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Heme Positively Regulates the Expression of β-Globin at the Locus Control Region via the Transcriptional Factor Bach1 in Erythroid Cells

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Abbreviations: kb, kilobases; DMSO, dimethylsulfoxide; MEL, mouse erythroleukemia; FCS, fetal calf serum; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; MAREs, Maf-recognition elements; PBS, phosphate buffered saline; LCR, locus control region; HS, hypersensitive sites; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PVDF, poly(vinylidene difluoride); ALA, δ-aminolevulinic acid; ALA-S2, erythroid specific δ-aminolevulinic acid synthase. SA, succinylacetone; DFO, desferrioxamine:

Abstract

The transcription factor Bach1 hetero-dimerizes with small Maf proteins, to repress Maf recognition element (MARE) -dependent gene expression. The repressor activity of Bach1 is inhibited by the direct binding of heme. To the investigate involvement of Bach1 in the heme-dependent regulation of the expression of the β-globin gene, mouse erythroleukemia (MEL) cells were cultured with succinylacetone (SA), a specific inhibitor of heme biosynthesis, and the level of β-globin mRNA was examined. A marked decrease of β-globin mRNA in SA-treated cells was observed, and was reversed by the addition of hemin. An iron chelator, desferrioxamine, also lowered the level of β-globin mRNA. The heme-dependent expression of β-globin is a transcriptional event since the expression of the human β-globin gene promoter-reporter gene containing the micro-locus control region (μLCR) was inhibited when human erythroleukemia K562 cells and MEL cells were cultured with SA. Hemin treatment restored the decrease in promoter activity caused by SA. The control of the μLCR-β-globin promoter reporter gene by heme was dependent on DNase I-hypersensitive site 2 which contains MARE. Transient expression of Bach1 suppressed the μLCR activity, and this repressor activity was cancelled by treatment with hemin. The expression of a mutated Bach1 lacking heme-binding sites led to a loss in the heme-responsiveness of the μLCR . The MARE-binding activity of Bach1 in K562 and MEL cells increased upon SA-treatment, and the increase was diminished by the treatment with hemin. Furthermore, during erythroid differentiation of MEL cells, the MARE-binding activity of Bach1 decreased while simultaneously, the NF-E2 activity increased. We propose that heme positively

regulates the β-globin gene expression by blocking the interaction of Bach1 with the MARE in the LCR in erythoid cells.

Introduction

The biochemical role of heme is related to either the transport or the utilization of oxygen and therefore, heme exerts regulatory effects on various cell functions that sense oxygen. In addition, oxygen is an essential requirement of heme biosynthesis. In yeast, heme regulates the expression of genes involved in the respiratory chain, heme biosynthesis and oxidative stress, at the transcriptional level, via heme-responsive transcription factors HAP1 and HAP2/3/4/5p (1). 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 (2-4). Hemin treatment also increases both the number of transferrin receptor and ferritin content (5,6). Thus, heme plays a key role in the coordinated expression of several genes during erythroid differentiation.

The human globin gene cluster spans a region of 70 kb containing five developmentally regulated genes including ε, γ_G, γ_A, δ, and β. The entire region is controlled by the micro-locus control region (μLCR) (7). Namely, high–level expression of globin genes requires the μLCR, an upstream region that enhances globin gene transcription and insulates the locus from the influence of flanking elements (8.9). Evidence from chickens and transgenic mice indicates that switching of the globin genes involves competition between globin promoters for enhancement by the μLCR (9). The

human and mouse LCR located upstream of the ε-globin gene consist of 4 and 6 DNase I hypersensitive sites (HS), respectively (10). Targeted disruption of HS in both mouse culture cells and mice has revealed that the μLCR is not required to initiate or maintain open chromatin and basal transcription, suggesting that the main function of the μLCR is the enhancement of transcription (9,11). Each HS contains AP-1, Sp-1, and/or GATA-like sites. The AP-1 like sites in the mouse HS 1-4 are closely related to the Maf-recognition elements [MAREs: TGCTGA(T/GT)TCAGCA] (12). Various leucine-zipper proteins including p45 NF-E2, Nrf1, Nrf2 and Bach1 can interact with MAREs as heterodimers with the small Maf proteins (13). HS2 is sufficient to confer high level and tissue-specific expression on a linked β-globin gene of transgenic mice (14). Two tandem MAREs strongly contribute to the overall activity of HS2 (15,16).

Among MARE-associated proteins, Bach1 is unique, in that it has a BTB/POZ domain (17). Bach1 forms a multivalent DNA binding complex, raising the possibility that it can act as an architectural component that is simultaneously able to crosslink multiple MAREs, resulting in the repression of transcription (18,19). Bach1 is a heme-binding protein and the DNA binding activity is negatively regulated by heme (20). Furthermore, Bach1 is involved in the regulation of heme oxygenase-1 (HO-1), a rate-limiting enzyme of heme catabolism. The heme-mediated inhibition of Bach1 confers heme-inducibility upon HO-1 (21). These observations suggest that Bach1 is involved in the regulation of other heme-responsive genes besides HO-1. We were particularly interested in the fact that the enhancer activity of the LCR is induced by hemin-treatment of erythroid cells (15,16). To clarify whether Bach1 regulates β-globin gene

expression, we examined heme-dependent expression of the β-globin gene in human erythroleukemia K562 and MEL cells, and the regulation of the interaction of Bach1 with the LCR by heme. Here we demonstrated that heme positively regulates the β-globin gene expression by disrupting the interaction of Bach1 with the LCR.

Materials and Methods

Materials--- [γ-^{32}P] ATP and poly (dI-dC) were purchased from Amersham-Pharmacia Co. Restriction endonucleases and DNA modifying enzymes were obtained from Takara Co. and Toyobo Co. Nylon membranes (Biodiene Type B) were products of Pall Co. The transfection reagent Lipofectamine was from Invitrogen Co. Ltd. Anti-c-Fos antibodies were purchased from Oncogene Science Co. Antibodies for Bach1 and anti-MafK were as previously described (21). All other chemicals were of analytical grade.

Plasmids- An 838-bp human β-globin gene promoter from BGT14LCR (22) was cloned into the *Bgl*II-*Hind* III site of pGL3B, resulting in pGLβp. A 3 kb *Not*I-*Sal*I fragment of the μLCR cassette was also isolated and inserted into a blushed *Sac*I site of pGLβp, to give pGLCRhβ. A reporter plasmid pGLCRhβHS2mut, lacking the tandem MAREs within the mouse HS2, was constructed as follows. Using pGLCRhβ (19) as a template, flanking regions of the tandem MAREs were amplified by PCR. Primers used were:5'-GAATTCGCCGCTCCCACCTCCAGCTTAGGGTGTGTGCCCAGATGTTC-3' (a) and 5'-CGAGCCCGGGCTAGCACGCGTA-3' (b) for one side, and 5'-CTGGAGGTGGGAGCGGCGAATTCGCTTGAGCCAGAAGGTTTGCTTAG-3' (c) and 5'-CTCAGAGCCTGATGTTAATTTAGC-3' (d) for the other side. Amplified DNA were purified, mixed, and subsequent PCR was carried out using the above primers (b)

and (d). The resulting HS2 DNA lacked the tandem MAREs where *EcoR*I site was introduced instead. The mutated HS2 DNA was digested with *Spe*I and used to replace the SpeI fragment, containing the wild-type HS2 DNA, on the pGLCRhβ. The resulting pGLCRhβHS2mut carried the HS4, HS3, and the mutated HS2 fused with the β-globin promoter and firefly luciferase gene. Mammalian expression vectors carrying wild type Bach1 and its derivative lacking the heme-binding sites (Bach1mCP1-6) were described previously (17,20).

Cell Cultures--- Mouse Balb/ 3T3 cells and MEL cells were grown in DMEM supplemented with 7% FCS and antibiotics. Human erythroleukemia K562 cells were grown in RPMI 1640 medium supplemented with10% FCS and antibiotics.. To reduce the level of heme in cells, the cells were incubated in medium supplemented with 7% FCS in the presence of 1 mM SA for 16h. The amounts of heme and porphyrin in the cells were determined as previously described (23). For the differentiation of MEL cells, the cells were cultured with 2% DMSO for 72h and then collected.

Reporter Assay- Cells were transfected with the reporter plasmids pGL3B, pGLβp, pGLCRhβ and pGLCRhβHS2mut, and pRL-CMV (Promega Co.) using a Lipofectamine reagent, according to the manufacturer's recommendation. The cells were incubated with 1 mM SA for 16h after the transfection and washed twice with PBS. They were then lysed in a Reporter lysis buffer (Promega Co.), the lysate was centrifuged and the supernatants were assayed for luciferase. The *Photinus* and *Renilla* luciferase assays were performed according to the protocol for the Dual Luciferase Assay System (Promega Co.). Transfection efficiency was normalized on the basis of

Renilla luciferase activity.

Gel- Shift Assay-Nuclear extracts were prepared by the method of Schneider et al. (24) with some modifications. Briefly, MEL cells (1 x 10^7) and K562 cells (1 x 10^7) left untreated or treated with 1mM SA for 16h were washed with 10 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl, and then lysed with 10 mM HEPES, pH 7.9, containing 0.6% Nonidet P-40, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. The homogenates were centrifuged and the nuclear pellet was resuspended in 100 µl of 20 mM HEPES, pH 7.9, containing 400 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. The nuclear extracts were obtained by centrifugation at 4℃. Nuclear extracts were also obtained from MEL cells cultured with 2% DMSO for 48h and 72h. The oligonucleotide probe containing the MARE/NF-E2 site from the chicken β-globin enhancer was described previously (25) and was end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. 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 DTT, 0.2 mM PMSF and 100 µg/ml poly(dI-dC)(26). After a 10-min incubation at 37° C, the reaction mixture was loaded onto 4% polyacrylamide gels containing 50 mM Tris, 380 mM glycine and 1 mM EDTA, pH 8.5, and electrophoresed at 100 V at room temperature. The gels were exposed to X-ray film at –80 ℃.

RNA blots----Total RNA was isolated from the cells by the guanidium isothiocyanate method (23). The RNA (20 µg) was applied to a 1% agarose gel, electrophoresed, and subsequently transferred onto a nylon membrane (Biodiene typeB) for hybridization

with biotin-labeled antisense β-globin and actin RNAs. The filters were hybridized and washed, according to the method of Suzuki et al. (27). Hybridized RNA was incubated with alkaline phophatase-conjugated avidin, washed and examined for positive signals with a Luminol reagent (Roche Mol. Bio. Inc.). The RNA concentration was quantified using an Advantec DMU-33c densitometer.

Results

Effects of SA and hemin on β-globin mRNA in MEL cells. SA is known as a competitive inhibitor of ALA dehydratase, the second enzyme of the heme biosynthetic pathway. MEL cells were cultured in the presence of 1 mM SA or an iron chelator, DFO (100 μM) for 16h, after which the amount of heme in the cells was measured. The heme content of SA- and DFO-treated cells decreased to about 70% and 45%, respectively, of that of control cells (Fig. 1). Whereas the expression of globin genes is markedly induced upon the differentiation of MEL cells treated with DMSO, α- and β-globin are known to be synthesized at lower levels in uninduced MEL cells. To examine whether SA or DFO causes any change in the β-globin expression in the absence of the differentiation-inducer DMSO, Northern blot analysis of β-globin mRNA in uninduced cells was carried out. As shown in Fig. 2A, β-globin mRNA in SA-treated cells decreased to about 40%. Since the level was restored on the addition of 50μM hemin, the effect of SA was through its inhibition of heme synthesis. Treatment of the cells with 100 μM DFO also resulted in a decrease of β-globin mRNA. Again, culture of the cells with 50 μM hemin reversed the decrease caused by DFO. When MEL cells were induced to differentiate with 2% DMSO for 48h, β-globin mRNA was

expressed (Fig. 2B). The increase in β-globin mRNA upon the differentiation of MEL cell was markedly inhibited when the cells were cultured with 1 mM SA in addition to DMSO. These obstacles were reversed by culture with 50 μM hemin, suggesting that the expression of β-globin mRNA in MEL cells is controlled by heme irrespective of whether cells are induced to differentiate or not.

To clarify whether the heme-dependent expression of β-globin mRNA is regulated at the transcriptional level, we investigated the effect of SA on the human β-globin gene promoter/LCR reporters in human erythroleukemia K562 cells. The reporter plasmid pGLβp contains the upstream 836bp of the human β-globin gene while pGLCRhβ was made by inserting human μLCR (28) into pGLβp (19). The reporter activity was weak in pGLβp-transfected K562 cells and no change in the activity was observed on treatment with 1 mM SA or 50 μM hemin for 16h (Fig. 3A). The reporter activity was strengthened when pGLCRhβ was transfected, consistent with the enhancer activity of the μLCR. The μLCR-dependent reporter gene activity decreased to 60% when the culture was treated with 1 mM SA. This loss of reporter activity was restored upon the addition of 50 μM hemin. To confirm these observations in other cells, MEL cells were transfected with pGLβp and pGLCRhβ, and effects of SA and hemin on the reporter activity were also examined. As shown in Fig. 3B, the decrease in pGLCRhβ activity caused by SA, and its cancellation by hemin, was similar to that in K562 cells. In contrast, when Balb/ 3T3 cells were examined, neither the μLCR-dependent activation nor the SA-dependent inhibition of the β-globin gene reporter was observed (Fig. 3C). Thus, heme induces the expression of the β-globin gene by stimulating the

enhancer activity of the β-globin LCR in erythroid cells.

Involvement of HS2 of the LCR in the regulation of hemin-dependent expression in K562 and MEL cells ---- Among the DNase I-hypersensitive sites (HSs), HS3 and HS4 harbor single MAREs, and HS2 contains tandem duplicated MAREs. It was shown previously that hemin induces the enhancer activity of the tandem MAREs of HS-2 (previously referred to as the NF-E2 site) in K562 cells (15,16). To verify that the tandem MAREs are involved in hemin-dependent regulation of the reporter gene, activities of a reporter plasmid pGLCRhβHS2mut carrying the mutated MAREs of HS2 in the μLCR were compared using cells treated with SA or hemin. As shown in Fig. 4A, the basal activity of pGLCRhβHS2mut in K562 cells was significantly less than that of the wild type control pGLCRhβ. Moreover, the reporter activity of pGLCRhβHS2mut was not affected by SA or hemin. When MEL cells were induced to differentiate with 2% DMSO for 24h, the reporter activity of pGLCRhβ was markedly increased as compared with that in uninduced cells (Fig. 4B). The reporter activity of pGLCRhβ was inhibited by SA and the effect of SA was cancelled by simultaneously adding hemin. In contrast, the reporter activity of pGLCRhβHS2mut did not change significantly in the presence of SA or SA plus hemin (Fig. 4B). These results indicate that a deficiency of heme in the cells leads to the suppression of the enhancer activity of the LCR and that this regulation by heme involves the tandem MAREs within the HS2.

Regulation of hemin-dependent expression in erythroid cells by Bach1

The above results confirm previous observations that the tandem MAREs were essential for the DMSO-induced expression of the μLCR reporter gene. Bach1 interacts

with the tandem MAREs in vitro and competes for the sites with p45 NF-E2 (18). Since Bach1 acts as a repressor of MARE-dependent transcription and its DNA binding activity is inhibited by hemin (18,20,21), it may be involved in the regulation of the enhancer activity of HS2 by heme. To examine whether Bach1 affects the hemin-dependent activity of the μLCR, the reporter activity was measured in K562 cells in the presence of the wild-type and heme-insensitive Bach1-expression plasmids. The Bach1 mutant (Bach1mCP1-6) carrying multiple changes in the heme-binding cysteine-proline (CP) motifs does not bind heme and its DNA binding activity is not inhibited by heme (20,21). As shown in Fig. 5A, the reporter activity of pGLCRhβ was suppressed by the wild type Bach1. Consistent with the previous results using a synthetic reporter and HO-1 reporter (20,21), the repressor activity of Bach1 was lost by treating cells with hemin (Fig. 5A). In contrast, the repressor activity of Bach1mCP1-6 was not affected by hemin treatment. As shown in Fig. 5B, the repressor activity of Bach1 further dropped in SA-treated cells. Unlike the case for the wild type Bach1, the reporter activity was not further reduced by SA in Bach1mCP 1-6 expressing cells (Fig. 5B). When Bach1 was expressed in MEL cells, a heme-dependent regulation of the LCR activity, similar to that in the case of K562 cells, was observed (data not shown). These results suggest that Bach1 regulates the heme-dependent activity of the HS2 enhancer.

Finally, we conducted a conventional gel-shift assay using a synthetic oligonucleotide probe containing a MARE from the chicken β-globin 3' enhancer to examine whether the DNA binding activity of endogenous Bach1 is indeed regulated by heme. To characterize the binding of Bach1 and p45 NF-E2, the MARE probe was

incubated with nuclear extracts of untreated and DMSO-treated MEL cells. As reported previously (18), we observed two specific retarding bands (Fig. 6A, lane 2). The upper band disappeared upon preincubation of the nuclear extracts with anti-MafK and Bach1, indicating that the binding complex was composed of Bach1 and small Maf protein. The DNA binding activity of the Bach1/small Maf complex was slightly and markedly decreased in 48h- and 72h-treated cells, respectively (Fig. 6A). The lower band, NF-E2 (p45 and small Maf heterodimer), intensified upon DMSO-induced differentiation. The specific band corresponding to Bach1 intensified when MEL cells were treated with 1 mM SA (Fig. 6B). The DNA binding activity of Bach1 was decreased by treating the cells with SA plus 50 μM hemin. In this experiment, we failed to detect the lower band in control MEL cells, but a weak band was found on longer exposure. In DMSO-treated MEL cells, SA caused an increase in the Bach1 activity, but inhibited the increase in the NF-E2 activity (Fig. 6A). When nuclear extracts from K562 cells were used, the probe containing MAREs exhibited a binding pattern similar to that seen with MEL cells (Fig. 6C).

Discussion

The present study showed that Bach1 plays an important role in the differentiation of erythroid cells and directly regulates the expression of β-globin, mediated by hemin. The expression of β-globin mRNA was reduced when the synthesis of heme in uninduced and DMSO-treated MEL cells was blocked by treatment with SA or DFO. Exogenously added hemin restored the level of β-globin mRNA. These findings were similar to previous observations that the expression of globin chains in MEL and human

erythroid progenitor cells was reduced when these cells were cultured with SA (29,30). Using a promoter assay of β-globin, the present study confirmed that the expression of β-globin mRNA is regulated by hemin at the transcriptional level. There are also many reports that heme induces erythropoiesis by the coordinate expression of α-, and β-globin, transferrin receptors, ferrochelatase and ALAS2 (4,30,31). In erythroid cells, it is reported that heme acts as on not only a transcription factor NF-Y for ferritin synthesis (32) but also kinases including the heme-regulated inhibitor kinase HRI (33). Heme is well known to regulate the synthesis of globin chains at the translational step, mediated by HRI. We have shown for the first time that the hemin-dependent activation of β-globin expression at the transcriptional level occurred at the HS2 of the LCR where is interacted with Bach1.

Analysis by atomic force microscopy revealed large looped DNA structures between MAREs located in different regulatory elements within the human β-globin LCR formed by Bach1/MafK heterodimer (19). Based on the observation that the formation of these loops required the Bach1 BTB/POZ protein interaction domain (19), Bach1 would function as an architectural factor and cause the repression of the enhancer activity of the LCR. We confirmed that Bach1 suppresses the activity of the μLCR and strongly suggest that the heme-binding ability of Bach1 contributes to the hemin-dependent induction of the LCR enhancer (Fig. 5). Consistent with previous findings of the formation of a multimetric and multivalent DNA complex with the ability to bind multiple MAREs simultaneously (18) and that β-globin LCR holocomplex can be formed by structurally connecting the MAREs present in HS2, HS3, and HS4 of the

LCR (9,19), Bach1 contributes to the generation of a multiprotein complex as a repressosome among the HS of the LCR (34). We have now showed the physiological role of the interaction of Bach1 with HS2 in the regulation of the expression of β-globin.

Recently chromatin immunoprecipitation experiments with anti-MafK and anti-p45 NF-E2 showed that the LCR was occupied by small Maf proteins in uninduced MEL cells where the synthesis of globin is suppressed (35) and p45 NF-E2 was recruited to the LCR as well as the active globin promoters on erythroid differentiation. Although Bach1 and NF-E2 share MAREs and effectively compete with them (18), the apparent binding of these molecules in cells is different. Namely, the present staudy and a previous study (18) showed that the DNA complex with Bach1 was down-regulated at the late stage of DMSO-induced MEL cell differentiation while the NF-E2 complex was induced to form by DMSO treatment. Bach1 may be involved in the assembly of the LCR complex at the early stages of hematopoietic cell differntiation, while p45 NF-E2 contributes to the activation of globin genes *per se* at the late stage. In fact, the treatment of MEL cells with DMSO increased NF-E2 activity significantly (36), which is supported by the present observation that the reporter activity of pGLCRhβ in DMSO-induced MEL cells was markedly increased as compared with that in uninduced cells (Fig. 4B). Thus, the induction of the p45 NF-E2 binding activity during erythroid differentiation implies that the replacement of the Bach1 associated with small Maf molecules with the NF-E2 complex occurs dependent on the stage of the differentiation. Based on the fact that the level of intracellular heme was markedly elevated in DMSO-treated cells, and a marked decrease in Bach1 activity was observed in hemin-treated

cells, heme contributes to the loss of function of Bach1 during erythroid differentiation. This is supported by the finding that the expression of Bach1 and Bach1mCP1-6 led to a decrease in the activity for the LCR enhancement, but the hemin-dependent restoration of the μLCR activity was only observed in wild type Bach1-expressing cells (Fig. 5). Similarly, a recent study (21) clarified the mechanisms involved in the induction of heme oxygenase-1 by hemin. Namely, Bach1 represses the expression of heme oxygenase-1 under physiological conditions, and an increased level of heme displaces Bach1 from the enhancers by inhibiting DNA binding, allowing activators to bind the enhancers. Thus, heterodimers of small Maf and p45 NF-E2 related activators including Nrf2 are most likely the form binding to the heme oxygenase-1 enhancers upon trancriptional activation.

We now provide evidence of a new function of heme directly regulating the transcription of genes. For a long time, it has been considered that intracellular heme has a significant role in the transcriptional up-regulation of several erythroid-specific proteins, including globin chains, transferrin receptors, ferritin, and enzymes of the heme biosynthetic pathway (3). We identified the LCR β-globin gene as one of the targets of Bach1 where heme acts as a positive regulator of the transcription of the β-globin gene, by regulating the interaction of Bach1 with the MARE region of the μLCR (HS2). We propose that transcriptional regulation of β-globin is a direct sensing of heme levels during the terminal differentiation of erythroid cells. Since Bach1 is ubiquitously expressed in a variety of tissues, there may be activation systems for various genes involving the replacement of the repressor Bach1 with some enhancer

protein, the event being triggered by heme.

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Fig. 1. Heme content of SA- or DFO-treated MEL cells. MEL cells were cultured with or without 1 mM SA or 100 μM DFO for 16h. The determination of heme in the cells was carried out, as described in Materials and Methods. Data are the average of 3 independent experiments (Bars=S.D.).

Fig. 2. Northern blots of β-globin in SA- or hemin-treated MEL cells. A. MEL cells were treated with or without 1 mM SA, 1 mM SA plus 50 μM hemin, 100 μM DFO or 100 μM DFO plus 50 μM hemin for 16h. Total RNA was collected, separated by electrophoresis, transferred onto a membrane, and hybridized with the biotin-labeled probe specific for mouse β-globin (Upper panel). B. MEL cells were also cultured in the presence of 2% DMSO alone or plus 1 mM SA for 48h.

Fig. 3. Transcriptional activity of the human LCR β-globin gene promoter in K562 (A), MEL (B) and Balb/ 3T3 (C) cells. The cells were transfected with pGL3 Basic (Promega Co.), pGLβp or pGLCRhβ vectors and cultured in the presence of the indicated compound for 16h. Luciferase activity was measured and normalized to the *Renilla* luciferase activity. Data are the average of 5 independent experiments (Bars=S.D.).

Fig. 4. Effect of deletion in MAREs of the HS2 on the transient activity of the human LCR β-globin gene in K562 (A) and DMSO-induced MEL cells (B). The cells were transfected with a luciferase reporter plasmid containing the human LCR β-globin promoter or one containing the mutated HS2 (pGLCRhβHS2mut). The culture of K562 cells and MEL cells with 2% DMSO for 24h, and the measurement of luciferase activity were as above. Data are the average of 4 independent experiments (Bars=S.D.).

Fig. 5. Transcriptional activity of wild type and mutated Bach1 for the LCR β-globin gene in K562 cells. (A) K562 cells were co-transfected with pGLCRhβ vector and plasmids carrying the wild type Bach1 or Bach1 mutations in the cystein-proline motifs (Bach1mCP1-6) (0.5-1.5 μg). The cells were cultured with or without 50 μM hemin. (B) pGLCRhβ was also transfected with Bach1 or Bach1mCP1-6 (1.0 μg) into K562 cells. The cells were treated with 0.25-0.5 mM SA for 16h. The reporter gene assay was carried out, as above. Data are the average of 3 separate experiments.

Fig. 6. Gel-shift assay of the Bach1-MARE binding activity in induced and uninduced MEL cells, and K562 cells. A. MEL cells were cultured with 2% DMSO for the indicated time. MEL (B) and K562 (C) cells were also treated with the indicated compound for 16h. Nuclear extracts were prepared, as described. The pre-incubation of nuclear extracts with antibodies for Bach1, MafK, and c-Fos was performed at 4° C for 30 min. A reaction mixture containing a radiolabeled MARE probe was prepared with 4 μg of each nuclear extract. The positions of Bach1- and p45 NF-E2-DNA complexes are shown by an arrow and arrow head, respectively.

Fig. 1

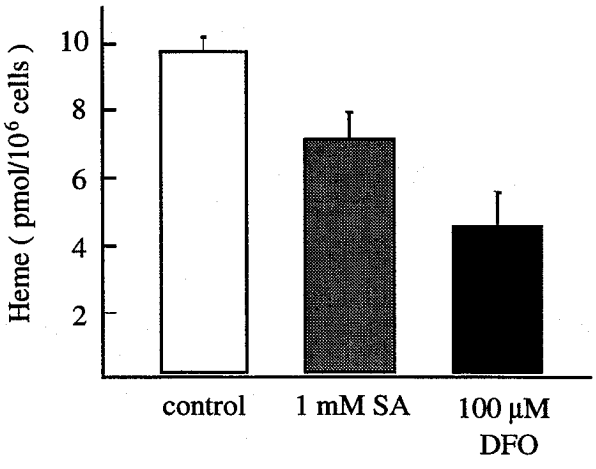


Fig. 2

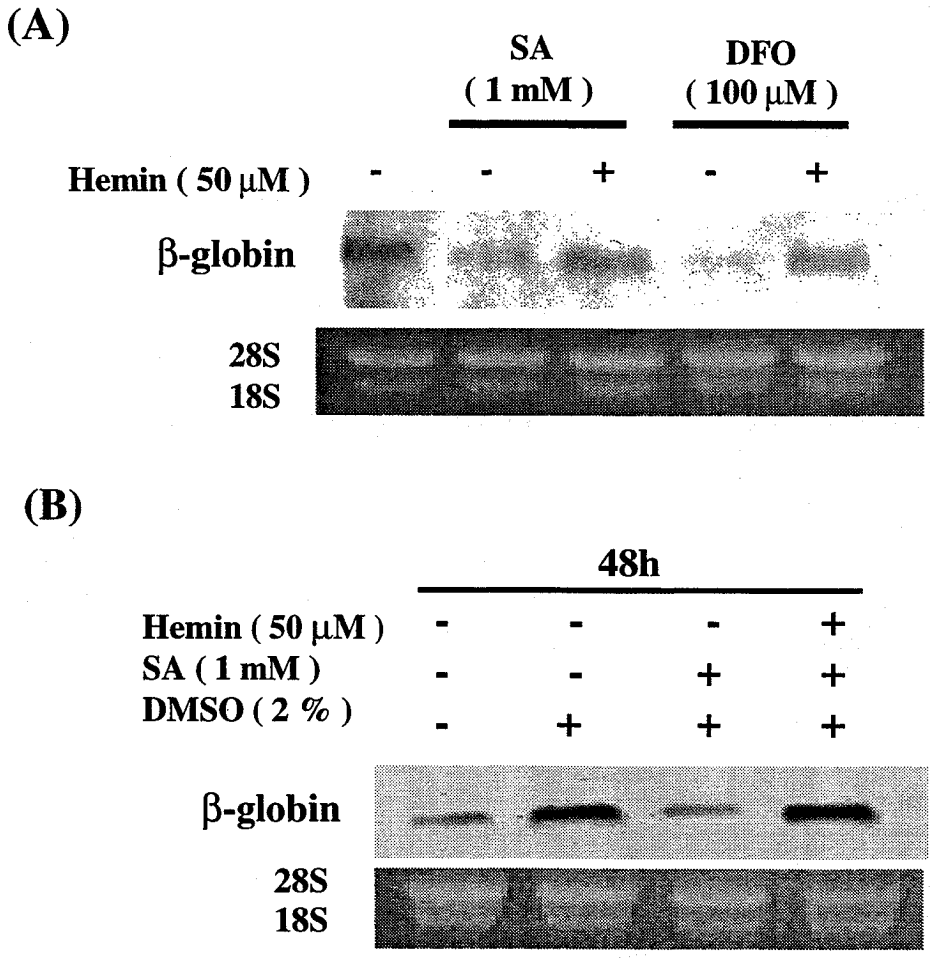

(A)
SA
(1 mM)
DFO
(100 μM)
Hemin (50 μM)
- - + - +
β-globin
28S
18S
(B)
48h
Hemin (50 μM) - - - +
SA (1 mM) - - + +
DMSO (2 %) - + + +
β-globin
28S
18S

Fig. 3

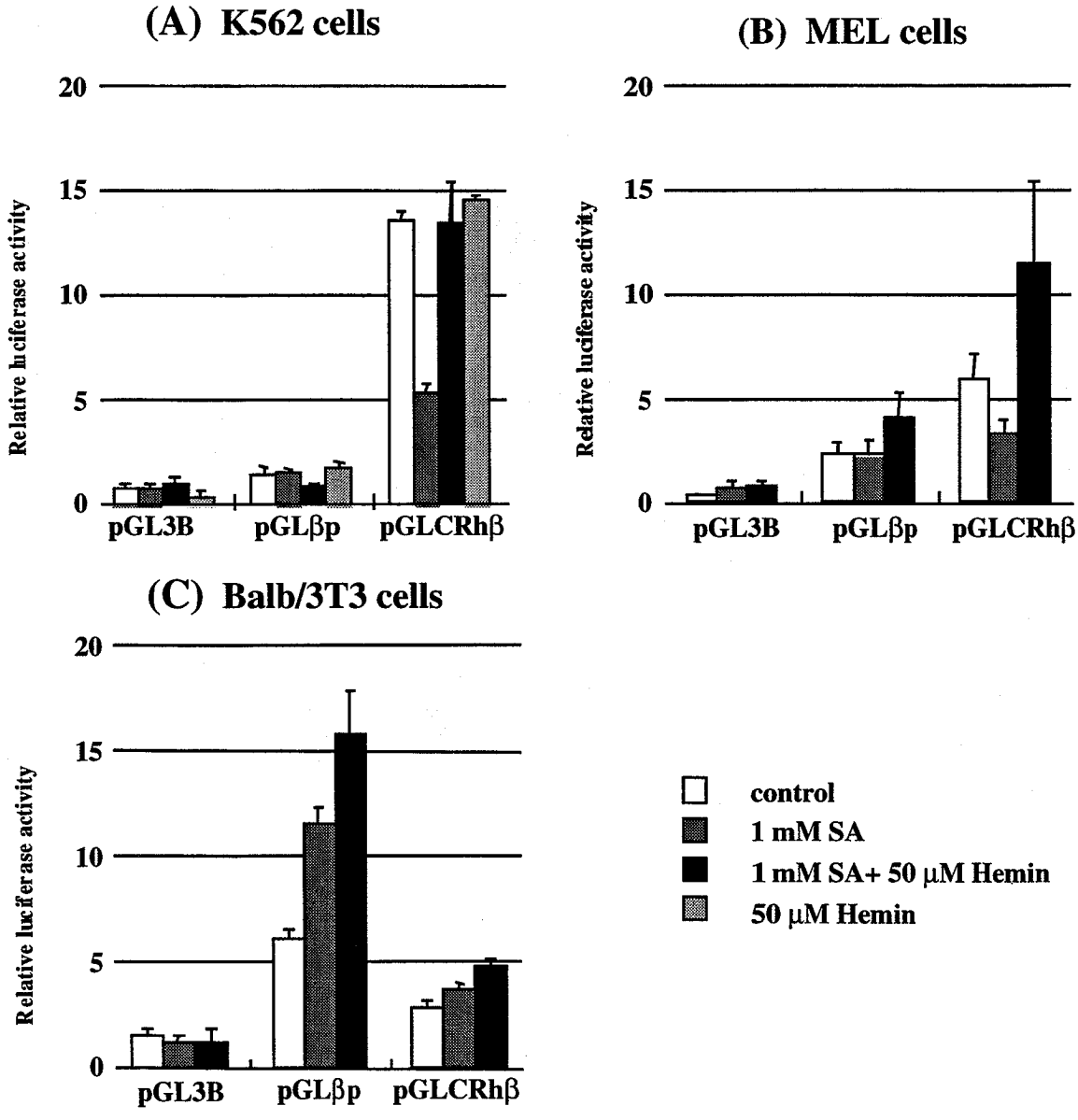

(A) K562 cells
(B) MEL cells
(C) Balb/3T3 cells
Relative luciferase activity
20
15
10
5
0
pGL3B
pGLβp
pGLCRhβ
control
1 mM SA
1 mM SA+ 50 μM Hemin
50 μM Hemin

Fig. 4

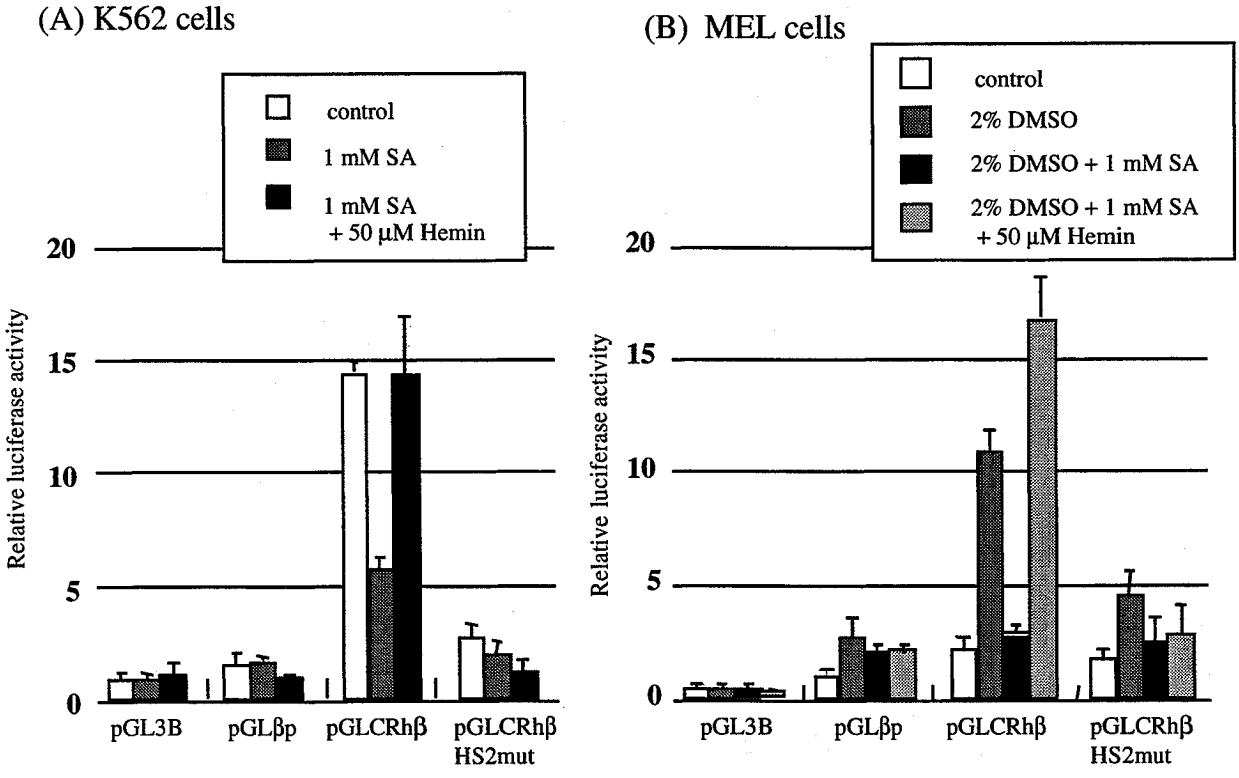

(A) K562 cells
control
1 mM SA
1 mM SA
+ 50 μM Hemin
Relative luciferase activity
20
15
10
5
0
pGL3B
pGLβp
pGLCRhβ
pGLCRhβ
HS2mut
(B) MEL cells
control
2% DMSO
2% DMSO + 1 mM SA
2% DMSO + 1 mM SA
+ 50 μM Hemin
Relative luciferase activity
20
15
10
5
0
pGL3B
pGLβp
pGLCRhβ
pGLCRhβ
HS2mut

Fig. 5

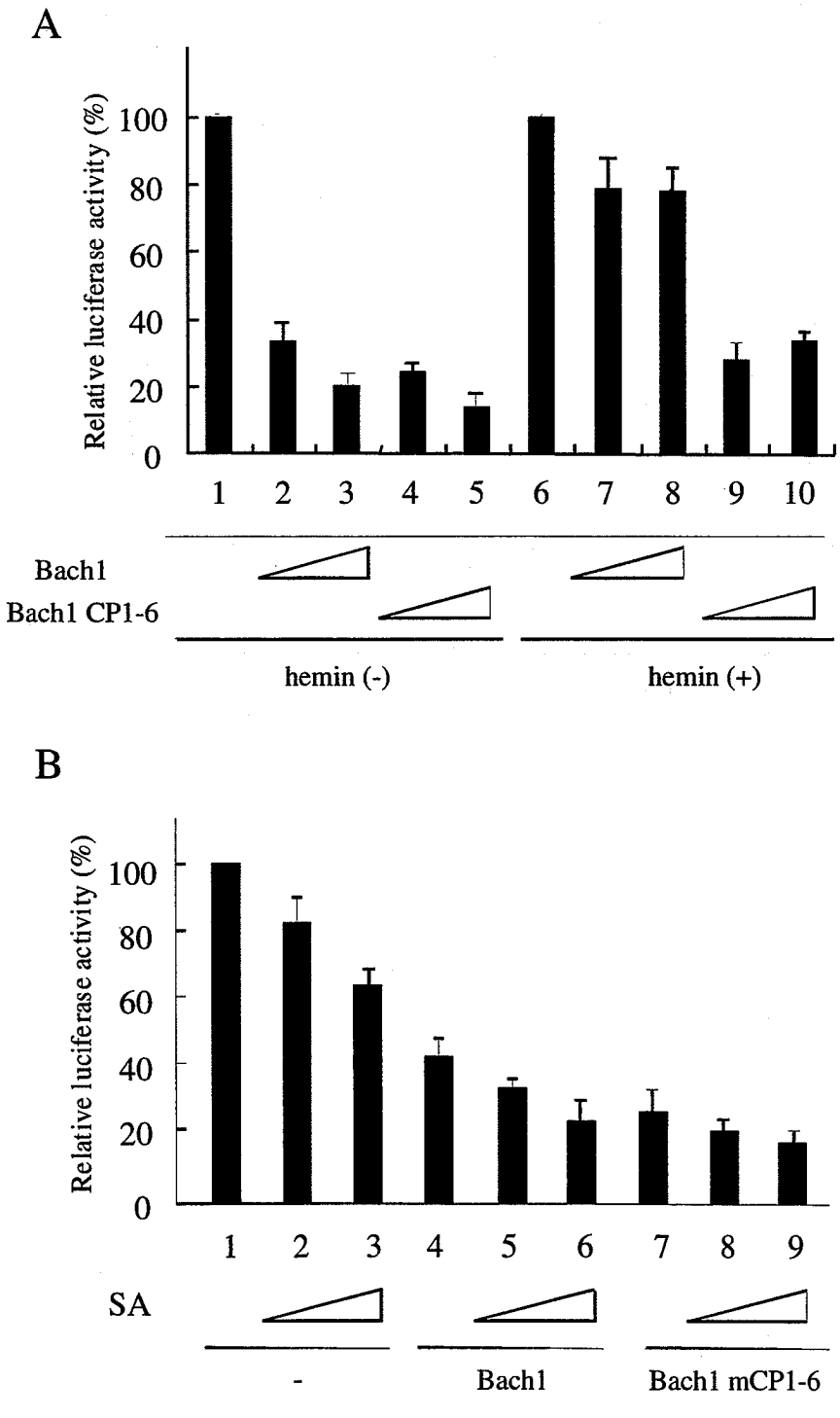
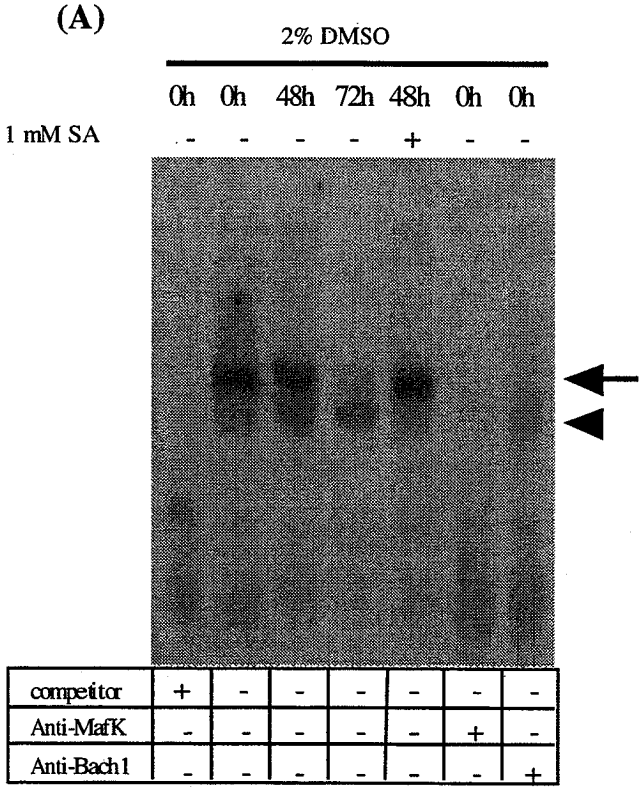
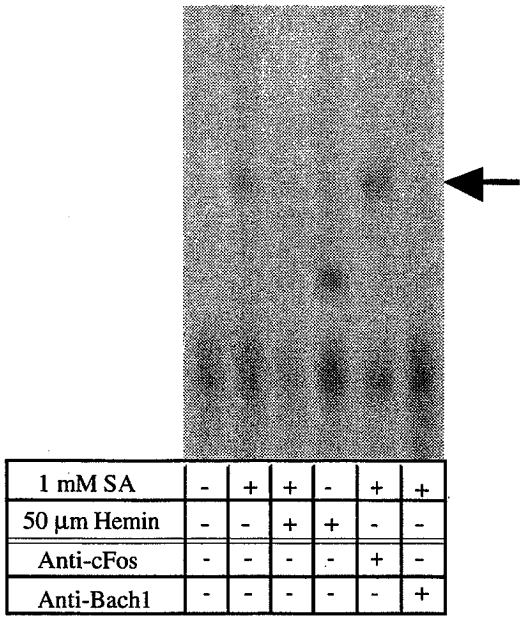

A
Relative luciferase activity (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Bach1
Bach1 CP1-6
hemin (-)
hemin (+)
B
Relative luciferase activity (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9
SA
-
Bach1
Bach1 mCP1-6

Fig. 6

(A)



(B) MEL cells



(C) K562 cells