J. Biochem. 132, 427–431 (2002)

Expression of the Oxidative Stress–Regulated Transcription Factor Bach2 in Differentiating Neuronal Cells[1]

Hideto Hoshino* and Kazuhiko Igarashi†,2

*Department of Biotechnology, Graduate School of Agriculure and Life Sciences, The University of Tokyo, Tokyo 113-8657; and †Department of Biomedical Chemistry, Hiroshima University Graduate School of Biomedical Sciences, Kasumi 1-2-3, Hiroshima 734-8551

Received June 6, 2002; accepted June 29, 2002

Bach2 is an oxidative stress–regulated transcription factor and functions as a repressor of gene expression directed by the TPA-response element, the Maf recognition element, and the antioxidant responsive element. To investigate the possibility that these enhancers are regulated in a tissue-specific manner, we analyzed expression of Bach2 during differentiation of neural cells. Bach2 was induced upon neuronal differentiation of P19 embryonic carcinoma cells, while its related factor Bach1 did not show significant change. By using affinity-purified anti-Bach2 antibodies, expression of Bach2 in mouse embryos was determined. High levels of Bach2 antigen were found in differentiating neuronal and lens cells in day 12.5 embryos. Consistent with the fact that subcellular localization of Bach2 is regulated by nuclear export in cultured cells, extensive Bach2-staining was found in the cytoplasmic regions of developing neuronal and lens cells. These results suggest that Bach2 regulates AP-1– and Maf–dependent gene expression during development of neuronal and lens cells and that its activity may be regulated by nuclear export in these cells.

Key words: AP-1, lens, nuclear export, oxidative stress.

The dimeric AP-1 transcription factor complexes control cell proliferation, differentiation, and apoptosis by regulating gene expression upon exposure to various stimuli (*1*). Originally, four different sub-classes of basic-leucine zipper (bZip) dimers were identified (*i.e.*, AP-1, ATF/CREB, C/EBP, and Maf) based upon the DNA sequences they bind (*2*). Dimers of the oncoprotein v-Maf and its related factors bind to the Maf recognition element or MARE (*3, 4*). Since MARE embeds a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–response element (TRE), it is also a binding site for the Jun and Fos family members (*4*). In neural systems, some of the AP-1 factors are categorized as immediate early gene products and are supposed to reprogram gene expression toward various stimuli including synaptic activities (*5*). In addition, Maf family members are involved in the development of neural systems. For example, MafB is required for proper segmentation of hindbrain in mice (*6*).

The bZip factor Cap'n'Color (CNC) is a segment-specific selector controlling the identity of cephalic segments in *Drosophila* (*7*). In mammals, CNC-related proteins, the nuclear factor-erythroid 2 (NF-E2) p45 and its related factors Nrf1, -2, and -3 form heterodimers with the small Maf proteins (*i.e.*, MafF, MafG, and MafK), bind to versions of MARE, and thus activate transcription (*8–12*). In a yin-yang scenario typical in biological systems, the Maf-CNC system is opposed by repressors Bach1 and Bach2. Like NF-E2 p45 and others, Bach1 and Bach2 form heterodimers with the small Maf proteins in order to bind to MARE. However, the resulting heterodimers repress MARE-dependent transcription in transfection assays (*13*). Thus, there appears to be a complex transcriptional antagonism regulating MARE-dependent gene expression. Activity of Bach2 is regulated by cytoplasmic compartmentation and conditional nuclear accumulation (*14*). Under normal cell culture conditions, Bach2 is exported from nuclei depending on Crm1 and accumulates within the cytoplasmic compartment. Oxidative stress inhibits nuclear export of Bach2, causing its nuclear accumulation. Overexpression of Bach2 in NIH3T3 cells sensitizes cells toward oxidative stress and results in massive apoptosis upon oxidative stress (*15*). These observations suggest that Bach2 constitutes a cell lineage-specific system that couples oxidative stress and cell death, and that inhibition of TRE, MARE, and ARE upon oxidative stress may be critical determinants for apoptosis. The presence of many cooperative and antagonistic factors that affect the related enhancers may indicate that different quantities of these factors will result in formation of different types of dimers that regulate gene expression in distinct ways from each other.

While expression of Bach2 among established cell lines is specific to B lymphoid cells and neural cell lines (*13, 16*), there is no detailed report about its expression in primary neural tissues. We describe here analysis of Bach2 expression during *in vitro* differentiation of P19 embryonic carcinoma (EC) cells as well as in mouse embryos. The results

[1] This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and grants from Yamanouchi Foundation for Research on Metabolic Disorders and the Naito Foundation, Japan.

[2] To whom correspondence should be addressed. E-mail: igarak@hiroshima-u.ac.jp

Abbreviations: ARE, antioxidant responsive element; bZip, basic-leucine zipper; EC, embryonic carcinoma cells; MARE, maf recognition element.

suggest a close association of Bach2 with neuronal and lens differentiation.

MATERIALS AND METHODS

Neural Differentiation of P19EC Cells—The neural differentiation of P19 EC cells was performed according to the published method (*17*). In brief, undifferentiated P19 EC cells were maintained in α-MEM supplemented with 10% FBS. The cells were trypsinized and aggregated in bacterial petri-dishes for 3 days in the presence of 0.5 μM all-*trans* retinoic acid (RA). The aggregates were washed twice with RA-free medium and cultured in a tissue culture dish for 3 days.

Reverse Transcription-PCR—Total RNA was prepared at each time point with RNA zolBTM (TEL-TEST). cDNA was synthesized by random hexamer priming, and PCR was carried out with specific primers for Bach2 (J2020F: 5′-CGCTGTCGAAAGAGGAAGCTGGAC-3′, and J2209R; 5′-CCTGGATCTGCTCTGGACTCTGGA-3′), Bach1 (A1-1: 5′-CGGATAATTTCGCTCTCACG-3′, and A1-2035R: 5′-GGAAAGCGGGCAGTCGG-3′), MAP2 (MAP2F: 5′-GAAGGAAAGGCACCACACTG-3′, and MAP2R: 5′-GCTGGCGATGGTGGTGGG-3′), and β-actin (β-actin-1: 5′-CTGGGAGGAGAAAAGCTACC-3′, and β-actin-2: 5′-AAAGCTGGTCCAGCCGTTCC-3′). All PCRs were carried out in 20-μl reactions using 1.25 U of ExTaq DNA polymerase (Takara) and 20 pmol of primer with a thermal cycler (Perkin Elmer 9600) and a profile of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with 25 cycles. Products were resolved on 2% agarose gels, transferred onto ZetaProbe membranes (Bio-Rad) and hybridized with radiolabeled DNA fragments specific for each taget cDNA.

Immunoblotting Analysis—Whole protein was extracted from whole embryos at E8.5 and E9.5 stages, whole neural tubes surgically separated from embryos between E10.5 to E13.5, and brains of E15.5 embryos. Whole cell extracts were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (Waters) and processed for reaction with the antiserum against Bach2 (F69-2 (*13*)). Secondary anti–rabbit IgG antibodies conjugated with horseradish peroxidase (Zymed) were used to detect immune complexes with the enhanced chemiluminescence system (Amersham).

Affinity-Purification of Anti-Bach2 Antibodies—Maltose binding protein (MBP) and MBP-Bach2 fusion protein (*13*) were cross-linked to HiTrap affinity beads (Amersham). Anti-Bach2 antiserum (F69-2) was first applied onto the MBP column, and the flow-through fraction was applied onto the MBP-Bach2 column. Bound antibodies were eluted using low pH buffer.

Immunohistochemical Analysis for E12.5 Mouse Embryo Sections—Mouse embryos were analyzed at E12.5. Embryos were fixed in 4% *para*-formaldehyde, 0.5% glutaraldehyde in phosphate-buffered saline at 4°C for 2 h, embedded in OCT compound (Tissue-Tek), and sectioned at 8 μm thickness using cryostat. For immunohistochemistry, affinity-purified anti-Bach2 antibodies were used at 1:5 dilution followed by application of an Elite ABC kit (Vectstain). Diaminobenzidine was used as a chromogen, and methyl green was used to counterstain.

RESULTS

Expression of Bach2 during Neural Differentiation of P19 Cells—To examine expression of Bach2 during differentiation of neural cells, we used the induced differentiation system of P19 EC cells *in vitro*. RNA was isolated at various time points after induction of neural differentiation, and expression levels of Bach2 mRNA were determined by RT-PCR (Fig. 1A). While expression of Bach2 mRNA was very low in uninduced P19 EC cells, it was highly induced upon induction of differentiation. The time course of induction was very similar to that of microtuble-associated protein 2 (MAP2) mRNA, which is expressed specifically in neuronal cells. In contrast, Bach1 mRNA, a closely related factor of Bach2, did not change during the *in vitro* differ-entiation of P19 EC cells. Rather, Bach1 mRNA showed a transient decrease during the aggregation phase of the neural differentiation. As expected, the induction of Bach2 mRNA was reflected in the protein levels: immunoblot analysis of cell extracts using antiserum specific to Bach2 revealed that Bach2 protein accumulated upon induction of neuronal differentiation (Fig. 1B). These results suggest that induction

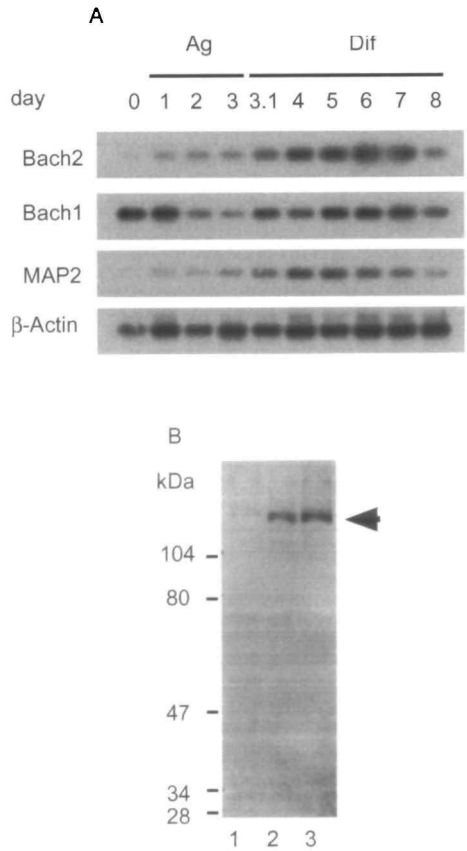

Fig. 1. **Expression of Bach2 during neural differentiation of P19 EC cells.** (A) RNA was isolated at indicated time points during differentiation of P19 cells and analyzed for expression of each gene by RT-PCR analysis. Ag, aggregation phase; Dif, neural differentiation phase. (B) Bach2 protein levels were detected by immunoblot analysis of protein extracts prepared from P19 cells under normal culture conditions (lane 1), aggregation phase (lane 2), and neural differentiation phase (lane 3).

of Bach2 is associated with differentiation of neuronal cells.

Expression of Bach2 during Development of Mouse Embryos—To verify the above observations, we examined expression of Bach2 mRNA and protein during development of mouse embryos. RNA and protein were prepared from whole embryos (E8.5 and E9.5) or CNS regions (E10.5–15.5). Upon RT-PCR, Bach2 mRNA was clearly induced during development of the nervous system (Fig. 2A). To investigate expression of Bach2 at the protein level, we utilized the antiserum against Bach2 [F69-2 (*13*)]. By using cell extracts prepared from Bach1- or Bach2-expressing cells, we showed previously that the anti-Bach2 antiserum reacts with Bach2 but not with Bach1 (*13*). Upon immunoblot analysis of protein extracts, the anti-Bach2 antiserum detected two bands that were induced markedly during the neural differentiation (Fig. 2B). These two bands may represent post-translational modification of Bach2 or alternatively spliced forms of Bach2. The upper band of Bach2 was detected at earlier time points than the lower band. Amounts of both bands were strongly induced around the E13.5 time point.

To further examine the correlation of Bach2 expression and neural development, we carried out immunohistochemical analysis using affinity-purified anti-Bach2 antibodies. In E12.5 embryos, specific signals detected with the anti-Bach2 antibodies were mainly restricted to the CNS regions, as shown in Fig. 3 (A–D). The medulla oblongata, posterior region of parietal cortex, and spinal cord showed strong signals (indicated with arrowheads in B and D). Within the spinal cord, differentiating cells in the mantle layer expressed high levels of Bach2, whereas neuronal precursor cells in the ventricular layer did not express detectable levels of Bach2 (E, F). Coronal sections revealed that Bach2 was expressed strongly in the lateral ganglionic eminence (G) but not in the corresponding ventricular or subventricular zones, which give rise to cells of the lateral ganglionic eminence. The anatomical positions of the Bach2-expressing cells suggest that they were differentiating neuronal cells that had already withdrawn from the cell cycle. Some regions of the parietal cortex showed strong signals (G, H, and I). In these regions, Bach2-positive cells were observed through the whole layer (I). Along the third ventricle, immunoreactivity was detected mainly in the mantle layer but not in the ventricular region (H).

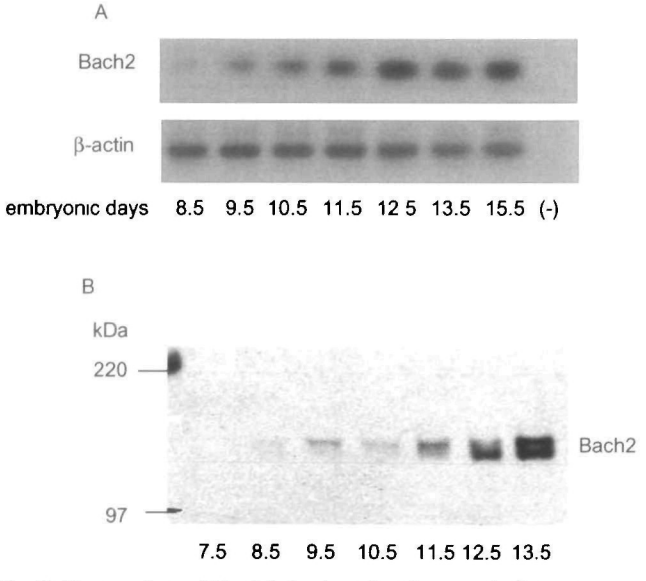

Fig. 2. **Expression of Bach2 during development of mouse embryos.** (A) RNA was isolated from whole embryos (E8.5 and 9.5), neural tissues including spinal cord (E10.5–13.5), and brain (E15.5), and Bach2 mRNA levels were compared by RT-PCR. Amounts of template cDNA were calibrated using β-actin mRNA. As a negative control, PCR reactions without template cDNA were carried out [indicated with (–)]. (B) Protein extracts were prepared from whole embryos (E7.5–9.5) and neural tissues including spinal cord (E10.5–13.5). Expression of Bach2 was compared by immunoblotting analysis. Positions of marker proteins are indicated at the left side.

Bach2 was also expressed in dorsal root ganglia (DRG, E and F). Since DRG is derived from neural crest, these observations suggest that the induction of *bach2* expression is a common event during neuronal differentiation in both central and peripheral nervous systems.

A high level of expression was also observed in the posterior lens fiber cells but was completely absent in the anterior lens epithelial cells (Fig. 3, G and J; see also Fig. 4, B and C). This expression pattern is similar to that of c-Maf, which is essential for lens development (*18–20*). Expression of Bach2 was not detected in the developing retina at this stage.

Cytoplasmic Localization of Bach2—We showed previously that activity of Bach2 is regulated by nuclear import and export (*14*). Thus, under normal culture conditions, Bach2 accumulates within the cytoplasmic regions due to efficient nuclear export in transfected neuronal cells and fibroblasts. However, there is no evidence that such regulation is operative *in vivo*. A closer examination of the immunohistochemical results shown above revealed that cytoplasmic regions and neuronal axons of cells within the lateral ganglionic eminence were stained intensely with the antibodies (Fig. 4A). Some cells within these regions showed a Bach2 signal exclusive to the cytoplasmic region (Fig. 4A, indicated with arrowheads). The staining of the cytoplasm and axons was judged to be a Bach2-derived signal because of the following reasons: first, in immunoblotting analysis of brain extracts prepared from mice embryos (see Fig. 2B), the affinity-purified antibody reacted with two bands, both of which also reacted with another independent anti-Bach2 antibody (data not shown); and second, the antibody staining was confined to the regions where Bach2 mRNA was detected by *in situ* RNA hybridization (data not shown). Besides cells in the lateral ganglionic eminence, lens fiber cells also showed a marked cytoplasmic staining (Fig. 4D). The cytoplasmic staining was judged to be specific, since the control serum did not give such a signal (compare B and C).

DISCUSSION

In this study, we examined expression of Bach2 during neural differentiation of P19 EC cells *in vitro* and development of neural systems in mice. We took advantage of affinity-purified Bach2-specific antibodies to carry out immu-nohistochemical analysis. This allowed us to determine the localization of Bach2 in primary tissues. Our results show that expression of Bach2 is specific to neural tissues in de-veloping mouse embryos and that, as envisaged from our previous observations *in vitro* (*14*), the function of Bach2 is regulated by subcellular compartmentation.

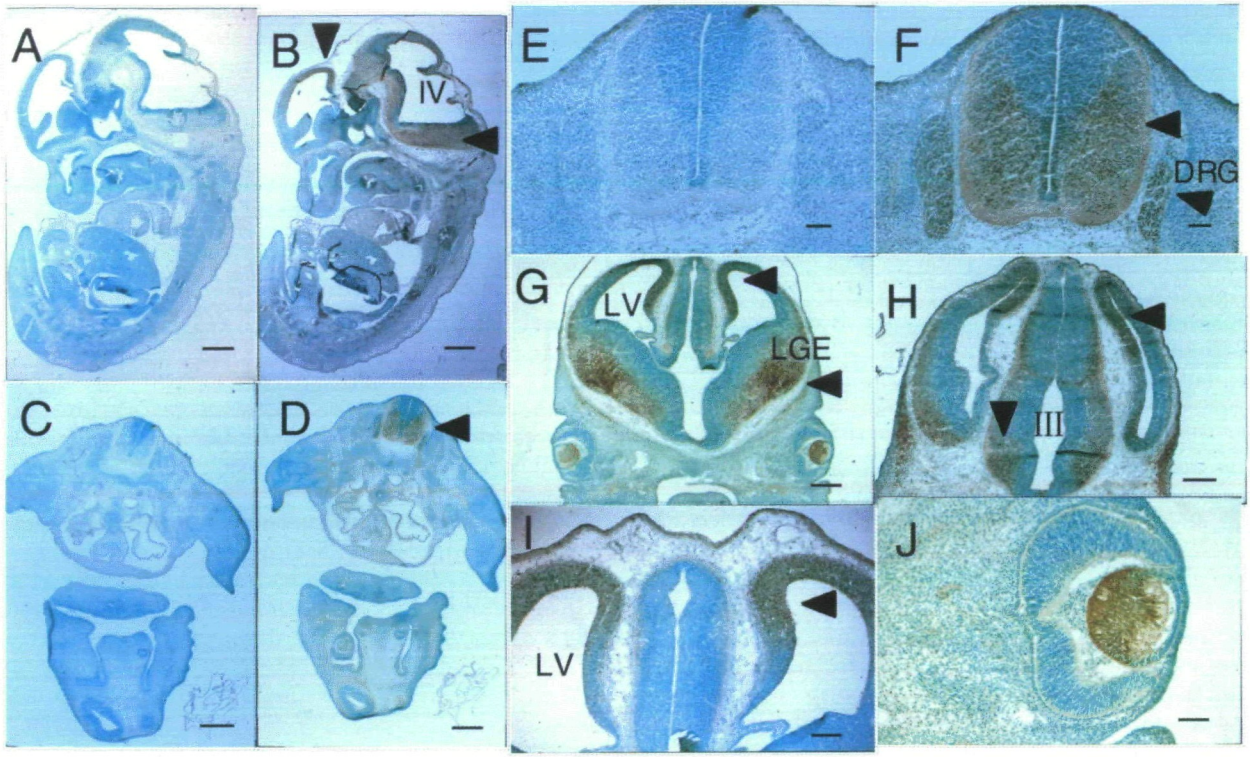

Fig. 3. **Immunohistochemical analysis of Bach2 expression in E12.5 mouse embryos.** Sections of E12.5 embryos were reacted with anti-Bach2 (B, D, F–J) or control antibody (A, C, E). Sagittal sections (A and B) and transverse sections (C and D) show specific signals confined within the nervous system (indicated with arrowheads). E and F show higher magnification images of spinal cord. Panels G, H, I, and J show coronal sections at a low (G and H) or high (I and J) magnifications. Specific staining signals are indicated with arrowheads. III, the third ventricle; IV, the fourth ventricle; LV, lateral ventricle; LGE, lateral ganglionic eminence; DRG, dorsal root ganglia. Scale bars indicate 0.4 mm in A–D, 0.1 mm in E and F, 0.2 mm in G and H, and 0.05 mm in I and J.

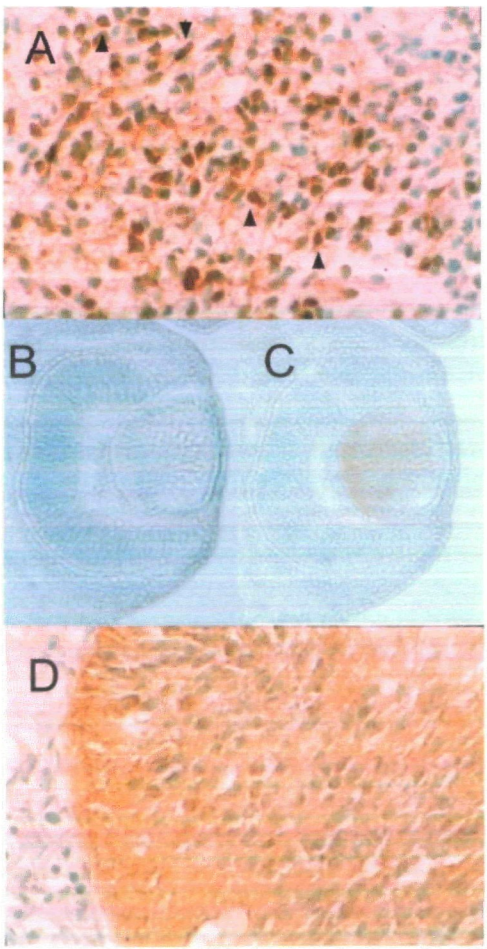

Fig. 4. **Localization of Bach2 protein in cells.** (A) A high magnification image of the LGE region. In some cells, Bach2 is mainly localized in cytoplasm (arrowheads). (B and C) Images of eye regions reacted with the control (B) or anti-Bach2 antibodies (C). (D) A high magnification image of lens. Bach2 protein is mainly localized in cytoplasm in lens fibers.

AP-1 proteins are involved in various neural processes such as development, differentiation, transynaptic stimulation, degeneration, and regeneration. Such diverse functions of AP-1 proteins may be due to the presence of various reinforcing and/or antagonizing activators and inhibitors of AP-1 (*5*). Our present results suggest that Bach2 represses TRE, ARE, and MARE-dependent gene expression upon differentiation of neural cells. Furthermore, since Bach2 can be either nuclear or cytoplasmic, Bach2-mediated repression may be regulated by cellular or environmental cues. At present, oxidative stress is known to inhibit nuclear export of Bach2 (*14*). This raises the interesting possibility that intracellular redox status affects gene expression in neuronal cells during differentiation. In this regard, there are several reports suggesting that oxidative stress signals might participate in differentiation (*21–23*). For example, over-stimulation of glutamate receptors, which play important roles in activity-dependent differentiation of neuronal cells, causes oxidative stress (*24*). Such an oxidative stress may affect gene expression through transcription factors like Bach2. It is also possible that subcellular localization of Bach2 is regulated by signals other than oxidative stress as well. Future analyses of Bach2 may shed light on novel aspects of relationships among oxidative stress, gene expression, and differentiation of neuronal and lens cells.

We would like to thank Dr. Masayuki Yamamoto for discussion and encouragement.

REFERENCES

1. Shaulian, E. and Karin, M. (2001) AP-1 in cell proliferation and survival. *Oncogene* **20**, 2390–2400
2. van Dam, H. and Castellazzi, M. (2001) Distinct roles of Jun:Fos and Jun:ATF dimers in oncogenesis. *Oncogene* **20**, 2453–2464
3. Kataoka, K., Igarashi, K., Itoh, K., Fujiwara, K.T., Noda, M., Yamamoto, M., and Nishizawa, M. (1995) Small Maf proteins heterodimerize with Fos and potentially act as competitive repressors of NF-E2 transcription factor. *Mol. Cell. Biol.* **15**, 2180–2190
4. Kataoka, K., Noda, M., and Nishizawa, M. (1994) Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol. Cell. Biol.* **14**, 700–712
5. Herdegen, T. and Waetzig, V. (2001) AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration. *Oncogene* **21**, 2423–2437
6. Cordes, S.P. and Barsh, G.S. (1994) The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025–1034
7. Mohler, J., Mahaffey, J.W., Deutsch, E., and Vani, K. (1995) Control of *Drosophila* head segment identity by the bZip homeotic gene cnc. *Development* **121**, 237–247
8. Andrews, N.C., Erdjument-Bromage, H., Davidson, M.B., Tempst, P., and Orkin, S.H. (1993) Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* **362**, 722–728
9. Chan, J.Y., Han, X.-L., and Kan, Y.W. (1993) Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. *Proc. Natl. Acad. Sci. USA* **90**, 11371–11375
10. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* **367**, 568–572
11. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y.W. (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the β-globin locus control region. *Proc. Natl. Acad. Sci. USA* **91**, 9926–9930
12. Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N., and Yamamoto, M. (1999) Molecular cloning and functional characterization of a new Cap'n'collar family transcription factor Nrf3. *J. Biol. Chem.* **274**, 6443–6452
13. Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M., and Igarashi, K. (1996) Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol. Cell. Biol.* **16**, 6083–6095
14. Hoshino, H., Kobayashi, A., Yoshida, M., Kudo, N., Oyake, T., Motohashi, H., Hayashi, N., Yamamoto, M., and Igarashi, K. (2000) Oxidative stress abolishes leptomycin B-sensitive nuclear export of transcription repressor Bach2 that counteracts activation of Maf recognition element. *J. Biol. Chem.* **275**, 15370–15376
15. Muto, A., Tashiro, S., Tsuchiya, H., Kume, A., Kanno, M., Ito, E., Yamamoto, M., and Igarashi, K. (2002) Activation of Maf/AP-1 repressor Bach2 promotes apoptosis and its interaction with PML nuclear bodies. *J. Biol. Chem.* **277**, 20724–20733
16. Muto, A., Hoshino, H., Madisen, L., Yanai, N., Obinata, M., Karasuyama, H., Hayashi, N., Nakauchi, H., Yamamoto, M., Groudine, M., and Igarashi, K. (1998) Identification of Bach2 as a B-cell-specific partner for small Maf proteins that negatively regulate the immunoglobulin heavy chain gene 3′ enhancer. *EMBO J.* **17**, 5734–5743
17. Jones-Villeneuve, E.M., McBurney, M.W., Rogers, K.A., and Kalnins, V.I. (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**, 253–262
18. Kim, J.I., Li, T., Ho, I.-C., Grusby, M.J., and Glimcher, L.H. (1999) The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Proc. Natl. Acad. Sci. USA* **96**, 3781–3785
19. Kawauchi, S., Takahashi, S., Nakajima, O., Ogino, H., Morita, M., Nishizawa, M., Yasuda, K., and Yamamoto, M. (1999) Regulation of lens fiber cell differentiation by transcription factor c-Maf. *J. Biol. Chem.* **274**, 19254–19260
20. Sakai, M., Imaki, J., Yoshida, K., Ogata, A., Masushima-Hibiya, Y., Kubori, Y., Nishizawa, M., and Nishi, S. (1997) Rat maf related genes: specific expression in chondrocytes, lens, and spinal cord. *Oncogene* **14**, 745–750
21. Buttke, T.M. and Sandstrom, P.A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today* **15**, 7–10
22. Camhi, S.L., Lee, P., and Choi, A.M. (1995) The oxidative stress response. *New Horiz.* **3**, 170–182
23. Jacobson, M.D. (1996) Reactive oxygen species and programmed cell death. *Trends Biochem. Sci.* **21**, 83–86
24. Murphy, T.H., Miyamoto, M., Sastre, A., Schnaar, R.L., and Coyle, J.T. (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* **2**, 1547–1558