibited *in vitro* in the presence of heme (Figure 2D, lanes 2–5). A Bach1 derivative (Bach1mCP1-6) carrying multiple mutations in the heme-binding cysteine–proline (CP) motifs does not bind to heme (Ogawa *et al.*, 2001). The binding of Bach1mCP1-6 to E2 was found to be insensitive to heme (Figure 2D, lanes 6–9). We therefore compared Bach1 and Bach1mCP1-6 in transfection assays in the presence or absence of added hemin in the medium. As shown in Figure 2E, both the wild-type and mutant forms of Bach1 repressed the HO-1 reporter in the absence of hemin (columns 1 and 2). While repression of HO-1 reporter activity by Bach1 was suppressed by hemin as described above, repression by Bach1mCP1-6 was not affected (Figure 2E, columns 3 and 4). These proteins were expressed at similar levels in the transfected cells, as determined by immunoblot assays of cell extracts (Figure 2F). Taken together, these results suggest that increased levels of heme inactivate the repressor Bach1 by suppressing its DNA-binding activity.

Generation of bach1-deficient mice

To investigate the role of Bach1 in the *ho-1* gene regulation *in vivo*, the *bach1* gene was disrupted in mice by homologous recombination in embryonic stem (ES) cells. The targeting vector (Figure 3A) was designed to cause a deletion of the entire second exon encoding the initiation methionine and the BTB/POZ domain (Sun *et al.*, 2001) that is essential for the proper biochemical function of Bach1 (Igarashi *et al.*, 1998; Yoshida *et al.*, 1999). Two independent heterozygous ES clones transmitted the mutation to the germ line. Bleeding heterozygous
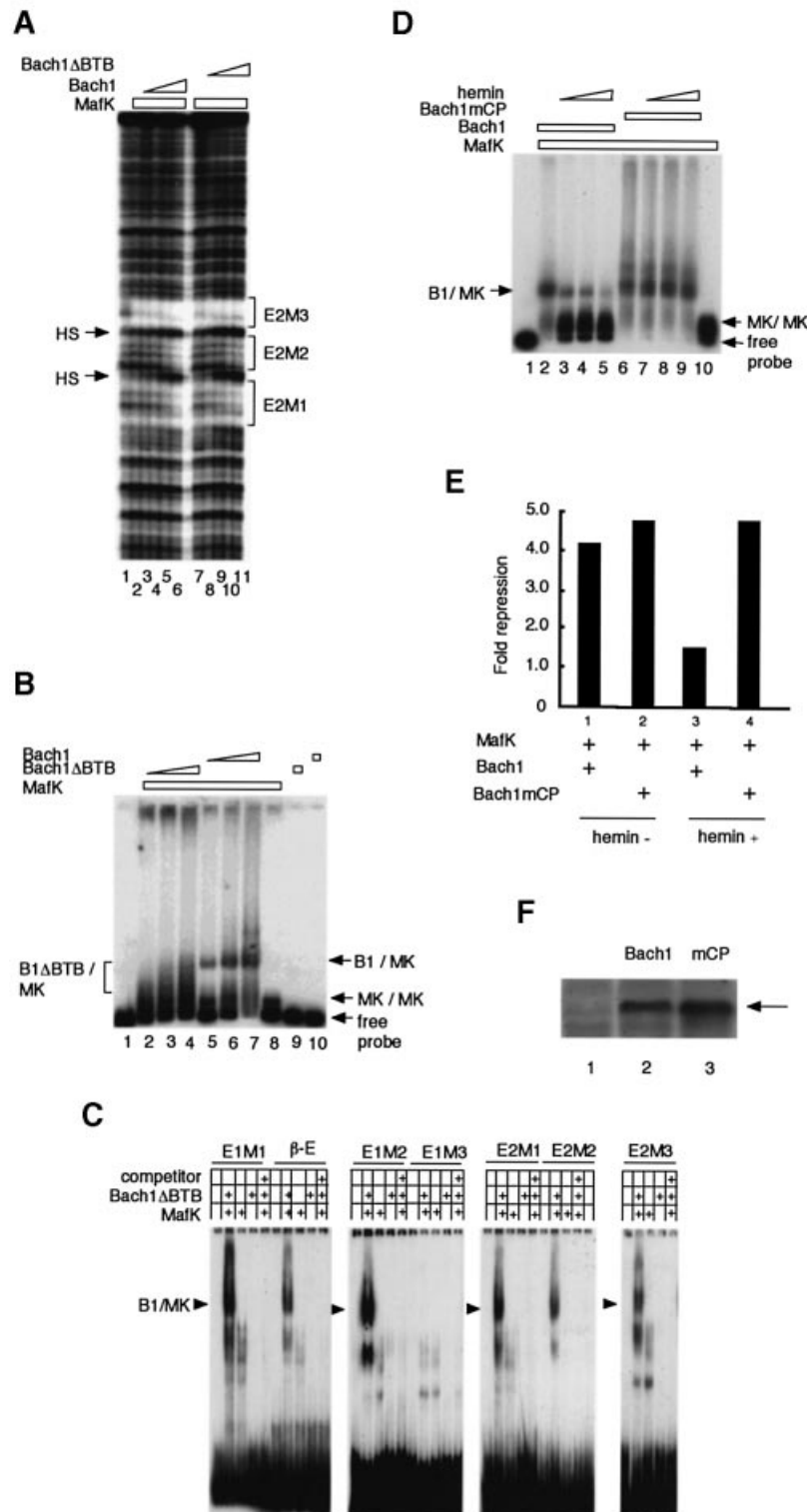
Fig. 2. Heme-regulated binding of Bach1 to the HO-1 enhancers. (**A**) Footprinting assays were carried out using 562 bp E2 DNA in the presence of 50 ng of MafK (lanes 2–11) and increasing amounts (40, 80, 120, 240 ng) of Bach1 (lanes 3–6) or Bach1ΔBTB (lanes 8–11). Protected regions were identified by sequencing reactions and are shown at the right (E2M1-3). HS indicates hypersensitive sites. (**B**) EMSA was carried out using the 562 bp E2 DNA in the presence of 50 ng of MafK (lanes 2–8) and increasing amounts (40, 80, 160 ng) of Bach1 (lanes 5–7) or Bach1ΔBTB (lanes 2–4). Bach1 and Bach1ΔBTB (160 ng) were examined in the absence of MafK (lanes 9 and 10). Complexes were separated on 1% agarose gels. (**C**) Binding of MafK (50 ng) and Bach1ΔBTB (40 ng) to individual MARE-like sites was examined using indicated oligonucleotide probes. Complexes were separated on 4.5% polyacrylamide gels. Heterodimeric complexes are indicated with arrowheads. (**D**) Agarose gel EMSA was carried out as in (B) using recombinant MafK and Bach1 (lanes 2–5), Bach1mCP1-6 (lanes 6–9) or MafK alone (lane 10). Hemin was added to the reactions at 0, 0.25, 0.75 or 1.5 μM. (**E**) The wild-type HO-1 reporter plasmid was transfected into 293 cells with MafK-, Bach1- or Bach1mCP1-6-expression plasmids in indicated combinations in the presence or absence of 10 μM hemin. Fold-repression was calculated as: (reporter activity without effectors)/(reporter activity with effectors). (**F**) Accumulation of Bach1 and Bach1mCP1-6 within transfected cells was compared by immunoblotting analysis.
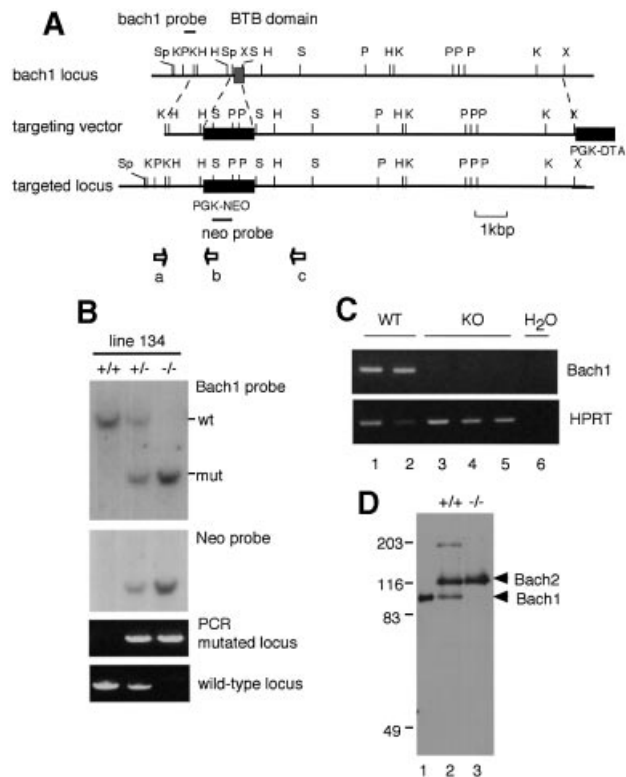
Fig. 3. Genetic ablation of *bach1* in mice. (**A**) The *bach1* genomic structure sorrounding the BTB domain-coding exon that includes the initiation methionine is indicated in the top line. The targeting vector is indicated in the second line. The targeted allele is indicated in the third line. DNA fragments used as probes for Southern blotting analysis are shown above and below the lines. Primers for PCR screening are indicated with arrows a–c. (**B**) Southern blot hybridization of the 5′ and neo probes to *Pst*I-digested genomic DNA prepared from wild-type, heterozygous and homozygous mutant mice. The 5′ probe hybridized with a 6 kbp (wild-type allele) or a 2.6 kbp (targeted allele) *Pst*I DNA fragment. The neo probe hybridized with the same 2.6 kbp *Pst*I DNA fragment on the targeted allele. Detection of wild-type and mutated alleles by PCR are shown below the Southern blots. (**C**) Expression of Bach1 mRNA in thymus was examined by RT–PCR using two (*bach1*^{+/+}) or three (*bach1*^{–/–}) mice. (**D**) Protein extracts from thymus of *bach1*^{+/+} or *bach1*^{–/–} were analyzed for expression of Bach1 using antiserum raised against Bach2 that is weakly reactive with Bach1. Extracts of Qt-6 cells transfected with a Bach1 expression plasmid was loaded in lane 1.

bach1^{+/–} mice produced viable and fertile homozygous null *bach1*^{–/–} progeny. The genotypes were determined by Southern blotting analysis using the 5′ probe and the Neo probe, as well as by a PCR analysis (Figure 3B). The *bach1*^{–/–} mice were born in the expected Mendelian ratio. The *bach1*^{–/–} mice were fertile and appeared grossly normal in size and morphology. To ensure that the targeted mutation abolished Bach1 mRNA expression, we carried out RT–PCR. RNA was isolated from bone marrow and thymus, where Bach1 is highly expressed (Igarashi *et al.*, 1998), and was analyzed using a primer set that amplified the bZip-coding region. We could not detect any accumulation of Bach1 in these RNA samples (Figure 3C; data not shown). Furthermore, we verified a loss of the Bach1 antigen upon immunoblotting of thymus extracts using an antiserum that reacts with Bach2 and weakly with Bach1 (Figure 3D). These results indicate that Bach1 is dispens-
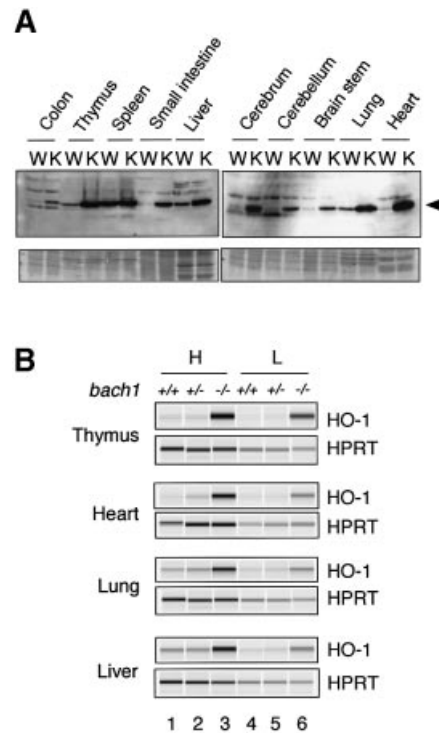
Fig. 4. Expression of HO-1 in *bach1*-deficient mice. (**A**) Protein extracts of indicated tissues of *bach1*^{+/+} or *bach1*^{–/–} (shown by W and K, respectively) mice were analyzed for expression of HO-1 by immunoblot assays (upper panels). HO-1 is indicated by an arrowhead. Membranes were stained for protein with amido black to verify equal loading (lower panels). (**B**) HO-1 and HPRT mRNA levels in thymus, heart, lung and liver from various genotypes (indicated above the panels) were compared by RT–PCR. To ensure linearity of amplification, 3-fold dilutions of cDNA (lanes 1–3 and 4–6, and indicated with H and L at the top) were used as templates. Images were recorded using the Agilent BioAnalyzer 2100.

able for development and reproduction in mice. Thus, these *bach1*^{–/–} mice provide an ideal genetic tool to test the possible involvement of Bach1 in *ho-1* gene regulation.

Expression of ho-1 in the absence of Bach1

To investigate expression of HO-1 in *bach1*^{–/–} mice, we prepared protein extracts from various organs and determined the HO-1 protein levels by immunoblotting. As shown in Figure 4A, HO-1 protein was expressed at much higher levels in the *bach1*^{–/–} mice than the wild-type mice in various organs including thymus, heart and lung. One exception was in the spleen, which is involved in degradation of hemoglobin-derived heme and expresses HO-1 at high levels under normal physiological conditions. HO-1 was expressed at similarly high levels in the spleen from wild-type and *bach1*-deficient mice. These results raise the possibility that *ho-1* is indeed disregulated in the absence of Bach1.

We next determined HO-1 mRNA levels by RT–PCR. To quantify PCR products, we used a microchip electrophoresis and imaging system. HO-1 mRNA was expressed at much higher levels in thymus, heart, lung and liver in the *bach1*^{–/–} mice as compared with organs from the wildtype or heterozygous mutant mice (Figure 4B). The differential levels varied among the tissues. There were >10-fold differences in the thymus and heart between the
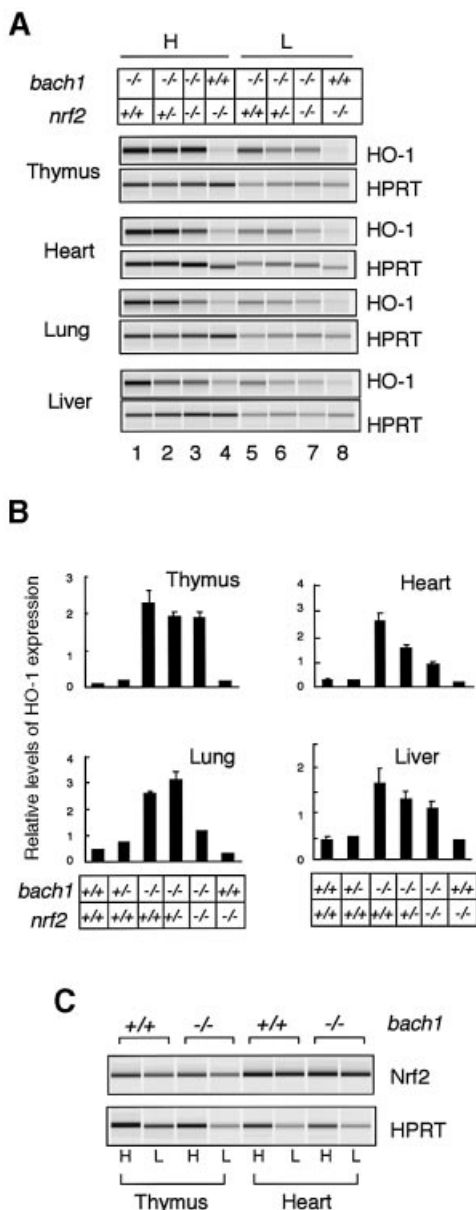
Fig. 5. Nrf2-dependent and -independent expression of *ho-1*. (**A**) HO-1 and HPRT mRNA levels were compared as described in Figure 4 using RNA from various tissues with the indicated genotypes. H and L at the top indicate reactions with 3-fold different amounts of templates. Images were recorded using the Agilent BioAnalyzer 2100. (**B**) Relative levels of HO-1 mRNA were determined and corrected for HPRT mRNA levels by PCR and BioAnalyzer 2100 analysis. Results are the mean of at least two mice for each genotype, with the SEM indicated. (**C**) Expression of Nrf2 mRNA in thymus and heart was compared between wild-type and *bach1*-deficient mice by PCR. Similar results were obtained using two mice for each genotype.

mutant and wild-type animals, whereas there were smaller but significant differences in the liver and lung (see Figure 5B). Similar results were obtained in a real-time PCR analysis (data not shown). These results indicate that Bach1 is a repressor of *ho-1* gene expression in a variety of organs. Thus, these data suggest that low-level HO-1 expression in the wild-type mice under normal physiological conditions is due to repression by Bach1 rather than a lack of activation.

Nrf2 readily activates ho-1 in the absence of Bach1

Our evolving model would currently predict that some cellular activators become constitutively active in the absence of Bach1. Since Nrf2 activates *ho-1* (Itoh *et al.*, 1997; Alam *et al.*, 2000a,b; Ishii *et al.*, 2000; Kataoka *et al.*, 2001), increased HO-1 expression in the *bach1$^{-/-}$* mice may be due to the activation by Nrf2. This possibility was investigated by constructing compound mutant mice carrying various combinations of *bach1* and *nrf2* mutations. Expression levels of HO-1 mRNA were compared by RT–PCR (Figure 5A and B). Unexpectedly, the high level of HO-1 expression in the absence of Bach1 in thymus was independent of Nrf2, since the loss of Nrf2 function did not affect the HO-1 expression levels. In contrast, expression in the heart, lung and liver was clearly dependent on Nrf2. As the *nrf2* gene dosage decreased, the levels of HO-1 mRNA in these organs decreased concomitantly. However, in the absence of Bach1, the levels did not return to the basal level (i.e. in the wild-type mice; Figure 5B). These results suggest that the activator activity of Nrf2 in HO-1 expression is redundant with other activators, and that the extent of this redundancy varies between tissues. Since *ho-1* is expressed at high levels in the *bach1$^{-/-}$* mice, Nrf2 might be induced in these mice. However, we did not find this to be the case (Figure 5C). Accordingly, these results establish that low levels of *ho-1* expression under normal conditions are due to repression by Bach1.

Ho-1 enhancer occupancy by small Mafs during repression and activation

The results of overexpression studies suggest that small Maf proteins can regulate MARE-directed genes in both positive and negative ways depending on the balance between small Mafs and their partner activators, like NF-E2 p45 and Nrf2 (Igarashi *et al.*, 1994; Motohashi *et al.*, 1997, 2000). However, the present results indicate that Bach1 is essential for repression of *ho-1* under physiological conditions. To assess the involvement of small Maf proteins in *ho-1* repression by Bach1 in its chromatin environment, we determined the binding of small Maf proteins to the *ho-1* enhancer E2 using chromatin immunoprecipitation (ChIP) assays with anti-small Maf antibodies. As shown in Figure 6A and B, the binding of small Maf proteins to the E2 enhancer was clear in both the wild-type and *bach1*-deficient thymocytes. The anti-small Maf antibodies did not enrich exon 1 of *ho-1* or the RAG2 gene, verifying the selectivity of the immunoprecipitation. In light of other observations described here, these results are consistent with a model in which the small Maf proteins participate in both repression and activation of *ho-1*. Their partner molecule for repression is Bach1, whereas that for activation is Nrf2 (or another activator). We are currently investigating whether Bach1 binds directly to *ho-1*.

Discussion

Repressors can restrict expression of their target genes to certain physiological conditions or cell types. Accordingly, activators can induce gene expression only in the absence of repressor function (Herschbach and Johnson, 1993). This model should be tested experimentally by
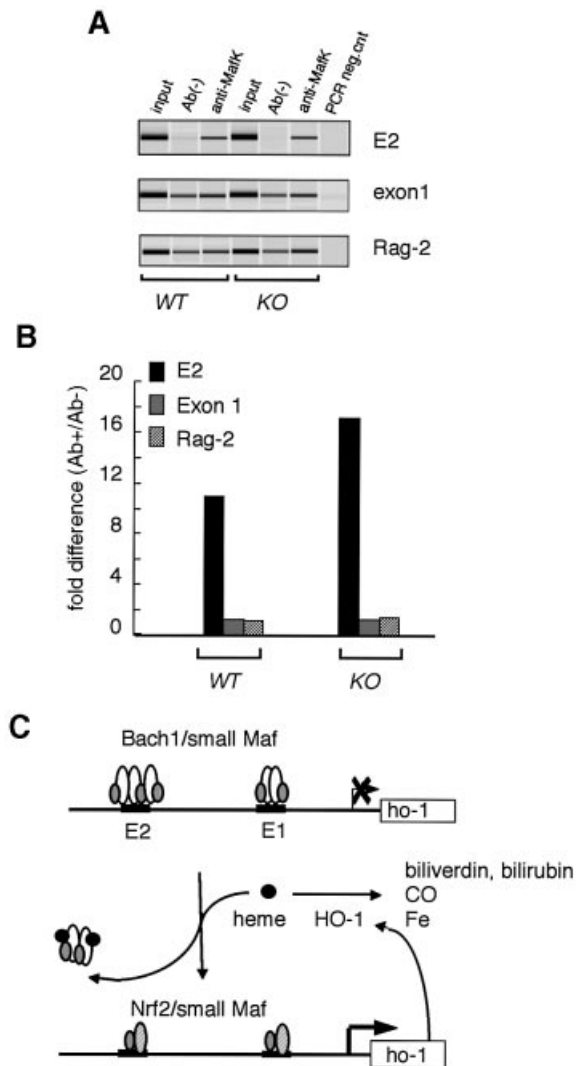
Fig. 6. Binding of small Maf to *ho-1* enhancers *in vivo*. (**A**) Gel images showing PCR amplification of E2, exon 1 and Rag-2 gene fragments recovered in the absence or presence of anti-small Maf antibodies. Chromatin was prepared from the wild-type (WT) or *bach1*-deficient (KO) thymi. PCR products without DNA template are shown in the right-hand lanes. (**B**) Fold-enrichment of each genomic DNA region by the small Maf antibodies was determined using the Agilent BioAnalyzer 2100. Results are mean of two experiments using two mice per each genotype. (**C**) A hypothetical model describing the regulation of *ho-1* by Bach1 and heme. Besides MafK, other Maf-related factors may also serve as partners for Bach1. Bach1 makes enhancers inaccessible to Nrf2 and other activators by binding to them. Repression by Bach1 is alleviated upon increase of heme levels, allowing expression of HO-1 followed by degradation of heme.

examining transcriptional readout of a natural target gene on a chromatin environment. However, such an approach has been difficult in higher eukaryotes because we do not have good combinations of well-defined repressors and target genes. The results described here provide *in vivo* functional evidence for the sequence-specific repressor Bach1 regarding *ho-1* regulation on native chromatin. The results also provide a distinct mechanism of temporal reversible repression, different from stable gene silencing.

Our main conclusion is that expression of *ho-1* is regulated through antagonism of transcription activators and the repressor Bach1 (Figure 6C). We suggest that, while expression of *ho-1* is repressed by Bach1 under

normal physiological conditions, increased levels of heme displace Bach1 from the enhancers by inhibiting DNA binding, allowing activators to bind to the enhancers. Contrary to what might have been expected, alleviation of repression, rather than activation of activators like Nrf2, is the critical step in *ho-1* induction, since ablation of *bach1* resulted in constitutive expression of *ho-1*. It is noteworthy that the expression levels of *ho-1* in tissues like thymus, intestine, liver, lung and heart from the *bach1*-deficient mice were comparable to those in spleen of wild-type or *bach1*-deficient mice (Figure 4). *ho-1* is induced in spleen under normal conditions due to high levels of heme derived from red blood cells. Thus, it appears that the expression levels in the *bach1*-deficient tissues are comparable to those induced by various stimuli, including heme.

The anti-small Maf antiserum enriched E2 DNAs from both repressed and activated *ho-1* locus, indicating that small Maf proteins participate in both repression and activation of *ho-1*. We suggested previously that homo-dimers of the small Maf proteins repress transcription, whereas heterodimers generated with NF-E2 p45 or Nrf2 activate transcription (Igarashi *et al.*, 1994; Motohashi *et al.*, 1997, 2000). The results of ChIP assays and from *bach1*-deficient mice together provide *in vivo* evidence that small Maf proteins repress *ho-1*, dependent on Bach1. On the other hand, since the small Maf proteins are devoid of a transcription activation domain (Fujiwara *et al.*, 1993; Igarashi *et al.*, 1994; Nagai *et al.*, 1998), heterodimers of small Maf and NF-E2-related activators including Nrf2 are most likely the binding form to the *ho-1* enhancers upon transcriptional activation. Thus, MARE-dependent regu-lation of *ho-1* involves activator and inhibitor subunits as well as shared small Maf subunits. Since there are multiple MAREs in E1 and E2, and Bach1 generates a multivalent DNA-binding complex through the BTB domain-mediated oligomer formation (Yoshida *et al.*, 1999), Bach1 may nucleate generation of a multiprotein complex like a repressosome (Courey and Jia, 2001) on the enhancer regions. Taken together, the results described here suggest that the Bach1/small Maf complex may render the enhancers inaccessible to activators by changing higher order structure of the enhancer regions. At present, however, it remains to be elucidated how Bach1 represses the *ho-1* enhancers. It may also cause changes in histone modification by recruiting co-repressors. In any case, generation of an inactive enhancer complex by Bach1 may play more active roles in inducible *ho-1* expression through inhibiting spurious transcription in an otherwise competent chromatin environment. In contrast, repression involving tightly condensed chromatin structure is suitable for fixing gene repression (Moazed, 2001), and may not be compatible with rapidly inducible expression. It should be noted that the repressive structure generated by Bach1 may yield easily to activators once the repressor is inactivated by increased levels of heme.

Coupling of key metabolic activities and gene expression is a major strategy to co-ordinate cellular homeostasis. Recent examples include co-enzyme NADH, which binds to the transcription factor NPAS2 and regulates expression of lactate dehydrogenase (Rutter *et al.*, 2001). To our know-ledge, however, the Bach1–*ho-1* system is the first example in higher eukaryotes that involves a direct regulation of a transcription factor for an enzyme gene by its substrate. The

results obtained with NPAS2 and Bach1 indicate that metabolic control of gene expression in higher eukaryotes involves simple regulatory strategies in which transcription factors directly sense co-enzymes like NADH and heme. We propose that transcriptional regulation of *ho-1* involves a direct sensing of heme levels by Bach1, generating a simple feedback loop by the substrate, as in the *lac* operon. According to the genome database, MAREs within E1 and E2 are well conserved between human and mouse (DDBJ/EMBL/GenBank accession Nos are Z82244 and AC084823, respectively). Furthermore, CP motifs are also conserved between human and mouse Bach1 (Ogawa *et al.*, 2001). Thus, a similar regulation of *ho-1* by Bach1 and heme is expected to operate in human cells as well.

Materials and methods

Transfection assays

The HO-1 reporter plasmids were as described previously (Alam *et al.*, 2000b). NIH 3T3 cells or 293 cells were transfected with reporter plasmids using FuGene6 (Roche) and cultured for 24 h before harvesting cells. Where indicated, hemin was added to the culture medium at 10 µM for the last 4 h. Cell lysates were prepared using the Luciferase Assay System (Promega) following the supplier's protocol. Luciferase activities were measured with a Biolumat Luminometer (Berthold). Firefly luciferase activity was normalized for transfection efficiency as determined by control sea pansy luciferase activity (Muto *et al.*, 1998). Three independent experiments, carried out in duplicate, were performed and the results are averaged and illustrated with standard errors.

Gel mobility-shift and footprinting assays

Sequences of the oligonucleotide probes are available upon request. EMSA and footprinting assays were carried out as described previously (Igarashi *et al.*, 1998). The probe for agarose gel EMSA and footprinting was a 562 bp E2 DNA isolated by PCR using primers (5′-AAT-TCGAATTCCCAGCGGTGAAAGC-3′ and 5′-AATTCAAGCTTCAA-TGCAGAAAGGCATACT-3′).

Construction of targeting vector

Analysis of genomic λ phage clones of mouse *bach1* was described previously (Sun *et al.*, 2001). The *bach1*-targeting vector (pB1TV) was constructed by subcloning a cDNA containing the neomycin resistance gene driven by the PGK promoter (PGK-neo) between a 1.2 kb *Kpn*I–*Sph*I 5′ genomic DNA fragment and an 11 kb *Sac*I–*Xho*I 3′ DNA fragment (see Figure 3A). A cDNA containing the diptheria toxin A fragment driven by the PGK promoter (PGK-DTA) without a poly(A) signal was then subcloned at the 3′ region to complete the construct (see Figure 3A).

Generation of bach1-deficient mice

E14 ES cells (1×10^7 cells) were electroporated with 20 µg of pB1TV DNA linearized with *Not*I and selected in media containing 300 µg/ml of G418 (Gibco-BRL) as described previously (Itoh *et al.*, 1997). Correctly targeted ES clones were identified by PCR analysis as described below. Correct targeting events in these clones were verified by Southern blotting analysis, and were injected into C57B6J blastocysts. Chimeric mice were crossed with C57B6J to identify those capable of transmitting the targeted *bach1* allele to offspring. Heterozygous knockout mice were mated to obtain homozygous knockout mice. To generate compound knockout mice, homozygous *bach1* knockout mice were mated with heterozygous *nrf2* knockout mice (Itoh *et al.*, 1997) to obtain compound heterozygous mice. The compound heterozygous mice were mated, and pups were genotyped by PCR at the time of weaning.

DNA analysis

To screen for recombinant ES clones and mice, PCR was carried out using a primer set that amplifies the 5′ junction region as a 1.6 kbp DNA. The primers were located outside the short arm or within the PGK-neo, respectively. Southern blotting analysis of genomic DNA isolated from tail was carried out using a 0.4 kbp *Pst*I–*Kpn*I genomic DNA fragment (see Figure 3A) as a probe. A 0.6 kbp *Sac*I–*Pst*I fragment of neomycin resistance gene (see Figure 3A) was used to rule out random integration of the targeting vector.

RT–PCR analysis

Preparation of RNA and cDNA from various tissues was carried out as described previously (Muto *et al.*, 1998). Primers to amplify HO-1 cDNA were 5′-ACATCGACAGCCCCACCAAGTTCAA-3′ and 5′-CTGACG-AAGTGACGCCATCTGTGAG-3′. Primers to amplify HPRT and Bach1 mRNA were as described previously (Igarashi *et al.*, 1998; Muto *et al.*, 1998). PCR products were analyzed using the BioAnalyzer 2100 and DNA 500 chip (Agilent). Serial 3-fold dilutions of cDNA were used to ensure linearity of amplification.

ChIP assay

Chromatin fixation and purification procedures were as described previously (Sawado *et al.*, 2001), without carrying out CsCl equilibrium centrifugation. In brief, single cell suspensions of thymocytes ($1–2 \times 10^8$) were prepared as described and fixed by adding 11% formaldehyde for 5 min at room temperature. Cells were then sonicated to prepare chromatin suspensions of ~300 bp DNA length. Immunoprecipitations were carried out using anti-small Maf antibodies (Igarashi *et al.*, 1995) as described previously (Sawado *et al.*, 2001). PCRs were carried out using ExTaq DNA polymerase (TAKARA) for 32 cycles (E2 primer set) or 35 cycles (other primer sets). Amounts of products were determined as above using the BioAnalyzer 2100 and DNA 500 chip (Agilent). PCRs were carried out using the following primers: E2, 5′-GGGCTAGCATGC-GAAGTGAG-3′ and 5′-AGACTCCGCCCTAAGGGTTC-3′; exon 1, 5′-GGGTGCTGCACTCCATGAG-3′ and 5′-GCTTGGTTTGCTGA-CACTTGC-3′; Rag2, 5′-CAACCATCACAGGGGTGCAG-3′ and 5′-GCCTACAGATGTTCCAGTGAG-3′.

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