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Small Maf Proteins Heterodimerize with Fos and May Act as Competitive Repressors of the NF-E2 Transcription Factor

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The *maf* oncogene encodes a bZip nuclear protein which recognizes sequences related to an AP-1 site either as a homodimer or as heterodimers with Fos and Jun. We describe here a novel *maf*-related gene, *mafG*, which shows extensive homology with two other *maf*-related genes, *mafK* and *mafF*. These three *maf*-related genes encode small basic-leucine zipper proteins lacking the *trans*-activator domain of v-Maf. Bacterially expressed small Maf proteins bind to DNA as homodimers with a sequence recognition profile that is virtually identical to that of v-Maf. As we have previously described, the three small Maf proteins also dimerize with the large subunit of NF-E2 (p45) to form an erythroid cell-specific transcription factor, NF-E2, which has distinct DNA-binding specificity. This study shows that the small Maf proteins can also dimerize among themselves and with Fos and a newly identified p45-related molecule (Ech) but not with v-Maf or Jun. Although the small Maf proteins preferentially recognize the consensus NF-E2 sequence as heterodimers with either NF-E2 p45, Ech, or Fos, these heterodimers seemed to be different in their transactivation potentials. Coexpression of Fos and small Mafs could not activate a promoter with tandem repeats of the NF-E2 site. These results raise the possibility that tissue-specific gene expression and differentiation of erythroid cells are regulated by competition among Fos, NF-E2 p45, and Ech for small Maf proteins and for binding sites.

The *maf* oncogene was identified by structural analysis of the genome of the AS42 avian transforming retrovirus (26, 40). It encodes a nuclear basic-leucine zipper (bZip) protein which can form a homodimer through its zipper structure (23). Recently, we reported that the v-Maf homodimer specifically recognizes two relatively long palindromic DNA sequences, TGCTGACTCAGCA and TGCTGACGTCAGCA, at roughly equal efficiency (24). The middle parts of the two consensus binding sequences for Maf are identical with two well-characterized binding sequences of the AP-1 transcription factor, the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE; TGACTCA) and the cyclic AMP-responsive element (CRE; TGACGTCA), respectively. We therefore named the two types of recognition elements TRE-type Maf recognition elements (MAREs) and CRE-type MAREs. It was also recently revealed that Maf forms heterodimers with the two major components of AP-1, Fos and Jun (24, 27, 28). These heterodimers preferably bind to asymmetric DNA sequences consisting of the two consensus binding sequences of Maf homodimer and AP-1 (24). Thus, Maf and the two AP-1 components are suggested to interact with each other in a cooperative or inhibitory way in association with their recognition sequences by forming heterodimers of altered binding specificities.

Like many other proto-oncogenes, the c-*maf* gene is a member of a gene family. To date, four *maf*-related genes, *mafK*, *mafF*, *mafB*, and *nrl*, have been reported (14, 22, 53). Their gene products are closely related to v-Maf especially in the structure of the DNA-binding domain, suggesting conservation of the binding sequence specificities. The products of the *mafK* and *mafF* genes, however, lack the amino-terminal domain of c-Maf/v-Maf (14). Our recent structure-function studies showed that the amino-terminal two-thirds of the Maf molecule, which is absent in the small Maf family proteins, confers the protein's *trans*-activating activity (25). As expected from their structure, the small Maf family proteins can act as negative regulators of transcription as homodimers. On the other hand, the small bZip proteins can heterodimerize with another bZip protein, NF-E2 p45, to form a major transcriptional activator of erythroid cell-specific genes, NF-E2 (2, 3, 20, 38).

In this study, we describe the identification and characterization of a new member of the small *maf* subfamily, *mafG*. Each small Maf protein, including MafG, was found to be able to form homodimer and intrasubfamily heterodimers which can efficiently bind to the MARE probes. The three small Maf proteins also form heterodimers with Fos and an NF-E2 p45-related molecule, Ech, but not with Jun, v-Maf, and MafB proteins. The Fos-small Maf and Ech-small Maf heterodimers showed binding specificities closely similar to those of the p45-small Maf complexes. Unexpectedly, however, the bZip heterodimers were seemed to associate with distinct *trans*-activating potentials. Coexpression of Fos and small Maf proteins failed to activate expression of a reporter gene which contains three tandem binding sites for NF-E2. These results suggest the importance of the relative concentration of these

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bZip factors in the regulation of growth and differentiation of hematopoietic cells.

MATERIALS AND METHODS

Molecular cloning of the *mafG* gene. A chicken genomic DNA library was constructed by partial digestion with *Sau*3AI and size fractionation of chicken genomic DNA followed by ligation to λ Charon40A vector *Bam*HI arms. From this library, three genomic clones of the chicken *mafG* gene were cloned with a 0.65-kb *mafK*-specific DNA probe excised from plasmid pSaeK-1 as described previously (14). To isolate cDNA clones of the *mafG* gene, the following four cDNA libraries were screened. A chicken embryo fibroblast cDNA library and the RBC-4 anemic hen reticulocyte cDNA library were prepared as described previously (52, 56). The BV4 cDNA library (46) constructed from poly(A)$^+$ RNA of 10-day-old chicken embryos was kindly provided by D. Engel. A chicken brain cDNA library was purchased from Clontech (Palo Alto, Calif.). Plasmid pSaeG-1, from which the DNA fragment used as a *mafG*-specific probe was excised, was constructed by subcloning a 0.7-kb *Stu*I-*Eco*T22I genomic DNA fragment (nucleotides 274 to 991) into the polylinker of the pGEM plasmid vector.

Production of bZip proteins in *Escherichia coli*. For the production of maltose-binding protein (MBP)-fused bZip proteins in *E. coli*, we used the expression vector pMAL-c or its derivative, pMAL-cRI (New England Biolabs, Beverly, Mass.). MBP–v-Maf and MBP–v-Jun were synthesized as described previously (24). To construct an expression plasmid for the MBP-MafG fusion protein, a 0.7-kb *Stu*I-*Eco*T22I fragment was excised from a *mafG* cDNA. The *Eco*RI linker was ligated to the *Stu*I site and cleaved with *Eco*RI, and the resulting fragment was ligated to *Eco*RI-*Pst*I-digested pMAL-cRI vector DNA. In the case of MafK and MafF, a 0.7-kb *Mse*I-*Eco*T22I fragment of the *mafK* gene and a 0.4-kb *Mse*I-*Nae*I fragment of the *mafF* gene were converted to *Eco*RI-*Hin*dIII fragments by treatment with the Klenow fragment of DNA polymerase I and subsequent addition of linkers. The resultant fragments were recloned between *Eco*RI and *Hin*dIII sites of the pMAL-cRI vector. Recombinant fusion proteins in which the amino-terminal 10 (MafG) or 11 (MafK and MafF) amino acid residues were replaced by the *E. coli* MBP sequence were expected to be produced from these constructs. *E. coli* SG12036, which lacks the *lon* protease gene, was used to express these MBP-fused small Maf proteins. Molecular cloning and the structure of the chicken cDNA for Ech, a newly identified bZip factor structurally related to the NF-E2 large subunit, will be described elsewhere (21). The bZip domain of chicken Ech (carboxy-terminal 141 amino acid residues) was also produced as an MBP-fused protein. These MBP-fused proteins were purified by amylose resin affinity chromatography as described previously (24).

EGMSA. For in vitro transcription and translation, we used a MEGAscript kit (Ambion, Austin, Tex.) and wheat germ extract from Promega (Madison, Wis.). The electrophoretic gel mobility shift assay (EGMSA) was performed as described previously (24). To make *mafG* mRNA in vitro, a 0.72-kb *Stu*I-*Eco*T22I fragment of the *mafG* gene (nucleotides 274 to 991) was excised from a cDNA clone λ10 and converted to a *Hin*dIII-*Mlu*I fragment by adding an *Mlu*I linker to the 3′ end and the following synthetic oligonucleotide to the 5′ end:

```
          BspHI
5′-AGCTTCCATCATGACGACCCCCAACAAAGGAAACAAGG
     AGGTAGTACTGCTGGGGGTTGTTTCCTTTGTTCC
       MetThrThrProAsnLysGlyAsnLys
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This fragment was then subcloned into a plasmid vector. A 0.74-kb *Bsp*HI-*Mlu*I fragment which contains all of the coding region was excised from this plasmid clone and replaced with the *Nco*I-*Mlu*I fragment of pRAM-GEM (23), a subclone of the v-*maf* gene. The structures of the plasmids used as templates for the *mafK* and *mafF* mRNAs were described previously (14). The Ech protein synthesized in vitro is an amino-terminally truncated form and contains the carboxy-terminal 157 amino acid residues including the bZip domain.

Point mutants of the small *maf* family genes (*mafK R22E*, *mafF R22E*, *mafG R22E*, *mafK L2PM4P*, *mafF L2PM4P*, and *mafG L2PM4P*) were constructed by the method of Kunkel et al. (29). The carboxy-terminally truncated version of small Maf proteins, MafKΔ, MafFΔ, and MafGΔ, were generated by cleaving the DNA templates by appropriate restriction enzymes which recognize the carboxy-terminal coding sequence of each. The restriction enzymes and their locations (nucleotide numbers in parentheses) are as follows: for *mafK*, *Eco*O109I (369); for *mafF*, *Sse*8387I (339); and for *mafG*, *Bst*XI (676). The nucleotide positions in the *mafK* and *mafF* genes are indicated by the numbering system used in our previous publication (14).

Three genes, designated χ*mafK*, χ*mafF*, and χ*mafG*, which encode chimeric proteins of v-Maf and the small Maf proteins were constructed by replacing the 3′ half of the v-*maf* gene in pRAM-GEM with the whole coding sequence of the small *maf* family genes. The *Pt* form of the v-*maf* gene contains almost all of the v-*maf* coding sequence with the exception of the amino-terminal 18 amino acid residues (23). pRAM-GEM is a subclone of v-*maf Pt* in pGEM-4 and was used to synthesize the v-*maf* mRNA in this analysis. To construct the chimeric genes, plasmid pRAM-GEM was digested with *Bss*HII, and an *Nco*I linker was ligated to the fragment to convert the *Bss*HII site (nucleotide 509 of v-*maf*) to an *Nco*I site. This fragment was then digested with *Mlu*I. The 3′ half of the v-*maf* gene sequence excised by *Bss*HII and *Mlu*I was replaced with the coding sequences of the small *maf* family genes, which were excised from their parent clones by double digestion with *Mlu*I and either *Nco*I or *Bsp*HI. Predicted products of these constructs are chimeric proteins containing the amino-terminal 153 amino acid residues (i.e., amino acid residues 19 to 171) of v-Maf Pt, followed by insertion of two amino acid residues (Val-Thr) derived from the synthetic oligonucleotide sequence and the whole polypeptide sequences of the small Maf proteins. The template plasmids for v-*jun* and v-*fos* mRNAs were constructed as previously described (24).

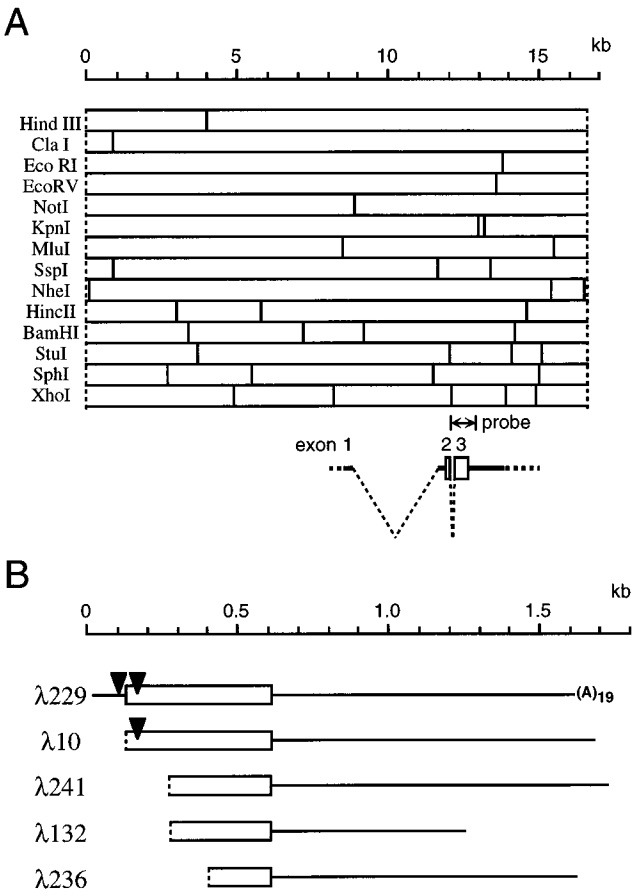

FIG. 1. DNA clones of the chicken *mafG* gene. (A) Restriction endonuclease map of a chicken *mafG* genomic DNA clone, λ602. The organization of the *mafG* gene was determined by comparison of the nucleotide sequence of the genomic clone with those of cDNA clones and is presented at the bottom. Solid lines indicate the position of the cDNA contig. Open boxes represent the coding sequence of the *mafG* gene. The 0.7-kb *Stu*I-*Eco*T22I fragment was used as a *mafG*-specific probe and is indicated below the restriction map. (B) Structures of the *mafG* cDNA clones. Solid lines represent the extent of cloned cDNA segments. The open boxes represent the extent of each of the predicted open reading frames within the cDNA clones. Closed triangles indicate positions of the introns. Two clones, λ229 and λ236, were isolated from the BV4 chicken embryo cDNA library. λ132, λ10, and λ241 were identified from a chicken brain cDNA library, a chicken embryo fibroblast cDNA library, and the RBC-4 cDNA library prepared from anemic hen reticulocyte, respectively.

Promoter constructs and transfections. An expression plasmid for *mafG* was constructed by excising the *Mlu*I fragment from the subclone used as a template for in vitro transcription and subcloning it into the *Bss*HII site of pEF*Bss*HII, which we previously constructed (22) from the pEF-BOS vector (34). Similarly, expression plasmids for *mafK*, *mafF*, and chicken v-*fos* (39) were constructed by excising coding sequences from the template plasmids for in vitro transcription by digestion with *Mlu*I or *Bss*HII and by recloning into pEF*Bss*HII. The chicken v-Fos expressed in this experiment is an amino-terminally truncated form but still contains two *trans*-activator domains of Fos defined by Abate et al. (1) and retains its cell transformation potential (unpublished observation). Within the expressed protein, in contrast to the case of mouse v-Fos and mouse c-Fos, chicken v-Fos does not contain any structural changes from its cellular counterpart, chicken c-Fos (13, 39). The luciferase reporter gene was constructed by inserting three copies of oligonucleotide 25 (see Fig. 5) upstream of the minimal

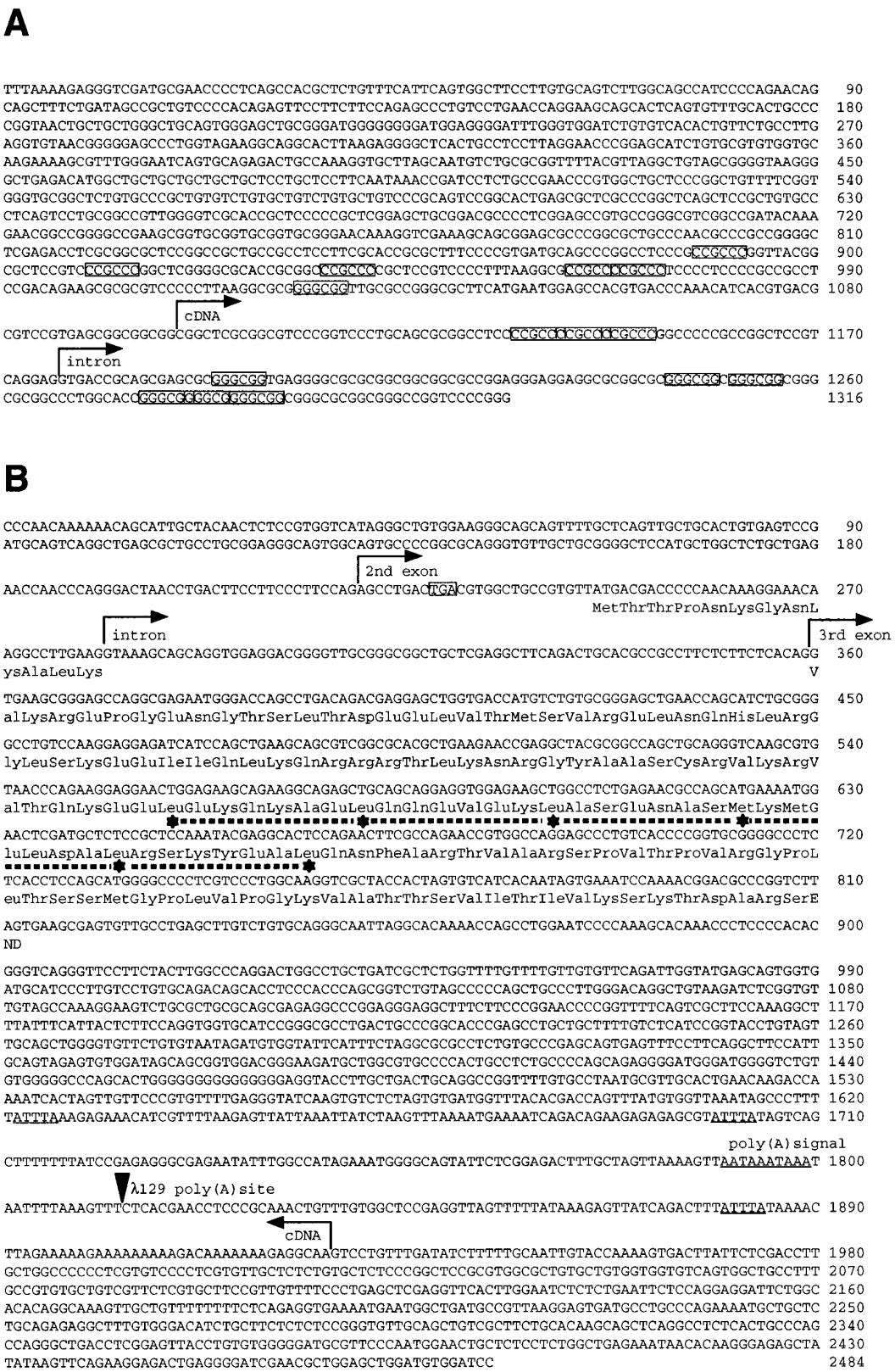

FIG. 2. Nucleotide and deduced amino acid sequences of the chicken *mafG* gene. (A) Putative first exon and flanking sequences of the *mafG* gene. Fifteen copies of GC box sequences, CCGCCC and GGGCGG, found in this region are boxed. The 5′ terminus of the cDNA clone and the putative splicing donor site for the first intron are indicated by arrows. (B) Second and third exons and flanking sequences. The predicted amino acid sequence is shown under the corresponding nucleotide sequence. Splicing junctions determined by comparison of cDNA and genomic DNA sequences are indicated by arrows. The leucine zipper structure is indicated by asterisks and dotted lines. An in-frame termination codon found just before the initiator methionine codon is boxed. Two contiguous poly(A) signal sequences and three ATTTA sequences, possibly involved in the selective degradation of the mRNA (50), are underlined.

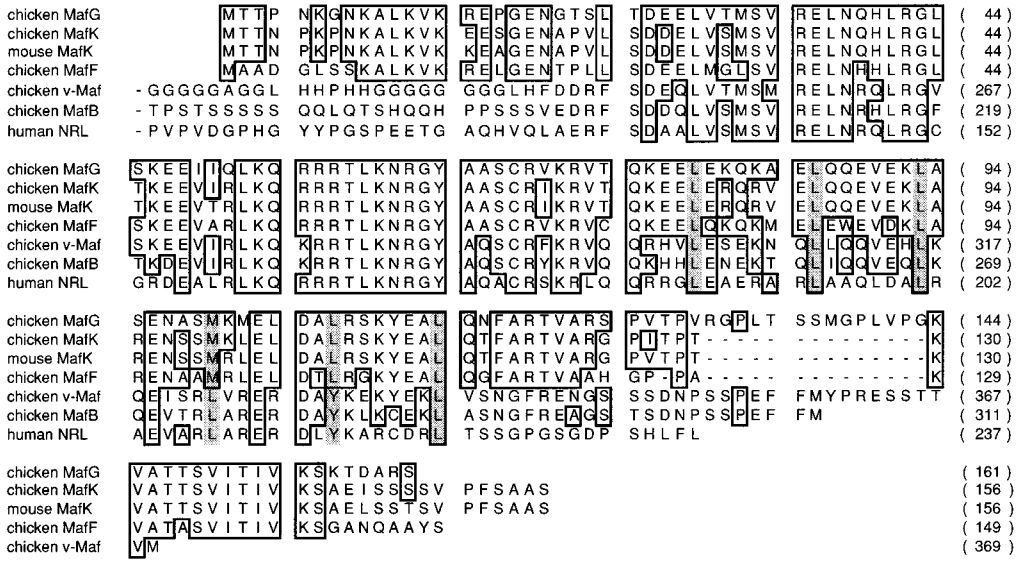

FIG. 3. Alignment of the predicted amino acid sequences of Maf family proteins. For the three large Maf proteins (v-Maf, MafB, and NRL), only the carboxy-terminal half of each molecule is shown. Amino acid residues conserved with respect to the MafG protein are boxed. The hydrophobic heptad repeats of leucine zipper structures are shaded. Numbers at the right indicate amino acid numbers. Dashes indicate the absence of the corresponding amino acids. Sequence information for v-Maf (40), MafB (22), chicken MafK and MafF (14), mouse MafK (3, 19), and human NRL (53) was from the indicated references.

promoter of the rabbit β-globin gene (RBGP). QT6 quail fibroblast cells (36) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections were performed as described previously (20).

Nucleotide sequence accession numbers. The nucleotide sequence of the chicken *mafG* gene has been deposited in the GSDB, DDBJ, EMBL, and NCBI DNA databases under accession numbers D28601 and D28602.

RESULTS

Isolation and structural characterization of *mafG*. Through an attempt to isolate genomic DNA clones for a *maf*-related gene, *mafK*, using the *mafK* cDNA as a probe, we had previously isolated a member of the *maf* gene family, *mafF* (14). Similarly, we identified an additional related gene by analyzing the genomic clones isolated by screening with the *mafK* probe. Restriction endonuclease mapping and blot hybridization analyses of three overlapping genomic DNA clones suggested that these clones are derived from neither the *mafK* nor the *mafF* gene. Figure 1A shows the restriction map of one of the genomic clones, λ602. Partial sequence analysis verified that this clone represents a locus of a novel *maf*-related gene. To confirm that this gene, which we named *mafG*, is indeed transcriptionally active and to clarify its whole structure, we isolated cDNA clones corresponding to this gene. As shown in Fig. 1B, we isolated five *mafG* cDNA clones by using the 0.7-kb *Stu*I-*Eco*T22I fragment (Fig. 1A) as a probe. The *mafG* gene consists of at least three exons, similar to the *mafK* and *mafF* genes. The nucleotide and predicted amino acid sequences of *mafG* are shown in Fig. 2. Although one of the cDNA clones, λ229, contained a poly(A) stretch at its 3′ terminus and this poly(A) stretch was preceded by two contiguous copies of a polyadenylation consensus sequence (AATAAA), three other cDNA clones (λ10, λ241, and λ236) were found to extend beyond this polyadenylation site (Fig. 1B), suggesting the presence of an alternative poly(A) addition site further downstream. RNA blot analyses showed that the chicken *mafG* gene is expressed as a transcript of 3.4 kb in various tissues (20, 38a). The cDNA contig (1.7 kb) covers only about half of the major transcript. Considering the fact that both chicken *mafK* and *mafF* genes contain long 3′ untranslated regions (UTRs), it seems likely that the missing parts of the cDNA mainly correspond to the 3′ UTR sequence of the transcript. Consistent with this idea, the first exon resides in a typical CpG island (16), which frequently hosts promoters for constitutively expressed eukaryotic genes (Fig. 2A). Sequence in this region is dominated by GC-rich sequence, including more than 10 copies of consensus binding sequences for the Sp-1 transcription factor (GGGCGG and CCGCCC). A possible initiator methionine in the second exon is preceded by a termination codon (Fig. 2B) and shows excellent coincidence with the amino termini of MafK and MafF (Fig. 3). We found three copies of an mRNA-destabilizing signal sequence (ATTTA) (50) in the 3′ UTR of the cDNA (Fig. 2B).

The predicted MafG protein shares highest identity with MafK and MafF throughout the molecule except for the carboxy-terminal region (Fig. 3). In this region, MafG has a unique insertional sequence of 14 amino acid residues (residues 130 to 143). It is evident from this comparison that the three small Maf proteins correspond to the carboxy-terminal half of the v-Maf molecule and lack the amino-terminal putative *trans*-activator domain of v-Maf. In the bZip domain, especially in the DNA-binding domain, all Maf family proteins are extensively conserved, suggesting they share the same DNA-binding specificity.

DNA-binding specificities of the small Maf proteins. To test the ability of the small Maf proteins to bind to MARE sequences, we translated the Maf family proteins and their mutated forms in vitro and subjected them to EGMSA. Figure 4A schematically represents structures of the mutated forms of the Maf family proteins used in this study. In the case of MafG, we synthesized a carboxy-terminally truncated version, designated MafGΔ (lane 13), and its derivatives for the binding assay because intact MafG protein was insoluble under the conditions of EGMSA (lane 10). All of these proteins were efficiently synthesized in vitro in the presence of [^{35}S]methionine, as judged by sodium dodecyl sulfate-gel electrophoresis and fluorography (data not shown). As shown in Fig. 4B, all of the

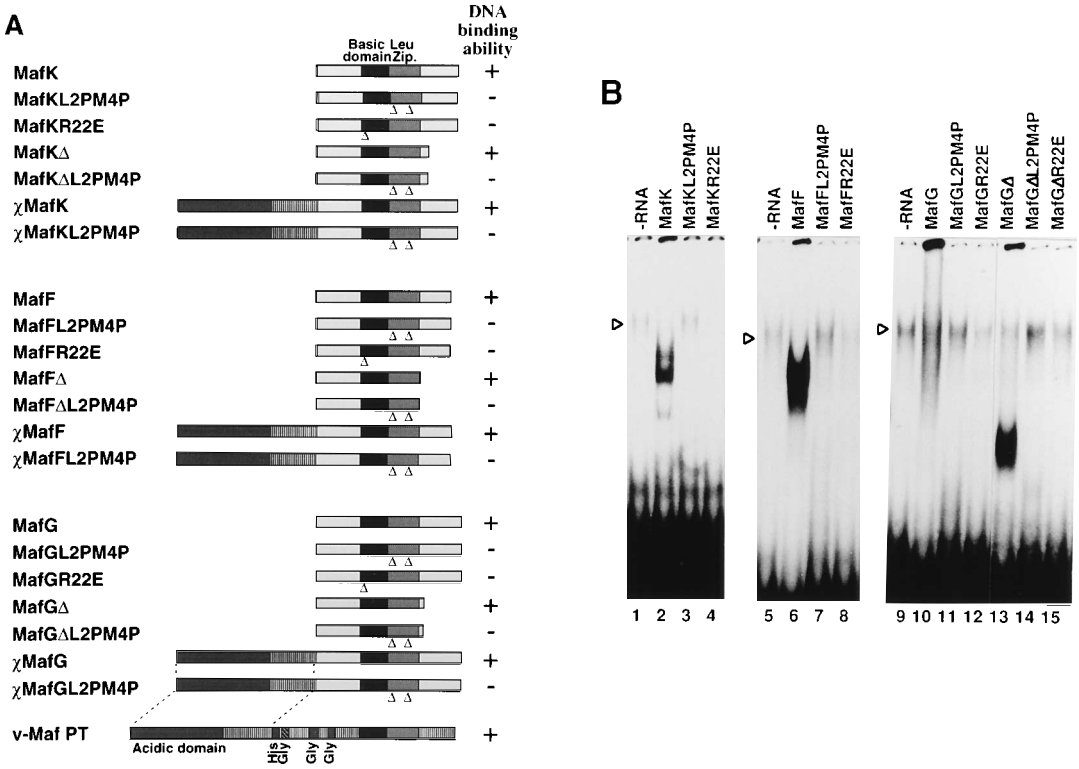

FIG. 4. DNA-binding activities of in vitro translated small Maf proteins. (A) Schematic representation of small Maf proteins and their mutants. Mutation sites in the substitution mutants are indicated by open triangles. v-Maf PT is a nearly full-length version of v-Maf (23). DNA-binding activities of these proteins are summarized at the right. (B) DNA-binding abilities of small Maf proteins and their mutants. Oligonucleotide 1, which contains the TRE-type MARE, was used as a probe. An open triangle indicates a retarded band of endogenous binding activity to this probe in the wheat germ extract used for in vitro translation.

small Maf proteins efficiently bind to the TRE-type MARE probe (lanes 2, 6, and 13). The mutant proteins, containing a substitution in the putative DNA-binding domain of a conserved arginine residue with a glutamate (MafK R22E, MafF R22E, and MafGΔ R22E), were defective in DNA binding (lanes 4, 8, and 15). Three other mutant proteins, MafK L2PM4P, MafF L2PM4P, and MafGΔ L2PM4P, each of which contains two substitutions of hydrophobic residues for proline residues in the zipper domain, also failed to recognize the MARE probe, probably because of their inability to form dimers (lanes 3, 7, and 14). The amino acid residues substituted in these mutants correspond to two mutants of v-Maf, R22E and L2PL4P, which were shown to be defective in DNA binding (24). From these observations, we conclude that the small Maf family proteins also recognize the specific binding sequences for v-Maf through their bZip DNA-binding domains.

To further characterize DNA-binding specificities of the small Maf proteins, we synthesized the bZip part of the four Maf proteins and v-Jun in *E. coli* as fusions with MBP and subjected them to EGMSA by using a series of oligonucleotide probes (Table 1). As shown in Fig. 5A to D, the bacterially synthesized Maf family proteins efficiently bound both of the two consensus binding sequences for Maf, TRE- and CRE-type MAREs (oligonucleotides 1 and 2; lanes 2, 20, and 21). Various substitutions in the consensus sequences reduced the binding of all four Maf proteins in the same manner, indicating similar binding specificities of the Maf family members. In binding specificities, v-Jun showed a profile different from that of the Maf family proteins (Fig. 5H).

We recently identified a gene which encodes a chicken bZip protein structurally related to mouse NF-E2 large subunit (p45) especially within the bZip domain. Interestingly, like expression of the gene for NF-E2 p45, expression of this novel gene, *ech*, is abundant in hematopoietic cells (21). As the NF-E2 p45 subunit efficiently heterodimerizes with the small Maf proteins and recognizes the NF-E2 sequences, we tested whether the Ech molecule also can associate with the small Maf proteins and examined DNA-binding specificities of the heterodimeric complexes. To this end, the bZip part of the chicken Ech protein was also synthesized in *E. coli* as a fusion protein with MBP for EGMSA. In the absence of small Maf family proteins, we detected no DNA binding of this MBP-Ech fusion protein to any of the probes (data not shown). Addition of MBP-Ech to MBP-MafK generated a band which migrates slightly faster than an MBP-MafK homodimer band (Fig. 5E). This band most likely represents the Ech-MafK heterodimer. Expectedly, heterodimerization with Ech greatly stimulated the binding of MafK to some probes, including the NF-E2 site probe (lane 1). In the cases of MafF and MafG, the heterodimers with Ech run at a position indistinguishable from that of the homodimers in this assay. Nevertheless, similar changes in DNA-binding specificities for these Maf family proteins were observed upon addition of an excess amount of Ech (Fig. 6F and G).

Small Maf proteins form dimers among themselves. We have previously shown homodimer formation of MafK and MafF by chemical cross-linking (14). In addition, in this study we have shown that integrity of the leucine zipper structure is necessary for the DNA-binding activities of all three small Maf family proteins, indicating that each binds to the MARE sequence as a homodimer. To test whether the three small Maf

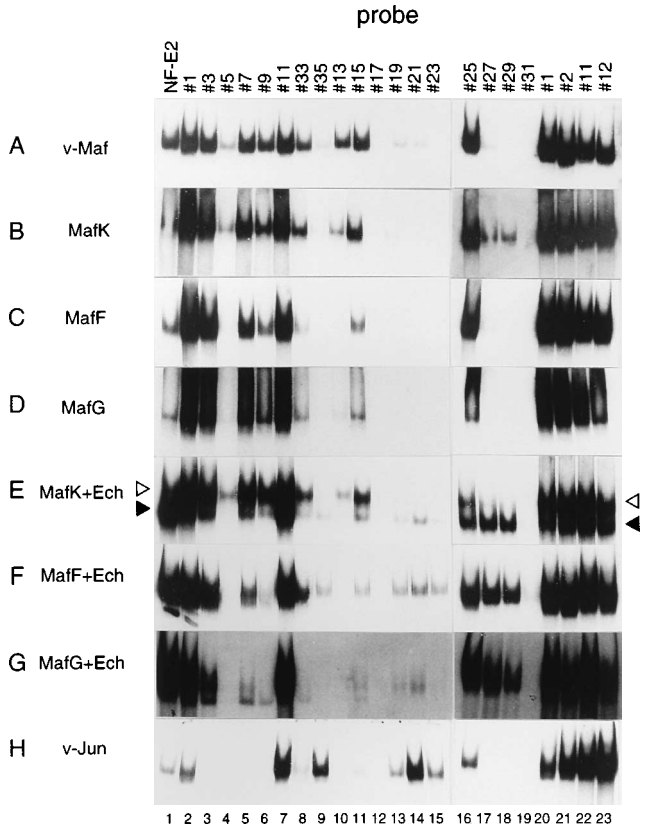

FIG. 5. DNA-binding specificities of the Maf family proteins. The four Maf family proteins, chicken Ech, and v-Jun were all bacterially synthesized as fusion proteins with MBP. The following proteins were mixed with the series of ^{32}P-labeled probes and subjected to EGMSA: (A) MBP–v-Maf, (B) MBP-MafK, (C) MBP-MafF, (D) MBP-MafG, (E) MBP-MafK plus MBP-Ech, (F) MBP-MafF plus MBP-Ech, (G) MBP-MafG plus MBP-Ech, and (H) MBP–v-Jun. Nucleotide sequences of the probes used in this experiment are shown in Table 1. In the case of the mixture of MBP-MafK and MBP-Ech, two retarded bands of MBP-MafK homodimer and MBP-Ech–MBP-MafK heterodimer could be separated and are indicated by open and closed triangles, respectively. In the cases of MBP-MafF and MBP-MafG, an excess amount of MBP-Ech was added and analyzed to show binding specificities of the heterodimers.

TABLE 1. Synthetic oligonucleotides used for EGMSA[a]

Probe	Sequence	No. of mismatches from consensus sequence of:		
		Maf	Jun	NF-E2
NF-E2	TGCTGAGTCACTG	3	2	0
1	TGCTGACTCAGCA	0	2	1
3	TGCTGATTCAGCA	1	3	2
5	TGCTGGCCCAGCA	2	4	3
7	TGCTTACTAAGCA	2	4	3
9	TGCCGACTCGGCA	2	4	3
11	TGATGACTCATCA	2	0	1
33	TGTTGACTCAACA	2	2	2
35	TGGTGACTCACCA	2	2	1
13	TACTGACTCAGTA	2	2	2
15	GGCTGACTCAGCC	2	2	2
17	TGAGGACTCCTCA	4	2	3
19	TAATGACTCATTA	4	0	2
21	GGATGACTCATCC	4	0	2
23	CAATGACTCATTG	6	0	3
25	TGCTGACTCATCA	1	1	0
27	TGCCGACTCATCC	3	2	1
29	TGCCGACTCATTG	4	2	1
31	TGCCGGCTCATTG	5	3	2
2	TGCTGACGTCAGCA	0	2	
12	TGATGACGTCATCA	2	0	

[a] The nucleotide sequence of only the unique middle part of each probe is shown. The whole structure of oligonucleotide 1 is

5′-TCGAGCTCGGAATTGCTGACTCAGCATTACTC-3′
3′-TCGAGCCTTAACGACTGAGTCGTAATGAGAGC-5′

Nucleotides matching MAREs are underlined. Oligonucleotides 1 and 2 contain TRE- and CRE-type MAREs, respectively. Numbers of mismatches from the consensus sequences for Maf (TGCTGA[C/G]TCAGCA and TGCTGACGTCAGCA), Jun (ATGA[C/G]TCAT and ATGACGTCAT), and NF-E2 (TGCTGA[C/G]TCA[C/T]) are shown.

proteins form intrasubfamily heterodimers, we constructed several additional variants of the small *maf* family genes and translated them in vitro. Predicted products of these variant genes are schematically represented in Fig. 4A. In this experiment, since multiple bands were produced in EGMSA when RNAs coding for intact MafK and MafF were translated in vitro (Fig. 4B, lanes 2 and 6), we synthesized carboxy-terminally truncated forms of the small Maf proteins and used them for the analysis. The in vitro-synthesized truncated MafK and MafF, which are designated MafKΔ and MafFΔ, gave single discrete bands in the same assay (Fig. 6A and B, lanes 3). We also constructed chimeric genes by inserting the 5′ half of the v-*maf* coding sequence in front of the entire coding sequences for the small Maf proteins (Fig. 4A). The in vitro-translated chimeric proteins, χMafK, χMafF, and χMafG, efficiently bound the MARE probes and produced a more slowly migrating band in EGMSA (Fig. 6A to C, lanes 2).

Homodimer formation of MafK can easily be identified by subjecting cotranslated MafK molecules of different lengths, MafKΔ and χMafK, to EGMSA. As expected, in addition to the homodimer bands, a band of intermediate mobility most likely derived from the MafKΔ-χMafK heterodimer was also seen (Fig. 6A, lane 4). When one of the two proteins was replaced with a mutant carrying two substitutions in the leucine zipper motif (MafKΔL2PM4P or χMafKL2PM4P), the nucleoprotein complex of intermediate mobility disappeared (lanes 5 and 6). Similarly, homodimer formation of MafF (Fig. 6B, lane 4) and MafG (Fig. 6C, lane 4) was also observed. The formation of three forms of intrasubfamily heterodimers, MafF-MafK, MafF-MafG, and MafK-MafG, was clearly demonstrated by using the same approach (Fig. 6D to F, lanes 4). All of these associations required integrity of the leucine zipper motifs (Fig. 6D to F, lanes 5 and 6).

Small Maf proteins heterodimerize with Fos but not with Jun. We previously found that v-Maf heterodimerizes with Fos and Jun nuclear oncoproteins (24). We have also recently shown that v-Maf and MafB are unable to associate with the small Maf family proteins (22). In this study, we analyzed combinations of small Maf proteins with AP-1 proteins. As shown in Fig. 7, the MafKΔ homodimer band (lane 1) migrated much faster than the v-Jun homodimer band (lane 5). If these two proteins form a heterodimer, an additional band of intermediate mobility would be detected when the mixture of MafK and v-Jun is used for EGMSA. However, no additional band was observed with the mixture, indicating the inability of MafK to associate with v-Jun (lane 6). Similarly, the two other small Maf proteins were also found to be unable to associate with v-Jun (lanes 7 and 8). As a control, we included the mixture of v-Maf and v-Jun in this EGMSA and found that the association of these two proteins is reproducible (lane 9). It is possible that the small Maf proteins can heterodimerize with Jun but the heterodimers fail to recognize the probe used in this experi-

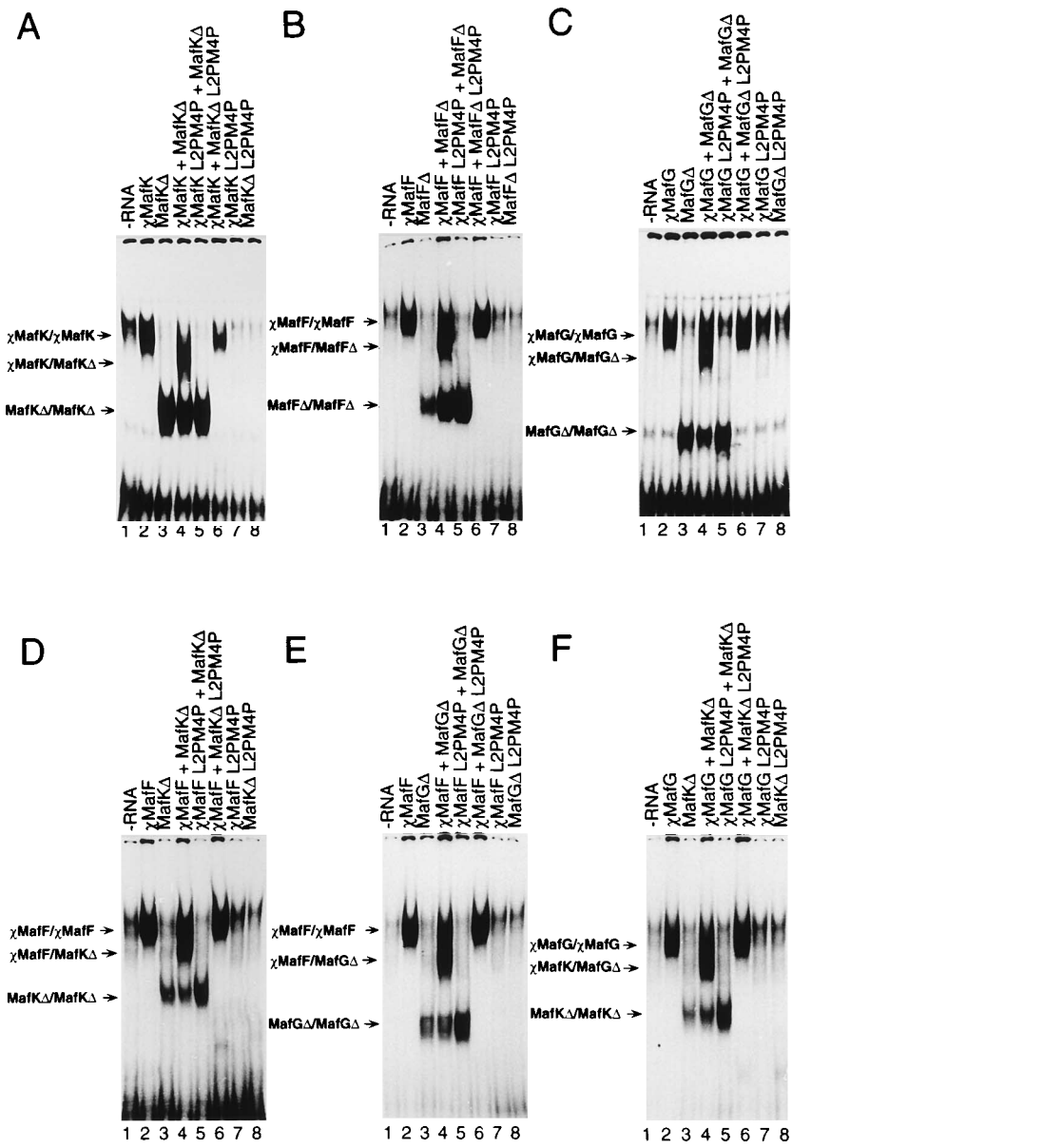

FIG. 6. The small Maf proteins form homodimers and intrasubfamily heterodimers. Small Maf family proteins of different lengths indicated above the lanes were cotranslated and subjected to EGMSA to examine the formation of MafK homodimer (A), MafF homodimer (B), MafG homodimer (C), MafF-MafK heterodimer (D), MafF-MafG heterodimer (E), and MafK-MafG heterodimer (F). The small Maf family proteins can form these functional homodimers and heterodimers, as indicated by arrows.

ment. Such a possibility is, however, very unlikely because Jun and small Maf proteins, as homodimers, efficiently recognize the probe used in this study.

In contrast to the results with v-Jun, we found that cotranslation of mRNAs for MafG and Fos produced an additional band in EGMSA which represents the Fos-MafG heterodimer (Fig. 7, lane 13). Heterodimer formation with Fos was also observed for MafK and MafF, albeit with apparently reduced efficiency (lanes 11 and 12). EGMSA experiments with various mutants demonstrated that the leucine zipper domain is essential for the association of small Maf proteins with Fos (data not shown). Heterodimer formation of Fos with small Maf proteins was not detected when each protein was translated separately and subsequently mixed before EGMSA (data not shown). As separately synthesized small Mafs and Ech (or NF-E2 p45) can form heterodimers under the same conditions, the affinity of small Maf proteins to Ech and p45 seems higher than that to Fos (see Discussion). Compatibility of dimer formation among the Maf family proteins, Fos, Jun, NF-E2 p45, and Ech is summarized in Table 2.

We next compared DNA-binding specificities of Fos-MafK and Ech-MafK heterodimers by subjecting the cotranslated mixtures of the bZip proteins to EGMSA, using the series of oligonucleotide probes (Fig. 8A and B). The Fos-MafK heterodimer was found to have profiles of DNA-binding specificity similar to those of the Ech-MafK complex. Interestingly, both Fos-MafK and Ech-MafK heterodimers most efficiently recognized two probes (NF-E2 probe and probe 25), both containing the complete consensus binding sequence for NF-E2. Both heterodimers bound in a similar manner to most of

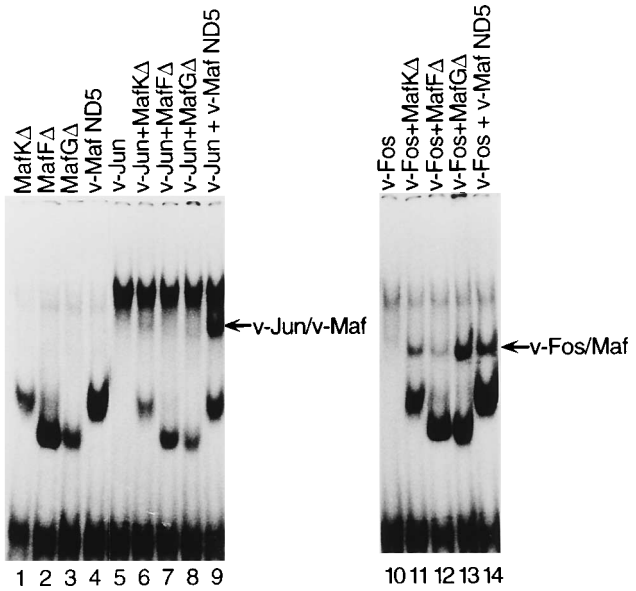

FIG. 7. Small Maf proteins associate with v-Fos but not with v-Jun. mRNAs for the small Maf proteins, v-Jun, v-Fos, or an amino-terminal deletion mutant of v-Maf (v-Maf ND5) (23) were translated in vitro for EGMSA. Oligonucleotide probe 11 was used for this assay because of its high affinity to both Maf and Jun (see Fig. 5).

the other probes, suggesting competitive binding of these complexes to overlapping DNA sequences. However, the Fos-MafK heterodimer showed greatly reduced affinity with two probes (probes 27 and 29), each of which contained only one mismatch from the NF-E2 consensus sequence and still efficiently bound to the Ech-MafK heterodimer. Results of competition experiments also supported this conclusion (Fig. 8C and D). Addition of nucleotides 27 and 29 as competitors significantly inhibited binding of the Ech-MafK heterodimer to the labeled probe (probe 11) but inhibited binding of the Fos-MafK heterodimer to the same probe, albeit with reduced efficiency. In similar experiments, mouse NF-E2 p45–small Maf complexes showed binding specificities indistinguishable from those of Ech-small Maf heterodimers (data not shown).

***trans* suppression by small Maf proteins and the effect of Fos.** We have previously shown that the three small Maf proteins act as sequence-specific negative regulators of transcription, but in the presence of p45, they can act as transactivators (20). To examine whether the *trans*-repression properties of small Maf homodimers could be modulated by association with

TABLE 2. Compatibility of homo- and heterodimer formation among Maf family proteins and other proteins[a]

Protein	Compatibility with:						
	Maf	MafB	Small Maf	Fos	Jun	p45	Ech
Maf	+	+	−	+	+	NT[b]	NT
MafB		+	−	+	−	NT	NT
Small Maf			+	+	−	+	+
Fos				−	+	NT	NT
Jun					+	NT	NT
p45						−	NT
Ech							−

[a] Along with our previous results (22, 24), dimer formation specificities of Maf family proteins, Fos, Jun, NF-E2 p45, and Ech are summarized.
[b] NT, not tested.

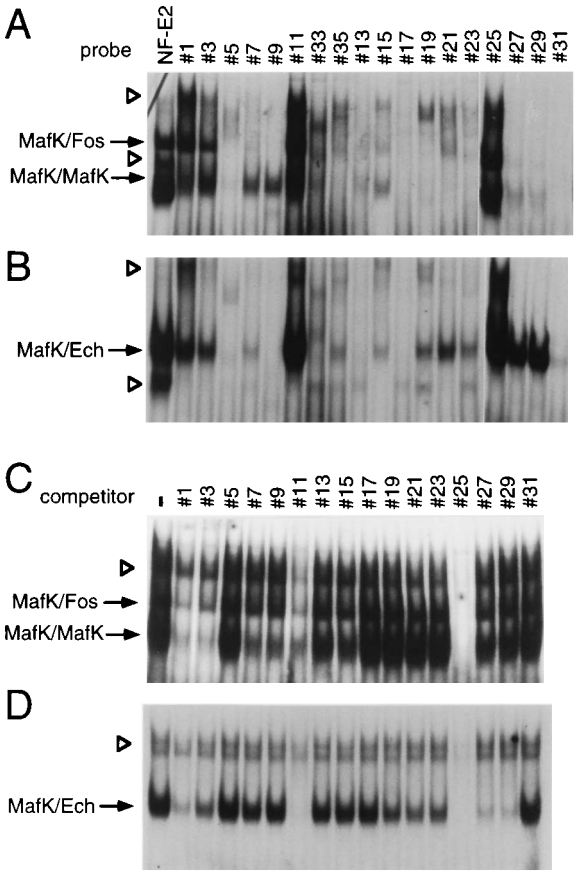

FIG. 8. Comparison of DNA-binding specificities of Fos-MafK and Ech-MafK heterodimers. An aliquot of a cotranslated mixture of MafK and v-Fos (A) or MafK and Ech (B) was mixed with ^{32}P-labeled oligonucleotide probes as indicated and subjected to polyacrylamide gel electrophoresis. Retarded bands of MafK homodimer, Fos-MafK heterodimer, and Ech-MafK heterodimer are indicated by arrows. The retarded bands of endogenous DNA-binding activities in the wheat germ extract used for translation are indicated by open triangles. Nucleotide sequences of the probes used in this experiment are summarized in Table 1. In panels C and D, to the constant amount of ^{32}P-labeled oligonucleotide 11, unlabeled oligonucleotides indicated at the top were added as competitors, and the mixtures were incubated with an in vitro-translated mixture of MafK and Fos (C) or MafK and Ech (D) and then subjected to gel electrophoresis. Competitor nucleotides were used at a 40-fold (C) or 200-fold (D) molar excess of the labeled nucleotide.

Fos, we analyzed the expression of 3×#25-RBGP, a reporter plasmid which contains the firefly luciferase gene under the control of three tandem copies of the oligonucleotide 25 sequence inserted 5′ to RBGP. Oligonucleotide 25 was used as a target sequence in the reporter plasmid because the heterodimers of small Maf proteins with both Fos and p45 most efficiently bind to this sequence.

Significant endogenous *trans*-activating activity was observed with this reporter plasmid in the QT6 cells used for the cotransfection assay, as was the case for transfection of a similar reporter (pRBGP2) into NIH 3T3 cells (20). More than 50-fold-higher luciferase activity was detected with the 3×#25-RBGP construct than with a reporter construct containing RBGP alone (pRBGP3). In agreement with our previous analysis using NIH 3T3 cells, ~90% of this endogenous activity was suppressed by the expression of MafF or MafG in QT6 cells (Fig. 9).

Our previous study indicated that coexpression of NF-E2 p45 markedly stimulated expression from a similar reporter

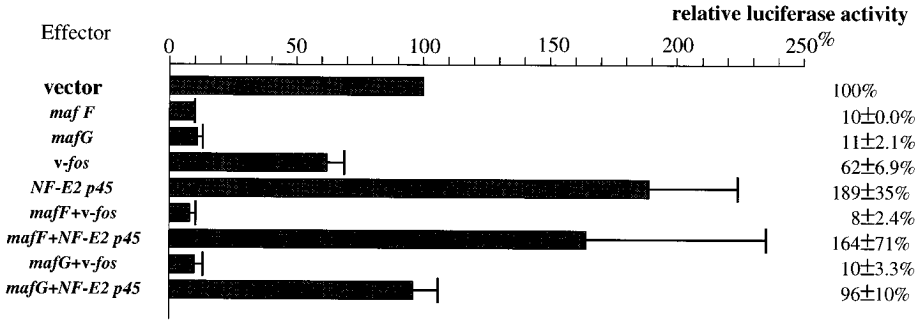

FIG. 9. Fos can act as a negative regulator of transcription in combination with the small Maf proteins. QT6 cells were cotransfected with the reporter and indicated effector plasmids. Luciferase activity of the 3×#25-RBGP plasmid in the absence of any effector plasmid was set at 100%, and relative activities obtained by cotransfection are shown. Bars represent means ± standard errors.

plasmid when reporter expression was suppressed by the presence of either MafK or MafF (20). By heterodimerization with small Maf proteins, p45 could bind to the NF-E2 site and acted as a *trans* activator of reporter gene expression. Since Fos-small Maf heterodimers were shown to bind to oligonucleotide 25 (Fig. 8), we expected that coexpression of Fos with the small Maf proteins would also stimulate transcription from the test promoter by competition with suppression by the small Maf homodimers. Contrary to our expectation, however, coexpression of Fos with the small Maf proteins failed to rescue the reporter gene expression from this construct (Fig. 9), indicating that the Fos-small Maf heterodimers are defective in *trans* activation.

DISCUSSION

In this study, we have shown that three small bZip proteins structurally related to Maf nuclear oncoprotein, including a newly identified protein, MafG, are able to form homodimers, intrasubfamily dimers, and heterodimers with an NF-E2 p45-related molecule, Ech, and Fos nuclear oncoprotein. However, the small Maf proteins could not associate with v-Maf or Jun. These bZip dimers recognize partially overlapping DNA-binding sequences related to an AP-1 site. Thus, these bZip dimers could compete for binding, depending on the target DNA sequences.

The NF-E2 transcription factor is a key regulator of erythroid cell-specific gene expression (31, 33) and is composed of two bZip proteins. NF-E2 p45 and the newly identified related molecule, Ech, are expressed in hematopoietic cells (2, 7, 21) and can associate with either of the small Maf proteins to form dimers which preferentially recognize the consensus sequence of NF-E2 as described previously (3, 20) and as shown in this study. Recently, we observed that the Ech protein shows much stronger *trans*-activating potential than p45 (21). Therefore, Ech-small Maf heterodimers may be functionally more important than p45-small Maf heterodimers in activating erythroid cell-specific gene expression and regulating hematopoietic cell differentiation. In addition, two other p45-related molecules, Nrf1/LCR-F1/TCF11 (6, 8, 30) and Nrf2 (35), have been identified. These two molecules show ubiquitous expression patterns but may play some regulatory role in hematopoietic cell-specific gene expression.

On the other hand, the *fos* and *jun* genes are known to be immediate-early genes, and their products form the AP-1 transcription factor, whose deregulated overexpression induces cell transformation (reviewed in references 12 and 55). The apparent similarity in DNA-binding specificities of these bZip dimers and the direct interaction between the small subunit of the differentiation-inducing factors (small Maf proteins) and the AP-1 component (Fos) are intriguing because terminal differentiation of erythroleukemia cells into the erythroid lineage is known to be inhibited by treatment with TPA, a potent inducer of AP-1 (41). Enforced overexpression of Jun family proteins in the Friend murine erythroleukemia cell line is also known to inhibit its terminal differentiation into the erythroid cell lineage (43). Notably, not only c-Jun but also two *jun*-related gene products, JunB (45) and JunD (18, 42), which are known to have markedly decreased activities in transcriptional activation and cell growth stimulation (5, 9, 17, 18, 42, 48), are equally effective in inhibition of differentiation of erythroleukemic cells (43). Furthermore, a recombinant avian retrovirus which expresses both v-*jun* and v-*erbB* was reported to induce morphological transformation of bone marrow cells of erythroid and megakaryocytic lineages (15). NF-E2 is known to be expressed in erythroid and megakaryocytic lineages, suggesting the functional importance of this transcription factor in these hematopoietic cell lineages. It is thus plausible that, at least in hematopoietic cells, the Jun proteins contribute to cell transformation through inhibition of differentiation by competing with transcription factors essential for cell differentiation (Fig. 10), not by activating growth-stimulatory genes, similar to the dominant negative effect of the v-ErbA against c-ErbA (the thyroid hormone receptor) and the retinoic acid receptor (11, 47, 49).

Hematopoietic cells are known to express c-*fos* at high levels (10, 12, 37). TPA treatment of K562 erythroleukemic cells rapidly induces an increase in AP-1 activity and a simultaneous decrease of NF-E2 DNA-binding activity (32, 51). Interestingly, decrease of NF-E2 activity correlates well with down-regulation of transcription driven by an erythroid cell-specific promoter of the human porphobilinogen deaminase gene, which contains a typical NF-E2-binding site (31). In these studies, NF-E2 was distinguished from AP-1 as a faster-migrating band in a gel shift assay using the AP-1/NF-E2-binding site as a probe (32, 51). In the light of the present study, we speculate that the results of these previous studies may reflect an interaction of Fos with small Maf proteins in competition with the functional NF-E2 complexes. Although small Maf proteins appear to bind p45 and Ech with higher affinity than Fos, the presence of a large excess of Fos may overcome the differences in affinity. Indeed, TPA treatment of mouse erythroleukemic cells greatly enhanced expression of the c-*fos* gene and of mRNAs for MafK, NF-E2 p45, and c-Jun (unpublished observation). Consistent with our observations, Auwerx et al. (4) reported that TPA treatment of a human erythroleukemic cell line induces c-*fos* mRNA about ninefold, while the induc-

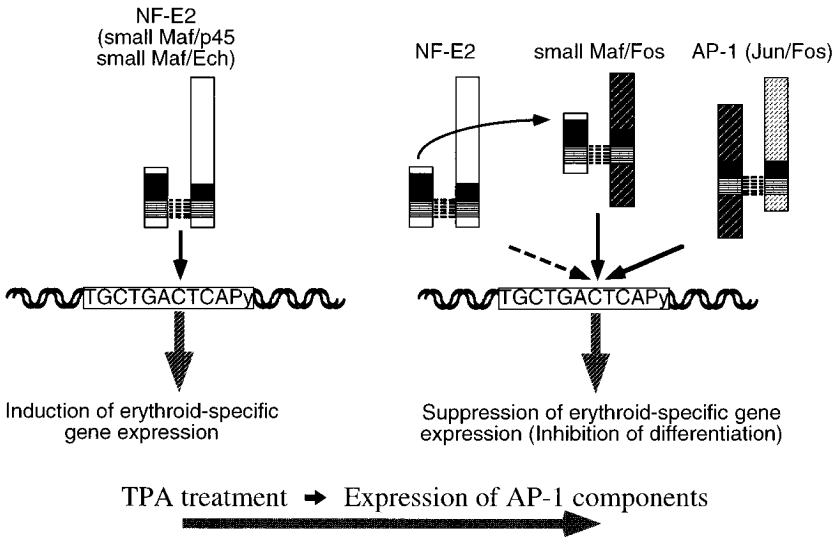

FIG. 10. Model for the regulation of tissue-specific gene expression and differentiation of erythroid cells by NF-E2 and AP-1. Overexpression of AP-1 proteins (Fos and Jun) depletes the functional NF-E2 complexes (NF-E2 p45–small Maf and Ech-small Maf heterodimers) and generates Fos-small Maf heterodimers. The inactive Fos-small Maf heterodimers and AP-1 complexes compete with NF-E2 for binding to the binding sites and thus prevent erythroid cell-specific gene expression and differentiation.

tion of c-*jun* is only twofold. It is therefore very likely that in the presence of an excess amount of c-Fos compared with its preferable partner, c-Jun, most of the small Maf molecules are present as heterodimers with c-Fos (Fig. 10). Suppression of the porphobilinogen deaminase gene promoter during TPA treatment might be due to occupation of the NF-E2 site by the Fos-small Maf heterodimers, which have been shown to be transcriptionally inactive. Consistent with this hypothesis, constitutive expression of the c-*fos* gene has also been reported to inhibit erythropoietin-induced differentiation of the SKT6 murine erythroleukemia cell line (54). To further explore the molecular mechanisms of differentiation control of hematopoietic cells, quantitation of the amounts of Maf proteins, AP-1 proteins, NF-E2 p45, and their related proteins before and after treatment of the cells with inducers or inhibitors of differentiation will be necessary. This analysis is currently under way, using mouse erythroleukemic cells.

Whereas all three small *maf* family genes are expressed not only in hematopoietic cells but also in wide variety of tissues (14), expression of NF-E2 p45 is limited in hematopoietic cells (2, 3, 7, 21). In other tissues, most of the small Maf molecules may be present as homodimers and may act as negative regulators of transcription. A transient increase of Fos in response to growth-stimulatory signals could modify the binding specificities of small Maf proteins. Alternatively, ubiquitously expressed p45-related molecules such as Nrf1/LCR-F1/TCF11 (6–8, 30) and Nrf2 (35) may act as heterodimeric partners of the small Maf proteins. Since these molecules do not efficiently form homodimers and are significantly homologous with p45, it seems likely that these molecules also form heterodimers with the small Maf proteins and specify the binding of the heterodimers to DNA sequences related to the NF-E2 site. The strong endogenous *trans*-activating activity found in QT6 cells and NIH 3T3 cells with the 3× NF-E2 site–RBGP reporter plasmid (Fig. 9) might be due to the endogenous activity of such complexes.

Heterodimers of many bZip proteins, including Maf, Fos, Jun, NF-E2 p45, and their related molecules, can recognize sequences related to an AP-1 site. These complexes could differentially affect the expression of target genes. Such interactions of tissue-specific factors and nuclear proto-oncogene products could be the basis for intricate fine tuning of transcriptional control of many cellular genes and cell differentiation. This study also raises the possibility that overexpression of the bZip nuclear oncoproteins participates in the cell transformation process by functionally antagonizing the key regulators of differentiation.

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ADDENDUM IN PROOF

After this paper was accepted, we noticed the possibility that the *ech* gene is a chicken counterpart of the human *nrf2* gene, as the predicted amino acid sequence of the Ech protein is about 64.8% identical to that of Nrf2. However, the expression profiles of the two genes are substantially different. Further studies are necessary to determine whether the two genes are cross-species homologs.

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