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MafB, a New Maf Family Transcription Activator That Can Associate with Maf and Fos but Not with Jun

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We have identified a new member of the *maf* oncogene family and named it *mafB*. This gene is expressed in a wide variety of tissues and encodes a protein of 311 amino acids containing a typical bZip motif in its carboxy-terminal region. In the bZip domain, MafB shares extensive homology not only with v-Maf but also with other Maf-related proteins. As expected from its structure, MafB forms a homodimer through its leucine repeat structure and specifically binds Maf-recognition elements (MAREs). In addition, MafB forms heterodimers with v-Maf and Fos through its zipper structure. However, unlike v-Maf, MafB fails to associate with Jun. Transient cotransfection assays revealed that both v-Maf and MafB act as transactivators for a promoter linked to MAREs, although MafB is less potent than v-Maf. As is the case for the c-*maf* gene, overexpression of the *mafB* gene induces transformation of chicken embryo fibroblasts in vitro. Through formation of numerous bZip dimers, the Maf family proteins along with the AP-1 components should provide great diversity in transcriptional regulation for a wide variety of genes.

The *maf* oncogene was initially identified from an acutely oncogenic avian retrovirus, AS42, which induces musculoaponeurotic fibrosarcoma in vivo and transformation of chicken embryo fibroblasts (CEFs) in vitro (27, 38). Sequence analysis and subsequent studies of the *maf* oncogene showed that its product, v-Maf, contains a typical bZip DNA-binding motif (33, 46) in its carboxy-terminal region and localizes predominantly in the nucleus of the virus-infected cells (24, 38). Recently, we determined the preferred DNA sequences for v-Maf by use of binding site selection techniques: v-Maf recognizes two relatively long palindromic sequences, TGCT GACTCAGCA and TGCTGACGTCAGCA, at roughly equal efficiencies. Notably, the central parts of these two sequences are identical to the consensus binding sequences for the AP-1 transcription factor, namely, the phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element (TRE) (TGACTCA) and the cyclic AMP-responsive element (CRE) (TGACGT CA). We named these two recognition sequences for Maf TRE-type and CRE-type MAREs (Maf recognition elements), respectively (25). In addition, we demonstrated that Maf interacts with both of the major components of AP-1, Fos and Jun, to form heterodimers with distinct but overlapping target sequence specificities. The cross-family heterodimers preferentially recognize asymmetrical DNA sequences consisting of half of each of the recognition consensus sequences of Maf and AP-1 (25).

The AP-1 transcription factor is well-known to be a heterogeneous collection of dimers composed of products of *jun* and *fos* family genes (3, 5, 11, 47). For the c-*jun* gene, two related genes, *junB* and *junD*, in the mammalian genome have been identified (18, 40, 41). As expected from their structural similarity, the three Jun family proteins (c-Jun, JunB, and JunD) form homo- and heterodimers among themselves or with Fos family proteins and recognize TRE and CRE (37, 42). However, they differ from one another in several aspects. For instance, c-*jun* and *junB* are known to be immediate early genes whose expression is rapidly and transiently induced by cell growth-stimulatory signals (3, 41, 47), whereas the expression of *junD* is constitutive and is scarcely affected under the same conditions (15, 18, 40). In adult tissues and during embryogenesis, these *jun* family genes are expressed at different sites (15, 18, 35, 40, 48). In addition, their protein products have different biochemical and physiological properties. Ryseck and Bravo (42) have reported subtle differences in the DNA-binding properties of the members of the Jun family. c-Jun is reported to be most potent in induction of transcription and oncogenic cell transformation (6, 7, 9, 18). JunB is known to be a poor stimulator of transcription and induces cell growth to a lesser extent (7, 9, 10, 43). The third member, JunD, also has a reduced activation effect in transcription (18). Even under the control of a strong retroviral promoter, structural alterations in the coding sequence are reported to be essential for the *junD* gene to acquire a cell-transforming ability (7, 16, 17, 39).

Like the *jun* oncogene, *maf* is a member of a family of related genes. We have so far reported two *maf*-related genes, *mafK* and *mafF* (13). Recently, we found that the amino-terminal domain of v-Maf is associated with its transactivation function (26). Interestingly, the two *maf*-related genes encode small bZip proteins, MafK and MafF, which are highly homologous to Maf within the bZip domain but lack the amino-terminal transactivator domain of Maf. In addition, we recently identified the *mafG* gene, which also encodes a small bZip protein (23). These three small Maf proteins form homodimers and recognize the MARE sequences (23). Furthermore, these small Maf proteins preferentially associate with another, structurally unrelated bZip protein, NF-E2 p45, and act as erythroid-specific transcription factors (1, 2, 20). c-*maf* and *maf* family genes are expressed in a wide variety of tissues, although another *maf*-related gene, designated *NRL*, was originally

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identified as a neural retina-specific gene (45). Like v-Maf, NRL protein has been reported to associate with Maf, Jun, and Fos (30).

In this paper, we report the isolation and primary characterization of the *mafB* gene, a novel member of the *maf* oncogene family. Its product, MafB, shares extensive homology in its primary structure with v-Maf, especially in the carboxy-terminal bZip domain and the amino-terminal transactivator domain. MafB readily dimerizes with itself and with Maf and efficiently recognizes TRE- and CRE-type MAREs. Both Maf and MafB also efficiently dimerize with Fos oncoprotein. Unlike Maf, however, MafB fails to associate with Jun. We also show that both proteins act as sequence-specific transactivators, although MafB is significantly less potent in transactivation than v-Maf.

MATERIALS AND METHODS

Molecular cloning. A partial *mafB* cDNA was isolated by screening a λ phage cDNA library from CEFs with a v-*maf*-specific DNA probe under relaxed hybridization conditions as described previously (13). Longer cDNA clones were isolated from a random-primed cDNA library prepared from CEFs. The pSaeB-2 plasmid, from which the 0.8-kb DNA fragment used as a *mafB*-specific probe was excised, was constructed as follows. The 0.9-kb *Nco*I-*Mse*I fragment (nucleotides 2100 to 2992) of a *mafB* cDNA clone was subcloned into the polylinker sequence of pUC-9 plasmid. From this subclone, a region of about 120 bases which contains two clusters of CAC trinucleotides was deleted to avoid nonspecific hybridization by double digestion with *Apa*I and *Stu*I, blunting of the ends with T4 DNA polymerase, and self-ligation.

A genomic library was constructed from *Bam*HI arms of λ Charon 40A vector (12) and chicken genomic DNA partially digested with *Sau*3AI. By using the *mafB*-specific probe excised from pSaeB-2, a genomic DNA clone of the chicken *mafB* gene was obtained from the genomic DNA library. The entire insert of the genomic clone λB8 was excised by utilizing *Sal*I sites in the polylinker sequences of λ Charon 40A and subcloned into the *Sal*I site of pUC-9 vector. The subclone, named pB815, was used for further structural analyses.

Synthesis of MafB protein in *Escherichia coli* cells. To examine the DNA-binding specificity of MafB, the bZip domain of MafB was synthesized as a fusion with maltose-binding protein (MBP) by utilizing an *E. coli* expression vector, pMAL-c (New England Biolabs). To prepare the MafB expression plasmid, plasmid pRAM-B-GEM (see below) was digested with *Bst*XI, treated with T4 DNA polymerase, and digested with *Eco*RI. The resulting 0.37-kb fragment was subcloned between *Stu*I and *Eco*RI sites of the pMAL-c plasmid. The expected product from this construct contains the carboxy-terminal 117 amino acid residues of MafB. Preparation of MBP-fused v-Maf and v-Jun proteins and gel retardation assays were performed as described previously (25).

In vitro synthesis of proteins. pRAM-B-GEM, used as a template for in vitro transcription, was constructed as follows. The *Nco*I-*Mse*I fragment (nucleotides 2100 to 2992) containing most of the coding sequence was excised from a *mafB* cDNA clone (λ45) and ligated to the 34-bp *Bal*I-*Nco*I fragment derived from the region just upstream of the translational initiator ATG codon of the v-*src* gene of Rous sarcoma virus, resulting in good agreement with the consensus sequence around the initiator methionine residue (31). The resulting *Bal*I-*Mse*I fragment was then converted to a *Bss*HII fragment by treatment with Klenow fragment of DNA polymerase I and addition of *Bss*HII linkers. The *Bss*HII fragment was then recloned into *Hinc*II and *Sma*I sites of plasmid pGEM-4. This construct was digested with *Eco*RI and was used as a template for in vitro transcription by T7 RNA polymerase. The RNA was translated by using wheat germ extract according to the instructions supplied by the manufacturer (Promega). The product from this RNA (MafB PT) is a truncated form of MafB which lacks the amino-terminal 18 amino acid residues of the intact MafB protein. Two substitution mutants of this truncated version of *mafB*, *mafB R22E* and *mafB L2PL4P*, were constructed by oligonucleotide-directed mutagenesis (32). In *mafB R22E*, the 232nd codon, for arginine (AGG), was converted to a glutamate codon (GAG). In *mafB L2PL4P*, two leucine codons (261 and 275) were converted to proline codons (CCC for codon 261 and CCG for codon 275). An amino-terminal deletion mutant, Δ*mafB*, was constructed by *Stu*I digestion of the pRAM-B-GEM plasmid, addition of *Nco*I linkers at the *Stu*I site, digestion with *Nco*I, and self-ligation. This construct, pRAMΔ*mafB*, lacks the *Nco*I-*Stu*I fragment of pRAM-B-GEM and encodes an amino-terminal-truncated MafB protein (ΔMafB) with the first two residues (Met-Ala) derived from the linker and the carboxy-terminal 184 amino acid residues of MafB.

χMafB is a product of a v-*maf*–*mafB* chimeric gene, designated χ*mafB*. This fused gene was constructed by replacing the 3′ half of the v-*maf* gene of pRAM-GEM with the corresponding 3′ part of the *mafB* gene sequence. In brief, the pRAM-GEM plasmid was digested with *Bss*HII, ligated to synthetic nucleotides to convert the *Bss*HII site (nucleotide 509 of v-*maf*) to a *Nco*I site, and partially digested with *Mlu*I. The 3′ half of the v-*maf* gene sequence excised by *Bss*HII and *Mlu*I was then replaced with a 0.45-kb *Nco*I-*Mlu*I fragment excised from pRAMΔ*mafB*. The resultant fusion gene is expected to encode a product of 341 amino acid residues which consists of the amino-terminal 153 amino acid residues of the PT form of v-Maf protein (amino acid residues 19 to 171 of v-Maf), 4 amino acid residues (Val-Thr-Met-Ala) derived from the linkers, and the carboxy-terminal 184 amino acid residues of MafB.

RNase protection assay. RNase protection analysis was performed with an RPAII RNase protection assay kit (Ambion Inc.). To make a *mafB*-specific cRNA probe for this assay, the pRAM-B-GEM plasmid DNA was digested with *Stu*I and used as a template for in vitro transcription with SP6 RNA polymerase. A c-*maf*-specific probe was prepared as described previously (13).

Eukaryotic expression and reporter plasmids. pEF *Bss*HII, a derivative of the pEF-BOS expression vector (36), was constructed as follows. To eliminate an AP-1 binding site within the replication origin of simian virus 40 from the pEF-BOS vector, a 0.2-kb *Hin*dIII fragment was deleted by *Hin*dIII digestion and self-ligation. Subsequently, the stuffer *Xba*I fragment was replaced by a *Bss*HII linker sequence to make pEF*Bss*HII. The coding sequence for MafB PT protein was excised from the pRAM-B-GEM plasmid and subcloned into a unique *Bss*HII site of the pEF*Bss*HII vector. Similarly, to express v-Maf, an *Mlu*I fragment which contains a translational initiation sequence linked to the coding sequence of v-Maf PT was excised from the pRAM plasmid (24) and subcloned into the *Bss*HII site of pEF *Bss*HII. A luciferase gene construct containing three copies of recognition sequences upstream of the minimal promoter of rabbit β-globin gene was used as a reporter plasmid (20). NIH 3T3 cells were grown in Dulbecco medium supplemented with 10% fetal calf serum. For transfection, the cells were plated at 1.6×10^5 cells per 60-mm-diameter dish. On the following day, the cells were transfected with 1 μg of reporter construct, 6 μg of various effector

plasmids, and 0.5 μg of the β-galactosidase expression vector (pEF/β-gal) by calcium phosphate coprecipitation. At 36 h posttransfection, cells were harvested and extracts were assayed with the luciferase assay system (Promega) as recommended by the supplier.

Transforming potential of *mafB*. To test the cell-transforming ability of the *mafB* gene, we utilized an avian retrovirus vector, pRV-2, constructed from provirus DNA of Rous sarcoma virus of subgroup D (44). This vector contains all sequences required for replication but has the v-*src* coding sequence replaced by an *Mlu*I linker sequence. We excised the *Bss*HII-*Bss*HII fragment which encodes the PT form of MafB from the pRAM-B-GEM plasmid and inserted it into the *Mlu*I site of the pRV-2 vector. Then, to recover the infectious virus containing the chicken *mafB* gene, CEFs were transfected with the resultant plasmid by the Polybrene-dimethyl sulfoxide method (29). Fertilized eggs were supplied by the Nippon Institute of Biological Science, Tokyo, Japan. CEFs were prepared and cultured as described previously (28).

Nucleotide sequence accession number. The nucleotide sequence of the chicken *mafB* gene has been deposited in the GSDB, DDBJ, EMBL, and NCBI databases under accession number D28600.

RESULTS

Cloning of the *mafB* gene. We previously used a v-*maf*-specific DNA probe to screen a cDNA library prepared from mRNA of CEFs under relaxed hybridization conditions and isolated 49 positive clones. From these cDNA clones, we identified the c-*maf*, *mafK*, *mafF*, and *mafG* genes (13, 20, 23). By further analyses of the remaining clones, we found that 12 clones had been derived from another novel *maf*-related gene which we named *mafB*. All 12 cDNA clones were much shorter than the mRNA size estimated by blot analysis of CEFs (data not shown). Structural analyses of the genomic and cDNA clones (Fig. 1) revealed that synthesis of the cDNA clones isolated from the oligo(dT)-primed cDNA library had been primed not from the poly(A) tail of mRNA but from internal adenine stretches in the 3′ noncoding region of the mRNA. We therefore decided to construct a new cDNA library, using the random hexamer-priming method to obtain additional cDNA clones.

Figure 1 schematically represents the structures of the DNA clones of the chicken *mafB* gene. By comparing nucleotide sequences of the cDNA and genomic DNA clones (Fig. 2), we found that the *mafB* gene is devoid of introns, at least in the region of the cDNA contig. In the region just upstream of the cDNA contig, one TATA box-like sequence (TATAAGA) and six recognition elements for the eukaryotic transcription factor Sp1 (22) (CCGCCC) were found to be clustered, suggesting a promoter function for this region. It is thus likely that the difference between the length of the cDNA contig (2,663 nucleotides) and the estimated length (3.2 kb) of *mafB* mRNA results mainly from a lack of the 3′ part of the mRNA. In the downstream region of this gene, as far as we have analyzed, no typical poly(A) signal sequence (AATAAA) was found.

The longest open reading frame of *mafB* could encode a protein of 311 amino acids. The sequence surrounding the putative initiator methionine codon has features of the consensus sequence deduced by Kozak (31). An in-frame termination codon was identified 261 bp upstream of the putative initiator codon. In addition, as evident from the alignment of amino acid sequences of Maf family proteins (Fig. 3), the amino-terminal region of MafB shows significant homology with the corresponding part of v-Maf (38) and NRL (45). On

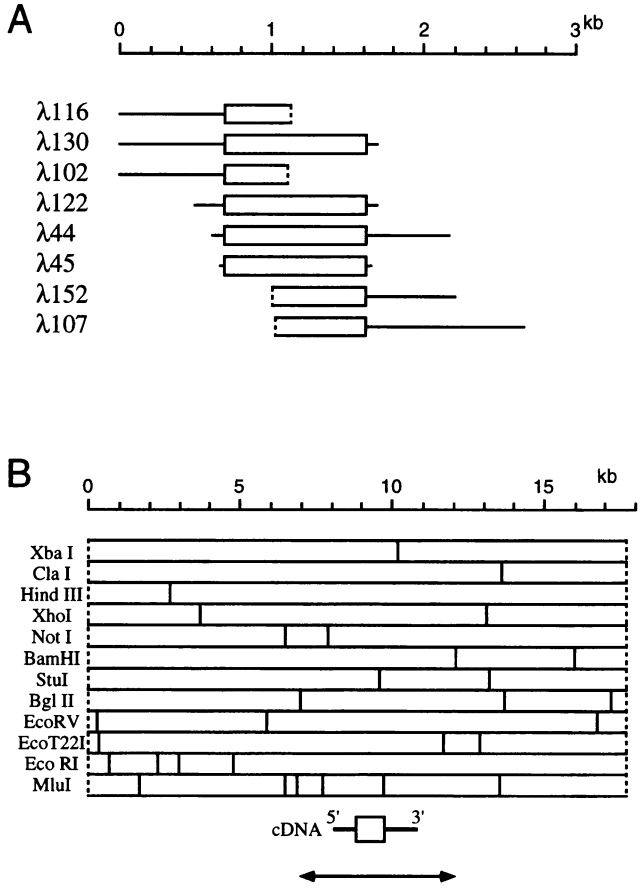

FIG. 1. cDNA and genomic DNA clones of the chicken *mafB* gene. (A) Schematic representation of the cDNA clones. Open boxes indicate positions of the coding sequence. (B) Restriction endonuclease map of the chicken *mafB* locus. The position of the cDNA contig is indicated at the bottom. The coding sequence of the *mafB* gene (open box) and the region subjected to nucleotide sequence analysis (arrow) are indicated.

the basis of these findings, we concluded that this methionine codon is the translational start site of MafB. Homology among the three Maf family proteins within their amino-terminal region suggests functional importance of this region. This region is rich in acidic amino acids and, as we suggested previously (24), may be important for transcriptional activation function. The three large Maf molecules are quite divergent from one another in the middle region. In this highly divergent region of MafB, two clusters of histidine residues may be functionally important, since the corresponding region of v-Maf contains three glycine stretches and one histidine cluster. In the carboxy-terminal bZip domain, MafB again shows extensive homology with the corresponding region of other Maf family members, including the small Maf proteins. We have previously shown that, in addition to the leucine zipper motif, a region of about 50 amino acid residues just upstream of the leucine zipper motif is essential for DNA-binding activity of Maf (25). The degree of homology among the Maf family proteins is especially high in this DNA-binding domain, suggesting similarity in DNA-binding specificities. In overall structure, v-Maf is the closest relative to MafB (51.8% identity) among the Maf family proteins thus far identified.

FIG. 2. Nucleotide sequence of the chicken *mafB* gene. Six GC boxes (boxed) and a TATA box sequence (underlined) located just before the 5′ terminus of the cDNA contig (boxed) are possible regulatory elements for expression of this gene. The deduced amino acid sequence is shown below the nucleotide sequence. The heptad repeat of hydrophobic amino acid residues (leucine zipper structure; stars and dotted lines) is shown.

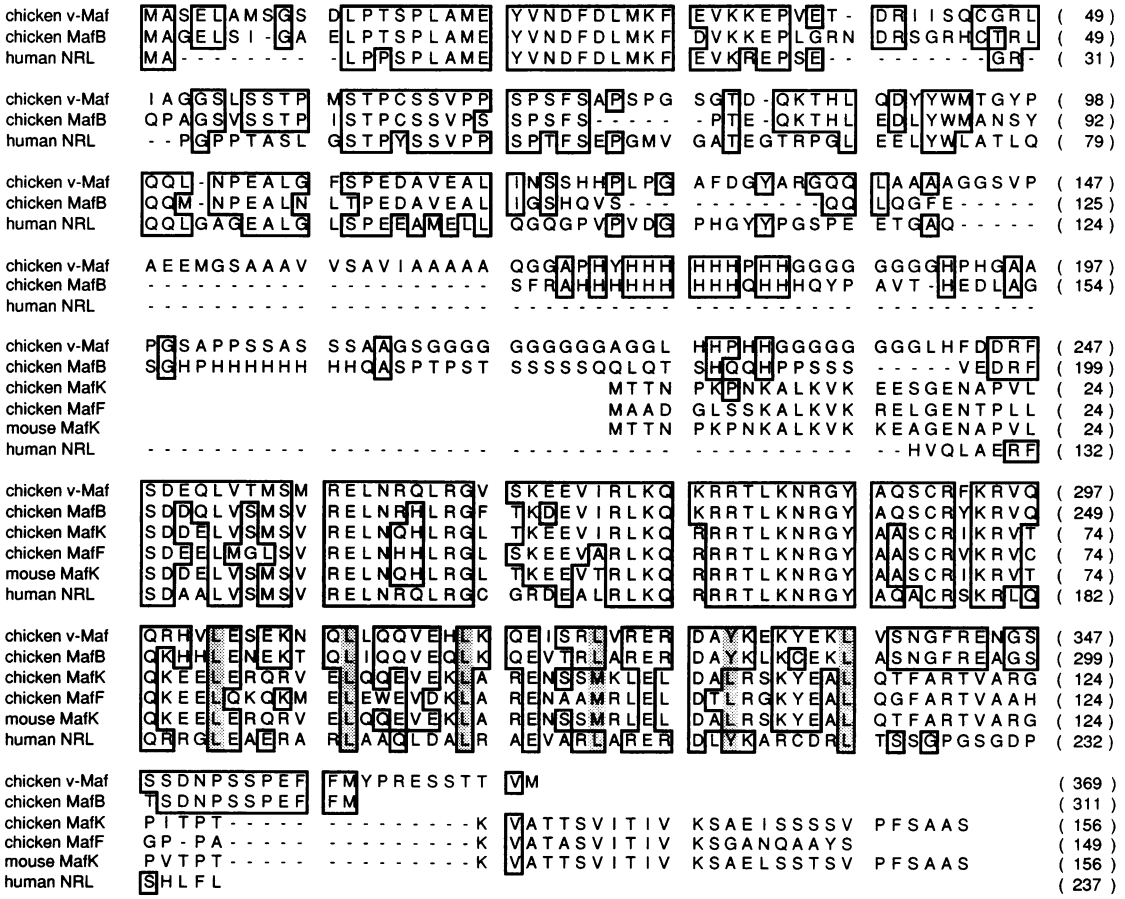

FIG. 3. Comparison of primary structures and deduced amino acid sequences of the Maf family proteins identified so far. Amino acids are indicated by single-letter code, and the numbers for the amino acid residues are shown at the right. Amino acid residues conserved with respect to the v-Maf protein are boxed. Sequence information for v-Maf (37), chicken MafK and MafF (13), mouse MafK (2), and human NRL (44) was published previously.

Tissue distribution of *mafB* mRNA. To study whether *mafB* is expressed differently according to cell types, we determined the levels of *mafB* mRNA in 15 different chicken organs by RNase protection assay. High levels of *mafB* transcripts were seen in the brain, thymus, lung, gut, mesenterium, spleen, kidney, ovary, and bursa (Fig. 4B). Expression in the stomach, liver, and testis was marginal, and no *mafB* mRNA was detected in the pancreas. On the other hand, c-*maf* mRNA expression is greatest in the kidney, followed by the brain, lung, gut, mesenterium, testis, and ovary (Fig. 4A). The spatial expression patterns of *mafB* and c-*maf* are clearly different from each other.

DNA-binding specificity of MafB. The extensive homology between v-Maf and MafB in their DNA-binding domain suggests that MafB recognizes the same sequence as v-Maf. To examine this possibility, we synthesized the bZip domain of MafB in *E. coli* cells and subjected it to a gel retardation assay, using a series of oligonucleotide probes which contain TRE- and CRE-type MAREs or their mutated versions. In parallel, we purified MBP-fused v-Maf and v-Jun proteins for gel retardation assay (Fig. 5). As expected, the oligonucleotides which contain TRE- and CRE-type MAREs, the optimal sequences for binding to Maf (oligonucleotides 1 and 2) (25), were shifted by MafB at the highest efficiency. Mismatches from the consensus sequences reduced the efficiency of binding to v-Maf and MafB proteins in the same manner. The binding

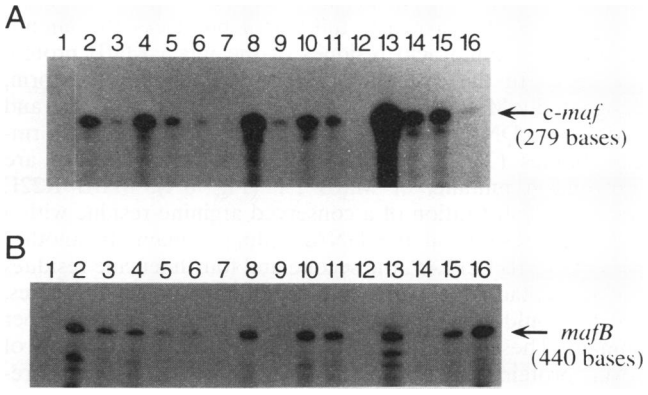

FIG. 4. Expression of c-*maf* and *mafB* genes in chicken tissues. A 10-μg sample of total RNA from each of the following adult chicken tissues was subjected to RNase protection assays to detect the presence of c-*maf* (A) or *mafB* (B) mRNA: brain (lanes 2), thymus (lanes 3), lung (lanes 4), heart (lanes 5), muscle (lanes 6), stomach (lanes 7), gut (lanes 8), liver (lanes 9), mesenterium (lanes 10), spleen (lanes 11), pancreas (lanes 12), kidney (lanes 13), testis (lanes 14), ovary (lanes 15), and bursa (lanes 16). Yeast tRNA (lane 1) was included as a negative control.

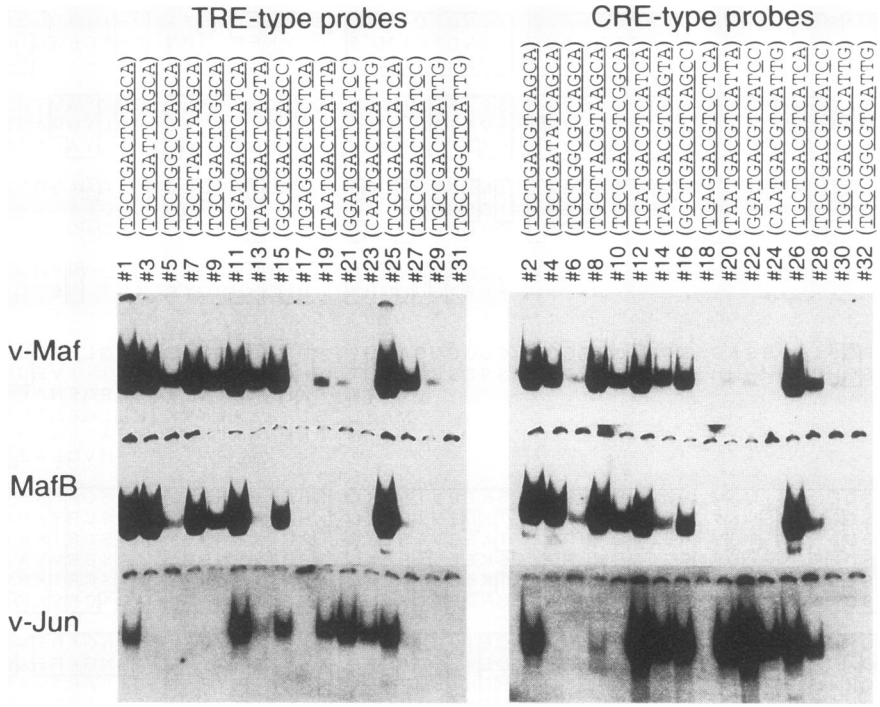

FIG. 5. Comparative analysis of the DNA-binding specificities of v-Maf, MafB, and v-Jun. The three bZip proteins prepared as fusion proteins with MBP were subjected to gel mobility shift assays with a series of oligonucleotide probes. The nucleotide sequence of only the middle, unique part of the probes is shown at the top. The whole structure of oligonucleotide 1 is

5′-TCGAGCTCGGAAT**TGCTGACTCAGCA**TTACTC-3′
3′-TCGAGCCTTA**ACGACTGAGTCGT**AATGAGAGC-5′

Oligonucleotides 1 and 2 contain the TRE- and CRE-type MAREs, respectively. Nucleotide matches with the MAREs are underlined. Other probes contain one to six mismatches from the consensus sequences.

specificity of Jun was clearly different from those of the two Maf family proteins.

To further confirm the above results and to examine the importance of the bZip structure for DNA-binding activity, we synthesized MafB protein or mutated forms by in vitro transcription and translation and subjected them to gel retardation assays. MafB PT is nearly full-length but lacks the amino-terminal 18 amino acid residues of the intact MafB protein (Fig. 6A). In the case of v-Maf, a similar truncated form, designated v-Maf PT, is as active as the full-length v-Maf and c-Maf in its DNA-binding, transactivating, and cell-transforming abilities (24–26). MafB R22E and MafB L2PL4P are substitution mutants of MafB PT (Fig. 6A). MafB R22E contains a substitution of a conserved arginine residue with a glutamate residue in the DNA-binding domain. In another mutant, MafB L2PL4P, the second and fourth leucine residues of the heptad repeat are substituted with proline residues, which should abolish the α-helical structure of the zipper domain. These mutations correspond to the two mutants of v-Maf protein (R22E and L2PL4P) which we previously reported to be defective in DNA binding (25). As expected, only MafB PT efficiently bound to the MARE probe (Fig. 6B, lane 2). The two substitution mutants of MafB failed to bind to the specific probe (Fig. 6B, lanes 3 and 4), indicating that integrity of the bZip structure is essential for the DNA-binding activity of MafB.

Dimer-forming specificity of MafB. To test the dimer-forming ability of MafB, we synthesized in vitro two MafB proteins with different lengths. However, probably because of translational initiation from internal methionines and/or premature termination, the MafB PT synthesized in vitro gave multiple bands in gel mobility shift assays (Fig. 6B, lane 2). Since it is well established that the leucine zipper domain is primarily responsible for the dimer-forming specificities of bZip proteins, we used in this particular experiment a v-*maf*–*mafB* chimeric gene designated χ*mafB*. As illustrated in Fig. 6A, the amino-terminal half of the chimeric χMafB protein is derived from v-Maf and the carboxy-terminal bZip domain is from MafB. In vitro-synthesized protein from chimeric gene χ*mafB* gave a single, lower-mobility band in a gel shift assay (Fig. 7A, lane 2). Another form of the *mafB* gene constructed for this experiment is Δ*mafB*, which encodes an amino-terminal-truncated form of MafB protein, ΔMafB. This protein lacks the amino-terminal half of the intact MafB protein but retains the bZip structure (Fig. 6A) and generated a faster-migrating band in a gel shift assay (Fig. 7A, lane 3). Two other mutants, χMafB L2PL4P and ΔMafB L2PL4P, are the derivatives of χMafB and ΔMafB, respectively, which contain two substitutions in the leucine zipper domain: the second and fourth leucine residues of the zipper structure were replaced with proline residues (Fig. 6A). These mutants were defective in DNA binding (Fig. 7A, lanes 7 and 8), probably because of their inability to form homodimers. When two MafB proteins of different lengths, χMafB and ΔMafB, were cotranslated, an additional band of intermediate mobility was observed in a gel mobility shift assay (Fig. 7A, lane 4). If one of the two MafB proteins was replaced with the respective leucine-to-proline substitution mutant (χMafB L2PL4P or ΔMafB L2PL4P), the

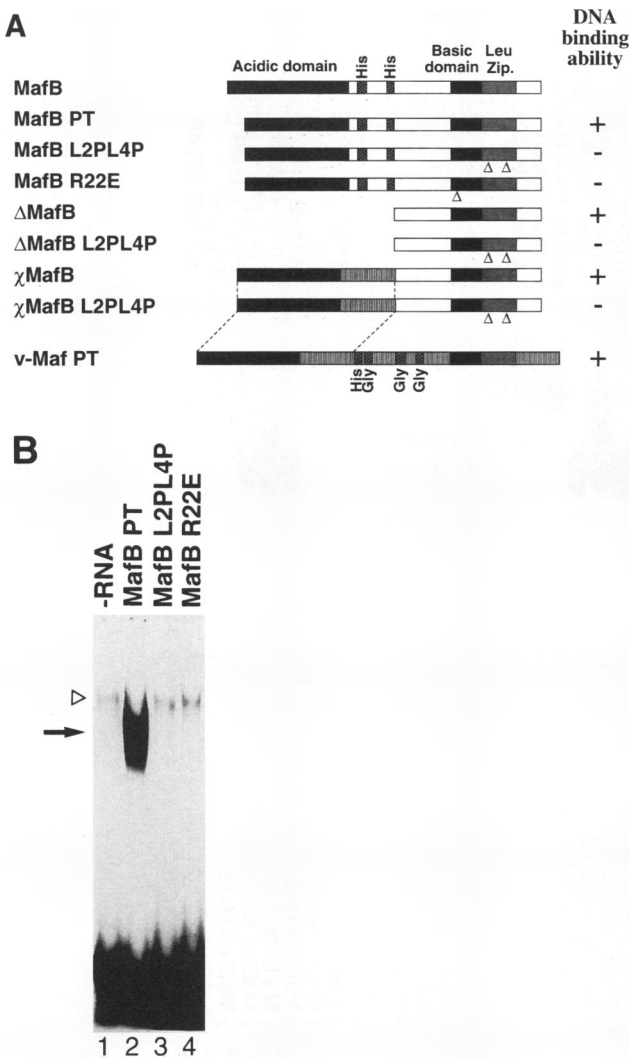

FIG. 6. DNA-binding ability of MafB protein synthesized in vitro. (A) Schematic representation of MafB mutants used in this study and their DNA-binding abilities. MafB PT is a nearly full-length version of MafB. ΔMafB is an amino-terminal truncated form. χMafB is a chimeric protein which consists of an amino-terminal part derived from v-Maf PT and a carboxy-terminal bZip part from MafB. Mutation sites in their substitution mutants, MafB L2PL4P, MafB R22E, ΔMafB L2PL4P, and χMafB L2PL4P, are shown (open triangles). The amino-terminal acidic region, basic DNA-binding domain, leucine zipper dimerization domain, and clusters of histidine or glycine residues are also indicated. (B) MafB PT and its substitution mutants, MafB L2PL4P and MafB R22E, were synthesized in vitro and subjected to a gel mobility shift assay. Oligonucleotide 1, which contains the TRE-type MARE, was end labeled and used as a probe. A retarded band of DNA-binding activity in the wheat germ extract used for in vitro translation (open triangle) and the retarded band of MafB PT (arrow) are indicated.

additional band disappeared, indicating that MafB forms a homodimer through the heptad repeat of the hydrophobic residues (Fig. 7A, lanes 5 and 6). Similarly, when we analyzed the cotranslated mixture of ΔMafB and either v-Maf or v-Fos, an additional band which should be derived from the v-Maf–MafB or Fos-MafB heterodimer was detected (Fig. 7B and C, lanes 4). Replacement of these bZip proteins with the leucine zipper mutant proteins (v-Maf L2PL4P, ΔMafB L2PL4P, and Fos L3P) completely abolished the heterodimer formation (Fig. 7B and C, lanes 5 and 6), confirming that these bZip proteins also interact through their zipper structures. As we reported previously (25) and as shown in Fig. 7D, lane 4, v-Maf efficiently heterodimerizes with Jun. However, we failed to detect the association of MafB with Jun (Fig. 7D, lane 5). In this experiment, comigration of a band of the possible MafB-Jun heterodimer with either of the homodimers is highly unlikely, because we confirmed defectiveness of MafB in association with Jun by analyzing a cotranslated mixture of MafB PT and v-Jun. Similar to the case for the mixture of ΔMafB and v-Jun, we could not detect a band of MafB PT–v-Jun heterodimer (data not shown). Moreover, in the absence of the target DNA sequences, amylose resin bound to a bacterially synthesized MBP-fused v-Jun protein efficiently precipitated in vitro-synthesized v-Maf and v-Fos proteins but failed to associate with MafB (data not shown).

A similar gel retardation assay showed that MafB also lacks the ability to form dimers with three small Maf family proteins, MafK, MafF, and MafG (Fig. 7F, lanes 7 to 9). The three small Maf proteins failed to dimerize not only with MafB but also with v-Maf (Fig. 7E, lanes 7 to 9). In conclusion, through its leucine zipper motif, MafB forms dimers with itself, v-Maf, and Fos but not with Jun or small Maf proteins. Noticeably, Jun can heterodimerize with v-Maf but not with MafB.

Transactivator potential of v-Maf and MafB. To examine the transactivation potential of v-Maf and MafB, we used a rabbit β-globin basal promoter-luciferase construct containing three copies of the binding sequence for Maf proteins in a transient cotransfection assay. In this experiment, to eliminate the background activity in the cells probably derived from AP-1, we used the oligonucleotide 7 sequence (TGCTTAC TAAGCA) as the target binding site of the reporter plasmid. As shown in Fig. 5, this sequence includes two mismatches from the TRE-type MARE (TGCTGACTCAGCA) but is still able to be recognized efficiently by Maf proteins but not by AP-1 (Jun). In a number of transfections, the median level of transactivation by the v-*maf* expression constructs was about 20-fold higher than that observed in control transfections without the v-*maf* gene (Fig. 8). When the upstream element was replaced by oligonucleotide 17, which differs from the MARE by four bases, no transactivation by v-Maf was detected, indicating that transactivation by Maf is fully dependent upon the presence of its recognition sites in the reporter. A v-Maf mutant, v-Maf L2PL4P, which contains two amino acid substitutions in its leucine zipper motif and is defective in homodimer formation and DNA binding, failed to activate the expression of the reporter gene. Compared with v-Maf, MafB activated the expression of the test gene with greatly reduced efficiency: only a two- to threefold increase in luciferase activity over the basal level was observed. The difference in transactivation potential between v-Maf and MafB cannot be attributed to the possible differences in stability or expression level of these proteins, since the activity of v-Maf was partially inhibited by coexpression of MafB, probably by competitive binding to the recognition sites (Fig. 8).

Phenotypes of CEFs infected with retroviruses carrying v-*maf* or *mafB*. We have previously shown that overexpression of the c-*maf* gene induces transformation of CEFs (24). To examine the transforming ability of the *mafB* gene, we used the same vector system to produce a variant of Rous sarcoma virus which carries the *mafB* gene instead of the v-*src* gene. Infection of CEFs with the recombinant retrovirus induced focus formation in liquid culture and colony formation in soft agar suspension culture (Fig. 9). The frequency of colony formation after infection with this virus was equivalent to or slightly

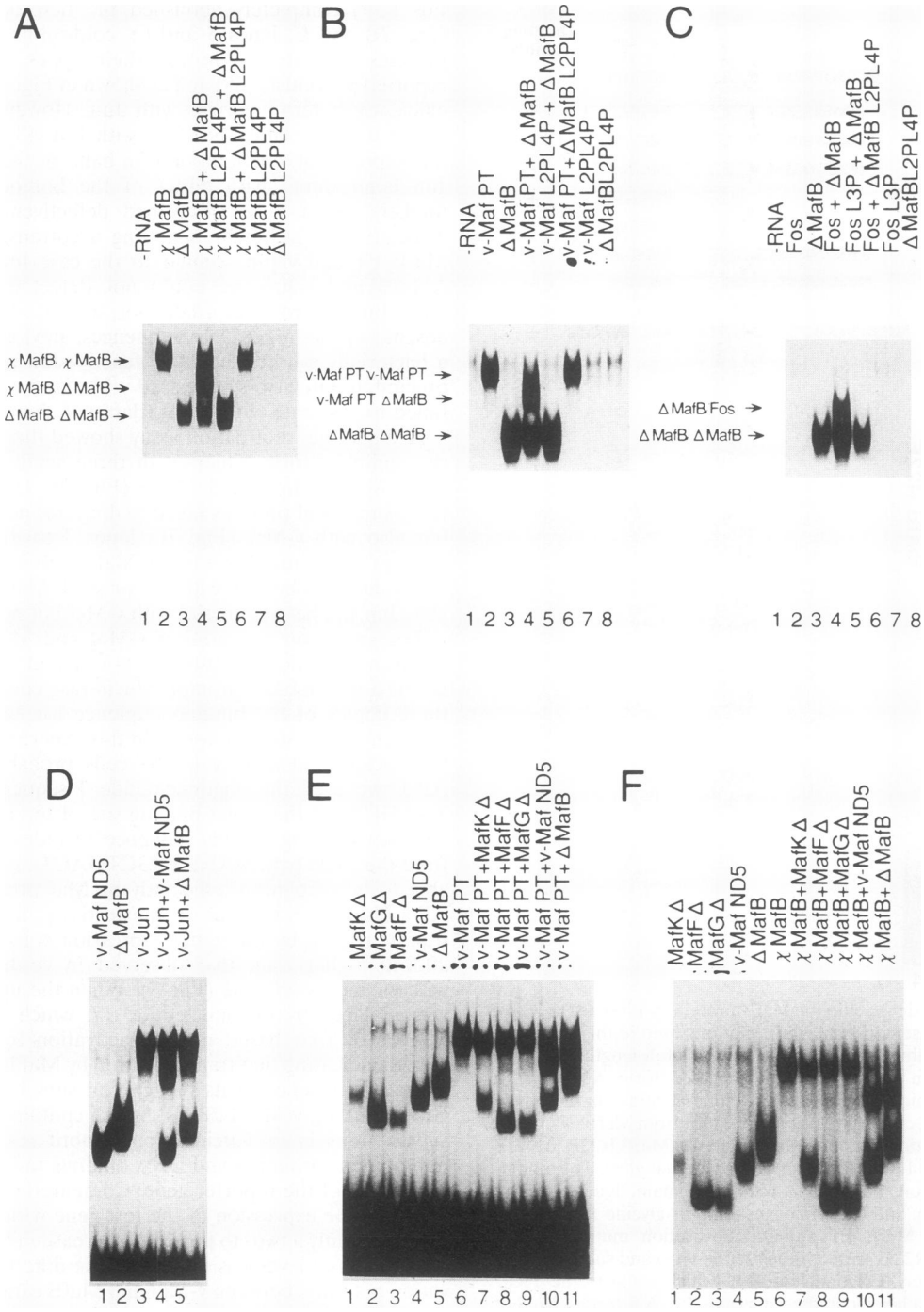

FIG. 7. Dimer-forming specificity of MafB. Two MafB proteins of different lengths, MafB PT and ΔMafB (Fig. 6A), other Maf family proteins (v-Maf, MafK, MafF, and MafG), and AP-1 proteins (Fos and Jun) were synthesized either separately or simultaneously, as indicated, by successive in vitro transcription and translation. The resulting complexes were analyzed by electrophoretic band shift, using oligonucleotide probe 1, which contains the MARE consensus motif. For the lysates including either Fos or Jun, oligonucleotide 11 was used as a probe, because of its high affinity for both Maf and AP-1 proteins. (A) Homodimer formation of MafB. (B) Heterodimer formation between v-Maf and MafB. (C) Association of MafB with v-Fos. (D) Inability of MafB to associate with v-Jun. (E and F) Defective formation of heterodimers between three small Maf proteins and v-Maf and MafB, respectively.

less than that after infection with the v-*maf* virus; one milliliter of culture fluid from the CEFs fully transformed with the *mafB* recombinant virus induced 2×10^3 to 10×10^3 colonies after a 3-week incubation. The colonies were morphologically similar to those induced by v-*maf*, though smaller on average.

DISCUSSION

In this article, we report the identification and primary characterization of *mafB*, a novel member of the *maf* oncogene family. Amino acid sequences of the Maf family proteins are

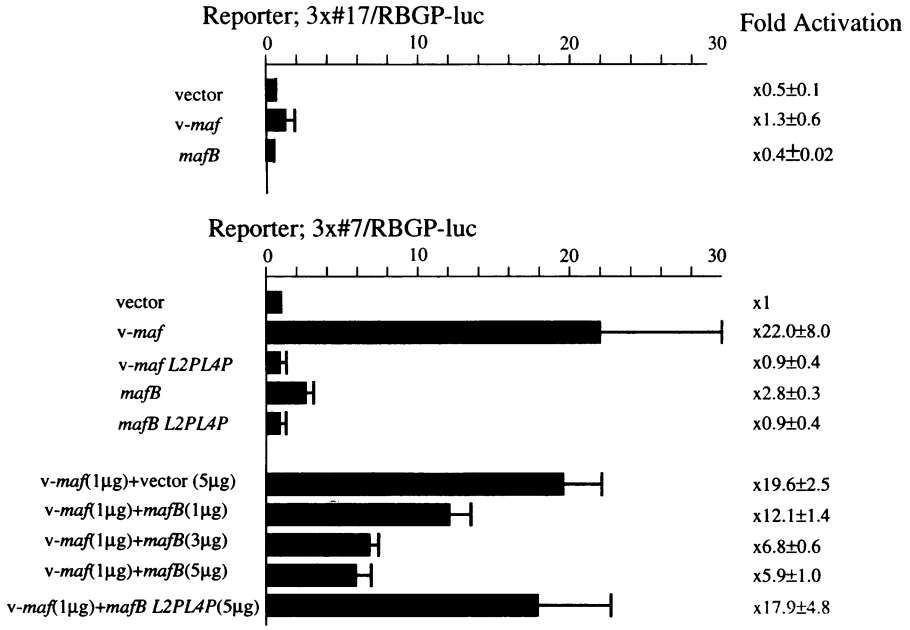

FIG. 8. Comparison of transactivating potentials of v-Maf and MafB. NIH 3T3 cells were cotransfected with a rabbit β-globin basal promoter (RBGP)-luciferase gene reporter plasmid associated with a triplicated binding sequence for Maf. As the Maf recognition sequence, oligonucleotide 7, which is efficiently recognized by Maf but not by AP-1, was used. A reporter plasmid integrated with oligonucleotide 17 in place of oligonucleotide 7 was used as a negative control. The reporter plasmid DNAs were transfected into NIH 3T3 cells along with effector plasmids including v-*maf* or *mafB*. Transfection of the corresponding amount of the pEF*Bss*HII vector with the RBGP-luciferase construct with a triplicated oligonucleotide 7 sequence (3×#7 oligo-RBGP-luc) served as a onefold-induction level. Results shown represent averages of at least three independent experiments.

particularly conserved within the putative DNA contact domain, suggesting similarities in DNA-binding specificities. Indeed, as we have shown in this study, Maf and MafB proteins exhibit the same sequence specificity. Among the *maf* family members, however, three genes, designated *mafK*, *mafF*, and *mafG* (small *maf* subfamily members), are clearly different from c-*maf* and *mafB* in the following respects. First, the small *maf* subfamily genes encode products which are small and lack the amino-terminal transactivator domain present in Maf and MafB. Second, under the control of a strong viral promoter, c-*maf* and *mafB* are able to transform CEFs efficiently, but the small *maf* genes are much less efficient in cell transformation (13). Third, exon-intron organizations of the three small *maf* subfamily genes are clearly different from that of *mafB*. The chicken small *maf* genes seem to consist of three exons: a noncoding first exon, a short second exon containing the initiator methionine codon, and a long third exon (13). In contrast, the chicken *mafB* gene is devoid of introns, at least within its coding sequence. Our preliminary characterization of the chicken and human c-*maf* genes suggests that the structural organization of the c-*maf* gene is similar to that of the *mafB* gene. Finally, the small Maf proteins are different from Maf and MafB in their specificity in dimer formation. The small Maf proteins associate most efficiently with the large subunit of an erythroid-specific transcription factor, NF-E2 (20). In contrast, under the same conditions, Maf and MafB do not associate with this preferred partner of the small Maf proteins, NF-E2 p45 (20).

MafB is similar to Maf in its primary structure, dimer-forming specificity, and cell-transforming ability. Maf, MafB, and another member of the Maf family, NRL, are highly homologous to one another not only in their bZip domain but also in the amino-terminal acidic domain, suggesting a strong selective pressure to maintain function. Also in the leucine zipper domain, which is responsible for the specificity of dimer formation (33, 46), v-Maf and MafB share significant sequence homology (75% identity). Consistent with this high degree of similarity, both v-Maf and MafB form homodimers and form dimers with Fos but not with small Maf proteins or NF-E2 p45. Unexpectedly, however, MafB differs from Maf in its ability to form a dimer with Jun: Jun is able to dimerize with Maf but not with MafB. As we reported previously, the DNA-binding specificity of v-Maf is affected by heterodimer formation with the components of AP-1 (25). The levels of expression of c-*jun* and c-*fos* are rapidly elevated by many growth-stimulatory signals (3). Unlike c-*fos*, however, c-*jun* and its related genes are known to be expressed to some extent in a wide variety of tissues in a tissue- and stage-specific manner without cell growth stimuli (15, 18, 35, 40, 41, 48). It is thus plausible that the expression of the Jun family proteins differentially affects the function of Maf and MafB proteins.

In addition to dimer-forming specificity, Maf and MafB also differ in their transactivation potentials. MafB is less efficient than v-Maf in transactivation. Possibly, MafB may not be an intrinsically less effective transactivator but may be unable to associate with other bZip transcription factors to form the most potent heterodimer forms. Consistent with reduced transactivation potential, MafB exhibits a somewhat weaker transforming ability than v-Maf. Overexpression of the *mafB* gene in CEFs induced colonies slightly smaller than those induced by v-*maf*. These properties of the *mafB* gene and its product are reminiscent of a *jun*-related gene, *junB*. JunB is indistinguishable from Jun in its DNA-binding specificity but is less potent in transactivation (9, 10, 43). Moreover, in soft agar suspension culture, CEFs overexpressing JunB form smaller colonies at a reduced frequency compared with the cells

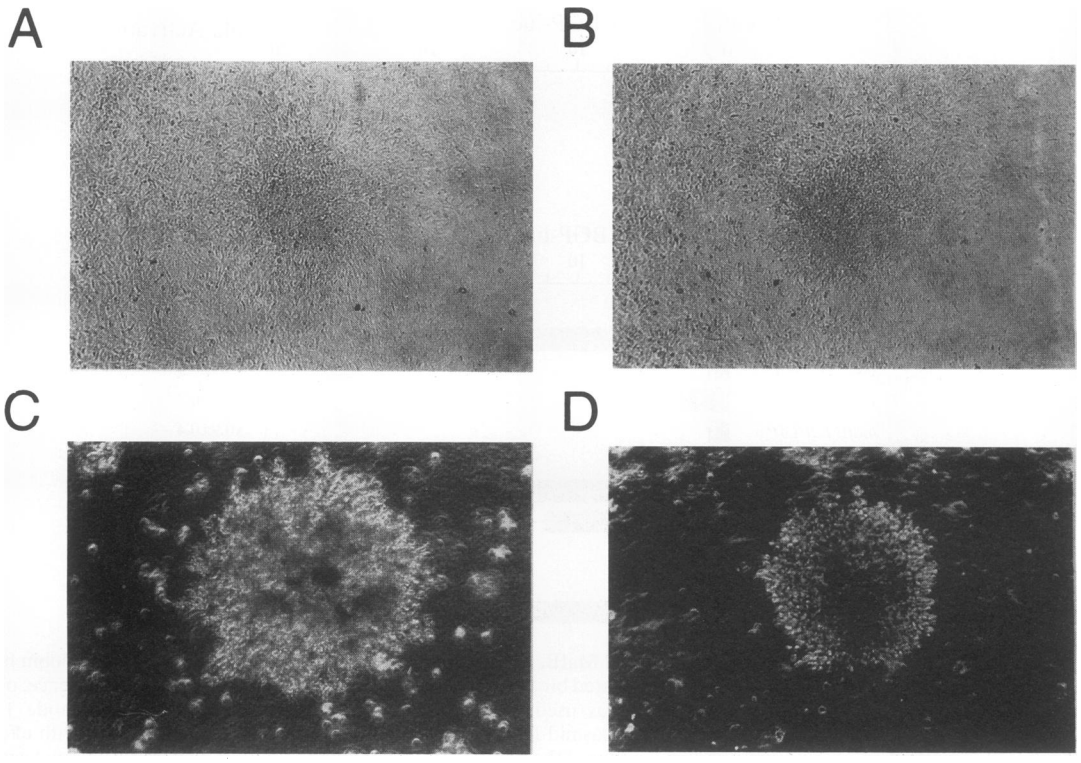

FIG. 9. Comparison of morphologies of typical foci and colonies of CEFs transformed by v-*maf* or *mafB*. Typical foci induced by overexpression of v-Maf PT (A) and MafB PT (B) are shown at the same magnification. Colonies in soft agar suspension culture induced by v-Maf PT (C) and MafB PT (D) are shown at the same magnification.

overexpressing c-Jun (7). These bZip proteins associated with reduced transactivation properties may be important in fine-tuning of expression of many cellular genes.

This study reveals a further complexity in transcriptional cross talk between Maf family proteins and the AP-1 components, Fos and Jun. Heterodimer formation between the Maf family proteins and other members of the Jun and Fos family proteins remains to be tested. Furthermore, Maf family proteins may also associate with other bZip proteins, including the ATF/CREB family transcription factors, some of which are known to associate with Jun and Fos (4, 8, 14, 19, 21, 34). Depending on the relative concentrations of many bZip proteins, numerous combinations of dimers will form, which should affect the transcriptional status of the cells in different ways.

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