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Heme-dependent up-regulation of the α-globin gene expression by transcriptional repressor Bach1 in erythroid cells☆

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Abstract

The transcriptional factor Bach1 forms a heterodimer with small Maf family, and functions as a repressor of the Maf recognition element (MARE) in vivo. To investigate the involvement of Bach1 in the heme-dependent regulation of the expression of the α-globin gene, human erythroleukemia K562 cells were cultured with succinylacetone (SA), a heme biosynthetic inhibitor, and the level of α-globin mRNA was examined. A decrease of α-globin mRNA was observed in SA-treated cells, which was restored by the addition of hemin. The heme-dependent expression of α-globin occurred at the transcriptional level since the expression of human α-globin gene promoter–reporter gene containing hypersensitive site-40 (HS-40) was decreased when K562 cells were cultured with SA. Hemin treatment restored the decrease of the promoter activity by SA. The regulation of the HS-40 activity by heme was dependent on the NF-E2/AP-1 (NA) site, which is similar to MARE. The NA site-binding activity of Bach1 in K562 increased upon SA-treatment, and the increase was diminished by the addition of hemin. The transient expression of Bach1 and mutated Bach1 lacking CP motifs suppressed the HS-40 activity, and cancellation of the repressor activity by hemin was observed when wild-type Bach1 was expressed. The expression of NF-E2 strengthened the restoration of the Bach1-effect by hemin. Interestingly, nuclear localization of Bach1 increased when cells were treated with SA, while hemin induced the nuclear export of Bach1. These results indicated that heme plays an important role in the induction of α-globin gene expression through disrupting the interaction of Bach1 and the NA site in HS-40 enhancer in erythroid cells.

Keywords: Erythroid differentiation; α-Globin; Heme; Bach1, NF-E2

Heme is a key molecule that plays an important role in either transport or utilization of molecular oxygen, as it is the prosthetic group of heme proteins. In addition, oxygen is an essential requirement of heme biosynthesis. In prokaryotes and yeast, heme is known to participate in gene regulation by functioning as a ligand for the transcription factor [1,2]. In mammals, heme has a profound effect on the proliferation and differentiation of hematopoietic progenitors. Heme not only is incorporated as a structural component of hemoglobin but also causes an increase in the expression of globin as well as enzymes of the heme biosynthetic pathway in erythroid cells [3–6]. The treatment of erythroid cells with hemin (the ferric chloride salt of heme) also increases both the number of transferrin receptor 1 and the intracellular level of ferritin [7,8]. Thus, heme plays a key role in the coordinated expression of several genes during the differentiation of erythroid cells.

The human α-like globin gene cluster is located at the tip of the short arm of chromosome 16 and includes the

☆ *Abbreviations:* ChIP, chromatin immunoprecipitation; CP, cysteine–proline; DMSO, dimethyl sulfoxide; DNase, deoxyribonuclease; Erk, extracellular signal-regulated kinase; FCS, fetal calf serum; HO, heme oxygenase; HS, hypersensitive site; LCR, locus control region; MARE, Maf recognition element; MEL, mouse erythroleukemia; NA, NF-E2/AP-1; SA, succinylacetone.

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embryonic ζ, fetal/adult α2, α1, and θ [9]. It produces α-like globins, which are combined with β-like globins to form hemoglobins, and its mutants cause α-thalassemia, which is one of the most common genetic diseases. High-level expression of the α-like globin genes depends on *cis*-acting DNA elements located 40 kb upstream of the ζ-globin mRNA cap site, which is called HS-40, the deoxyribonuclease (DNase) I hypersensitive site (HS) [10]. The HS-40 consists of specific sequence motifs including a GT motif, three GATA-1 binding sites, and two NF-E2/AP-1 (NA) sites, 5′NA and 3′NA [10–12]. The NA sites in the human HS-40 enhancer are closely related to the Maf recognition elements (MARE) [13]. Various leucine zipper proteins including p45 NF-E2, Nrf1, Nrf2, Bach1, and Bach2 can interact with MARE as heterodimers with small Maf proteins [14].

Among MARE-interacting proteins, Bach1 is unique in that it has a BTB/POZ domain [15], which plays a role in forming a multivalent DNA-binding complex and has been implicated in the regulation of the chromosomal structure, resulting in the repression of transcription [16,17]. Transcriptional factor Bach1 represses the activity of the β-globin gene locus control region (LCR) in erythroid cells [16]. Bach1 is a heme-binding protein, and the DNA-binding activity of Bach1 is negatively regulated by heme-binding [18]. Bach1–heme interaction is mediated by evolutionarily conserved heme regulatory motifs, including the cysteine–proline (CP) dipeptide sequence in Bach1 [18]. We previously reported that heme positively regulates the expression of β-globin at the MAREs via the Bach1 in erythroid cells [19]. To clarify whether the expression of α-globin gene is regulated by transcriptional repressor Bach1, we examined the role of Bach1 in heme-dependent expression of the α-globin gene with human erythroleukemia K562 and mouse erythroleukemia (MEL) cells. Here we report low expression of the α-globin gene in heme-depleted cells and the up-regulation of the expression by heme. We further show that hemin treatment induces the decrease of the interaction of Bach1 with the NA site of the HS-40 in heme-depleted cells. The importance of Bach1 on the expressions of several genes during erythroid differentiation will be discussed.

Materials and methods

Materials. [γ-^{32}P]ATP (6000 Ci/mol) and poly(dI–dC) were purchased from Amersham Biosciences. Transfection reagent Lipofectamine was from Invitrogen. Restriction endonucleases and DNA-modifying enzymes were obtained from Toyobo and Takara. Antibodies for p45 NF-E2 and actin were purchased from Santa Crutz Biotechnology. Antibody for c-Fos was obtained from Oncogene Science. Antibodies specific for Bach1 and MafK were described as previously [20,21]. All other chemicals were of analytical grade.

Cell culture. Human erythroleukemia K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. MEL cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% FCS and antibiotics. K562 and MEL cells were treated with 1 mM succinylacetone (SA) and/or 50 μM hemin, a ferric chloride salt of heme, for 16 h. For the differentiation of MEL cells, the cells were cultured with 2% dimethyl sulfoxide (DMSO) for 48 h and then collected.

Plasmids. The regions containing the 452-bp promoter and the HS-40 enhancer of the human α1-globin gene [10,22] were amplified by PCR. Primers used were: 5′-AACTCGAGTCGATGCGCGTC CAGC-3′ (A) and 5′-AAAAGCTTGGTGGGTTCTCTCTGAGT-3′ (B) for the promoter region and 5′-AAGAGCTCTGGAACCTAT CAGGGA-3′ (C) and 5′-AAACGCGTCTCTCAGATAAACAG GAG-3′ (D) for HS-40 enhancer. The α1-globin promoter was cloned into the *Xba*I–*Hin*dIII site of pGL3 Basic, resulting in pGLαp. A *Sac*I–*Mlu*I fragment of HS-40 enhancer was isolated and inserted into the *Sac*I–*Mlu*I site of pGLαp, named pGLαHS. Reporter plasmids pGLαHS5′NAM, pGLαHS3′NAM, and pGLαHSDM were constructed as follows. In the first step of PCR, we used pGLαHS plasmid as a PCR template. Primer pairs used were primer (C) as a 5′ primer, the mutagenic primer m5′NAF (5′-GCCACCAAAACTCAGTGCT-3′), and primer (D) as a 3′ primer, and the mutagenic primer m5′NAR (5′-AGCTGAGTTTTGGTTGGC-3′) for 5′NA mutation. On the other hand, in the preparation of 3′NA mutation, we used m3′NAF (5′-AGGACTGCAAAGTCATCCTGT-3′) and m3′NAR (5′-ACAG GATGACTTTGCAGTCCT-3′) as mutagenic primers. In the second step, we used (C) and (D) primer pairs to amplify the full-length HS-40 region with mutation, and the DNA fragments were purified, sequenced, and inserted into pGLαp, to give pGLαHS5′NAM and pGLαHS3′NAM. Double mutation on 5′NA and 3′NA was prepared from 5′NA mutation used as a PCR template, and was cloned and named pGLαHSDM. The p45 NF-E2 expression plasmid was constructed as follows. The full-length p45 NF-E2 was inserted into the *Xba*I site of eukaryotic expression vector pEF-MCIneo [23]. Mammalian expression plasmids carrying wild-type Bach1 and its derivative lacking the heme-binding sites (Bach1mCP1-6) were described previously [18].

Northern blot analysis. Total RNA was isolated from cultured cells by the guanidium isothiocyanate–phenol chloroform extraction method [24]. Total RNA (20 μg) was loaded in 1% formaldehyde agarose gel electrophoresis and transferred to nylon membrane. Hybridization, washing, and detection were carried out as described previously [19]. The intensity of hybridization signals and the RNA concentration were quantified using an Advantec DMC-33c densitometer.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) was carried out as described previously [25]. Briefly, to fixation, approximately 1×10^8 K562 cells were treated with 1% formaldehyde for 10 min at room temperature. Immunoprecipitations were performed for overnight at 4 °C with specific antibodies, followed by isolation of immunoprecipitates with protein A–Sepharose beads. DNA fragments were extracted with phenol/chloroform and ethanol was precipitated. PCR was carried out as described previously [19]. The DNA fragment of HS-40 enhancer was amplified with primers (C) and (D) as above. After PCR, products were electrophoresed on 6% polyacrylamide gels.

Immunoblot analysis. Whole cell extracts, cytoplasmic, and nuclear fractions were prepared as described [26]. The samples of treated or untreated MEL cells were subjected to SDS–PAGE and electroblotted onto poly(vinylidene difluoride) membrane. Immunoblotting was carried out with anti-Bach1, anti-p45 NF-E2, anti-c-Fos, and anti-actin as the primary antibodies.

Luciferase assay. Cells were transfected with the reporter plasmids pGL3B, pGLαp, pGLαHS, pGLαHS5′NAM, pGLαHS3′NAM, and pGLαHSDM, and pRL-CMV (Promega, Madison, WI) using a Lipofectamin reagent, according to the manufacturer's recommendation. After transfection, cells were cultured with SA and/or hemin for 16 h and washed twice with PBS. They were then lysed in a Reporter lysis buffer (Promega), the lysate was centrifuged, and the supernatants

were assayed for luciferase. The luciferase activity was carried out as described previously [19]. All transfection experiments were carried out at least three times independently, and the averages are shown together with standard deviations.

Gel-shift assay. Nuclear extracts were prepared from treated- and untreated-K562 cells (1×10^7) as described previously [27]. The two probes for gel shift assay were synthetic double-stranded 5′NF-E2/AP-1 (5′-CCAACCATGACTCAGTGC-3′; the core sequence underlined) and 3′NF-E2/AP-1 (5′-GACTGCTGAGTCATCCTG-3′) located in HS-40 [10]. The nuclear extracts were incubated with ^{32}P-labeled probe (35,000 cpm) in a reaction buffer containing 25 mM Hepes, pH 7.9, 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethyl sulfonyl fluoride, and 100 μg/ml poly(dI–dC) [26] at 37 °C for 10 min. After incubation, the reaction mixture was loaded onto 4% polyacrylamide gels containing 45 mM Tris–borate and 1 mM EDTA, pH 8.0, and electrophoresed at 100 V at room temperature. The gels were dried and exposed to X-ray film at −80 °C.

Results

Effects of SA and hemin on α1-globin mRNA in K562 and differentiated MEL cells

SA is a specific inhibitor of δ-aminolevulinic acid dehydratase, the second enzyme of the heme biosynthetic pathway. To examine the effects of SA and hemin on α1-globin mRNA expression, we carried out Northern blot analysis. The α1-globin mRNA was significantly decreased when human erythroleukemia K562 cells were cultured in the presence of 1 mM SA for 16 h, and this decrease was cancelled by the addition of 50 μM hemin (Fig. 1A). When MEL cells were differentiated with 2% DMSO for 48 h, α1-globin mRNA was induced (Fig. 1B). This increase in the expression of α1-globin mRNA was markedly decreased when the cells were treated with 2% DMSO plus 1 mM SA. Furthermore, the decrease caused by the inhibition of heme synthesis was restored by the addition of 50 μM hemin to the medium, suggesting that the expression of α1-globin mRNA in erythroid cells is controlled by heme in both uninduced and induced cells.

It is reported that the DNaseI hypersensitive site located at 40 kb upstream of the ζ-globin mRNA cap site, HS-40, a-major regulatory element (Fig. 2) [9], plays an important role in the α-globin gene cluster expression. The HS-40 enhancer consists of at least five functional motifs that are bound with factors in vivo. In an erythroid-lineage- and developmental stage-specific manner [10]. These motifs include a GT motif, two GATA-1binding sites, and two NF-E2/AP-1-binding sites, named as 5′NA and 3′NA [10–12]. The NA motif(s) can be recognized by a number of different factors including the more ubiquitously expressed AP-1, small Maf protein, erythroid-enriched-NF-E2, Bach1, and Bach2 [14,28,29]. To clarify whether the heme-dependent expression of α1-globin mRNA is regulated at the transcriptional level, we investigated the effects of SA and hemin on the human α-globin gene promoter–reporter

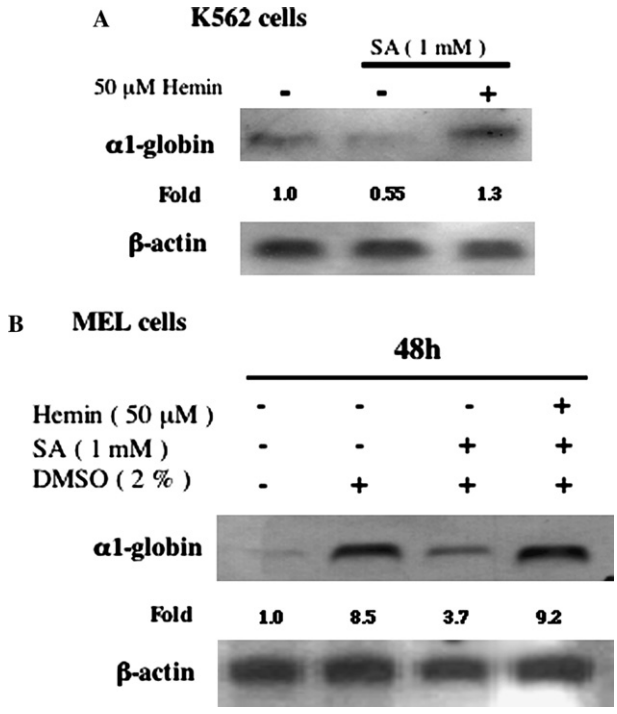


Fig. 1. Effect of SA and hemin on the expression of α1-globin mRNA in K562 and MEL cells. (A) Northern blots of α1-globin in SA- or hemin-treated K562 cells. K562 cells were treated with or without 1 mM SA plus 50 μM hemin for 16 h. (B) MEL cells were cultured in the presence of 2% DMSO alone or with SA for 48 h. Total RNA was collected, separated by electrophoresis, transferred onto a nylon membrane, and hybridized with the biotin-labeled probe specific for mouse α1-globin mRNA (upper panel) and β-actin mRNA as an internal control (lower panel). The levels of the α1-globin mRNA are indicated as the fold induction relative to the respective RNA of the untreated K562 cells, after calibration against the level of the β-actin mRNA.

gene in K562 cells. The reporter plasmid pGLαp contains the upstream 452-bp of the human α1-globin gene, and pGLαHS was made by inserting human HS-40 α-globin enhancer into pGLαp. The reporter activity of pGLαp was weak and no change in the activity was observed on treatment with 1 mM SA or 50 μM hemin for 16 h (Fig. 3A). The reporter activity was high in pGLαHS-transfected K562 cells, thereby indicating that the enhancer activity of the HS-40. HS-40-dependent reporter activity was decreased to 60% when K562 cells were treated with 1 mM SA. This decrease was cancelled and up-regulated by the addition of 50 μM hemin. We previously reported that the expression of β-globin by heme was regulated at the MARE site, which is very similar to NAs in HS-40, within LCR on β-globin gene promoter [19]. To clarify heme-response elements in the HS-40 region, we examined the mutation construct of the two NA sites in HS-40 (Fig. 3B). As shown in Fig. 3C, no changes were detected in the activity of pGLαHS3′NAM (3′NAM) compared with that of pGLαHS, whereas the basal activity of pGLαHS5′NAM (5′NAM) markedly decreased, and the loss of reporter activity by SA was not restored by the addition of hemin. The reporter

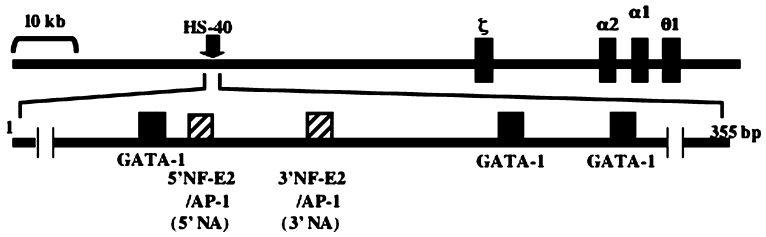


Fig. 2. The linkage map of human α-like globin gene cluster. The transcriptional factor binding motifs of HS-40 are shown below the map.

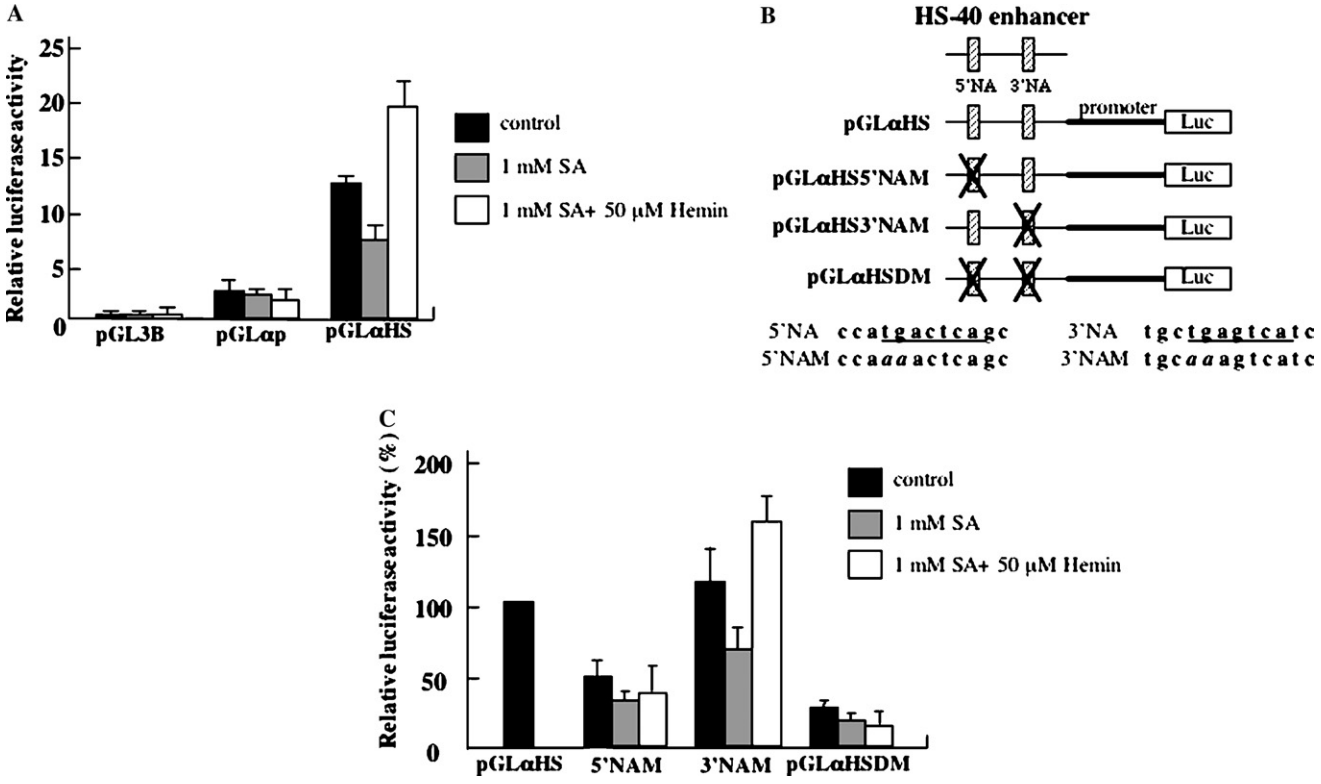


Fig. 3. Regulation of human α-globin HS-40 enhancer by heme. (A) Transcriptional activity of the human α-globin HS-40 enhancer in K562 cells. The cells were transfected with pGL3 Basic (Promega), pGLαp or pGLαHS plasmids and cultured in the presence of the indicated compound for 16 h. (B) Mutation constructs of two NA sites in HS-40 enhancer. Mutations were constructed as described in Materials and methods. Comparison of normal NA sites and NAs carrying mutation within HS-40 enhancer is shown. Core sequences of NA sites are underlined. (C) Reporter activity of HS-40 carrying mutation on each NA site. K562 cells were transfected with pGLαHS, pGLαHS5′NAM (5′NAM), pGLαHS3′NAM (3′NAM), and pGLαHSDM plasmids and cultured with 1 mM SA or 1 mM SA plus 50 μM hemin for 16 h. Luciferase activity was measured and normalized to the *Renilla* luciferase activity.

activity of pGLαHSDM containing mutations at two NAs was decreased to the level of the pGLαp activity. The activity did not change significantly by the treatment with SA or SA plus hemin. These results indicate that a decrease of the level of heme in the cells leads to suppression of the enhancer activity of the HS-40, and the 5′NA site in the HS-40 contributes to heme-dependent regulation.

Heme counteracts the repression of Bach1 on enhancer activity of HS-40

We previously reported on transcriptional factor Bach1which can bind to heme, and that heme-dependent regulation of the expression of β-globin gene was mediated by Bach1 [19]. We then examined whether Bach1 affects the heme-dependent activity of the HS-40 enhancer. Fig. 4A shows the HS-40 activity in Bach1-expressing K562 cells. Wild-type Bach1 suppressed activities of three reporter plasmids, pGLαHS, 5′NAM, and 3′NAM. The repressor activity of Bach1 was counteracted by the addition of hemin. Bach1 mutant (Bach1mCP1-6) carrying multiple mutations in the heme-binding CP motifs, which does not bind to heme [18], was expressed. The mutant also suppressed the reporter activity but the repressor activity was not affected by hemin treatment (Fig. 4B). No changes were detected in the reporter activity of pGLαHSDM with or without hemin.

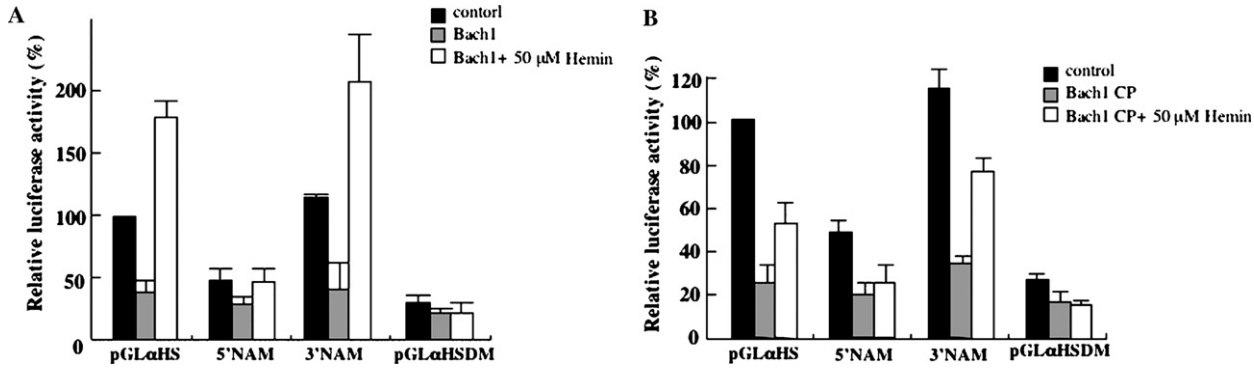


Fig. 4. Effect of wild-type Bach1 and mutated Bach1 for the HS-40 enhancer activity in K562 cells. K562 cells were co-transfected with each reporter plasmid vector (pGLαHS, 5′NAM, 3′NAM or pGLαHSDM) and plasmids carrying the wild-type Bach1 (A) or the mutated Bach1mCP1-6 (B). The cells were cultured with or without 50 μM hemin for 16 h. The reporter assay was carried out as above. Data are the average of three separate experiments.

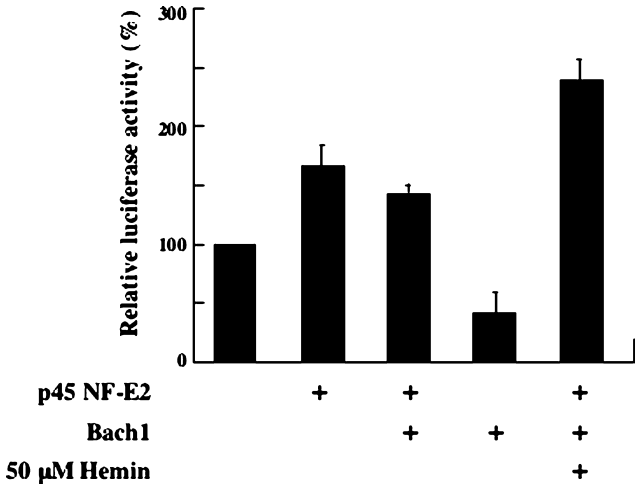
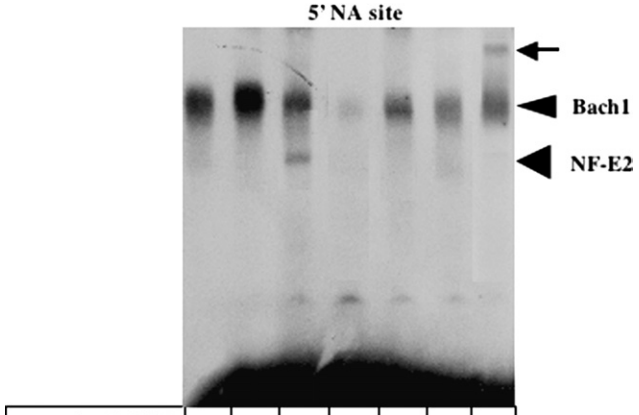


Fig. 5. Relation between Bach1 and p45 NF-E2 in HS-40 enhancer. K562 cells were co-transfected with the wild-type reporter along with 0.5 μg of each expression plasmids for Bach1 and p45 NF-E2 as indicated. The cells were cultured with or without 50 μM hemin. Reporter gene assay was carried out, as above. Data are average of three independent experiments.

We next examined the regulatory relationship between p45 NF-E2 and Bach1 by co-transfection assay. As shown in Fig. 5, NF-E2 (p45 and small Maf heterodimer) activated the HS-40 enhancer in the absence of exogenous Bach1. When co-expressed, furthermore, NF-E2 overcame Bach1 repression and activated the reporter activity. Hemin treatment further strengthened the active effect of NF-E2. These data showed that Bach1 suppresses the activity of the HS-40 enhancer via two NA sites and that NF-E2 plays an important role as an antagonist of the suppressor Bach1 to activate the enhancer activity of HS-40 for α-globin gene.

Bach1 and NF-E2 bind to NA site in α-globin HS-40 enhancer

The above results revealed that two NAs were essential for the heme-dependent regulation of the HS-40 reporter gene expression via Bach1 and NF-E2. To further verify the binding of Bach1 and NF-E2 to NA in the HS-40, we carried out a conventional gel-shift assay using the oligonucleotide DNA probe corresponding to the NA site (Fig. 6). The specific band, NF-E2, which was supershifted with anti-p45 NF-E2 antibodies (Fig. 6, lane 7), was only detected with hemin-treated cells (lane 3). On the other hand, Bach1-DNA complex increased in SA-treated cells and was decreased by anti-Bach1 (lanes 2 and 5). Furthermore, anti-Bach1 had no effect in hemin-treated cells (lane 6). When the 3′NA probe was used for gel-shift assay, similar results with 5′NA probe were obtained (data not shown).



1 mM SA	-	+	+	-	+	+	+
50 μM Hemin	-	-	+	-	-	+	+
competitor	-	-	-	+	-	-	-
Anti-Bach1	-	-	-	-	+	+	-
Anti-p45	-	-	-	-	-	-	+

Fig. 6. Gel-shift assay of extracts from K562 cells treated with SA and hemin. K562 cells were cultured with 1 mM SA and 50 μM hemin for 16 h. Nuclear extracts were prepared as described. The pre-incubation of nuclear extracts with antibodies for Bach1 was performed at 4 °C for 30 min. A reaction mixture containing radiolabeled probe was prepared with 4 μg of each nuclear extract. Bach1 and NF-E2 indicate the Bach1–DNA and NF-E2–DNA complexes, respectively. The supershifted band by anti-p45 is indicated by an arrow.

Bach1 and NF-E2 bind to HS-40 enhancer of the α-like globin gene cluster in vivo

Finally, to investigate the recruitment of Bach1 and NF-E2 to the HS-40 in K562 cells treated with SA and hemin, we carried out the ChIP assay. The presence of the HS-40 in chromatin immunoprecipitates was analyzed by semi-quantitative PCR using specific pairs of primer spanning the HS-40 of α-globin gene (Fig. 7). An increase in the binding of Bach1 on HS-40 was detected in SA-treated cells and this increase was cancelled by the addition of hemin, while the NF-E2 binding was increased in the cells treated with hemin. In the immunoprecipitation using anti-MafK antibodies, the binding of MafK to HS-40 was decreased in SA-treated cells. Then we examined changes in nuclear localization of Bach1 transcription factor when cells were treated with SA and hemin for 16 h, using Western blot analysis (Fig. 8). In untreated cells, Bach1 was detected in both cytosolic and nuclear fractions. Interestingly, the nuclear accumulation of Bach1 was increased upon SA-treated cells, and furthermore, the Bach1 was exported from nuclear to cytosol by hemin treatment. In the case of transcriptional activator p45 NF-E2, which heterodimerizes with small Maf protein and binds to MARE in β-globin LCR, we observed that the nuclear localization was reduced in SA-treated cells and the p45 NF-E2 increased in nuclei by the addition of hemin. No change was detected in the nuclear localization of c-Fos. These results revealed that heme regulated the binding of the transcription factor Bach1 and NF-E2 to HS-40 in vivo.

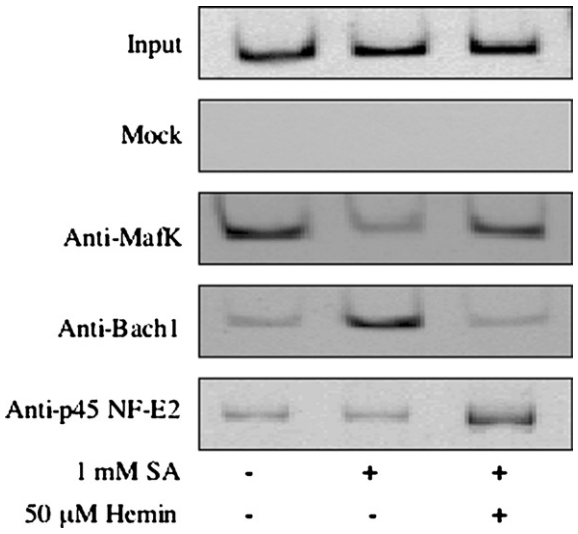


Fig. 7. In vivo detection of HS-40 occupancy by Bach1 and NF-E2 using chromatin immunoprecipitation. Chromatin immunoprecipitation was performed with lysates from K562 cells treated with or without 1 mM SA or 1 mM SA plus 50 μM hemin. Input corresponds to PCR containing 0.5% of the total amount of chromatin used in immunoprecipitation reactions. Mock immunoprecipitation with irrelevant and control antibodies (anti-ferrochelatase) is shown. PCR was performed to amplify HS-40 with 25 cycles, where the amount of the products increases linearly at each cycle.

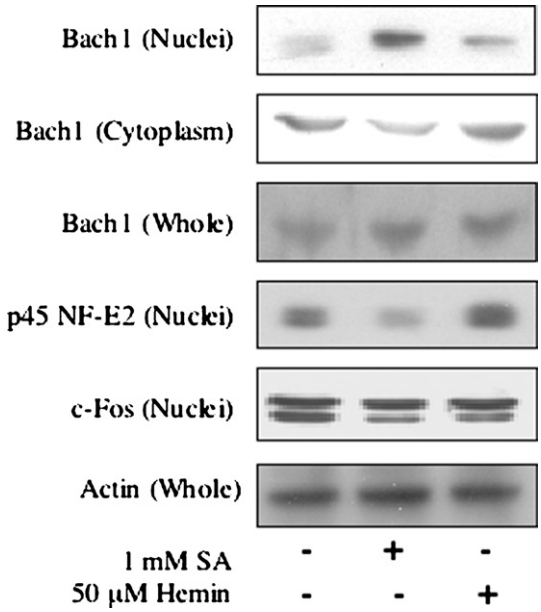


Fig. 8. Nuclear localization of Bach1 in SA- or hemin-treated K562 cells. Whole cell extracts, cytoplasmic and nuclear extracts were prepared from K562 cells treated with 1 mM SA or 50 μM hemin for 16 h. Each extract was separated, blotted, and subjected to Western analysis using antibodies against Bach1, NF-E2, c-Fos, and actin.

Discussion

Here we demonstrated that Bach1 regulates the heme-dependent expression of α-globin gene in human and mouse erythroid cells. α-Globin mRNA expression was decreased in K562 cells and DMSO-induced MEL cells when heme biosynthesis was inhibited by treatment with SA, and this decrease was restored by the addition of hemin. The promoter assay of the α-major regulatory element, HS-40, revealed that the expression of α-globin gene is regulated directly by hemin at the transcriptional level. There were several reports that heme increases the expression of α- and β-globin, transferrin receptor 1, ferrochelatase, and erythroid-specific δ-aminolevulinic acid-synthase [3–8], resulting in the prompting of erythroid differentiation. K562 cells have served as a model to research hematological cell differentiation and are a suitable material for study on the α-globin gene expression during erythroid differentiation because K562 cells do not express the adult β-globin gene [30]. In addition to its effect on hemoglobin synthesis, treatment of K562 with hemin resulted in up-regulation of the expression of erythroid markers, such as the Kell–Cellano blood group antigens, M and Pra antigens of glycophorin, A and the P1 antigen [31,32]. Although mechanisms involved in the induction of various genes by hemin are unknown, we found that the HS-40 enhancer confers

to the activation of the transcription of α-globin gene by heme in K562 cells.

The HS-40 enhancer is the major *cis*-acting regulatory element responsible for the developmental stage- and erythroid lineage-specific expression of the human α-like globin genes, the embryonic ζ and the fetal/adult α2/α1. The HS-40 consists mainly of three GATA-1 binding sites and two NA sites (Fig. 2). These five transcriptional binding motifs play different roles in erythroid differentiation. Analysis by site-directed mutagenesis [33,34] revealed that these HS-40 enhancer motifs, 5′NA and three GATA-1 sites, positively regulate the ζ-globin gene and the α-globin gene promoter activities in the early and/or late stage of erythroid differentiation. Our observations showed that the promoter activity decreased and the up-regulation by treatment of hemin was lost when the HS-40 activity was examined with mutated NA, 5′NAM (Fig. 3). Wen et al. [34] reported that a 1-bp mutation in the 3′NA motif, which was expected to abolish the function of NF-E2 but not that of AP1, decreased α-globin promoter in DMSO-induced MEL cells. However, the enhancer activity of HS-40 with 3′NAM carrying 2-bp mutations did not change in both untreated and hemin-treated K562 cells, but dual mutations containing 5′NAM and 3′NAM markedly reduced the activity of HS-40 enhancer (Fig. 3). These observations suggest that affinities of transcriptional factors bound to 3′NAM differ from that of 1-bp mutation in the 3′NA motif or that the functions of 3′NA may be different depending on the species of the cell. The present data indicate that the 5′NA site of HS-40, but not the 3′NA, contributes to the heme-dependent up-regulation of the enhancer activity.

Both NA motifs of HS-40 are similar to MAREs. The MAREs can be recognized by several transcriptional factors, including NF-E2, AP-1, small Maf family or other proteins such as Nrf1, Nrf2, Nrf3, Bach1, and Bach2 [14,28,29]. The present study showed the binding of Bach1 to NA sites by gel-shift assay (Fig. 6) and this was supported by observations that the HS-40 activity was decreased by the expression of Bach1 (Fig. 4). Heme-dependent enhancement of the HS-40 activity was accompanied by the decrease of the suppressive activity of Bach1 with heme. We previously reported [18] that heme bound specifically to Bach1 caused the decrease of its DNA-binding activity. The expressions of wild-type Bach1 and mutated Bach1mCP1-6 in the promoter assay led to the decrease of the activity of the α-globin gene enhancer, but hemin-dependent up-regulation of the HS-40 activity was only found when the wild-type Bach1 were expressed in the K562 cells (Fig. 4). There is a recent study [21] showing the mechanisms of heme-dependent induction of heme oxygenase-1 (HO-1): namely, Bach1 bound to the MAREs of the HO-1 enhancer and suppressed the HO-1 expression in untreated cells. Increased level of heme leads to the release of Bach1 from enhancers by inhibiting DNA-binding and allows activators, including Nrf2 to bind to the enhancers.

A report appeared showing that Bach1 was excluded from nuclei to cytoplasm when the cells were treated with cadmium, which inhibited the binding of Bach1 to the *cis*-element of the HO-1 gene [35]. The cadmium-induced nuclear export of Bach1 was mediated by Crm1/exportin-1, as well as extracellular signal-regulated kinase (Erk) 1/2 activity, via the cytoplasmic localization signal of the C-terminal region within Bach1 [35]. The present data showed that Bach1 in the nuclear fraction increased in SA-treated K562 cells while it decreased in hemin-treated K562 cells. Furthermore, we also showed that Bach1-dependent suppression of the HS-40 activity through NA sites was cancelled by the addition of hemin. There were several reports that hemin increases the activities of several transduction pathways, including Erk1/2 [36] and soluble guanylate cyclase-protein kinase G in K562 cells [37], although mechanisms involved in the export form nuclei of Bach1 by heme have not been demonstrated.

Transcriptional activator NF-E2 comprises the hematopoietic-specific subunit p45 and the more ubiquitously expressed small Maf protein p18/MafK [38,39]. Using ChIP assay, we found that the intracellular level of heme regulates recruitments of NF-E2 and Bach1 to the HS-40, and that NF-E2 and Bach1 compete for binding to NA sites. We observed that p45 NF-E2 increased its binding to the HS-40 in vivo and activated the enhancer activity of α-globin gene in hemin-treated K562 cells (Figs. 5 and 7). On the other hand, the present and previous studies [19] showed that Bach1 bound to the HS-40 enhancer as well as the β-globin LCR in vivo, and suppressed the expression of the globin genes. During DMSO-induced MEL cell differentiation, NF-E2 may switch in the occupancy of Bach1 in the α- and β-globin gene enhancers, leading to the production of the globins gene per se later stages. It has been recently shown that intracellular levels of heme control the exchange of small Maf protein partners in not only the differentiation-associated control of β-globin expression but also the stress-responsive control of HO-1 [20]. Similar to the induction of β-globin gene, the increased level of heme promotes the displacement of Bach1 from the MafK-occupied HO-1 enhancers, which is followed by the binding of transcriptional activator Nrf2 instead of p45 NF-E2 to these elements [21].

It is evident that the β-globin LCR is one of the targets of Bach1 where heme acts as a positive regulator of the β-globin gene transcription by regulating the interaction of Bach1 with the MAREs in the HS2 site [19,40]. We also found that the 5′NA site in HS-40 of α-globin gene is one of the target elements of Bach1 where heme plays a crucial role in the expression of the α-globin gene

by disrupting the interaction between Bach1 and the NA site in the HS-40 enhancer during erythropoiesis. Thus, heme acts as an inducer in the expressions of α-globin gene via the HS-40 and β-globin gene via HS2, thereby enhancing the synthesis of adult hemoglobin at a late stage of differentiation in erythroid cells. In addition to erythropoiesis, heme positively regulates the differentiation of several tissue cells, including neural [41], adipotic [42], and muscular cells [43]. During cell differentiations, heme regulates the expression of various tissue specific genes. We therefore propose that the interaction of Bach1–heme plays important roles in control of gene expression.

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