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Heme Oxygenase-1 Gene Enhancer Manifests Silencing Activity in a Chromatin Environment Prior to Oxidative Stress

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ABSTRACT

The expression of heme oxygenase-1 (HO-1) is regulated by E1 and E2 enhancers, both of which contain multiple Maf recognition elements (MAREs). In living cells, MAREs are bound by Bach1/MafK heterodimers, hence maintaining a quiescent state of the HO-1 gene (hmox-1). However, in transient transfection assays, they act as transcriptional enhancers. Therefore MAREs may manifest their function only in a chromatin environment. By using NIH3T3 cell pools stably transfected with EGFP reporter genes driven by the wild-type or mutated E2 enhancer, we demonstrate that the E2 MAREs function as transcriptional silencers depending on the binding of Bach1/MafK heterodimer *in vivo* only in a chromatin environment. After cadmium treatment, they switched into transcriptional enhancers. Surprisingly, single MARE site did not exhibit such function. Furthermore, by using DNase I hypersensitivity assay, we demonstrate that simple chromatin condensations were not involved in the Bach1-mediated repression. We conclude that, in a chromatin environment, the E2 MAREs function as transcriptional silencers depending on binding of Bach1/MafK heterodimer. *Antioxid. Redox Signal.* 8: 60–67.

INTRODUCTION

HEME OXYGENASE-1 (HO-1) is an antioxidant defense enzyme that degrades pro-oxidant heme into iron, carbon monoxide, and biliverdin, and is essential for higher eukaryotes in order to cope with various aspects of cellular stress and to regulate cellular iron metabolism (26, 33, 37–39). Its expression is induced at the transcriptional level by its substrate heme (3, 4, 40, 47, 48)and regulated principally by two upstream enhancers, E1 and E2 (2, 45). Both enhancer regions contain multiple stress responsive elements (StRE) that also conform to the sequence of Maf recognition elements (MAREs) (18, 28). MARE is bound by a heterodimer of the Cap'n'Collar (CNC) families and small Maf families (5, 12, 17, 27, 28, 49). The heterodimer of NF-E2 related factor 2 (Nrf2) and small Maf (MafF, MafG, or MafK) binds to MARE and activates HO-1 expression (4, 18–21), while the heterodimer of BTB and CNC homology 1 (Bach1) and small Maf also binds to MARE and represses its expression (30, 31, 45). This competitive interplay between Nrf2 and Bach1 constitutes the fundamental mechanism of HO-1 regulation. Based on the results from chromatin immunoprecipitation experiments, we proposed the following model. Under normal conditions, the Bach1/MafK heterodimer binds to MARE and keeps a quiescent state of the HO-1 gene (*hmox-1*). In the presence of oxidative stress, such as heavy metals and heme, Bach1 is released from MAREs to which the Nrf2/MafK heterodimer binds (44, 45), leading to HO-1 induction and ultimately protection against oxidative stress. This model suggests that *in vivo* the *hmox-1* locus is situated in a chromatin

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environment that is permissive for activation but under normal conditions *hmox-1* MARE mediate transcriptional repression by recruiting Bach1. However, the presumptive repressive role of the *hmox-1* MAREs has not yet been elucidated. As previously reported, the *hmox-1* E1 and E2 enhancers function as strong transcriptional enhancers without any repression in transient transfection assays (2, 18, 45). We therefore hypothesize that MARE might manifest its function as a transcriptional silencer *in vivo* only in a chromatin environment.

In general, enhancer regions are often spread over long stretches of DNA. Enhancers direct the assembly of multiple transcription factors in order to regulate transcription. Recently, transcription regulation is conceived to depend, at least in part, on changes in chromatin structure (10, 25, 42, 43). Covalent histone modification and nucleosome remodeling are important in locally altering chromatin structure, and these alternations depend on histone modifying enzymes such as acetyltransferases (HATs) complexes (6) and ATP-dependent chromatin remodeling complexes (50), respectively. Thus, both the assembly of transcription factors and changes in chromatin conformation are important for gene expression.

By using NIH3T3 cell pools stably transfected with EGFP reporter genes driven by wild-type or mutated *hmox-1* E2, we examined effects of E2 MAREs at a single cell level. We found that transcriptional activity of the wild-type E2 enhancer was lower than that of the mutated E2 under normal conditions. The activity of wild-type E2 is strongly induced by treating the cells with cadmium. This induction was severely diminished by mutations in the E2 MAREs. We also investigated the chromatin conformations of the *hmox-1* E2 enhancer and core promoter regions in thymocytes from wild-type and *Bach1* knock out mice in which *hmox-1* is repressed and activated, respectively (45). Surprisingly, chromatin conformation of these regions were hypersensitive to DNase I digestion, showing less condensation even when the gene was off in the wild-type cells. Our results clearly demonstrate that the E2 MAREs possess a dual function as both transcriptional silencers and enhancers *in vivo* chromatin environment. Moreover, the silencing function of MAREs depends on the binding of Bach1/MafK heterodimers to the enhancer region.

MATERIALS AND METHODS

Plasmids

Plasmids pd2EGFP-1 and pIRESpuro3 were obtained from Clontech (Mountain View, CA). Plasmids constructed in this study are shown in Fig. 1A or Fig. 2A. The mouse *hmox-1* core promoter was isolated by PCR using *hmox-1* clone (1). PCR were carried out using the following primers: 5'-GTTAAGGATCCTGGAGGCTTTGAAGAACCAC-3' and 5'-GTTAAGGATCCGCTATGCTCGAGACGGCTCT-3'. The mouse *hmox-1* E2 enhancer was isolated by PCR as described previously (45). The core promoter PCR product was digested with *Bam*HI and inserted into the *Bam*HI site of pd2EGFP-1. The resulting plasmid was digested with *Xma*I/*Afl*II and inserted into the *Xma*I/*Not*I site of pBluescript KS (+), to generate pBSHO-1EGFP. Because the *hmox-1* E2 includes three tandem MAREs, mutations were introduced into two or three MAREs of *hmox-1* E2 by site-directed mutagenesis using an altered sites *in vitro* mutagenesis systems (Promega, Madison, WI), resulting in pAlter1E2WT, pAlter1E2M2 or pAlter1E2M3. These plasmids were digested with *Eco*RI/*Hin*dIII and the E2 DNAs inserted into the *Bam*HI site of pTK-luc plasmid, presented by K. Umezono (Kyoto University), resulting in pE2WTTKluc, pE2M2TKluc, and pE2M3TKluc (Fig. 1A). The E2WT and E2M3 DNAs were also inserted into the *Sal*I site of pBSHO-1EGFP plasmid, resulting in pGFPE2WT and pGFPE2M3, respectively (Fig. 2A).

Transfection and luciferase assay

NIH3T3 cells were seeded in 12-well plates at 1×10^5 cells/well and incubated for 15 hours. The cells were transfected with pE2WTTKluc, pE2M2TKluc, or pE2M3TKluc reporter plasmids along with control sea pansy luciferase expression plasmid as an internal control by using the FuGENE6 transfection reagent (Roche, Basel, Switzerland). After 24 hours, cell lysates were prepared using the Luciferase Assay System (Promega) following the supplier's protocol. Luciferase activities were measured with a Biolumat Luminometer (Berthold, Dortmund, Germany), and normalized for transfection efficiency as determined by control sea pansy luciferase activities. Three independent experiments, carried out in duplicate, were performed and the results are averaged and illustrated with standard errors.

Cell culture and generation of stable cell pools

NIH3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) including 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Gibco, Los Angeles, CA). To generate NIH3T3 cell pools stably transfected with pGFPE2WT or pGFPE2M3, the cells were transfected with these plasmids along with pIRESpuro3 plasmid at a ratio of 20:3 using the FuGene6 (Roche). After 72 h, the transfected cells were selected with 3 μg/ml puromycin. After 4 days, the concentration of puromycin was increased to 7 μg/ml. This selection was continued until all assays were completed.

FACS analysis

NIH3T3 cells stably transfected with pGFPE2WT or pGFPE2M3 were seeded in 6-well plates at 2×10^5 cells/well and incubated for 15 h. $CdCl_2$ was then added to achieve a final concentration of 10 μ*M*, and the cells were incubated for an additional 24 h. After incubation, the cells were harvested by trypsinization and resuspended in 500 μl 3% FBS/phosphate-buffered serine (PBS). The cells were analyzed using a FACSCalibur (Becton Dickinson Bioscience, Franklin Lakes, NJ). The results were quantified using the Cell Quest software (Becton Dickinson Bioscience).

ChIP assay

Chromatin fixation and purification procedures were as described previously (35, 44). In brief, single cell suspension of stably transfected cells ($5–8 \times 10^6$) were prepared as previously described and fixed by adding formaldehyde to 1%

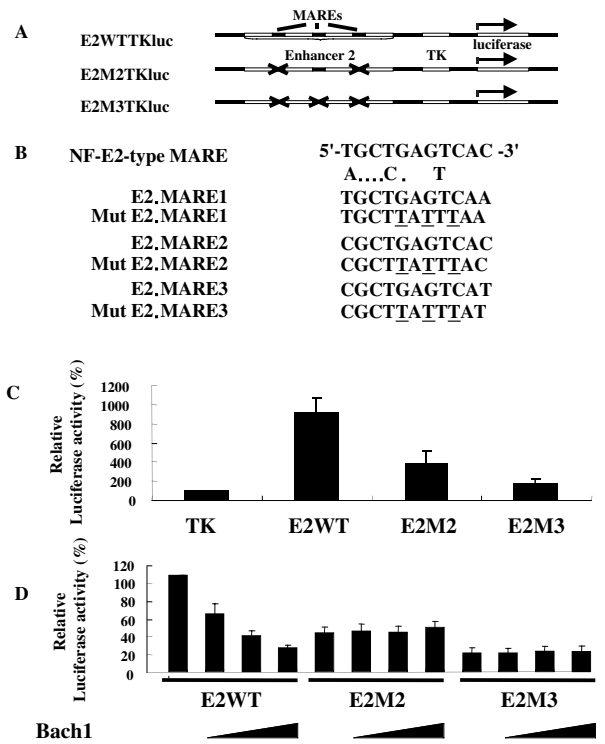

FIG. 1. Transcriptional activity of the *hmox-1* E2 enhancer depends on MAREs in transient transfection assays. (**A**) Schematic representation of the *hmox-1* E2 reporter plasmids pTKE2WT, pTKE2M2, or pTKE2M3. *Closed boxes* show MARE-like elements within the E2 enhancer. Mutations are represented by *cross symbols. Bent arrows* indicate transcription initiation site. TK and Luc indicate thymidine kinase promoter and luciferase coding regions, respectively. (**B**) The various mutations introduced into three MARE-like elements of *hmox-1* E2 are *underlined*. (**C** and **D**) NIH3T3 cells were transiently co-transfected with reporter plasmids (**C**), and increasing amounts (1, 3, 9 ng) of Bach1 expression plasmid (**D**).

final concentration for 10 min at room temperature. The cells were then sonicated to prepare chromatin suspensions of 300 to 1000-bp DNA in length. Immunoprecipitations were carried out using anti-Bach1 antibody (polyclonal rabbit antisera) as described previously (45). Normal rabbit serum was used as negative control. PCR reactions were carried out using Ex Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). PCRs were carried out using the following primer: E2, 5'-AGACTCCGCCCTAAGGGTTC-3' and 5'-GGAAACAGCTATGACCATG-3'. Primers of mcm5 were as described previously (44).

DNase I hypersensitive site assay

To prepare nuclei of wild type and *Bach1* knock out mouse thymocytes, 1×10^8 thymocytes were washed with ice-cold PBS and resuspended in RSB buffer (10 m*M* Tris-HCl, pH7.4, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1% Nonidet P-40). After 10 min incubation, the suspension was centrifuged (2500 rpm, 10 min, 4°C) to separate the nuclear fraction. Nuclei were digested with various concentrations (0, 0.011, 0.033, 0.1, 0.3, 0.9, or 2.7 u/μl) of DNase I for 18 min at 37°C. The reactions were stopped by incubating the mixture with stopping buffer (20 m*M* Tris-HCl, pH 7.4, 0.6 m*M* NaCl, 10 m*M* EDTA, 1% SDS, 2 mg/ml proteinase K) for 16 h at 50°C. The DNA was purified three times by phenol-chloroform extraction and ethanol precipitation. The DNA was then digested with 10 units of *Bam*HI or *Xba*I and treated with RNase for an additional 10 min at 37°C. After purification, the DNA was electrophoresed on 0.7% gel and transferred to Hybond-N^+ membrane (Amersham, Piscataway, NJ). Membranes were hybridized with DNA probes of 766-bp (*hmox-1* core promoter region) or 1.1-kbp (E2 region) (see Fig. 4A) and analyzed by Bioimaging Analyzer (Fuji BAS 2000, Tokyo, Japan).

RESULTS

MARE functions as a transcriptional enhancer in transient reporter assays

The *hmox-1* E2 includes three MAREs/StREs which are directly bound by several heterodimers of bZip proteins (15, 18, 45). To verify that these MAREs activate transcription in transient transfection assays, we placed E2 or E2 with double or triple MARE mutations (E2M2 and E2M3) in front of the core promoter of thymidine kinase (see Figs. 1A and 1B). These mutations are expected to abolish binding of any Maf heterodimers. When these reporter plasmids were transfected into NIH3T3 cells, the wild-type E2 enhancer supported a high level expression of luciferase (Fig. 1C). By mutating two or three MAREs, reporter expression was reduced progressively compared to pE2Wtluc (Fig. 1C). Whereas Bach1 represses HO-1 expression depending on the E1 and E2 enhancers, whether all three MAREs within the *hmox-1* E2 are important for Bach1-mediated repression has not been examined. When a Bach1-expression plasmid was co-transfected with pTKE2WTluc in NIH3T3 cells, Bach1 effectively repressed luciferase expression in a dose dependent manner (Fig. 1D). In contrast, both of the reporters with mutations showed less sensitivity to Bach1-mediated repression. Surprisingly, although E2M2 retained one MARE and its basal expression level was higher than E2M3, its response to Bach1 was completely lost like in the case of E2M3 (Fig. 1C and D). When combined, these results demonstrate that MAREs within the *hmox-1* E2 function as transcriptional enhancers in transient transfection assays, and the clustered MAREs are essential for Bach1-mediated transcription repression.

Dual functions of the HO-1 E2 MAREs as transcriptional silencer and enhancer in an in vivo *chromatin environment*

Endogenous *hmox-1* is repressed through binding of Bach1/MafK heterodimers to the E1 and E2 MAREs under normal conditions (44, 45). This contradicts the above and previous observations that MAREs within *hmox-1* E2 function as transcriptional enhancers in transient transfection assays. We therefore hypothesize that the transcriptional repression through the E2 MAREs is manifested *in vivo* only in a chromatin en-

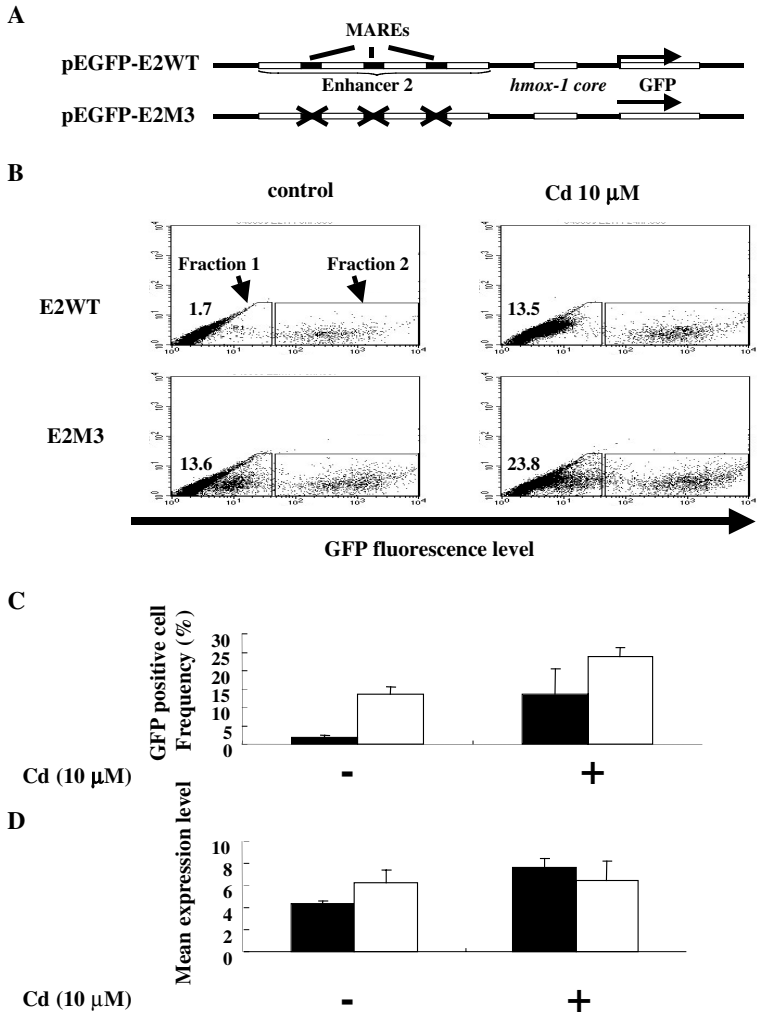

FIG. 2. Transcriptional activity of the *hmox-1* E2 enhancer depends on MAREs *in vivo* chromatin environment. (**A**) Schematic representation of reporter plasmids pGFPE2WT, or pGFPE2M3. E2 enhancer constructions with or without mutations are the same as in Figs. 1A and 1B. (**B**) Flow cytometry analysis of NIH3T3 cell lines stably transfected with pGFPE2WT or pGFPE2M3. The cells were incubated with 0 or 10 μ*M* $CdCl_2$ for 24 h. Then cells were harvested and resuspended in 3% FBS/PBS. Dot plots show GFP fluorescence (*x axis*) as a transcriptional activity of the integrated reporter plasmids. Numbers indicate the percentage of the cells with GFP expression in fraction 1. (**C**) The frequency of GFP positive cells in fraction 1 from E2WT cell pools (*filled columns*) and E2M3 cell pools (*open columns*). (**D**) Relative enrichment of the mean GFP fluorescence levels. *Filled* and *open columns* show E2WT and E2M3, respectively.

vironment. To explore this possibility, we constructed GFP reporter plasmids driven by E2 or E2M3 and the core promoter of mouse *hmox-1* (pGFPE2WT and pGFPE2M3, Fig. 2A), and subsequently generated NIH3T3 cell pools stably transfected with these plasmids. In order to avoid clone-to-clone variation, we evaluated promoter activity by assessing reporter GFP expression in the cell pools at a single cell level by FACS (Fig. 2B). In both cell pools, GFP-positive cells formed two fractions depending on their expression levels: lower expression cells (fraction 1) and higher expression cells (fraction 2). Because the reporter plasmids were randomly integrated in chromosomes, the GFP expression levels were affected, at least in part, by chromatin structure surrounding each integration site (positional effect) (36). Considering the relative GFP expression level, we surmised that fraction 2 included cells with a positive positional effect (i.e., DNA integrated near enhancers that are active in NIH3T3 cells). This conjuncture is also supported by the appearance of fraction 2 in both E2WT and E2M3 reporter cell pools. By analyzing the two fractions independently, we noticed that the E2M3 reporter gave a larger number of cells in fraction 1 than did E2WT (Fig. 2C). This may be due to leaky expression from the core promoter. Furthermore, whereas the reporter genes were affected by a positional effect in fraction 2, the mean GFP expression levels were higher in E2M3 than in E2WT cell pools (Fig. 2D). These results strongly suggest that the E2 MAREs function as transcriptional silencers in an *in vivo* chromatin environment under normal conditions.

To determine whether the E2 MAREs function as enhancers under oxidative stress, we examined the effects of cadmium (Cd) treatment, which is used to mimic oxidative stress (41). The percentage of GFP-expressing cells in fraction 1 was strongly increased after Cd treatment of E2WT cell pools. In contrast, the magnitude of change was much lower with E2M3 cell pools than E2WT cell pools (Figs. 2B and 2C). Although cells in fraction 2 were under a positive positional effect, the same tendency was also observed. The mean GFP expression level in fraction 2 strongly increased in E2WT cell pools, while it was not affected in E2M3 cell pools (Figs. 2B and 2D). These results strongly suggest that the repressive effect of the E2 enhancer is canceled after Cd treatment, converting its function to a transcriptional enhancer. Moreover, this dual function depends on the presence of MAREs.

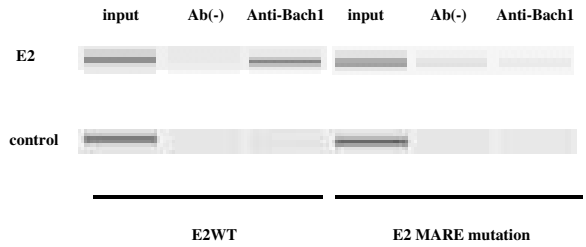

FIG. 3. The binding of Bach1 to the *hmox-1* E2 enhancer *in vivo*. ChIP assays were carried out with or without anti-Bach1 antibodies or control rabbit IgG. Chromatin was prepared from NIH3T3 cells stably transfected with pGFPE2WT or pGFPE2M3. Gel images show PCR products of E2 enhancer and mcm5 promoter using input and precipitated chromatin as template.

Bach1 bound to WT MAREs, but not to mutated MAREs

To confirm that the E2 enhancer integrated into chromatin was actually occupied by Bach1, we carried out chromatin immunoprecipitation assays using anti-Bach1 antibodies. To distinguish the E2 sequence of the reporter gene from endogenous E2 sequence, we used a primer that anneals to the vector sequence. Bach1 binding to the E2 MAREs of the wild-type reporter gene was clearly detected, while little Bach1 binding to the mutated E2M3 was detected (Fig. 3). Binding of Bach1 was not detected to genes such as mcm5 that do not contain MARE. These results clearly indicated that the repressive function of the E2 MAREs was dependent on Bach1 binding.

DNase I hypersensitivity of hmox-1 enhancer

Because transcription repressors often inhibit gene expression by inducing changes in chromatin structure, we investigated whether the repressive function of E2 MARE also involved changes in chromatin structure. We compared DNase I hypersensitivity (24) of the *hmox-1* promoter and the E2 enhancer regions using normal and Bach1-deficient mice. Bach1-deficient mice constitutively express high levels of HO-1 in various tissues including thymus (45). Nuclei were isolated from thymocytes and digested with various amounts of DNase I. As shown in Figure 4, we observed a similar extent of digestion in E2 (B) and core promoter (C) regions of *hmox-1* irrespective of the genotype. These results indicate that chromatin domains of the E2 enhancer and the promoter were packaged in a less condensed structure even when *hmox-1* is repressed by Bach1.

DISCUSSION

Gene expression results from the balance of two opposing forces, transcriptional activation and transcriptional repression. The importance of histone modification (6) and chromatin structure (50) in the regulation of eukaryotic gene transcription has become much more widely accepted. Thus, it is important to examine the physiological functions of transcriptional repressors such as Bach1 and its target DNA sequences in the context of a chromatin environment. By using NIH3T3 cell pools stably transfected with the *hmox-1* E2 reporter plasmids with or without mutations in MAREs, we showed that the E2 enhancer can function as a transcriptional silencer *in vivo* only in a chromatin environment. Importantly, this silencing function correlated well with binding of Bach1 to E2. Previously we had shown that the induction of HO-1 by cadmium or heme is achieved by removing Bach1 from the E2 enhancer (44–46). Our previous and present observations establish that, in a chromatin environment, the E2 MAREs repress transcription by recruiting Bach1 under normal conditions, whereas they activate transcription by recruiting Nrf2 or other activators in place of Bach1 upon oxidative stress.

To our knowledge, this study is the first report to demonstrate that MARE possesses the ability to repress transcription. We previously reported that the basal luciferase activity of the *hmox-1* reporter plasmid with deletions of the E1 and E2 enhancers was low compared with wild-type *hmox-1* reporter plasmid in transient transfection assays (45). However, under normal physiological conditions, *hmox-1* is repressed by binding of Bach1/MafK heterodimers to MAREs within the E1 and E2 enhancer regions. This apparent contradiction may be explained by a chromatin effect. Transient transfection assays do not necessarily reflect chromatin structure. Probably because MARE embeds a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) to which AP-1 proteins such as Jun and Fos bind to activate transcription (22), the *hmox-1* reporter plasmids may be affected by AP-1 or other bZip proteins in transient transfection assays. Under such conditions, endogenous Bach1 may not be able to bind to MARE efficiency enough to repress transcription. It is still not clear how Bach1 binds to MAREs of endogenous *hmox-1* or the integrated reporter gene dominantly over other transcriptional activators. However, unique characteristics of Bach1 and the clustering of the E2 MAREs suggest several interesting possibilities. The *hmox-1* E1 and E2 include tandem duplicated or triplicated MAREs (18, 45). In this study, we showed that E2M2, which retained only one MARE, functioned as a weak enhancer. However, it was not repressed by Bach1, suggesting that multiple MAREs are critical for the binding of Bach1. Along this line, we have previously reported that, among MARE-binding factors, Bach family is unique in that it possesses a BTB/POZ domain which interacts to form homo-oligomers (16, 31). The formation of Bach1/MafK hetero-multimers increases the specificity of their binding to tandem MAREs *in vitro* (51). In this regard, there is a possibility that endogenous Bach1 may dominantly bind to the *hmox-1* E1 and E2 MAREs because Bach1 oligomers can bind to the clustered MAREs in a co-operative manner. In addition, Bach1 may interact with proteins that modify chromatin structure and thus facilitate binding of Bach1 to MARE in chromatin.

We also demonstrated in this study that the chromatin structure of the *hmox-1* promoter and E2 enhancer regions was open even when the gene was transcriptionally inactive (Fig. 4). This is consistent with the finding that histones H3 and H4 of the promoter and E2 enhancer regions are hyperacetylated, irrespective of activation or repression (44). In general, chromatin structure is supposed to change between an open and closed conformation under activation and repression condi-

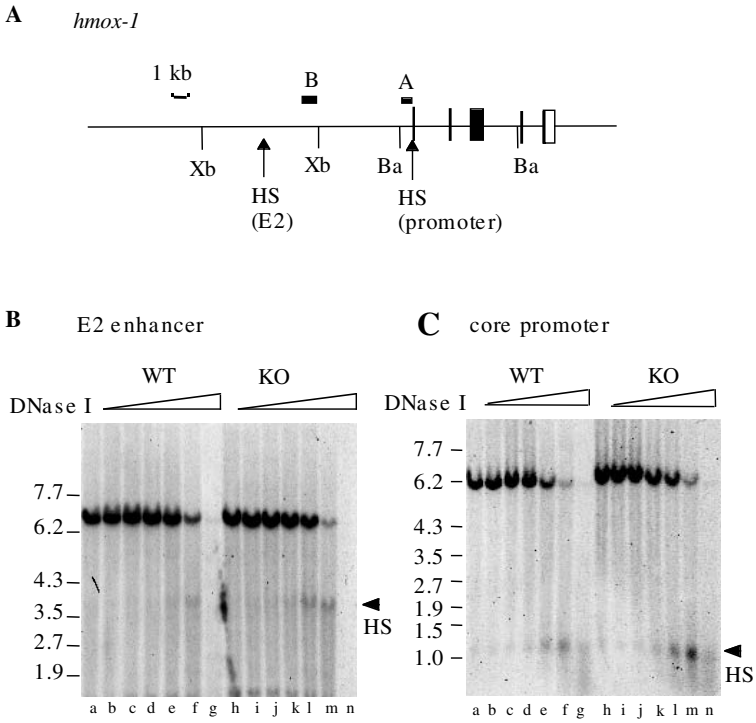

FIG. 4. The formation of the *hmox-1* core promoter region and the *hmox-1* E2 region DNase I hypersensitive site. (**A**) Schematic representation of the *hmox-1* gene locus region. *Black box* shows coding region. The *black bars A* and *B* show the probe used to detect the region of the core promoter and the E2 enhancer, respectively. The *arrows* show the hypersensitive sites (HS). Xb, XbaI; B, BamHI. (**B**) and (**C**) Representation of DNase I hypersensitive assays of the *hmox-1* core promoter region and the *hmox-1* E2 region. Bach1 wild-type or Bach1 knock-out cell nuclei were prepared and digested with various amounts of DNase I as described in Materials and Methods. The purified DNA was digested with either XbaI (E2 analysis) or BamHI (core promoter analysis) to produce a fragment, and hybridized with DNA probe B or A. Increasing DNase I concentrations are indicated above the lanes. The signals from the *hmox-1* core promoter and the E2 enhancer digested with DNase I on their hypersensitive sites are shown by *arrowheads* (HS).

tions, respectively (7, 23, 29). In contrast to such a widely accepted view, our results suggest that the Bach1-mediated repression of *hmox-1* involves a mechanism distinct from a simple chromatin condensation. Binding of Bach1 oligomers to the multiple MAREs may nucleate formation of a structure like a "chromatin hub" which was suggested for the β-globin locus control region (LCR) (8, 32).

We cannot exclude the possibility that the amount of integrated DNA is greatly different between E2WT and E2M3 cell pools. Indeed, larger numbers of GFP positive cells were detected in E2M3 cell pools compared to E2WT cell pools. However, we generated both stable cell pools simultaneously, and used all integrated cell pools to average cell-to-cell variation. After Cd treatment, GFP expression in fraction 1 was strongly activated in E2WT cell pools. Moreover, stimulation of enhancer activity by cadmium was evident in fraction 2 of E2WT cell pools, while cadmium effect was not evident in E2M3 cell pools. Thus, our study strongly suggests that the E2 MAREs possess the ability to suppress positional effect as shown previously with the β-globin LCR containing MAREs (9, 11, 13, 14, 34).

This study points to the causative relationship among MARE and gene silencing. To unveil the detailed mechanisms by which Bach1 achieves reversible, repressed state of target genes, it is necessary to identify proteins that function together with Bach1 and to investigate their activities on transcription regulation.

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ABBREVIATIONS

Bach1, BTB and CNC homology 1; bZip, basic region-leucine zipper; BTB, Broad complex/Tramtrack/Bric-a-brac; CNC, Cap'n-Collar; HO, heme oxygenase; HS, DNase I-hypersensitive site; LCR, locus control region; MARE, Maf recognition element; MEL, mouse erythroleukemia; NF-E2, nuclear factor-erythroid 2; Nrf, NF-E2-related factor; POZ, Poxvirus and zinc finger; StRE, stress response element; TRE, TPA-response element.

REFERENCES

1. Alam J, Cai J, and Smith A. Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5' sequences are required for induction by heme or heavy metals. *J Biol Chem* 269: 1001–1009, 1994.
2. Alam J, Camhi S, and Choi AM. Identification of a second region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. *J Biol Chem* 270: 11977–11984, 1995.
3. Alam J, Shibahara S, and Smith A. Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. *J Biol Chem* 264: 6371–6375, 1989.

4. Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, and Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 274: 26071–26078, 1999.
5. Andrews NC, Kotkow KJ, Ney PA, Erdjument-Bromage H, Tempst P, and Orkin SH. The ubiquitous subunit of erythroid transcription factor NF-E2 is a small basic-leucine zipper protein related to the v-maf oncogene. *Proc Natl Acad Sci USA* 90: 11488–11492, 1993.
6. Cheung P, Allis CD, and Sassone-Corsi P. Signaling to chromatin through histone modifications. *Cell* 103: 263–271, 2000.
7. Croston GE, and Kadonaga JT. Role of chromatin structure in the regulation of transcription by RNA polymerase II. *Curr Opin Cell Biol* 5: 417–423, 1993.
8. de Laat W, and Grosveld F. Spatial organization of gene expression: the active chromatin hub. *Chromosome Res* 11: 447–459, 2003.
9. Epner E, Reik A, Cimbora D, Telling A, Bender MA, Fiering S, Enver T, Martin DI, Kennedy M, Keller G, and Groudine M. The beta-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse beta-globin locus. *Mol Cell* 2: 447–455, 1998.
10. Felsenfeld G. Chromatin as an essential part of the transcriptional mechanism. *Nature* 355: 219–224, 1992.
11. Fraser P, Hurst J, Collis P, and Grosveld F. DNaseI hypersensitive sites 1, 2 and 3 of the human beta-globin dominant control region direct position-independent expression. *Nucleic Acids Res* 18: 3503–3508, 1990.
12. Fujiwara KT, Kataoka K, and Nishizawa M. Two new members of the maf oncogene family, mafK and mafF, encode nuclear b-Zip proteins lacking putative trans-activator domain. *Oncogene* 8: 2371–2380, 1993.
13. Grosveld F, van Assendelft GB, Greaves DR, and Kollias G. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* 51: 975–985, 1987.
14. Guy LG, Kothary R, DeRepentigny Y, Delvoye N, Ellis J, and Wall L. The beta-globin locus control region enhances transcription of but does not confer position-independent expression onto the lacZ gene in transgenic mice. *EMBO J* 15: 3713–3721, 1996.
15. He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AM, and Alam J. Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J Biol Chem* 276: 20858–20865, 2001.
16. Igarashi K, Hoshino H, Muto A, Suwabe N, Nishikawa S, Nakauchi H, and Yamamoto M. Multivalent DNA binding complex generated by small Maf and Bach1 as a possible biochemical basis for beta-globin locus control region complex. *J Biol Chem* 273: 11783–11790, 1998.
17. Igarashi K, Kataoka K, Itoh K, Hayashi N, Nishizawa M, and Yamamoto M. Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* 367: 568–572, 1994.
18. Inamdar NM, Y. I. Ahn YI, and J. Alam J. The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. *Biochem Biophys Res Commun* 221: 570–576, 1996.
19. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, and Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 275: 16023–16029, 2000.
20. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, and Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 236: 313–322, 1997.
21. Kataoka K, Handa H, and Nishizawa M. Induction of cellular antioxidative stress genes through heterodimeric transcription factor Nrf2/small Maf by antirheumatic gold(I) compounds. *J Biol Chem* 276: 34074–34081, 2001.
22. Kataoka K, Noda M, and Nishizawa M. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol Cell Biol* 14: 700–712, 1994.
23. Kornberg RD, and Lorch Y. Interplay between chromatin structure and transcription. *Curr Opin Cell Biol* 7: 371–375, 1995.
24. Kuo MT, Iyer B, and Schwarz RJ. Condensation of chromatin into chromosomes preserves an open configuration but alters the DNase I hypersensitive cleavage sites of the transcribed gene. *Nucleic Acids Res* 10: 4565–4579, 1982.
25. Lorincz MC, Dickerson DR, Schmitt M, and Groudine M. Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. *Nat Struct Mol Biol* 11: 1068–1075, 2004.
26. Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 37: 517–554, 1997.
27. Mohler J, Vani K, Leung S, and Epstein A. Segmentally restricted, cephalic expression of a leucine zipper gene during *Drosophila* embryogenesis. *Mech Dev* 34: 3–9, 1991.
28. Motohashi H, O'Connor T, Katsuoka F, Engel JD, and Yamamoto M. Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* 294: 1–12, 2002.
29. Narlikar GJ, Fan HY, and Kingston RE. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* 108: 475–487, 2002.
30. Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H, and Igarashi K. Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. *EMBO J* 20: 2835–2843, 2001.
31. Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M, Yamamoto M, and Igarashi K. Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol Cell Biol* 16: 6083–6095, 1996.
32. Patrinos GP, de Krom M, de Boer E, Langeveld A, Imam AM, Strouboulis J, de Laat W, and Grosveld FG. Multiple interactions between regulatory regions are required to stabilize an active chromatin hub. *Genes Dev* 18: 1495–1509, 2004.

33. Poss KD, and Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci USA* 94: 10925–10930, 1997.
34. Recillas-Targa F, Pikaart MJ, Burgess-Beusse B, Bell AC, Litt MD, West AG, Gaszner M, and Felsenfeld G. Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. *Proc Natl Acad Sci USA* 99: 6883–6888, 2002.
35. Sawado T, Igarashi K, and Groudine M. Activation of beta-major globin gene transcription is associated with recruitment of NF-E2 to the beta-globin LCR and gene promoter. *Proc Natl Acad Sci USA* 98: 10226–10231, 2001.
36. Schotta G, Ebert A, Dorn R, and Reuter G. Position-effect variegation and the genetic dissection of chromatin regulation in *Drosophila. Semin Cell Dev Biol* 14: 67–75, 2003.
37. Shibahara S. The heme oxygenase dilemma in cellular homeostasis: new insights for the feedback regulation of heme catabolism. *Tohoku J Exp Med* 200: 167–186, 2003.
38. Shibahara S, Muller R, Taguchi H, and Yoshida T. Cloning and expression of cDNA for rat heme oxygenase. *Proc Natl Acad Sci USA* 82: 7865–7869, 1985.
39. Shibahara S, Muller RM, and Taguchi H. Transcriptional control of rat heme oxygenase by heat shock. *J Biol Chem* 262: 12889–12892, 1987.
40. Shibahara S, Yoshida T, and Kikuchi G. Induction of heme oxygenase by hemin in cultured pig alveolar macrophages. *Arch Biochem Biophys* 188: 243–250, 1978.
41. Shukla GS, Shukla A, Potts RJ, Osier M, Hart BA, and Chiu JF. Cadmium-mediated oxidative stress in alveolar epithelial cells induces the expression of gamma-glutamylcysteine synthetase catalytic subunit and glutathione S-transferase alpha and pi isoforms: potential role of activator protein-1. *Cell Biol Toxicol* 16: 347–362, 2000.
42. Simpson RT. Nucleosome positioning: occurrence, mechanisms, and functional consequences. *Prog Nucleic Acid Res Mol Biol* 40: 143–184, 1991.
43. Struhl K. Chromatin structure and RNA polymerase II connection: implications for transcription. *Cell* 84: 179–182, 1996.
44. Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, and Igarashi K. Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. *Proc Natl Acad Sci USA* 101: 1461–1466, 2004.
45. Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, and Igarashi K. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J* 21: 5216–5224, 2002.
46. Suzuki H, Tashiro S, Sun J, Doi H, Satomi S, and Igarashi K. Cadmium induces nuclear export of Bach1, a transcriptional repressor of heme oxygenase-1 gene. *J Biol Chem* 278: 49246–49253, 2003.
47. Taketani S, Kohno H, Yoshinaga T, and Tokunaga R. The human 32-kDa stress protein induced by exposure to arsenite and cadmium ions is heme oxygenase. *FEBS Lett* 245: 173–176, 1989.
48. Tenhunen R, Marver HS, and Schmid R. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med* 75: 410–421, 1970.
49. Toki T, Itoh J, Kitazawa J, Arai K, Hatakeyama K, Akasaka J, Igarashi K, Nomura N, Yokoyama M, Yamamoto M, and Ito E. Human small Maf proteins form heterodimers with CNC family transcription factors and recognize the NF-E2 motif. *Oncogene* 14: 1901–1910, 1997.
50. Vignali M, Hassan AH, Neely KE, and Workman JL. ATP-dependent chromatin-remodeling complexes. *Mol Cell Biol* 20: 1899–1910, 2000.
51. Yoshida C, Tokumasu F, Hohmura KI, Bungert J, Hayashi N, Nagasawa T, Engel JD, Yamamoto M, Takeyasu K, and Igarashi K. Long range interaction of cis-DNA elements mediated by architectural transcription factor Bach1. *Genes Cells* 4: 643–655, 1999.

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2. Kati H. Pulkkinen, Seppo Yl##-Herttuala, Anna-Liisa Levonen. 2011. Heme oxygenase 1 is induced by miR-155 via reduced BACH1 translation in endothelial cells. *Free Radical Biology and Medicine* . [CrossRef]
3. Henna-Kaisa Jyrkk##nen, Suvi Kuosmanen, Merja Hein##niemi, Heidi Laitinen, Emilia Kansanen, Eero Mella-Aho, Hanna Leinonen, Seppo Yl##-Herttuala, Anna-Liisa Levonen. 2011. Novel insights into the regulation of the antioxidant response element mediated gene expression by electrophiles: induction of the transcriptional repressor BACH1 by NRF2. *Biochemical Journal* . [CrossRef]
4. Ken Itoh , Junsei Mimura , Masayuki Yamamoto . 2010. Discovery of the Negative Regulator of Nrf2, Keap1: A Historical Overview. *Antioxidants & Redox Signaling* **13**:11, 1665-1678. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
5. John D. Hayes , Michael McMahon , Sudhir Chowdhry , Albena T. Dinkova-Kostova . 2010. Cancer Chemoprevention Mechanisms Mediated Through the Keap1###Nrf2 Pathway. *Antioxidants & Redox Signaling* **13**:11, 1713-1748. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
6. A.K. MacLeod, M. McMahon, S. M. Plummer, L. G. Higgins, T. M. Penning, K. Igarashi, J. D. Hayes. 2009. Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes: demonstration that the KEAP1-NRF2 pathway, and not the BACH1-NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds. *Carcinogenesis* **30**:9, 1571-1580. [CrossRef]
7. Kazuhiko Igarashi , Jiying Sun . 2006. The Heme-Bach1 Pathway in the Regulation of Oxidative Stress Response and Erythroid Differentiation. *Antioxidants & Redox Signaling* **8**:1-2, 107-118. [Abstract] [Full Text PDF] [Full Text PDF with Links]
8. Dr. Jawed Alam . 2006. The Mammalian Cap and Collar Family of Transcription Factors. *Antioxidants & Redox Signaling* **8**:1-2, 39-42. [Citation] [Full Text PDF] [Full Text PDF with Links]