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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

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Members of the small Maf family (MafK, MafF, and MafG) are basic region leucine zipper (bZip) proteins that can function as transcriptional activators or repressors. The dimer compositions of their DNA binding forms determine whether the small Maf family proteins activate or repress transcription. Using a yeast two-hybrid screen with a GAL4-MafK fusion protein, we have identified two novel bZip transcription factors, Bach1 and Bach2, as heterodimerization partners of MafK. In addition to a Cap'n'collar-type bZip domain, these Bach proteins possess a BTB domain which is a protein interaction motif; Bach1 and Bach2 show significant similarity to each other in these regions but are otherwise divergent. Whereas expression of Bach1 appears ubiquitous, that of Bach2 is restricted to monocytes and neuronal cells. Bach proteins bind in vitro to NF-E2 binding sites, recognition elements for the hematopoietic transcription factor NF-E2, by forming heterodimers with MafK. Furthermore, a DNA binding complex that contained MafK as well as Bach2 or a protein related closely to Bach2 was found to be present in mouse brain cells. Bach1 and Bach2 function as transcription repressors in transfection assays using fibroblast cells, but they function as a transcriptional activator and repressor, respectively, in cultured erythroid cells. The results suggest that members of the Bach family play important roles in coordinating transcription activation and repression by MafK.

Protein interactions within and between various families of transcription factors play important roles in gene regulation during the development of multicellular organisms. Heterodimer formation by basic leucine zipper (bZip) proteins is typical of such interactions, conferring novel functions that differ from those of the individual proteins. Hence, to understand the roles of bZip proteins in gene regulation, it is crucial that the physiological partners for each factor be identified and that dimer formation as regulated spatially and temporally during embryogenesis be analyzed.

Transcription factor NF-E2, which was originally identified as an erythrocyte-specific DNA binding activity (45), is a heterodimeric factor that consists of a large and a small bZip subunit (1, 3, 33, 52). The large subunit of NF-E2 is p45, which belongs to the Cap'n'collar (CNC)-type bZip protein family (1, 8, 52). The mammalian CNC family includes p45 NF-E2, Nrf1/LCR-F1/TCF11 (7, 9, 43), and Nrf2 (47), the last being closely related to chicken ECH (34). These proteins and the *Drosophila* transcription factor CNC (46) show high structural similarity in their bZip domains as well as conservation of the preceding regions, referred to as the CNC domain (7). The other half of NF-E2 is contributed by the so-called small Maf family, which consists of MafK (also known as p18), MafG, and MafF (3, 21, 31, 33, 37). These small Maf family proteins possess a conserved basic region that is characteristic of the Maf family (21) but lack any canonical transactivation domain. Various dimeric combinations of the small Maf family proteins bind in vitro to a DNA sequence motif called T-MARE [TGCTGA(G/C)TCAGCA] containing a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element [TRE; TGA(G/C)TCA] (37, 38). Furthermore, the small Maf family proteins form heterodimers with p45 and ECH and the resultant heterodimers can bind to the NF-E2 consensus site [TGCTGA(G/C)TCA(T/C)] that is related to the T-MARE, whereas p45 and ECH show very low levels of DNA-binding activity by themselves (1, 31, 33, 34). In contrast, Nrf1/LCR-F1/TCF11 appears to bind to the NF-E2 site as a homodimer (7).

NF-E2 sites have attracted particular attention because of their presence in the locus control region (LCR) of the β-globin gene cluster. The β-globin LCR was initially recognized as a series of DNase I-hypersensitive sites and is composed of five DNase-hypersensitive sites (HS-1 to HS-5) (19, 68). In transgenic mice, the LCR was found to direct copy-number-dependent, position-independent gene expression (25). This activity is a result of redundant combinations of *cis* regulatory sites and is not attributable to a single regulatory site (6). In contrast, the very strong erythroid enhancer activity of the LCR has been mapped within HS-2 and established to be dependent on the tandem NF-E2 sites (6, 48, 53, 54, 69). However, mysteries regarding effector molecules of NF-E2 sites still abound, since (i) p45 NF-E2 is dispensable for functions of the β-globin LCR in erythroid cells (61), (ii) p45-related proteins that can bind to the NF-E2 sites, as summarized above, also exist and (iii) NF-E2 sites in HS-2 are occupied by proteins in hematopoietic cells even before commitment to erythroid lineage, suggesting

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that the program of β-globin activation is set in motion at an earlier stage (35).

Another feature of NF-E2 sites is that similar sequences are also frequently found in regulatory regions of various nonerythroid genes (38), suggesting that Maf- and CNC-related factors regulate gene expression through NF-E2-like sites not only in hematopoietic cells but also in a variety of systems. For example, antioxidant response elements contain NF-E2 site-like sequences (reference 71 and references therein). Our previous observation that the *mafK* gene is expressed specifically in neuronal, mesenchymal, and hematopoietic cells during mouse embryogenesis also suggests importance for the small Maf network in various developmental programs (50).

Previous studies showed that the small Maf family proteins are unique transcription factors which can switch from transcriptional repressors to activators, depending on the dimer compositions (31, 33, 37). For example, the small Maf-p45 heterodimers activate transcription whereas small Maf-Fos heterodimers exert a repressive effect. To understand the molecular mechanisms and roles of the small Maf interactor network, we set out to define constituent components. In this report, we describe the cloning and characterization of members of a novel bZip transcription factor family, Bach, which form heterodimers with MafK and bind to NF-E2 sites. In addition to a leucine zipper, the Bach family contains a BTB domain (Broad complex–Tramtrack–Bric-a-brac domain, also known as the POZ [poxvirus and zinc finger] domain), an emerging protein interaction motif (4, 10, 75). The Bach family appears to connect the MafK network with different regulatory networks or to different levels of transcriptional regulation through unique structural and functional features.

MATERIALS AND METHODS

Two-hybrid system. *Saccharomyces cerevisiae* two-hybrid screening was performed by the Matchmaker two-hybrid system (Clontech). To construct a bait plasmid, the entire open reading frame (ORF) of mouse MafK (31) was fused to the DNA binding domain of GAL4 on the pGBT9 plasmid (Clontech). A mouse day 17 post coitus (pc) embryonic Matchmaker cDNA library (Clontech) was transformed into the HF7c yeast strain along with the MafK bait plasmid. Approximately 2×10^7 double transformants were tested for their ability to grow on His^- medium. Of 950 His^+ transformants, 67 clones were scored positive for LacZ expression. To obtain larger cDNA clones, a mouse brain cDNA library in Lambda ZAPII (Stratagene) was screened by hybridization with cDNA fragments. Inserts of the positive phage clones were rescued into plasmids by in vivo excision, resulting in pBSA1-3 and pBSF69-J. Sequences of the cDNA clones were determined on both strands with an ABI Prism dye terminator cycle-sequencing ready reaction kit (Perkin-Elmer) and an automated DNA sequence analyzer (ABI, model 373A). Additional screening of the brain cDNA library with a *bach2* cDNA probe yielded another cDNA clone termed pBSF69-F.

To examine protein interactions within yeast cells, HF7c cells were transformed with various combinations of plasmids that express DNA binding domain (DBD)-tagged and activation domain (AD)-tagged molecules. Transformants were diluted in water and spotted onto His^- and His^+ media to test for activation of the GAL4-dependent *HIS3* reporter gene.

Plasmids. Expression plasmids of Bach1 and Bach2 were constructed as follows. Eukaryotic expression plasmid pGFP-c2 (Clontech) was digested with *Nhe*I and *Xho*I to remove the green fluorescent protein-coding region and circularized again, resulting in pCMVKM. The entire ORF of Bach1 was isolated as a *Sal*I fragment from pA1 and inserted into the *Sma*I site of pCMVKM, resulting in pCMV/Bach1. To construct pCMV/Bach2, the entire ORF of Bach2 was isolated as an *Xba*I-*Xho*I fragment from pBSF69-J and inserted into the *Sma*I site of pCMVKM. The F69-J and F69-F cDNAs were recombined at the internal *Apa*I site to construct a cDNA encoding a presumptive full-size F69-F-type ORF. The resulting composite cDNA was transferred into pCMVKM, resulting in pCMV/Bach2(F).

To construct prokaryotic expression plasmids, cDNA fragments of Bach1 and Bach2 that included the bZip-coding domains were inserted into pMal-c2 (New England Biolabs). The resulting plasmids encoded the carboxy-terminal 453 and 329 amino acids of Bach1 and Bach2, respectively, fused to the maltose-binding protein (MBP). The prokaryotic expression plasmids of chicken ECH and mouse MafK, the eukaryotic expression plasmid of mouse MafK, and the luciferase reporter plasmids pRGBP2 and pRGBP4 were described previously (30, 34). Expression plasmids for GAL4 DBD-tagged Bach1 were constructed as follows.

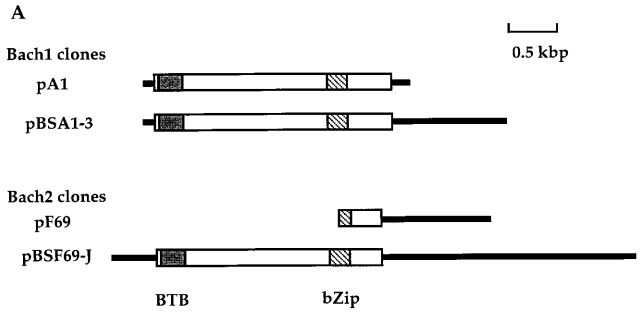


FIG. 1. (A) Schematic representation of Bach1 and Bach2 cDNA clones. Protein-coding domains are indicated by boxes. pF69 contained the leucine zipper-coding domain but lacked the basic region-coding domain. (B) Nucleotide and deduced amino acid sequences of *bach1*. (C) Nucleotide and deduced amino acid sequences of *bach2* cDNA. In-frame stop codons preceding the Bach2 ORF are underlined.

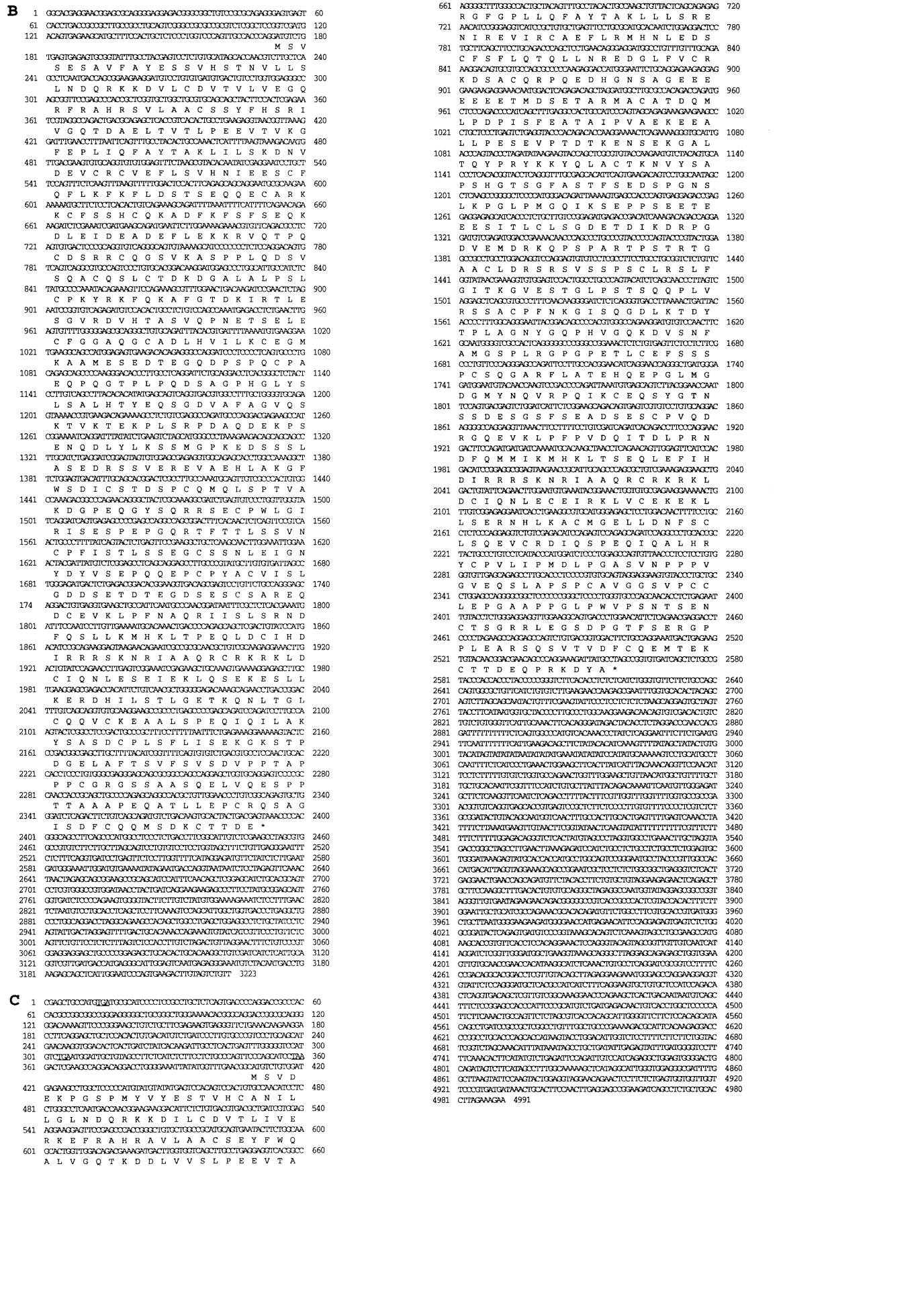
The *Sal*I fragment of pA1 was inserted into the *Bam*HI site of pGBT9 after filling in the relevant DNA ends. The resulting plasmid, pGBT/Bach1, encoded the entire Bach1 ORF fused to the DBD of GAL4. pGBT/Bach1 was digested with *Bgl*II and *Sal*I and recircularized after being filled in with T4 DNA polymerase. This treatment resulted in pGBT/Bach1ΔBS, with removal of the entire carboxy-terminal region of Bach1, leaving only the amino-terminal 163 residues of the predicted Bach1 polypeptide. To remove the bZip-coding domain from pA1, pA1 was digested with *Nco*I and recircularized, generating pA1ΔNc. This plasmid encoded the amino-terminal 287 residues of the predicted Bach1 polypeptide.

RNA blot hybridization. $Poly(A)^+$ RNAs were isolated from various cell lines or tissues of adult mice and embryos by the acid guanidinium thiocyanate-phenol-chloroform extraction method (12) and with oligo(dT)-latex beads (Takara). Approximately 2 to 3 μg of $poly(A)^+$ RNA was separated on 1% agarose–formaldehyde gels and transferred onto Zetaprobe membranes (Bio-Rad). Hybridization with ^{32}P-labeled DNA or RNA probes and subsequent washing was carried out as described previously (20). Probe DNA fragments were either the *Nco*I 1.4-kb DNA of *bach1* cDNA or the *Apa*I-*Hin*dIII 1.4-kb DNA of *bach2* cDNA.

Recombinant transcription factors and antisera. Expression and purification of MBP fusion proteins were carried out as previously described (38) with *Escherichia coli* SG 12036. The purified MBP-Bach fusion proteins were used to immunize Japanese White rabbits by the RIBI ImmunoChem adjuvant system after collection of preimmune sera. Three different antisera were obtained, and their specificities were examined by immunoblotting analysis with overproduced Bach1, Bach2, p45 NF-E2, and ECH proteins obtained by transfection of fibroblast cells. The first, raised against Bach1, was specific for Bach1. The second was raised against Bach2 and found to react with both Bach1 and Bach2. The third was raised against Bach2 and was specific for Bach2. These three antisera are referred to as anti-Bach1, anti-Bach panspecific, and anti-Bach2, respectively. Since none of these three reacted with p45 NF-E2 or ECH in an immunoblotting analysis or in an electrophoretic gel mobility shift analysis (EGMSA), we assumed that they do not react with known CNC family proteins. In EGMSA, each of the three antisera reacted with both Bach1 and Bach2.

Transient-transfection assay. The quail fibroblast cell line QT6 (49) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and seeded in 12-well dishes 24 h before transfection. QT6 cells were transfected with the reporter and effector plasmids by the calcium phosphate precipitation method as previously described (60). The chicken erythroid cell line HD3 (5) was maintained and transfected by the DEAE-dextran method as previously described (41). Luciferase assays were performed by the luciferase assay system (Promega) by following the supplier's protocol and with a Biolumat luminometer (Berthold). Transfection efficiencies were normalized with a co-transfected β-galactosidase expression plasmid, pENL. To analyze DNA binding activities generated by the effector plasmids in transfected QT6 cells, transfections were carried out with 2 μg of effector plasmids, 1.5 μg of pENL, and carrier plasmid DNA (a total of 8 μg of plasmids per 100-mm-diameter dish).

EGMSA. Nuclear extracts were prepared from transfected QT6 cells by the previously described protocol (2). Nuclear extracts of mouse embryo brain cells were as described previously (50). An oligonucleotide containing the chicken β-globin enhancer NF-E2 site (5′-TCGACCCGAAAGGAGCTGACTCATGC TAGCCC-3′) was labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. Oligonucleotides containing either the mutated β-globin enhancer NF-E2 site, the NF-E2 sites of the β-globin LCR, or the NF-E2 site-like sequence of the erythroid δ-aminolevulinate synthase gene promoter were as described previously (33). Binding reactions and electrophoresis were carried out as previously described (38) with bacterially synthesized proteins or nuclear extracts. Where indicated in the figures and figure legends, rabbit preimmune serum or antisera



were included in the binding reactions at 1/10 to 1/100 dilutions, and the reactions were incubated for 10 min on ice before addition of the probe.

Selection and amplification of binding sites. PCR-assisted DNA binding site selection was performed essentially as described previously (38). Briefly, a 55-bp degenerate oligonucleotide (5′-CATAGATGGATCCTCTGTN$_{18}$GGCTCAGA ATTCTCGAACC-3′) was converted to double-stranded DNA with Klenow DNA polymerase by being primed with the 16-bp reverse primer (5′-GGTTCG AGAATTCTGA-3′). The double-stranded degenerate DNA was purified on a polyacrylamide gel, recovered, and labeled at the 5′ end with ^{32}P. About 2 μg of the DNA was incubated with 2 μg of MBP-Bach2 in the binding reaction buffer, in a total volume of 100 μl for 10 min at 25°C. The reaction mixture was separated as in the EGMSA described above and processed for autoradiography. The upper half of the gel, which included a faint retarded band, was then excised. DNA was eluted from the gel, recovered, and subjected to PCR with the reverse primer and a forward primer (5′-CATAGATGGATCCTCT-3′). The resultant PCR product was purified on a polyacrylamide gel and used for the second selection. From the second selection onwards, we used 100 ng of labeled DNA for binding reactions. After four rounds of selection, selected DNA was cloned in pGEM-T (Promega) and sequences were determined. Sequences from random portions of the selected DNA were compiled to make a tally.

Immunoblot analysis. Nuclear extracts from transfected QT6 cells and brain cells of mouse embryos were separated with sodium dodecyl sulfate (SDS)-polyacrylamide gels; proteins were then transferred onto polyvinylidene difluoride membranes (Waters) and processed for reaction with the primary antisera against Bach proteins (1,000-fold dilution), and secondary antibodies were conjugated with horseradish peroxidase as described previously (26). Where indicated in the figures and figure legends, the antisera (5 μl) were preabsorbed with MBP (100 μg) or MBP-Bach2 (30 μg) for 2 h on ice. Detection of peroxidase activity was carried out by the enhanced chemiluminescence system (Amersham).

Nucleotide sequence accession number. The nucleotide sequence data described in Fig. 1 will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession numbers D86603 (Bach1) and D86604 (Bach2).

RESULTS

Molecular cloning of new bZip factors. Yeast two-hybrid screening (18) was used to identify proteins that bind to MafK protein. The bait plasmid expressed the full-length mouse MafK protein fused to the DBD of GAL4. From 2×10^7 yeast cells transformed with the bait plasmid and mouse day 17 pc embryo cDNA library plasmids, we isolated plasmids that activated reporter genes in conjunction with the bait plasmid. The majority of such plasmids were found to encode either NF-E2 p45 or Nrf2, verifying that the bait plasmid can detect interactions between MafK and its known heterodimerizing partners within yeast cells. Among the other plasmids, two (pA1 and pF69) were found to encode novel leucine zipper proteins, which we named Bach1 and Bach2 (see below), respectively. pA1 and pF69 plasmids failed to activate reporter gene expression when transformed into the reporter yeast strain in conjunction with a bait plasmid carrying mutations in the leucine zipper motif of MafK, indicating that the leucine zipper of MafK mediated the interactions (data not shown). The fusion proteins encoded by plasmids pA1 and pF69 also interacted with MafG in the two-hybrid system (data not shown).

In order to obtain larger cDNAs covering entire ORFs, we screened a newborn mouse brain cDNA library with the pA1 and pF69 cDNA fragments recovered from the two-hybrid screening. The resulting two cDNA clones, one for each probe, completely overlapped the cDNA fragments obtained from the two-hybrid screening (Fig. 1A). Their cDNA sequences and conceptual translations are shown in Fig. 1B and C. The *bach2* cDNA clone was found to contain an ORF which starts with a Kozak consensus site for initiation of translation (39), is preceded by an in-frame stop codon, and was predicted to encode a protein of 716 amino acid residues with a calculated molecular mass of 79 kDa. No preceding in-frame stop codon was noted for the ORF for *bach1* cDNA. However, on the basis of its structural similarity with Bach2, we tentatively assigned an initiation methionine to it, as shown in Fig. 1B, so that it would

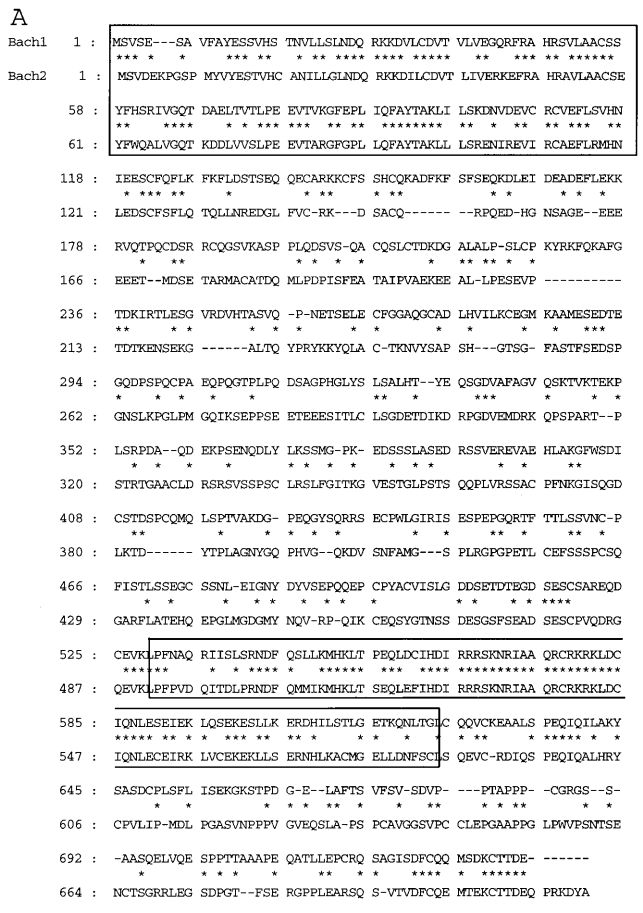
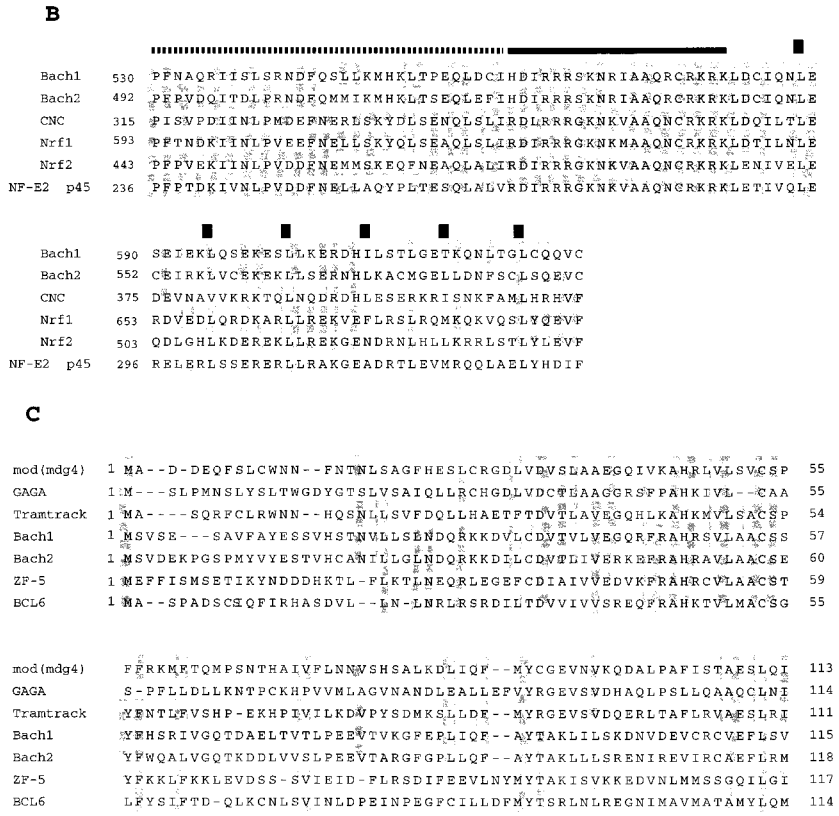


FIG. 2. (A) Comparison of amino acid sequences of Bach1 and Bach2. Identical amino acids are indicated by asterisks. The BTB and CNC-bZip domains are indicated by boxes. (B) Comparison of the bZip and surrounding regions of Bach1, Bach2, murine CNC family proteins, and *Drosophila* CNC. Amino acids conserved among at least three proteins are shaded. The CNC domain and basic region are indicated with broken and thick lines, respectively, and the leucine zippers are indicated by filled rectangles. (C) Comparison of the BTB domains of mod(mdg4), Tramtrack, the GAGA factor, Bach1, Bach2, ZF5, and BCL6. Amino acids conserved among at least four proteins are shaded.

conform to the Kozak consensus. Bach1 was predicted to consist of 739 amino acid residues with a calculated molecular mass of 81 kDa.

A comparison of the deduced amino acid sequences of Bach1 and Bach2 is shown in Fig. 2A, with the two proteins showing 38% identity. Bach1 and Bach2 contain well-conserved bZip domains most closely related to the CNC-type bZip domains among the known bZip factors (Fig. 2A and B). CNC family proteins are characterized by the presence of an additional conserved region preceding the bZip domain. However, the corresponding regions of Bach proteins were less conserved than those of murine CNC family members.

As shown in Fig. 2C, another hallmark of Bach proteins is the presence of a BTB domain (4, 75). As with previously described examples, the BTB domains of the Bach proteins were found to be located close to the amino termini. Most BTB domains characterized thus far are associated with zinc finger motifs (4, 75), and hence Bach proteins are the first transcription factors in which the BTB domains are associated with bZip domains. This characteristic defines them as belonging to a new family of bZip factors, and thus we adopted the name Bach for "BTB and CNC homology." Since BTB domains are



known to mediate protein-protein interactions, we speculate that they confer upon Bach proteins a specific function that is unique to them among known bZip transcription factors. Other than the similarity between the bZip and BTB domains, only limited similarity was observed between Bach1 and Bach2.

Expression pattern of *bach* genes. To begin to address the role of the Bach family in vivo, we examined the expression profiles of the two genes by RNA blotting (Fig. 3). *bach1* and *bach2* transcripts were detected as relatively long mRNAs whose sizes were estimated to be 8 and 11 kb, respectively. Because the cDNA sequences of *bach1* and *bach2* that were determined here lack an authentic polyadenylation signal at the 3′ ends, we assume that each mRNA possesses a relatively long 3′ untranslated region which might account for the length of the mRNA. *bach1* mRNA was detected in all of the tissues examined, including the spleen, with the small intestine expressing the highest level (Fig. 3A). In contrast, expression of *bach2* mRNA was essentially restricted to the brain and spleen. In the brain, expression of *bach2* but not *bach1* was found to be lower in the adult than in the neonate. Despite their expressions in adult spleen cells, *bach1* and *bach2* mRNAs were barely detectable in the livers of day 17 pc embryos, at which stage of development the liver is the major hematopoietic organ. During mouse embryogenesis, both of the *bach* RNAs were detectable in day 10, 13, and 17 pc whole embryos (Fig. 3A and B and data not shown).

Because the expression sites of MafK are in neuronal, mesenchymal, and hematopoietic tissues (50), we examined expression of *bach* genes in several cell lines representing these cell lineages. Among the hematopoietic cell lines tested, *bach1* mRNA was expressed abundantly in P815 (mastocytoma [16]), FDCP-1 (bipotential myeloid progenitor), M1 (myelomonocytic leukemic [29]), murine erythroleukemia (MEL) DS-19 (erythroleukemic [62]), and BW5147 (T-lymphoid) cells (Fig. 3B). The level of *bach1* mRNA was low in B-lymphoid BaF/3 cells (58). MEL cells are known to undergo terminal erythroid differentiation upon treatment with dimethyl sulfoxide (44). However, the level of *bach1* mRNA did not change significantly during this induced differentiation in vitro (data not shown). In contrast to the expression pattern of *bach1*, the level of *bach2* expression was high only in the M1 cells among the hematopoietic cell lines. In addition to the major mRNA, M1 cells expressed a less abundant and smaller mRNA species. This mRNA was also found to be expressed in the neuroblastoma cells (see below). The level of expression of *bach2* did not increase during the induced differentiation of MEL cells (data not shown). The specific expression of *bach2* in hematopoietic cell lines suggests a role of *bach2* in lineage-related gene regulation. Its expression in M1 but not in FDCP-1 suggests that *bach2* plays an important role during development of the monocyte/macrophage lineage.

Consistent with its widespread expression in mouse tissues, *bach1* mRNA was also evident in the C1300 neuroblastoma cells and NIH 3T3 cells (Fig. 3B). In contrast to *bach1*, *bach2* mRNA was significantly more abundant in C1300 cells than in NIH 3T3 cells. Comparison of the expression patterns of the *bach* genes in these cell lines with those of the *p45* and *nrf2* genes indicated that these MafK interactor genes showed overlapping but distinct expression profiles.

Formation of heterodimers with MafK. The primary structures of the bZip domains of Bach1 and Bach2 and their interactions with the leucine zipper of MafK suggested that Bach1 and Bach2, like NF-E2 p45, bind to a NF-E2 site by forming heterodimers with MafK. To test this possibility and to

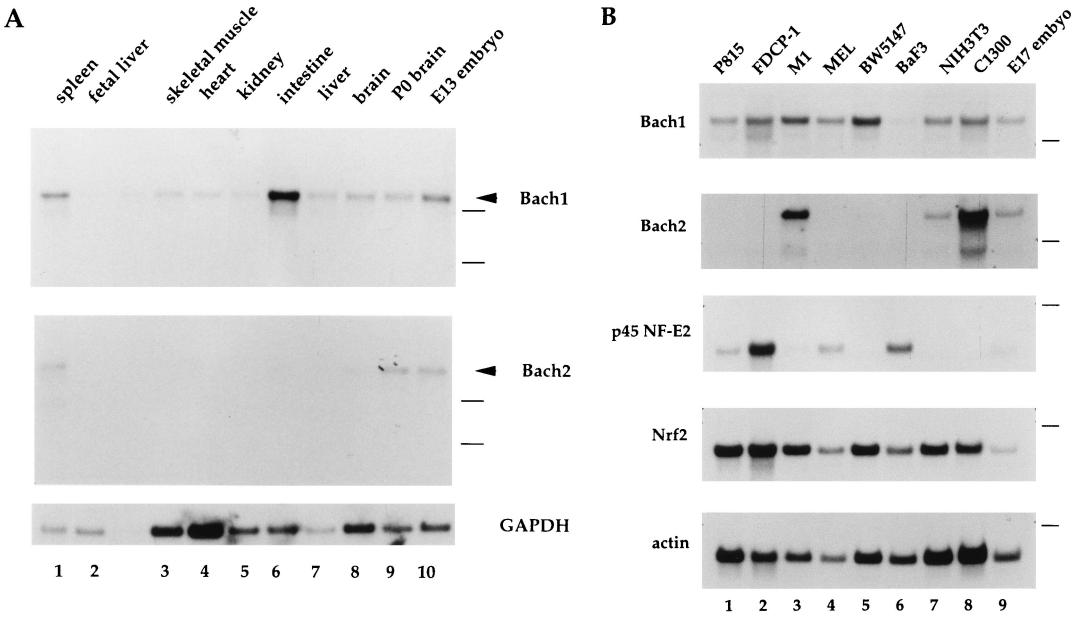


FIG. 3. Expression of *bach* mRNAs. Poly(A)$^+$ RNAs (2 to 3 μg), isolated from various sources, were hybridized with *bach1*- or *bach2*-specific RNA or DNA probes after separation on agarose-formaldehyde gels and transfer onto nylon membranes. RNA loadings were verified by hybridizing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH)- or β-actin-specific probes. (A) RNAs were isolated from tissues of adult mice (lanes 1 and 3 to 8), day 17 pc fetal liver cells (lane 2), newborn mouse brain cells (lane 9), or day 13 pc whole embryos (lane 10), as indicated above the lanes. Positions of 28S and 18S RNAs are indicated on the right side with bars. (B) RNAs were isolated from the indicated murine cultured cells (lanes 1 to 8) or from day 17 pc embryos (lane 9). Expressions of *p45 NF-E2* and *nrf2* mRNAs were also analyzed by subsequent hybridizations. Positions of 28S RNAs are indicated on the right.

confirm interactions between Bach proteins and MafK, each protein was expressed in *E. coli* as a MBP fusion and purified, and their DNA binding activities were then examined by EGMSA. Binding of MBP-MafK, MBP-ECH, which we employed as a control, MBP-Bach1, and MBP-Bach2 fusion proteins to the NF-E2 site of the chicken β-globin enhancer was barely detectable with 5 ng of protein (Fig. 4A, lanes 2, 4, 6, and 8). Addition of MafK to Bach resulted in strong DNA binding (lanes 5 and 7), as was the case with ECH (lane 3). Because formation of the complexes required both MafK and Bach fusion proteins, these complexes should be heterodimers of MafK and Bach proteins (see below). Formation of these complexes was efficiently inhibited by competition with the unlabeled chicken β-globin enhancer NF-E2 site probe (Fig. 4B, lanes 3 and 8). The NF-E2 site on the probe was essential for binding, since mutations within the NF-E2 site abolished binding (lanes 4 and 9). A DNA fragment containing the NF-E2 site from HS-2 of the β-globin LCR competed efficiently with the β-globin enhancer probe (lanes 5 and 10). In contrast, the NF-E2 site-related sequence from the erythroid δ-aminolevulinate synthase gene promoter, which cannot bind NF-E2 efficiently (1), barely competed with the probe (lanes 6 and 11). These results showed that the nucleoprotein complexes reflect specific interactions between the NF-E2 site and MafK-Bach heterodimers. The observed DNA recognition specificities of the Bach-MafK heterodimers are very similar to that of NF-E2 (1).

To compare the relative affinities, various amounts of Bach fusion proteins were incubated with the probe in the presence or absence of an excess amount of MafK fusion protein. As shown in Fig. 4C, Bach1 showed much stronger NF-E2 site binding activity in the presence than in the absence of MafK. On the other hand, Bach2 showed significant DNA binding activity by itself, especially when high doses of proteins were used in the binding reaction mixtures. However, in the presence of MafK, Bach2 bound to the probe preferentially as a heterodimer with MafK. These results suggested that in cells, the availability of MafK or other small Maf family proteins may govern the DNA binding forms and activities of Bach proteins. The results also suggested that one of the differences between Bach1 and Bach2 is their efficacy of DNA binding as homodimers.

Bach2 binds to a TRE sequence. Because homodimers and heterodimers of bZip proteins often bind to different sequences, we determined optimal binding sites for Bach2 by PCR-assisted selection of binding sites. We used the MBP-Bach2 fusion protein for this experiment, because the EGMSA experiments described above showed that Bach2 bound more actively to DNA than did Bach1 in the absence of MafK. After four rounds of selection, Bach2 exhibited clear binding preferences over a 7-bp region whose consensus sequence was identical to the TRE consensus sequence (Fig. 5A). In addition to the TRE sequence, Bach2 showed preference for purine at the −4 position and pyrimidine at the +4 position. The inverted repeat structure of the consensus sequence suggested that Bach2 bound to the selected sites as a homodimer, just like other bZip proteins. Comparison of the Bach2-selected sequences with the sequence of the NF-E2 site revealed that the Bach2 consensus site is identical to the last 8 of the 11 nucleotides in the NF-E2 site (Fig. 5A and B). On the other hand, the half site of the T-MARE is identical to the longer half of the NF-E2 site (38). These features of the Maf and Bach binding sites lead to the conclusion that, upon heterodimeric DNA binding, MafK and Bach proteins recognize the longer and shorter halves of the NF-E2 site, respectively. Andrews and colleagues reached a similar conclusion on the topology of NF-E2 subunits using a different experimental approach (3). The results suggest that the DNA recognition specificity of Bach2 is regulated by differential dimer formation with other bZip proteins like MafK.

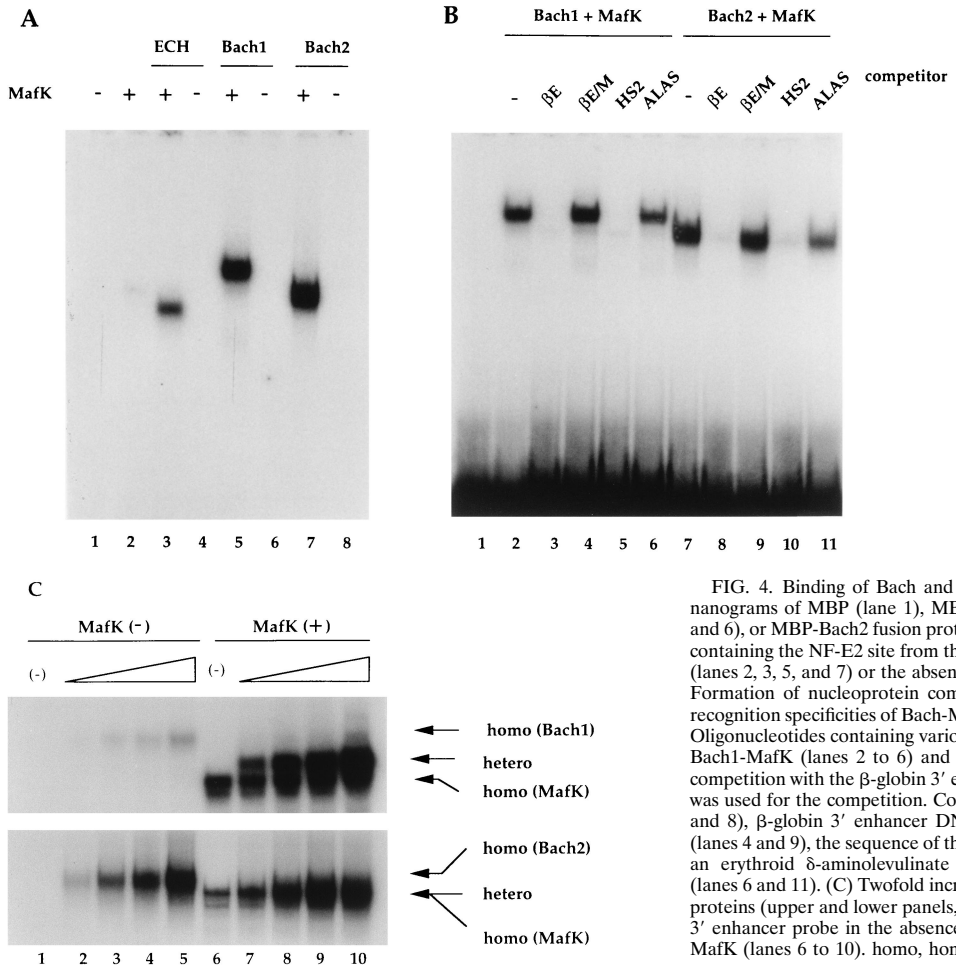


FIG. 4. Binding of Bach and MafK heterodimers to NF-E2 sites. (A) Five nanograms of MBP (lane 1), MBP-ECH (lanes 3 and 4), MBP-Bach1 (lanes 5 and 6), or MBP-Bach2 fusion proteins (lanes 7 and 8) was incubated with a probe containing the NF-E2 site from the chicken β-globin 3′ enhancer in the presence (lanes 2, 3, 5, and 7) or the absence of 5 ng of MBP-MafK (lanes 1, 4, 6, and 8). Formation of nucleoprotein complexes was examined by EGMSA. (B) DNA recognition specificities of Bach-MafK heterodimers were examined by EGMSA. Oligonucleotides containing various sequences were tested for their affinities for Bach1-MafK (lanes 2 to 6) and Bach2-MafK (lanes 7 to 11) heterodimers by competition with the β-globin 3′ enhancer probe. A 100-fold excess of cold DNA was used for the competition. Competitors were cold probe DNA (βE) (lanes 3 and 8), β-globin 3′ enhancer DNA with mutations in the NF-E2 site (βE/M) (lanes 4 and 9), the sequence of the mouse HS-2 NF-E2 site (lanes 5 and 10), and an erythroid δ-aminolevulinate synthase (ALAS) NF-E2 site-like sequence (lanes 6 and 11). (C) Twofold increments (0 to 40 ng) of Bach1 and Bach2 fusion proteins (upper and lower panels, respectively) were incubated with the β-globin 3′ enhancer probe in the absence (lanes 1 to 5) or presence of 50 ng of MBP-MafK (lanes 6 to 10). homo, homodimer; hetero, heterodimer.

Bach proteins participate in unique DNA binding complexes within cells. To determine if Bach and MafK form heterodimers in vivo, we expressed these proteins in fibroblast cells by transient transfection of expression plasmids, prepared nuclear extracts, and tested resultant binding activities to the NF-E2 site by EGMSA experiments. Expression of Bach1 or Bach2 alone resulted in nucleoprotein complexes with distinct mobilities (Fig. 6A, lanes 2 and 3). The amounts of these complexes increased significantly without a change in their mobilities when MafK was simultaneously expressed (lanes 5 and 6). Because formation of the Bach1 complex was inhibited by anti-Bach1 or anti-MafK antisera, it contained Bach1 and MafK (Fig. 6B). Formation of the Bach2 complex was inhibited by the anti-Bach2 antiserum (Fig. 6B). The complexes that were formed by expression of Bach proteins alone may contain the exogenous Bach and endogenous MafK or MafK-related proteins. Expression of Bach1 and Bach2 proteins within transfected cells was checked by immunoblot analysis utilizing the anti-Bach1 and anti-Bach2 antisera and the Bach-panspecific antiserum recognizing both Bach1 and Bach2 (Fig. 6C and data not shown). The estimated sizes of the overproduced Bach proteins were roughly 110 kDa and thus larger than their calculated molecular masses predicted from the ORFs. Such aberrant mobility of Bach proteins in denaturing protein gels might result from posttranslational modifications or the presence of clustered basic amino acid residues on Bach proteins. Taken together, these results indicate that Bach proteins form DNA binding complexes with MafK when they are overexpressed in cells.

A

	-5	-4	-3	-2	-1	0	1	2	3	4	5
G	7	13	2	25	1	24	1	1	1	3	5
A	2	10	0	0	18	2	1	2	21	2	3
T	2	1	24	1	1	0	24	0	0	10	1
C	11	2	0	0	6	0	0	23	0	5	11
	c	g	T	G	A	G	T	C	A	t	c

B

T-MARE half site	TGCTGAG/C
NF-E2 site	TGCTGAG/CTCAT/C
Bach-selected half site	G/CTCAt/c

FIG. 5. Selection of TRE-like sequences by Bach2. (A) The tally was compiled by aligning sequences of 26 independent clones such that the centers of palindromic sequences were occupied by a purine. The number of samples for each position varies, because whenever nonrandom portions of the DNAs overlapped with the aligned sequence, these sequences were excluded from the analysis. The consensus site shown below the tally was derived by selecting nucleotides at each position that were present in more than 70% (uppercase letters) or 50% (lowercase letters) of clones. (B) Comparison of the half sites of the Bach2 consensus site and the TRE-type Maf recognition element with the NF-E2 consensus site (2).

The next question we asked was whether Bach proteins function by forming heterodimers with MafK under physiolog-

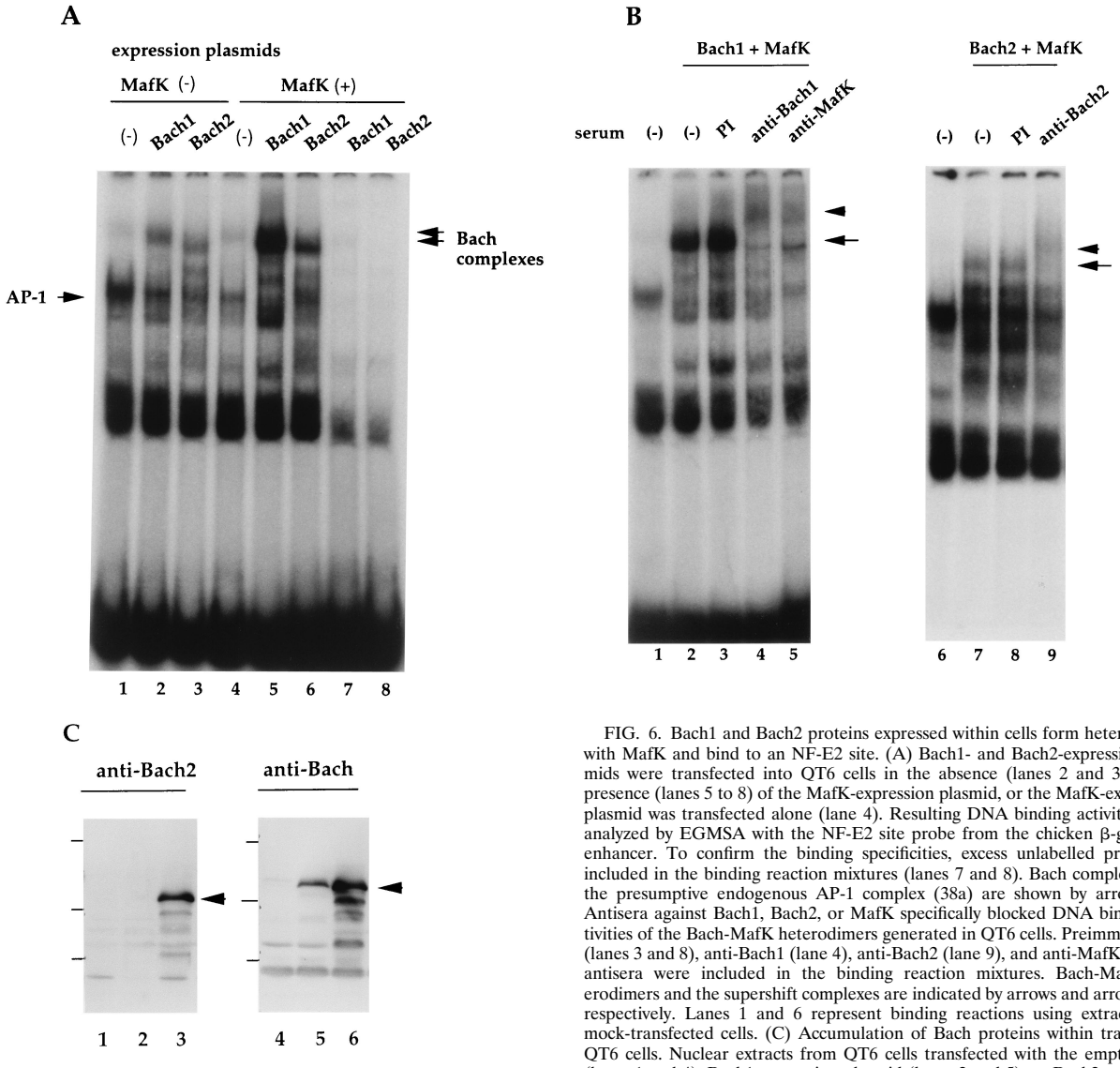


FIG. 6. Bach1 and Bach2 proteins expressed within cells form heterodimers with MafK and bind to an NF-E2 site. (A) Bach1- and Bach2-expression plasmids were transfected into QT6 cells in the absence (lanes 2 and 3) or the presence (lanes 5 to 8) of the MafK-expression plasmid, or the MafK-expression plasmid was transfected alone (lane 4). Resulting DNA binding activities were analyzed by EGMSA with the NF-E2 site probe from the chicken β-globin 3′ enhancer. To confirm the binding specificities, excess unlabelled probe was included in the binding reaction mixtures (lanes 7 and 8). Bach complexes and the presumptive endogenous AP-1 complex (38a) are shown by arrows. (B) Antisera against Bach1, Bach2, or MafK specifically blocked DNA binding activities of the Bach-MafK heterodimers generated in QT6 cells. Preimmune (PI) (lanes 3 and 8), anti-Bach1 (lane 4), anti-Bach2 (lane 9), and anti-MafK (lane 5) antisera were included in the binding reaction mixtures. Bach-MafK heterodimers and the supershift complexes are indicated by arrows and arrowheads, respectively. Lanes 1 and 6 represent binding reactions using extracts from mock-transfected cells. (C) Accumulation of Bach proteins within transfected QT6 cells. Nuclear extracts from QT6 cells transfected with the empty vector (lanes 1 and 4), Bach1-expression plasmid (lanes 2 and 5), or Bach2-expression plasmid (lanes 3 and 6) were separated by SDS-polyacrylamide gel, transferred onto a membrane, and reacted with the anti-Bach2 antiserum (lanes 1 to 3) and the Bach-panspecific antiserum (lanes 4 to 6). Bach proteins are indicated by arrowheads. Positions of molecular size markers (200, 97, and 69 kDa) are indicated on the left sides with bars.

ical conditions. In a previous study, we identified a novel NF-E2 site binding activity in brain extracts and showed that this binding activity was associated with MafK (50). However, dimeric partners of the NF-E2 site binding activity remained unclear. Because expression of both *bach1* and *bach2* mRNAs was noted in brain and C1300 neuroblastoma cells, we examined whether Bach1 and/or Bach2 participate in the NF-E2 site binding activity in brain cells. For this purpose, we employed the anti-Bach1 and anti-Bach2 antisera in an EGMSA. As shown in Fig. 7A, the NF-E2 site binding activity was inhibited by the anti-MafK antiserum (lane 6), which is consistent with our previous observation. The level of NF-E2 site binding activity was also decreased by the anti-Bach1 and anti-Bach2 antisera (lanes 3 and 4). Incubation of the brain extracts with the Bach-panspecific antiserum resulted in a supershifted complex (lane 5). Upon immunoblot analysis of the brain extract, the anti-Bach2 and the Bach-panspecific antisera reacted with a 136-kDa protein which is larger than Bach2 overproduced by transfection (Fig. 7B and data not shown; see Fig. 7C). However, the anti-Bach1 antiserum failed to detect specific antigen (not shown). This might be explained by its lower titer. The 136-kDa protein that reacted with the anti-Bach2 and the Bach-panspecific antisera was a Bach2-related molecule inasmuch as it reacted with the two independent antisera whose reactivities were absorbed by MBP-Bach2 fusion protein but not by MBP (Fig. 7C).

Alternative splicing generates brain cell-type Bach2. Because it was possible that the 136-kDa protein in brain cells represented a variant form of Bach2 generated by alternative splicing, we carried out another round of cDNA library screening. Sequence analysis of resulting cDNA clones revealed that one cDNA clone, termed F69-F, possessed a 369-bp insertion in front of the CNC-bZip domain (Fig. 8A). F69-F appeared to be a partial cDNA lacking the 5′ region of its corresponding mRNA. Comparison of the F69-F and F69-J sequences with

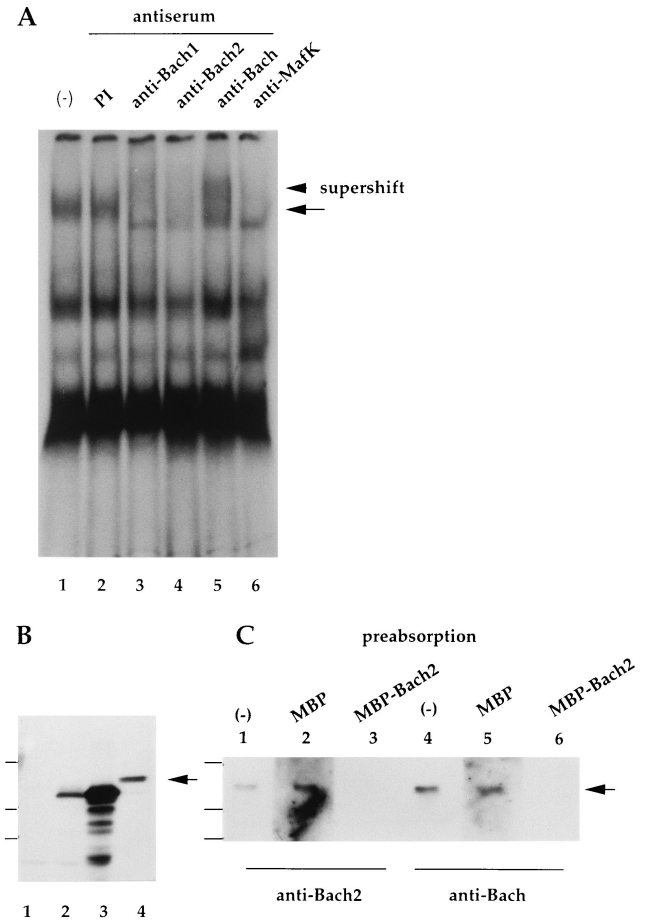


FIG. 7. Bach2 participates in an NF-E2 site binding complex within brain cells. (A) Nuclear extracts were prepared from the brains of day 17 pc mouse embryos, and NF-E2 site binding activity was analyzed by EGMSA with the chicken β-globin 3′ enhancer probe (lane 1). Preimmune (PI) (lane 2), anti-Bach1 (lane 3), anti-Bach2 (lane 4), and Bach-panspecific (lane 5) antisera were included in the binding reaction mixtures. The effect of the anti-MafK antiserum was also examined (lane 6). The positions of the specific binding activity and the supershift complex generated by the Bach-panspecific antiserum are indicated by an arrow and arrowhead, respectively. (B) Nuclear extracts from QT6 cells transfected with the empty vector (lane 1), Bach1-expression plasmid (lane 2), Bach2-expression plasmid (lane 3), and the day 17 pc fetal brain extract (lane 4) were analyzed for the presence of Bach proteins by immunoblotting with the Bach-panspecific antiserum as described for Fig. 6C. A reactive protein in the brain extract is indicated by an arrow. Size markers used were as indicated in the legend to Fig. 6C. (C) Specificities of the anti-Bach2 and Bach-panspecific antisera were examined in a preabsorption experiment. Each antiserum was preincubated with either buffer alone (lanes 1 and 4), excess MBP (lanes 2 and 5), or MBP-Bach2 (lanes 3 and 6) before performing immunoblot reactions with day 17 pc fetal brain extract. The position of the specific antigen is indicated by an arrow. Preabsorption of the antisera with MBP (lanes 2 and 5) resulted in a relatively high background for unknown reasons. Size markers noted by bars at the left are as indicated in the legend to Fig. 6C.

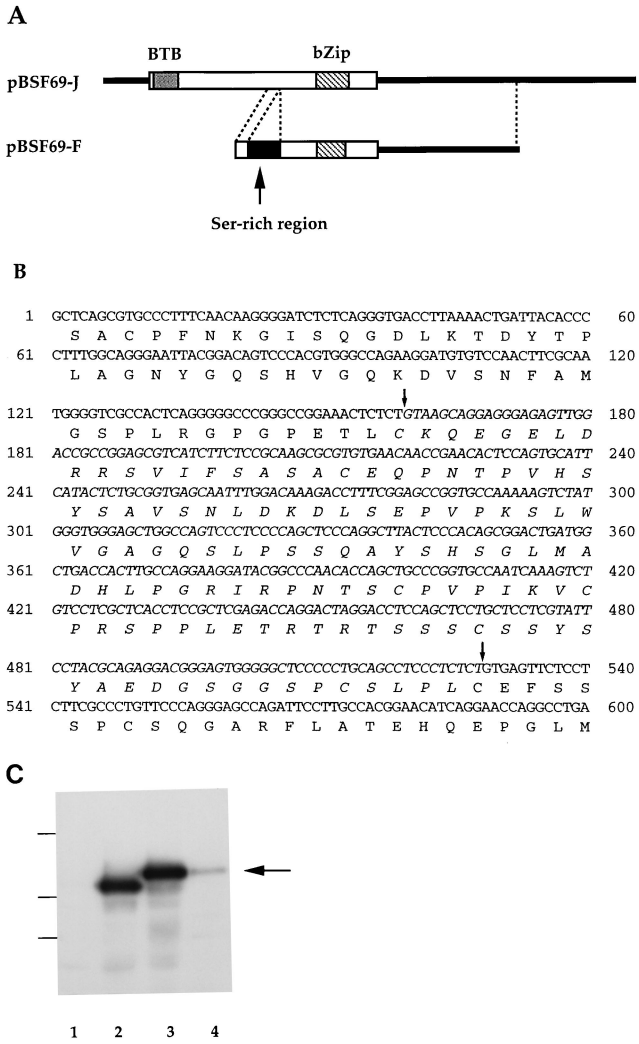


FIG. 8. (A) Schematic representation of two distinct Bach2 cDNA clones. Protein-coding domains are indicated by boxes. Correlation between the two cDNAs is indicated by the dotted lines. (B) Nucleotide and deduced amino acid sequences surrounding the F69-F-specific insertion. 5′ and 3′ junctions of the insertion are indicated by vertical arrows above the lines. (C) Nuclear extracts from QT6 cells transfected with the empty vector (lane 1), Bach2-expression plasmid (lane 2), Bach2(F)-expression plasmid (lane 3), and day 17 pc fetal brain extract (lane 4) were analyzed for the presence of Bach proteins by immunoblotting with the Bach-panspecific antiserum as described in the legend to Fig. 6C. A reactive protein in the brain extract is indicated by an arrow. Size markers used were as indicated in the legend to Fig. 6C.

the genomic DNA sequence revealed that the F69-F-specific insertion was generated by omitting splicing of an intron (27a). The results of RNA-PCR analysis showed that Bach2 mRNA with the F69-F-type insertion was prevalent in brain cells (data not shown). F69-F-specific insertion encodes a region that is rich in serine (Fig. 8B). To express Bach2 with the serine-rich region, a presumptive full-length cDNA was constructed by fusing the F69-F and F69-J cDNAs. Upon transfection into fibroblast cells, the composite cDNA expressed a protein with a mobility identical to that of the Bach2-related 136-kDa antigen in brain cells in a denaturing protein gel (Fig. 8C). These results suggested strongly that the Bach2-related antigen in the brain cells was actually a variant form of Bach2 generated by alternative splicing of mRNA. Taken together, the results point to an importance for the Bach-MafK network in gene regulation in the brain.

The BTB domain mediates protein interactions. Previous studies showed that BTB domains mediate protein interactions among various zinc finger proteins that contain this domain (4, 10). To examine whether the BTB domain of Bach1 was capable of mediating protein interactions, we used the yeast two-hybrid system. The entire Bach1 protein was fused to either the DBD or the AD of GAL4. None of the fusion proteins activated individually the GAL4-dependent *HIS3* reporter gene (Fig. 9). However, when the fusion proteins were

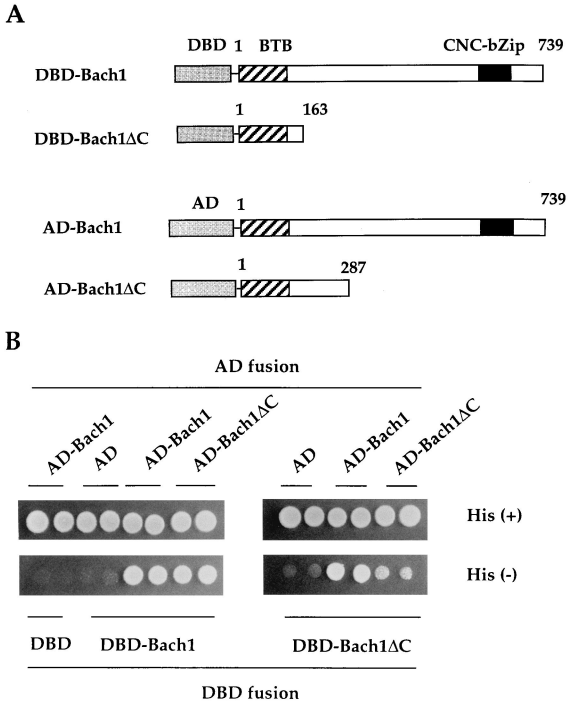


FIG. 9. The BTB domain of Bach1 mediates homophilic interaction within cells. (A) Schematic representation of Bach1 proteins that were fused to the DBD and AD of GAL4. (B) Various combinations of plasmids encoding DBD and AD fusions were introduced into the reporter yeast strain, and the resulting transformants were tested for the His^+ phenotype by spotting onto His^- or His^+ plates. Duplicate clones were tested for each transformation.

coexpressed, reporter gene expression was induced, resulting in histidine autotrophy. To determine whether the BTB domain was involved in the homophilic interaction, the amino-terminal region of Bach1, including the BTB domain, was fused to the DBD or AD and expressed in the reporter cells. The results indicated the bZip region in AD- or DBD-tagged Bach1 to be dispensable for the interaction. Furthermore, the amino-terminal region showed self-interaction, albeit to a weaker extent than those of the other combinations tested. These results indicated that the BTB domain of Bach1 functions as a protein interaction motif within cells.

Transcription regulation by Bach. Because one of the target sites of Bach proteins appears to be a NF-E2 site, we examined the regulatory activities of Bach proteins with a promoter containing NF-E2 sites. Cotransfection of either the Bach1- or Bach2-expression plasmid with the reporter plasmid in QT6 fibroblasts repressed promoter activity in a dose-dependent manner (Fig. 10A). Similar effects of the Bach-expression plasmids were observed in the presence of the MafK-expression plasmid (data not shown). Neither Bach1 nor Bach2 showed any significant effect on a reporter gene carrying mutated NF-E2 sites that could not bind NF-E2 or AP-1. Thus, the effects of Bach1 and Bach2 were specific for promoters with functional NF-E2 sites. Since functions of transcription factors may be dependent on the presence of specific cofactors, we carried out a similar transfection experiment using the HD3 chicken erythroid cell line. Unexpectedly, Bach1 and Bach2 showed distinct activities. Bach2 functioned as a transcriptional repressor, whereas Bach1 acted as a transcriptional activator (Fig. 10B). They showed no effect on the reporter gene carrying the mutated NF-E2 sites (data not shown), verifying that Bach proteins acted through the NF-E2 sites. These results clearly indicated that Bach1 can function as both an activator and a repressor of transcription, depending on the cellular context. Even though Bach2 functioned as a transcription repressor in these experiments, it might also function as a transcription activator in particular types of cells.

DISCUSSION

Previous studies implicated MafK in gene regulation during various developmental events, such as erythroid and neuronal cell differentiation (1, 32, 50). The present report describes the cloning and characterization of members of a novel family of bZip transcription factors, Bach1 and Bach2, that form heterodimers with MafK. In addition to their contribution to novel small Maf heterodimers, Bach1 and Bach2 may function to connect the small Maf protein network with different regulatory networks or different levels of transcriptional regulation through their unique structures and activities.

The intriguing and definitive feature of Bach proteins is the presence of both bZip and BTB domains. In *Drosophila melanogaster*, BTB domain proteins are involved in a variety of processes, including chromatin modeling, regional specification in the early embryo, metamorphosis, oogenesis, photoreceptor development, formation of neural connections, and limb development (14, 22–24, 27, 28, 63, 66, 70, 72, 73). Thus, BTB domain proteins play important roles in various developmental programs. However, little is known about BTB domain proteins in vertebrates, known examples of which include zinc finger proteins like ZFPJS (65), ZF5 (56), ZID (4), PLZF (11), and BCL6 (74). Among the BTB domain proteins identified thus far, those of the Bach family are the first demonstrating association with a bZip domain. This novel combination of BTB and bZip domains is presumably a reflection of unique functions not shared by other zinc finger and bZip protein families.

Some BTB domain proteins in *Drosophila melanogaster* regulate transcription by modulating chromatin structure. The *mod*(*mdg4*) gene imparts directionality on a chromatin insulator (22). The same gene is also known as the enhancer of position effect variegation gene, *E*(*Var*)*3-93D* (15). Another enhancer of position effect variegation gene, *Trithorax-like*, encodes a GAGA factor whose biochemical function is to disrupt nucleosomes in concert with the ATP-dependent nucleosome-remodeling factor (17, 66, 67). Because of its conservation among modifier genes of position effect variegation, the BTB domain has been suggested to mediate protein interactions responsible for establishing certain chromatin structures (15, 17). In this regard, it is noteworthy that the β-globin LCR contains multiple NF-E2 sites (reference 64 and references therein). Hence, an interesting question that stems from the present study is whether the Bach proteins contribute to the functions of the β-globin LCR to activate or repress expression of the locus.

The known biochemical function of BTB domains is to mediate homodimerization and/or heterodimerization with other proteins containing this domain (4, 10). Our findings for Bach1 are consistent with this. Bach1, and presumably Bach2, contains two independent motifs for oligomer formation, a leucine zipper and a BTB domain. While we do not know at present any specific heterotypic interactions or physiological targets, they would be expected by analogy with GAGA and Tramtrack (4). From the observations described in this report, we may infer that Bach proteins form heterodimers with MafK or other bZip proteins through the leucine zipper, bind to TRE- or NF-E2 site-related DNA sequences, and interact with another Bach protein or other proteins through the BTB do-

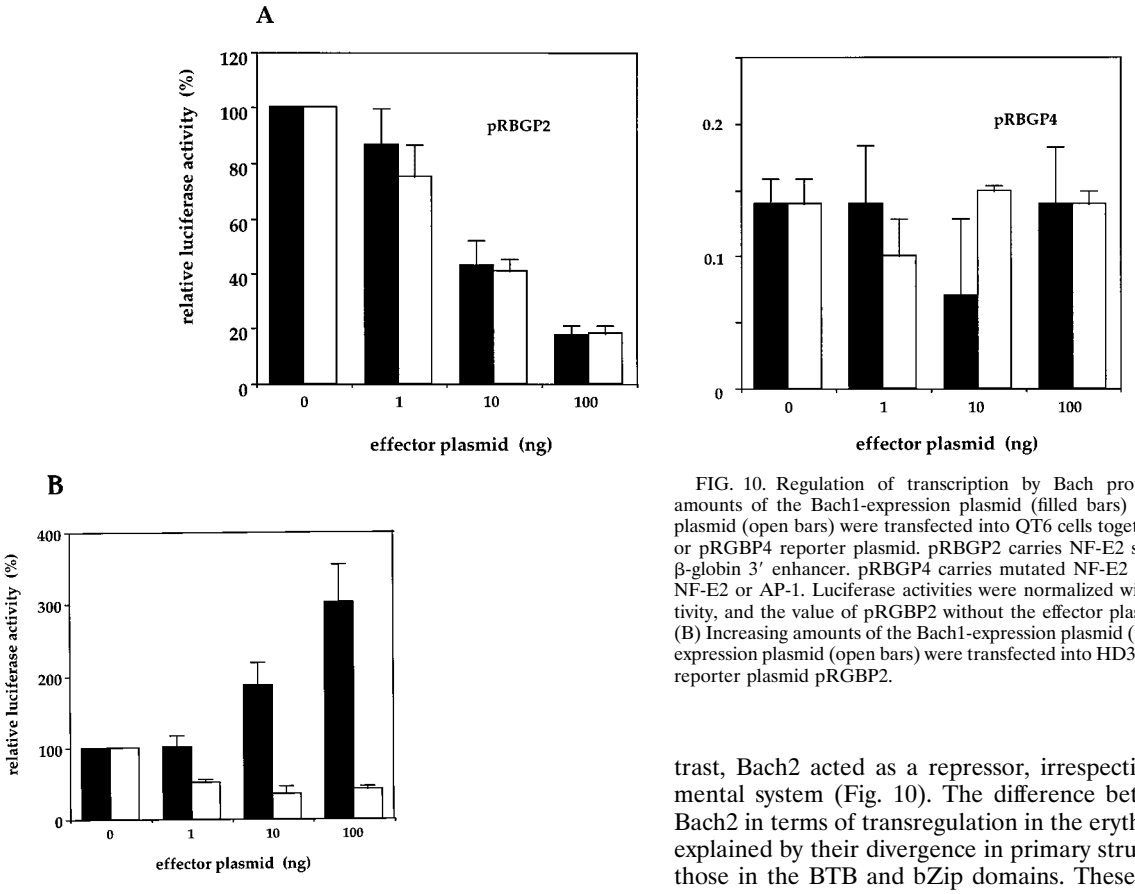


FIG. 10. Regulation of transcription by Bach proteins. (A) Increasing amounts of the Bach1-expression plasmid (filled bars) and Bach2-expression plasmid (open bars) were transfected into QT6 cells together with the pRGBP2 or pRGBP4 reporter plasmid. pRBGP2 carries NF-E2 sites from the chicken β-globin 3′ enhancer. pRBGP4 carries mutated NF-E2 sites that do not bind NF-E2 or AP-1. Luciferase activities were normalized with β-galactosidase activity, and the value of pRGBP2 without the effector plasmid was set at 100%. (B) Increasing amounts of the Bach1-expression plasmid (filled bars) and Bach2-expression plasmid (open bars) were transfected into HD3 cells together with the reporter plasmid pRGBP2.

mains. Our hypothesis that multiple interactions, with formation of multiprotein complexes on the NF-E2 sites, in turn execute unique regulatory roles is now testable.

In the above-described transient-transfection assays, both Bach1 and Bach2 acted as transcription regulators that were specific for a promoter with functional NF-E2 sites (Fig. 10). At present, there are two possibilities with regard to the DNA binding forms of Bach proteins in these experiments. They may bind to the target sites as homodimers or as heterodimers with endogenous small Maf or related proteins. The results of the EGMSA experiment (Fig. 6) support the latter alternative. In fibroblasts, both Bach1 and Bach2 repressed transcription. Because NF-E2 sites contain TRE and can bind AP-1 and related factors, repression of NF-E2 site-driven promoters can be achieved by competitive binding of proteins which do not possess intrinsic transactivation activity. Bach1 fused to the DNA binding domain of GAL4 did not activate expression of GAL4-dependent reporter genes in yeast cells (Fig. 9) or in fibroblast cells (30), indicating that Bach1 lacks general transactivation activity. Thus, competition for binding sites is likely to be one of the mechanisms by which Bach proteins repress transcription. However, it remains possible that Bach proteins exert their effects by interacting with other transcription factors. The repressor activities of Bach proteins are intriguing, since known CNC family proteins activate, rather than repress, transcription (7–9, 33, 34, 47). Thus, the Bach family may play important roles in coordinating transcriptional activation and repression by the small Maf family proteins.

In our transient-transfection assays using an erythroid cell line, Bach1 functioned as an activator of transcription. In contrast, Bach2 acted as a repressor, irrespective of the experimental system (Fig. 10). The difference between Bach1 and Bach2 in terms of transregulation in the erythroid cells may be explained by their divergence in primary structures other than those in the BTB and bZip domains. These structural differences would be expected to cause differential interactions of the proteins with other proteins, like coactivators, resulting in different regulatory properties. Our observations suggest that functions of the small Maf interactor network depend not only on deployment of different combinations of bZip proteins but also on the presence of other factors interacting with specific portions of the molecules.

During mouse embryogenesis, the *mafK* gene is expressed predominantly in hematopoietic, mesenchymal, and neuronal cells (50). The results of the two-hybrid screening indicated that, in the day 17 pc mouse embryo, at least four MafK interactors, namely, p45 NF-E2, Nrf2, Bach1, and Bach2, were present. Furthermore, their expression profiles suggested that all of them can participate in the MafK interactor network in hematopoietic cells. Another place where the Bach family may contribute to MafK function is in neuronal cells, as evidenced by the above-described demonstration that brain nuclear extracts contain a Bach2-related molecule which binds to the NF-E2 site as a complex with MafK (Fig. 7 and 8). Because C1300 neuroblastoma cells expressed high levels of *bach2* mRNA (Fig. 3), the presumptive Bach2-MafK complex was likely to have been derived from neuronal cells. Consistent with this inference, an in situ hybridization experiment showed that Bach2 mRNA was actually expressed in neural tubes in the day 11 pc mouse embryo (51). On the basis of these observations, we propose that Bach2 functions as a partner of MafK in neuronal cells. Whereas the NF-E2 site binding activity in brain cells was also reactive to the anti-Bach1 antiserum in the EGMSA experiment, the antiserum did not react with specific protein upon immunoblotting. Even though it is specific for Bach1 in an immunoblot analysis, the anti-Bach1 antiserum cross-reacts with Bach2 under the conditions of the

EGMSA experiment (not shown; see Materials and Methods), which could explain the effect of the anti-Bach1 antiserum apparent in Fig. 7A. Alternatively, the complex might actually contain Bach1 in addition to Bach2 and MafK. Interactions through the BTB domains would be expected to allow formation of such a ternary protein complex.

Several reports have pointed to important roles of the Maf family in the development of the brain. The *Kreisler* gene, which is a mouse homolog of *mafB*, is essential for hindbrain development (13, 36). Nrl, another *maf* family gene, is expressed in postmitotic neurons and is supposed to regulate retina-specific gene expression (42, 59). c-Maf was suggested to function in Purkinje cell-specific gene regulation (40). It is quite possible that Bach proteins regulate the functions of these Maf family proteins by competing for binding sites or by interacting directly with Maf proteins. Indeed, we could detect an interaction between c-Maf and Bach1 in the yeast two-hybrid system (55).

In conclusion, the results described in this report suggest multiple roles for Bach family proteins in coordinating transcription activation and repression by MafK and other Maf-related factors and underscore the importance of deciphering cross-talk among various bZip regulatory networks during cell proliferation and differentiation.

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