Molecular Cloning and Functional Characterization of a New Cap’n’ Collar Family Transcription Factor Nrf3*

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The NF-E2-binding sites or Maf recognition elements (MARE) are essential cis-acting elements in the regulatory regions of erythroid-specific genes recognized by the erythroid transcription factor NF-E2, composed of p45 and MafK. Recently, two p45-related factors Nrf1 and Nrf2 were isolated, and they are now collectively grouped as the Cap’n’ collar (CNC) family. CNC factors bind to MARE through heterodimer formation with small Maf proteins. We report here the identification and characterization of a novel CNC factor, Nrf3, encoding a predicted 73-kDa protein with a basic region-leucine zipper domain highly homologous to those of other CNC proteins. In vitro and in vivo analyses showed that Nrf3 can heterodimerize with MafK and that this complex binds to the MARE in the chicken β-globin enhancer and can activate transcription. Nrf3 mRNA is highly expressed in human placenta and B cell and monococyte lineage. Chromosomal localization of human Nrf3 is 7p14–15, which lies near the hoxa gene locus. As the genetic loci of p45, nrf1, and nrf2 have been mapped close to those of hoxc, hoxb, and hoxd, respectively, the present study strongly argues for the idea that a single ancestral gene for the CNC family members may have been localized near the ancestral Hox cluster and have diverged to give rise to four closely related CNC factors through chromosome duplication.

Regulation of biological processes is accomplished through interactions among various tissue- or developmental stage-specific transcription factors. For instance, the transcription factor NF-E2 is critical for erythroid-specific expression of the porphobilinogen deaminase gene (1). NF-E2 is composed of two subunits; the p45 component contains the Cap’n’ collar (CNC)1-type basic region-leucine zipper (bZip) domain, which is highly conserved with the bZip domain of the Drosophila transcription factor CNC (2). Recently, several mammalian transcription factors carrying the CNC/bZip domain were identified, such as Nrf1/LCR-F1/TCF11 (3–5), Nrf2/ECH (6, 7), Bach1, and Bach2 (8). The other subunit of the NF-E2 complex is composed of the small Maf proteins, members of the Maf proto-oncoprotein family (9, 10). To date, three small Mafs, MafF, MafG, and MafK, have been identified (11–14).

A homodimeric complex of small Maf proteins recognizes a DNA sequence motif called Maf recognition element (MARE, TGCTGAG/C/TCAGCA or TGCTGACGTCAGCA), which contains either a 12-O-tetradecanoylphorbol-13-acetate-responsive element (TGA(G/C)/TCA) or cAMP-responsive element (TGACGTCA) (15, 16). Homodimeric complexes of small Mafs repress transcription through binding to MARE, since they lack canonical trans-activation domains. On the other hand, CNC proteins require a small Maf protein for their DNA binding activities. A CNC-small Maf heterodimer complex binds to both the NF-E2 consensus sequence (TGCTGAG/C/TCAGCA) and MARE (in particular to the 12-O-tetradecanoylphorbol-13-acetate-responsive element-type MARE). These regulatory motifs are frequently observed in the regulatory regions of erythroid-specific genes, such as the locus control region (LCR) of β-globin genes, as well as in several non-erythroid gene promoters (17).

To assess the biological roles of the CNC family proteins, p45, nrf1 and nrf2 genes were disrupted individually (18–20). Whereas the loss of p45 function impaired megakaryocyte maturation and platelet formation, erythropoiesis in p45-null mice was normal. Nrf2 is essential for the induction of phase II detoxifying gene expression such as glutathione S-transferase and NADPH-quione oxidoreductase (NQ01) by phenolic antioxidants, but its loss (20), even in combination with p45 (21, 22), did not cause anemia. The results of the nrf1 gene disruption are somewhat controversial; one report showed early embryonic lethality (19), whereas another study showed loss of definitive hematopoiesis (23). These results indicate that compensation among CNC family proteins may exist in erythroid-specific gene regulation and that lineage-specific regulation of gene expression through MAREs appears to be accomplished by multiple CNC family proteins.

Fluorescence in situ hybridization (FISH) analyses revealed...
that the chromosomal localization of the p45, nrf1, and nrf2 genes are mapped close to the hoxC (12q13.1–13.3), hoxB (17q21), and hoxD (2q31) gene clusters, respectively (24), suggesting that the p45-related factors may be derived from a single ancestral gene through chromosome duplication, as is the case for the hox gene clusters. Since no CNC factor has been identified near the hoxA locus (7p14–15), this also implies the existence of an additional CNC factor. We report here the cloning and structure of Nrf3.

A New CNC Family Factor Nrf3

![Fig. 1. Cloning and structure of Nrf3. A, nucleotide sequence and deduced amino acid sequence of mouse Nrf3 cDNA. The CNC and basic domains and leucine residues in zipper domains are indicated by the dotted line, box, and circles, respectively. A polyadenylation consensus sequence is indicated by the thick underline. Two mRNA destabilizing sequences in 3'-UTR and one CNC factor-conserved amino acid sequence (DS-GLSL) are underlined. B, comparison of amino acid sequences of mouse and human Nrf3. Sequence information from human Nrf3 genomic sequence data of bacteria artificial chromosome clone (AC004520) is underlined.](image)
identification and characterization of this new CNC family member, Nrf3 (NF-E2 related factor 3)

EXPERIMENTAL PROCEDURES

Isolation of Nrf3—Expressed sequence tag (EST) cDNA fragment for Nrf3 (THC181377) was isolated by PCR using genomic DNA of HeLa cells as a template. 30 cycles of PCR were performed at 94 °C for 30 s; 60 °C for 30 s; 72 °C for 1 min. Primers used were 5'-GATATTTTTAG-TAGATTAAGAGATGACC-3' and 5'-GCACTTCATGAAAAAGTTGTGGC-3'.

Human and Mouse Nrf3 cDNA Cloning—A human placenta lgt11 cDNA library (a generous gift from Dr. Shigeru Taketani, Kansai Medical University) was plated on 150-mm Petri dishes at a density of 5 × 10⁴ plaque-forming units per plate, and 1 × 10⁶ plaques were screened with labeled EST fragment (25). To isolate mouse Nrf3, a mouse brain cDNA library (Stratagene) was screened using a 0.5-kbp HindIII fragment of human Nrf3 cDNA as a probe. Hybridization was carried out at 65 or 50 °C in 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10 mM EDTA, 1 × Denhardt’s solution, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Membranes were washed twice for 30 min at 65 °C in 0.1 × SSC and 0.1% SDS solution (human cDNA library screening) or 50 °C in 2 × SSC and 0.1% SDS solution (mouse cDNA library screening). Positive plaques were isolated and purified.

5′-Rapid Amplification of the cDNA End Analyses and DNA Sequencing—5′-Rapid amplification of the cDNA end analyses were carried out with the Marathon™ cDNA amplification kit (CLONTECH). DNA sequences were determined on both strands by the dideoxynucleotide method using an ABI377 automated sequencer (Perkin-Elmer).

Cell Culture and RNA Blot Analysis—NALL-1, NALM-6, BALM-2, NAMALWA, and RPMI8226 cells were gifts of Dr. Kenji Ohtani (Fujisaki Cell Center of Hayashibara Biochemical Laboratories, Inc.), and KOPT-K1, THP-6, and NALM-17 cells were gifts of Dr. Yasuhide Hayashi (University of Tokyo). MGS cells were a gift of Dr. Shinkichi Yokoyama (Yamagata University). KG-1, HL60, U937, and THP-1 were obtained from Japanese Cancer Resources Bank. Poly (A)⁺ RNA samples from various cultured cell lines were prepared by the guanidine-acidified phenol chloroform method (26) and purified with oligotex (dT)₃₀ column chromatography (Roche). RNA blots containing multiple human tissue RNAs were purchased from CLONTECH (2 mg of poly (A)⁺ RNA per sample). Radiolabeled probe was prepared from the 500-bp HindIII fragment of the human clone, SKhNrf3/1-1.

Construction of Expression Plasmids for Transient Transfection Assays—BosNrf1, BosNrf2, and BosNrf3 were generated by subcloning the 2.6-kbp HindIII/XbaI fragment from pcDNA1/Neo-Nrf1 (11), the 2.6-kbp BamHI/XbaI fragment from pcDNA1/Neo-Nrf2 (11), and the 2.5-kbp fragment from SKmNrf3–1, respectively, into the blunt-ended XbaI site of pEFBos (27).

The quail fibroblast cell line QT6 (28) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and plated onto 24-well dishes (5 × 10⁴ cells per well with 0.3 ml medium). 24 h before transfection by the calcium phosphate precipitation method (29). 10 ng of firefly luciferase (Luc) reporter plasmid, 50 ng of pEML (an internal control), and various combinations of effector plasmids were used (see figure legends). Luc reporter plasmids pRBP2 and pRBP4 were previously described (10). QT6 cells were washed twice with phosphate-buffered saline 12 h after transfection, fed with fresh media, and incubated for an additional 24 h. Luc activity was measured following the supplier’s protocol (Promega) with a Bioluminometer (Berthold). Assays were performed in triplicate in
independent transfection experiments, and the results were normalized with respect to pENL β-galactosidase activity.

Expression of CNC Proteins in Yeast Cells and Yeast Two-hybrid Analysis—Expression plasmids of GADNrf1, GADNrf2, and GADNrf3 were generated by inserting the 2.6-kbp DraI/XbaI fragment from pCDNANeo-Nrf1 (11), the 2.8-kbp BglII/XbaI fragment from pCDNANeo-Nrf2 (11), and the 1.8-kbp EcoRI fragment from SKhNrf3/1–1, respectively, into the appropriate sites of GAD424. GBD-MafK was generated by subcloning the 1.0-kbp BamHI/HindIII fragment from pQ630-MafK (11) into the blunt-ended ClaI site of GBD-pCIA which was created by inserting a ClaI linker (8-mer, 5'-ACAGTACG-3') into the EcoRI site of GBT9. Saccharomyces cerevisiae SFY526 were transformed with several combinations of plasmids by the lithium acetate method. Measurement of β-galactosidase activity of transformants was performed as described (30).

Expression of MBP-CNC-bZip and MBP-MafK in Escherichia coli—MBP/Nrf1, MBP/Nrf2, MBP/Nrf3, and MBP/MafK were constructed by inserting 500-bp PCR-generated BamHI/XbaI fragments from pCDNANeo-Nrf1, pCDNANeo-Nrf2, and SKhNrf3/1–1, and the 1.0-kbp BamHI/HindIII fragment from pQ630-MafK (11) into appropriate sites of pMAL-C2 (New England Biolabs). Primers used were 5'-CGGAGTACCAAGGAGCAAGGAGGACGAC Site 3'-SP6 primer (Nrf1), 5'- CGGATCCGCTCCATACGAAGAACAAAGC Site 3'-SP6 primer (Nrf2), and 5'- CGGATCCGACAAGGATGAGCTAGTAGTA-3' T3 primer (Nrf3). All constructs were confirmed by sequencing.

BL21(DE3) pLysS strain of E. coli was transformed with each expression vector and incubated overnight. Each culture was diluted 10-fold with fresh LB medium containing 100 μg/ml ampicillin and incubated for additional 2 h at 37 °C. The cells were harvested 3 h after the addition of isopropyl-1-thio-β-galactosidase at a final concentration of 0.2 mM and mildly sonicated in extraction buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Lysates were prepared by centrifugation at 5,000 × g for 20 min at 4 °C and loaded on columns of amylose resin (New England Biolabs). The columns were washed with extraction buffer and subsequently with buffer B (20 mM Hapes-NaOH (pH 7.9), 20 mM NaCl, 4 mM MgCl₂, 1 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Recombinant proteins were eluted with buffer B containing 10 mM maltose and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining (31).

Electrophoretic Gel Mobility Shift Analyses (EMSA) and Off-rate Kinetic Experiments Using MBP-CNC-Proteins—Oligonucleotides encoding the chicken β-globin enhancer MARE sequence (5'-TGGGCCTGAAAAGAGCATCCTATCATGAGCCC-3') were labeled with γ[32P]ATP by T4 polynucleotide kinase. The reaction mixture contained 20 μM Hepes-NaOH (pH 7.9), 1 mM EDTA, 60 mM NaCl, 1 mM dithiothreitol, 4 mM MgCl₂, 25 μg of poly(dI-dC) and poly(dA-dT)/ml, 100 μM of bovine serum albumin/ml, and 50 ng of purified proteins. The reactions were electrophoresed at 4 °C, 150 V on a 4.5% polyacrylamide gel in 0.5 × TBE (45 mM Tris-HCl (pH 8.2), 45 mM borate, and 1.25 mM EDTA). Competition assays using various cold probes were performed as described previously (8). In off-rate kinetic experiments, EMSA reaction mixture was first incubated with radiolabeled probe at 37 °C for 5 min. After the addition of cold double-stranded oligonucleotides (500-fold molar excess), an aliquot of the mixture was transformed and analyzed by polyacrylamide gel electrophoresis at several time points (described in figure legend). Amounts of binding complexes were quantified by an imaging analyzer (Fuji BAS-2000).

Chromosome Mapping by Fluorescence in Situ Hybridization—Metaphase spreads for FISH were prepared from phytohemagglutinin-stimulated normal human lymphocytes using a thymidine synchroni- zation/deoxybromouridine release technique. Nrf3 cDNA was labeled by nick translation with biotin-16-dUTP (Boehringer Mannheim). In situ hybridization was performed with the protocol of Inazawa et al. (32) with minor modifications. Briefly, for hybridization, 200 ng of probe was added to 10 ml of a hybridization mixture containing 50% formamide, 2× SSC, 10% dextran sulfate, and 20 μg/ml bovine serum albumin at 37 °C for 90 min. Slides were denatured at 70° formamide, 2× SSC at 72 °C for 3 min, dehydrated, and hybridized overnight in a moist chamber at 37 °C with the probe. The slides were washed in 50% formamide, 2× SSC and in 1× SSC at 37 °C for 10 min each, and then in 4× SSC at room temperature for 10 min. After washing, the slides were incubated with avidin-ﬂuorescein isothiocyanate at 37 °C for 50 min and then counterstained with DAPI in anti-fade solution. Analysis was carried out using an Olympus BX50-FLA fluorescence microscope and photographs were taken with Kodak Ektachrome ASA 400 film.

Nucleotide Sequence Data—The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank™ nucleotide sequence data bases with the following accession numbers, AB010812 (human) and AB013852 (mouse).

RESULTS

Isolation of a cDNA Clone Encoding a Novel CNC Family Member—Loci for the p45, nrf1, and nrf2 genes have been mapped near the hoxC, hoxB, and hoxD gene clusters, respectively (24), but no CNC factor mapping close to the hoxA locus had yet been identified, suggesting that a novel CNC factor may exist. We searched for CNC factors in the TIGR human
FIG. 3. DNA binding activity of Nrf1-, Nrf2-, and Nrf3-MafK in EMSA.
A, MBP, MBP-Nrf1, MBP-Nrf2, and MBP-Nrf3 proteins (50 ng) were incubated with chicken β-globin enhancer MARE in the presence (lanes 5–8) or absence (lanes 1–4) of MBP-MafK protein (50 ng). B, DNA recognition specificity of Nrf3-MafK complex was examined by competition assay. 100-fold excess competitors were added to the reaction. Competitors were double-stranded cold oligonucleotides containing wild type (βE, lane 3) or mutated β-globin 3'enhancer MARE (βE/M, lane 4), LCR HS-2 MARE (HS2, lane 5) and erythroid-specific 5-aminolevulinate synthase MARE (ALAS, lane 6). C, DNA binding affinities of CNC factor-MafK complexes were determined by off-rate kinetic experiments. After addition of 560-fold excess competitors, a portion of the binding mixture was taken at 0, 3, 6, 9, 15, 20, and 25 min time points, respectively, and separated by polyacrylamide gel electrophoresis. D, the dissociation curve of CNC factors-MafK complex from DNA. Intensity of the time 0 band was arbitrary assigned to 1, and other data were normalized on the basis of this. The values represent average of three independent experiments.
The cDNA sequence for human MafK was fused to the GAL4 DNA binding domain (GAD, green in hatched boxes). One of the Nrf2/ECH conserved domains, Domain 2 (7), is shown as hatched boxes.

To isolate the mouse homologue, we screened a mouse brain cDNA library using one of the human partial cDNA clones and obtained eight positive phage clones. As shown in Fig. 1A, the longest mouse cDNA clone encodes a 1983-bp open reading frame flanked by 237 and 339 bp of 5'- and 3'-untranslated regions (UTRs), respectively. Although no preceding in-frame translation initiation codon was noted in the 5'-UTR, we identified a termination codon 15 nucleotides upstream from 5'-terminal of this cDNA in the mouse genomic clone, suggesting that this protein is a member of the CNC family. We therefore named this factor Nrf3 (NF-E2 related factor-3).

In the bZip region, the basic region of Nrf3 was especially highly conserved with that of other CNC factors (see Fig. 2B), suggesting that Nrf3 may share their DNA binding specificity. A short region "DSGLSL" that is absolutely conserved among the CNC family members (24) is also present in Nrf3 (see Fig. 1A, underlined). The leucine-zipper sequence of Nrf3, however, was much more variable than those of other CNC factors.

DNA Binding Activity of Nrf3—The three CNC factors currently analyzed (i.e. p45, Nrf1, and Nrf2) have all been shown to bind MAREs as heterodimers with one of the small Maf proteins, but not as homodimers. Since one of these small Mafs, MafK (9, 11, 12), is highly coexpressed with Nrf3 (see below) in the placenta, these two factors are likely to be partners, at least in this tissue. We therefore examined whether a complex of Nrf3 and MafK could bind to the MARE of the chicken β-globin enhancer by EMSA.

Bacterially expressed MBP-fused bZip domains of the CNC proteins were tested for their ability to bind MARE. In the absence of MafK, no DNA binding was observed (Fig. 3A, lanes 2–4). Although a homodimer of MafK could bind the probe (lane 5), the affinity appeared to be much weaker than the CNC-MafK heterodimers (lanes 6–8). The binding of Nrf3-MafK was efficiently competed out with addition of cold double-stranded oligonucleotides encoding the MAREs of the chicken β-globin enhancer and the hypersensitive site 2 (HS-2) of human β-globin (Fig. 3B, lanes 3 and 5), but not by mutated MAREs (lane 4). Similarly, the MARE sequence of the erythroid 5-aminolevulinate synthase gene promoter, to which NF-E2 cannot efficiently bind,3 did not compete with the binding of the labeled probe (lane 6). These results demonstrate the specificity of the recognition for MAREs by the Nrf3-MafK heterodimer complex.

To assess binding affinities of the three CNC factors to MARE, a series of off-rate kinetic experiments was carried out (Fig. 3C). These CNC factors were mixed with MafK and radiolabeled MARE probe, and the mixtures were subjected to the addition of cold double-stranded oligonucleotides. The mixtures were then separated at several time points by polyacrylamide gel electrophoresis. Fig. 3D shows half-times of Nrf1-, Nrf2- and Nrf3-MafK-DNA complexes. The half-time of dissociation of Nrf1- and Nrf3-MafK complex from MARE was approximately 5 min, while that of Nrf2-MafK complex was within 3 min. These results suggest that Nrf1- and Nrf3-MafK complexes could bind the MARE probe more tightly than Nrf2-MafK complex in this experimental condition.

Nrf3-MafK Dimer Formation in Yeast Cells—We then examined dimerization affinities between Nrf3 and MafK using the Yeast strain SFY526 containing lacZ gene under the control of the GAL1 promoter was transformed with the plasmids indicated, and β-galactosidase activity was determined.

### Table I

| Yeast strain | GAD424 | GAD Nrf3 | GAD Nrf1 | GAD Nrf2 |
|-------------|--------|----------|----------|----------|
| GBT9        | 0.043  | 0.035    | 0.038    | 0.047    |
| GBT MaFK    | 0.048  | 61       | 1.1      | 167      |

Nrf3 Is a New Member of CNC Family—Inspection of the domain structure of the new mouse clone revealed the presence of a bZip domain which is homologous to that of other CNC transcription factors: 53, 56, and 50% identity, respectively, for p45, Nrf1, and Nrf2 (Fig. 2A), indicating that this protein is a member of the CNC family. We therefore named this factor Nrf3 (NF-E2 related factor-3).

2. A. Kobayashi, unpublished observations.

3. K. Igarashi and M. Yamamoto, unpublished observations.
yeast two-hybrid system. For this purpose the *S. cerevisiae* strain SFY526 was utilized which carries a lacZ reporter gene with binding sites for GAL4 (upstream activating sequence). Hybrid proteins of human Nrf1, Nrf2 (both full length), and Nrf3 (lacking the N-terminal) fused to the GAL4 activation domain (GAD) were used for "prey," whereas a fusion of human MafK to the GAL4 DNA-binding domain (GBD) was used as "bait" (Fig. 4). Dimerization activities between these chimeric proteins were determined by measuring the LacZ activity. As shown in Table I, control experiments using GBD alone and GAD-Nrf1, GAD-Nrf2, or GAD-Nrf3 did not exhibit LacZ reporter activity. Similarly, GBD-MafK and GAD424 plasmid (GAD alone) showed no LacZ activity. In contrast, considerable enhancement of LacZ activity was observed when the yeast cells were transformed with GBD-MafK and GAD-Nrf1, GAD-Nrf2, or GAD-Nrf3, indicating the presence of interaction between MafK and these CNC family members. GAD-Nrf2 and -Nrf3 showed much stronger enhancement of LacZ activity than GAD-Nrf1 did in this yeast interaction assay. These results thus show that MafK-Nrf2 and -Nrf3 may have stronger heterodimerizing activities than MafK-Nrf1 does, although there is a possibility that a part of the enhancement of LacZ activity is derived from the activation domain of CNC factors in the GAD constructs.

**Nrf3 Is a Transcriptional Activator**—The trans-activation activity of Nrf3 was examined by transient transfection assay using two reporter plasmids, pRBGP2, encoding three copies of chicken β-globin MARE, and pRBGP4, containing mutated binding sites (10). The reporter plasmids were transfected with various amounts of Nrf3, Nrf1, and Nrf2 expression plasmids into QT6 quail fibroblasts. Co-transfection of the Nrf3 expression plasmid with the pRBGP2 reporter resulted in activation of the reporter by approximately 4-fold (Fig. 5A, compare lanes 1 and 4) in a dose-dependent manner. This activation was not observed with the pRBGP4 reporter (Fig. 5B), demonstrating that the activation by Nrf3-MafK is dependent on binding to the MAREs. Furthermore, while the expression of high doses of MafK efficiently repressed the pRBGP2 reporter plasmid (Fig. 5C, lane 2), co-expression of Nrf3 overcame this MafK-mediated repression (see lanes 3–5), indicating that Nrf3 is a transcriptional activator.

Nrf2 has been shown to be the strongest trans-activator.
**Fig. 6. Expression profile of Nrf3.** A, RNA blot analysis with poly(A)$^{+}$ RNAs derived from human adult tissues. Positions of molecular markers are shown on both sides. B and C, RNA blot analysis using mRNA from various hematopoietic lineage cell lines. Positions of 28 S and 18 S rRNA are indicated by lines.

**Fig. 7. Chromosome assignment of the human nrf3 gene by FISH.** A, metaphase chromosome spread hybridized with Nrf3 probe, showing the presence of fluorescent signals at chromosome region 7p14–15 (arrowhead). B, the same chromosomes demonstrating Q bands with DAPI.
among the CNC factors (11), and this observation was reproducible in the present analysis (see Fig. 5A). We speculate that this may be either due to the stronger trans-activation activity of Nrf2 or the high affinity of Nrf2 for endogenous MafK than Nrf1 or Nrf3. Nrf3 showed strong enhancement of the LacZ reporter expression in the yeast two-hybrid assay (see Table I), suggesting that Nrf3 may activate transcription less efficiently than Nrf2 does in QT6 cells. Precise functions of Nrf3 activation, dimerization, and DNA binding domains, however, remain to be elucidated.

Expression Pattern of Nrf3—To investigate the functional role of Nrf3, the expression profile of human Nrf3 was determined by RNA blot analysis. High levels of Nrf3 mRNAs were detected in placenta and also in various other tissues, but at lower levels (Fig. 6A). In all cases, two distinct species of Nrf3 transcripts were observed.

We also examined the expression of Nrf3 in various hematopoietic cell lines. High levels of Nrf3 mRNA were detected only in a B cell line, Raji, and monocytic cell lines THP-1 and U937, whereas low levels of Nrf3 transcripts were widely present in all cases except CCRF-CEM and KG-1 (Fig. 6B). The expression of Nrf3 in the B cell lineage was intriguing and further examined using additional B and T cell lines. We found that Nrf3 is expressed in RPMI8226, a myeloma cell line and three Burkitt’s lymphoma cell lines, Raji, BALS-2, and NAMALWA, but not in pre-B cell lines, NALM-6, NALM-17, and NALL-1 (Fig. 6C). Nrf3 mRNA was also not expressed in two T cell lines, KOPT-K1 (Fig. 6C) and CCRF-CEM (Fig. 6B). These results suggest that Nrf3 may play an important role in B cell and monocyte lineages.

nrf3 Gene Is Mapped Close to HoxA Gene Cluster—We determined the chromosomal localization of nrf3 gene by FISH analysis of a total of 142 metaphase and prometaphase phytomem-agglutinin-stimulated lymphocytes. Fluorescent spots on one or both chromatids of chromosome 7 were observed in 94 metaphases (66.2%), and 25 plates (14.8%) displayed double signals on both homologues of chromosome 7. No significant signals were observed at any other chromosomal location. DAPI Q-banding was used to locate the probe on the short arm of chromosome 7. Localization of the hybridization signals was determined by comparing 10 selected FISH photos with the corresponding Q-band photos and assigned to 7p14–15 (Fig. 7, A and B, shown by arrowheads), where the hoxA locus is also mapped. In addition, a human bacteria artificial chromosome clone containing the nrf3 gene sequence was isolated from 7p15 (data not shown).

An evolutionary tree comparing bZip domain sequences among CNC family revealed that Nrf3 might have been the first derivative from an ancestral gene (Fig. 8) and that the CNC family may consist of two subfamilies, p45-related factors and the Bach family.

DISCUSSION

In this study, we isolated and characterized a new CNC family transcription factor Nrf3, which binds to MARE as a heterodimer with small Maf proteins and functions as a transcriptional activator. The nrf3 gene maps near the hoxA gene cluster. Since genes for p45, nrf1, and nrf2 are closely linked to the hoxC, hoxB, and hoxD clusters, the present result implies that CNC factors may be derived from a single ancestor, as is the case for the hox gene clusters. The evolutionary tree analysis and other results clearly indicate that the CNC family is composed of two distinctively related subfamilies, the p45-related and Bach subfamilies. It is interesting to note that members of the former subfamily all contain a conserved “DSGLSL” region, referred to as domain A (24), in the N termini.

The basic region of the Nrf3 bZip domain shows high structural similarity to those of other p45-related CNC factors (see Fig. 2B), suggesting that they recognize similar or overlapping DNA sequences. Indeed, EMSA showed that Nrf3-MafK binds to the MARE in the chicken β-globin enhancer, which is known to be a common target sequence of p45-, Nrf1-, and Nrf2-small Maf heterodimers. In yeast two-hybrid assays, Nrf3 (and Nrf2) enhanced the LacZ reporter expression to a much greater extent than Nrf1, suggesting that CNC factors differ in their ability to dimerize with small Maf proteins and hence in their DNA binding affinities (shown in Fig. 3, C and D). However, trans-activation activity of Nrf3 was weaker than that of Nrf1 and much weaker than that of Nrf2, indicating that the activation potentials also differ between factors. These divergences in heterodimer formation and trans-activation abilities of the CNC factors were most likely acquired during their evolution.

We recently demonstrated that phosphorylation is involved in the p45-dependent trans-activation process (35). The DNA binding and trans-activation activities of the p45-MafK complex appeared to be positively regulated by serine/threonine phosphorylation in mouse erythroleukemia cells. Since Nrf3 contains several phosphorylation consensus sites for protein kinase A, protein kinase C, and casein kinase II (data not shown), one plausible explanation of the above observation (i.e. Nrf3 exhibited strong activity in yeast assays but weak activity in transfection assays) is that the trans-activation activity of Nrf3 may be under the regulation of specific protein kinases in the cytokine signal cascade, and this regulation may be absent in the fibroblast transfection assays.

Recently, MafO was reported to interfere with trans-activation by Nrf1 in transfection studies using COS1 cells. Based on that observation, the authors (36) claimed that Nrf1 does not cooperate with MafG for trans-activation but may utilize a hypothetical partner molecule for this purpose in vivo. Although the discrepancy between their observation and ours (Fig. 5C and see Ref. 7) requires further testing, the proposition of new bZip partner molecule(s) for the CNC factors has not been examined yet. In this regard, we have extensively screened for “partner molecule(s) of CNC factors” using Nrf2/ ECH molecules in yeast two-hybrid assays, but to date have not isolated any suitable candidates with the expected function. Since we observed significant differences among the CNC factors in both yeast two-hybrid assays and EMSA, an alternative explanation for the difference in results may be the variation in the affinity of the CNC factors for the small Mafs.

We observed that Nrf3 and MafK were both abundantly expressed in placenta (this study and see Refs. 11 and 37). The placental giant cells produce a number of hormones, including four members of the prolactin/growth hormone family: placental lactogen I and II (PL-I and -II), proliferin, and proliferin-
related protein. A regulatory element recognized by AP-1 has been shown to be essential for the trophoblast-specific transcription of the mouse PL-I gene (38). Since the AP-1 binding sequence is completely integrated in MARE, the possibility exists that Nrf3 acts as a regulator of the PL-I gene through this site. To shed more light on the biological role of Nrf3, it is necessary to investigate the mRNA distribution by in situ hybridization and elucidate the Nrf3-positive cell types in placenta.

Nrf3 is also expressed abundantly in B cell and monocyte lineages. It is interesting to note that, in B cell development, Nrf3 mRNA is expressed after the pre-B cell stage, especially in Burkitt’s lymphoma cell lines. This expression profile is in clear contrast to that of Bach2; Bach2 is expressed in pro-B cell stages, and its expression is extinguished in the plasma cell stage.3 Thus, whereas Nrf3 and Bach2 share similar binding sequence specificity (i.e. both bind to MARE), they show reciprocally exclusive expression profiles during B cell development. Whether Nrf3 induces the expression of regulators of the class switch and B cell maturation remains to be clarified.

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