hMAF, a Small Human Transcription Factor That Heterodimerizes Specifically with Nrf1 and Nrf2

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A 1.6-kilobase pair full-length cDNA encoding a transcription factor, homologous to the Maf family of proteins, was isolated by screening a K562 cDNA library with the NFE2 tandem repeat probe derived from the globin locus control region. The protein, which was designated hMAF, contains a basic DNA binding domain and an extended leucine zipper but lacks any recognizable activation domain. Expressed in vitro, the hMAF protein is able to homodimerize in solution and shift the NFE2 tandem repeat probe. In addition to homodimers, hMAF can also form high affinity heterodimers with two members of the NFE2/CNC-bZip family (Nrf1 and Nrf2) but not with a third family member, p45-NFE2. Although hMAF/MAF homodimers and hMAF/Nrf1 and hMAF/Nrf2 heterodimers bind to the same NFE2 site, they exert functionally opposite effects on the activity of a linked γ-globin gene. In fact, whereas all hMAF/CNC-bZip heterodimers stimulate the activity of a γ-promoter reporter construct in K562 cells, the association into heterodimers that is induced by overexpressing hMAF inhibits the activity of the same construct. Thus variations in the expression of hMAF may account for the modulation in the activity of the genes that bear NFE2 recognition sites.

In the last decade, meticulous searches along the β-globin gene cluster have led to the identification of numerous regulatory DNA sequences located either in close proximity to the genes or at a distance in regions that were originally identified for their DNase I hypersensitivity (1–3). The latter regions, which are referred to individually as hypersensitive sites (from 5′-HS1 to HS4) and collectively as the locus control region of the β-globin gene cluster (4), contain elements with different functions such as enhancers (5–8), silencers (9, 10), origins of replication (11–13), and putative insulators (14). By a series of structural and functional experiments based on DNA-protein interactions (15–23) as well as selective mutagenesis and expression studies in cell lines and transgenic mice (17, 18, 22–27), several short DNA consensus sequences have been identified to bind regulatory proteins that represent the effectors of the activities ascribed to the locus control region. One such sequence (TGAGTCA) that is repeated twice in the core of the HS2 enhancer is recognized by proteins of the AP1 (28), cAMP-responsive element-binding protein, and NFE2/CNC-bZip families of transcription factors (29, 30) and is known as the NFE2/A1 consensus sequence. As the latter motif is frequently found along the globin clusters in DNA elements with enhancer activity, cloning p45-NFE2 (31–33) has raised much interest as it could provide useful insights on the transcription factor enhancement of the globin gene expression, which in turn might lead to novel therapeutic approaches for inherited hemoglobin diseases such as sickle cell and thalassemia syndromes.

Soon after the cloning of NFE2, we and others have cloned two more genes, NRF1 (also known as LCR-F1 and TCF11) (34–36) and NRF2 (37), which is highly related to NFE2, thus extending the NFE2 family to three members and predicting the existence of a fourth member on the basis of linkage with other large gene families (COL, INT, and HOX) on specific chromosomes (38). The three genes are highly homologous in the DNA binding domain and leucine zipper but as is usually the case among related transcription factors, they are completely different in the activation domain. The genes are also differently regulated as NFE2 is restricted to hematopoietic tissues, whereas NRF1 and NRF2 are ubiquitously expressed. Despite the dramatic decrease in the HS2 enhancer activity produced by the disruption of the NFE2 consensus enhancer sequence (5, 6), knockout of the p45-NFE2 gene in mice resulted in a disorder of megakaryocyte maturation but only minimally decreased the expression of the globin genes (39), suggesting that other factors are able to compensate for the lack of p45-NFE2 activity. Although Nrf1 and Nrf2 are ubiquitous factors, both are highly expressed in erythroid tissues and are able to transactivate globin gene promoters. Thus Nrf1 and Nrf2 could potentially compensate for p45-NFE2 function in the p45-NFE2 knockout mouse. Although this would suggest an evolutionary redundancy to protect and maintain the crucial body function of oxygen delivery, the observation that none of these factors bind DNA by itself and the identification of the p45-NFE2-associated protein p18 (40) raise the possibility that p45-NFE2 and the related factors might indeed play distinct functions by dimerization with their respective partners. Interestingly, the p45-NFE2 partner p18 belongs to another family of bZip transcription factors (the Maf family (41–43)) whose...
members in chickens have different levels of tissue expression and could therefore drive the functional specificity of the proteins with which they associate. Even though the small Maf proteins do not seem to have an activation domain, a recent report suggests that they are able to modulate the activity of p45-NFE2 according to their preferential association into homodimers or heterodimers, resulting in a negative or positive regulatory activity on the target genes, respectively (44).

These studies emphasize how our knowledge on the globin regulation would benefit from isolation of the proteins that associate with Nrf1 and Nrf2. It was also predictable on the basis of similarities in the dimerization domains among members of the NFE2 family that the partners for Nrf1 and Nrf2 could also be found within the family of the Maf oncoproteins. Here we describe the cloning of a small human MAF cDNA (hMAF) through recognition site screening of a K562 cDNA library with a probe derived from the NFE2 tandem repeat motif of HS2. Even though hMAF shares strong structural homology with the other small Maf proteins in its leucine zipper, it heterodimerizes specifically with Nrf1 and Nrf2 but not with p45-NFE2. As a consequence of heterodimerization, Nrf1 and Nrf2 acquire the ability to bind and stimulate the activity of the target promoters, whereas hMAF homodimers (lacking any activation domain) apparently repress transcription by keeping the heterodimers from binding to their recognition sites.

MATERIALS AND METHODS

Nucleic Acid Cloning and Analysis—A cDNA expression library was constructed in AgtU1 using the TlmeSaver cDNA kit (Pharmacia Bio-tech Inc.), and the mRNA derived from hemin-induced K562 cells was primed either with poly(dT) or random primers. Primary phase screening was done according to Singh et al. (45) using double strand concatemers of the tandem NFE2/AP1 repeat of HS2 (5′-GCACAGAATCT- GAGTCTAGTGAAGCTGCTG-3′) as the recognition site probe. Specificity of the binding was confirmed by the inability of the clones to bind a mutant version of the same oligonucleotide (5′-GCA- CGAATCTGAGTCTAGTGAAGCTGCTG-3′). Total RNA was prepared by the acid guanidium thiocyanate/phenol/chloroform extraction method, further purified to mRNA with poly(dT) spin columns (Pharmacia) and used in 1-μg aliquots to prepare poly(A)+ RNA with poly(dT) spin columns from Pharmacia, and the binding assay was performed according to Smith and Johnson (49). 250 ml of XL1Blue cells transformed with the plasmid were grown to A0.7–1 and induced with 1 mM isopropyl-1-thio- β-D-galactopyranoside at 37 °C for 3 h. Expression of the recombinant polypeptide was determined by loading in the sample buffer 200 μl of bacterial culture on SDS-PAGE protein gels stained in Coomassie Brilliant Blue R250. The whole culture was sedimented and resuspended in 0.2 ml of lysis buffer (50 mM KCl, 20 mM Hepes-KOH, pH 7.9, 2 mM dithiothreitol, 1 mM EDTA, 4 mg/ml lysozyme), lysed through 3 cycles of freeze and thaw, and spun at a relative centrifugal force of 150,000 × g in a 60T rotor (Beckman). 20% glycerol was added to the supernatant, and the solution was quickly frozen in liquid nitrogen. 1-ml aliquots of extracts were mixed with 25 μl of glutathione-Sepharose, incubated with shaking for 40 min at 4 °C, and washed twice with NENT + M buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8, 0.5% Nonidet P-40, 0.5% (w/v) nonfat dry milk). The proteins to be assessed for protein-protein interaction were [35S]methionine-labeled during in vitro translation with reticulocyte lysate. 12.5 μl of the in vitro translation products were incubated with either the chimera GST/hMAF or the wild-type GST protein as a negative control for nonspecific binding. After five washes in the binding buffer the agarose beads were heat-denatured in sample buffer and immediately applied to an SDS-PAGE gel. Equal amounts of each labeled protein were loaded on the gel as a size marker for electrophoretic migration.

Cell Culture and Transactivation Analysis—K562 cells were cultured in a 5% carbon dioxide atmosphere at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum as well as penicillin and streptomycin. Aliquots of 3 × 106 cells in a logarithmic growth phase were transfected in a 60-mm Petri dish with 20 μg of Lipofectin (Life Technologies, Inc.) per 5 ml of DNA, grown overnight in serum-free media, washed in phosphate-buffered saline, and transferred for an additional 40 h in complete medium with 40 μM freshly prepared hemin added. Cells were pelleted, washed in phosphate-buffered saline twice, resuspended in 100 μl of Triton lysis buffer (1% (v/v) Triton X-100, 20 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 1 mM dithiothreitol added immediately before use), and spun in a microcentrifuge at 4 °C and 10,000 rpm for 10 min. 10 μg of protein in 50 μM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 15 mM potassium phosphate, pH 7.8, 1 mM ATP, 1 mM dithiothreitol were assayed for luciferase activity on a Lumat LB9501 luminometer (Berthold and Wallac) by injecting 100 μl of 0.2 mM Luciferin/KCl. The expressor plasmids contained the human cDNA clones for h MAF, NRF1, NRF2, NFE2, and nNFE2 inserted into the EcoRI site of the pcDNA vector (Invitrogen), and the reporter gene was the WY4 construct in which the luciferase activity is driven by the HS2 core enhancer (same sequence used in the recognition site probe screening) and the AluI fragment of the Avg-globin promoter. The amount of DNA transfected was kept constant by the addition (when necessary) of the pcDNA vector as normalizing DNA. All values were normalized for efficiency of transfection by measuring identical amounts of protein extracts and by correcting for the activity of an internal control (the RSVCAT reporter gene) equally distributed among the different transfections. Cell extracts for CAT assays were prepared according to Gorman et al. (50), and CAT activity was determined with a liquid assay by analyzing the [14C]acetyl-CoA kinetic of diffusion in a nonpolar scintillation mixture. The luciferase activity was expressed as a percentage of the activity of the reporter gene in the absence of any expressor plasmid.

RESULTS

The recognition site probe screening yielded a dozen clones, 10 of which bound with clear specificity to the wild-type but not to the mutated NFE2 probe. Some of these clones have already to T mutation outside of the API consensus sequence that discriminated between NFE2 and API binding (NFM: 5′-TACAGAGCTCT-GAGTCTAGTGAAGCTGCTG-3′) (30).

Methylation Interference Analysis—For the methylation interference assay (48), the end-labeled probes were first methylated with dimethyl sulfate for 3 min at 20 °C and then subjected to a preparative electrophoretic mobility shift analysis. The bound and free bands were excised, and the DNA was electroeluted in agarose gel into DEAE strips (Schleicher & Schuell NA-45), purified by a phenol/chloroform extraction, ethanol-precipitated, dried, resuspended in 100 μl of 10% piperi- dine in water (v/v), and cleaved by whool min at 90 °C. After vacuum evaporation and 2 washes with 20 μl of 70% ethanol, equal counts/min amounts were applied to and resolved on 10% denaturing PAGE.

Glutathione S-Transferase Assay—The hMAF cDNA was subcloned in-frame with the glutathione S-transferase into the pGEX2 vector from Pharmacia, and the binding assay was performed according to In Vitro Transcription and Translation—In vitro transcription and translation reactions were performed with the TnT kit (Promega) using affinity column purified plasmid DNA (Qiagen) and unlabeled or 35S-labeled methionine from Amersham. Amount and quality of the protein products were controlled on denaturing SDS-PAGE. 1 Electrophoretic Mobility Shift Analysis—Electrophoretic mobility shift analyses were carried out as described previously (46). 10 μg of the protein extracts were incubated for 10 min at 4 °C with 10 fmol/50,000 cpm of a 32P-labeled double strand probe in the absence or (when using crude extracts) presence of 3 μg of nonspecific competitor poly(dI-dC) (Pharmacia). Reactions were electrophoresed in 5% acrylamide gels (50/1 cross-linking) in 50 mM Tris borate buffer at 10 V/cm, dried, and autoradiographed overnight at −80 °C. The DNA-binding proteins were produced mostly from rabbit reticulocyte lysates (TnT, Promega) by in vitro transcription and translation reactions from plasmid vectors carrying the following phase promoters: pET5a/MAF (Novagen), pcDNAI/NRF1, pcDNAI/NRF2, and pcDNAI/NFE2 adult (aNFE2) and fetal (fNFE2) isoforms (Invitrogen). K562 nuclear extracts were prepared as described previously (47). The probes used contained either the complete NFE2 tandem repeat of HS2 (5′-GCAACAGAATCT-GCTAGTGAAGCTGCTG-3′), the motif alone (NFE2 probe: 5′-GCAACAGAATCT-GCTAGTGAAGCTGCTG-3′), the right motif alone (API: 5′-GCTAAGTGAAGCTGCTG-3′), and the left motif with a G

1 The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
been reported (37), and here we present the cloning and character-
ization of one of the remaining clones.

cDNA Cloning—The primary screening yielded four overlap-
ping cDNA clones of 300, 550, 600, and 1631 base pairs. As
expected from an expression cloning procedure, all cDNAs con-
tained a functional DNA binding domain composed of at least
four heptads of the leucine zipper motif (51). The longest of the
cDNAs contained a relatively short open reading frame as well
as 5′- and 3′-untranslated regions (Fig. 1). Since the 3′-end of
the clone terminated with a putative poly(A) tail (a stretch of 23
adenines that was 14 nucleotides downstream from a poly(A)
addition signal (TATAAA)), the 3′-untranslated region is likely
to be complete. On the other hand, we found an in-frame stop
codon within the sequences upstream of the initiation codon
that will prevent the upstream extension of the internal open
reading frame, confirming that the upstream sequences are
truly untranslated and that the cDNA clone encodes a full
protein product. This conclusion is further supported by the
fact that the sequences surrounding the first ATG conform well
to the Kozak rule (52) for optimal initiation of translation.

Based on these observations the coding region for hMAF
begins at nucleotide 190 and terminates at nucleotide 676.
Other noteworthy features of the cDNA are the AT richness of
the 3′-untranslated region and the presence of several putative
destabilization signals (ATTTA). The latter feature suggests
that the mRNA may undergo rapid turnover in vivo (53–55).

Predicted Protein Structure—Translation of the open
reading frame embedded in our cDNA predicts a short protein of 162
amino acids and a molecular mass of 17.9 kDa. Since the amino
acid sequence comparison in the protein data base revealed homology with the oncogene v-Maf and especially with the
small Maf proteins, the clone was designated hMAF. Similar to
the other small Maf proteins, hMAF has a classical bZip do-
main that takes up most of the protein structure. The leucine
zipper is comprised of seven heptad repeats with the D position
of the α-helix occupied by five leucines and by two leucine
zipper-compatible highly conserved residues, a methionine in
the middle and a valine in the terminal heptad. Comparison
with the published small Maf protein sequences (Fig. 2) shows
the highest degree of homology (93.2%) with the recently cloned
chicken MafG (cMafG) (56) and the lowest homology with the
chicken MafF (64%) (41). Thus even though our clone is re-
ferred to as hMAF throughout the text and in the figures, it
should be considered the human homologue of cMafG. The
evolution of hMAF and cMafG from the other small Maf pro-
teins appears to be driven by an insertion of 14 amino acids
C-terminal to the leucine zipper and by a premature termina-
tion in the polypeptide chain resulting in the truncation of the
last eight residues.

hMAF Binding in Solution to the NFE2 Repeat Motif—The
full-length and partial clones of hMAF were subcloned into
prokaryotic (pET3a, Novagen) and eukaryotic (pcDNAI, In-
vitrogen) expression vectors flanked by phage RNA polymer-
ase promoters. Proteins were prepared by in vitro transcrip-
tion and translation and assayed in band-shift experiments for
their ability to bind probes derived from the core HS2 enhancer
containing either the full NFE2/AP1 tandem repeat or the
isolated left and right repeats. In a previous paper (5) we
presented evidence that the most 5′ (left) repeat had a more
extended consensus sequence and a higher binding affinity for
NFE2 than the 3′ (right) repeat. Thus we postulated that
in vivo NFE2 might be mainly recognizing the left repeat,
whereas members of the AP1 family were probably binding to
the right repeat. Both the full-length hMAF and a shorter
protein truncated at amino acid position 110 (hMAFΔ) were
able to bind strongly to the NFE2 tandem repeat probe (Fig.
3A, lane 1, band c and Fig. 3C, lane 15, band c), whereas
binding to a single NFE2 site required the complete hMAF
protein (Fig. 3B, lane 3 and Fig. 3C, compare lanes 5 and 9 to
lane 15, where the truncated hMAFΔ produced band c).
As the bZIP proteins bind only after dimerization, these results were taken as evidence that hMAF can form stable homodimers and bind DNA in solution. Overall, the binding and competition experiments with the full tandem repeat and with the isolated left and right motifs indicate that hMAF binds with the highest affinity to the full NFE2 tandem repeat and, when tested individually, to the left NFE2 repeat.

**hMAF Heterodimerizes with Nrf1 and Nrf2—**

Band-shift assays were also performed to assess the ability of hMAF to form heterodimers with the known members of the NFE2/CNC-bZip family. As p45-NFE2, Nrf1, and Nrf2 do not bind the DNA as heterodimers with the known members of the NFE2/CNC-bZip family, assays were also performed to assess the ability of hMAF to form heterodimers in band shifts of Nrf1 and Nrf2 (Fig. 3A, lanes 2, 5, and 7) shows that a second slower mobility complex is indeed formed only when hMAF is mixed and preincubated with Nrf1 and Nrf2 and is never observed when combined with NFE2 (either the fetal (nNFE2) or the adult (hNFE2) splicing isoform) as they never observed when combined with NFE2 (either the fetal (nNFE2) or the adult (hNFE2) splicing isoform). An even clearer result is obtained when the same experiments are repeated using truncated versions of the hMAF and Nrf1 proteins. An NRF1 cDNA spanning nucleotides 2184–3016 (Nrf1Δ) also cloned through recognition site screening and migrating slower than the p18/NFE2 complexes (barely seen in these extracts).

When the hMAF homodimers and hMAF/Nrf1 heterodimers bound to the tandem repeat are assayed for competition, the left (NFE2) and right (AP1) motifs competed efficiently, whereas the mutant left repeat NFM, which carries a T to G mutation in the NFE2 binding site thought to discriminate specifically among transcription factors NFE2 and AP1 (30), was unable to compete (Fig. 3B, lanes 4–6, and 8–10). These results indicate that both hMAF homodimers and hMAF/Nrf1 heterodimers have DNA binding affinities identical to the heterodimer p18-NFE2.

**Glutathione S-Transferase Assays**—We wanted to further confirm the selectivity of the hMAF/Nrf1 and hMAF/Nrf2 interactions with an independent assay based on the glutathione S-transferase fusion analysis. The complete hMAF cDNA fused in-frame with the glutathione S-transferase gene in the vector pGEX2T (Pharmacia) and expressed in bacterial cells was anchored to glutathione-Sepharose beads and assayed for the ability to retain [35S]methionine-labeled Nrf1, Nrf2, NFE2, and hMAF proteins. After stringent washings the only proteins retained in the beads were Nrf1, Nrf2, and hMAF (Fig. 4). Thus the preferential interactions as defined by band-shift assays were confirmed.
truncation variant that allowed homodimer formation. The bands corresponding to the hMAF Δ/Nrf1 Δ heterodimer (Fig. 3C, lane 13 and Fig. 3E, lane 4, band b) and to the hMAF Δ and Nrf1 Δ homodimers (Fig. 3C, lanes 14 and 15 and Fig. 3E, lanes 3 and 4, bands a and c, the latter separated in top and bottom sub-bands) were cut from a preparative gel and subjected to methylation-interference analysis. In the bottom sub-band formed by the Nrf1 Δ homodimer (N/NB) the protection of G residues on the tandem NFE2 repeat probe was more pronounced on the left motif, whereas the top sub-band (N/NT) showed protection on both repeats (Fig. 5B, lanes 2 and 3). The Nrf1 Δ/hMAF Δ heterodimer, on the other hand, produced a unique band with protection restricted to the left motif (Fig. 5B, lane 4), confirming that it preferentially binds to the 5′-NFE2 site of the tandem repeat. The lack of interference in the right motif may indicate that heterodimers binding to the left motif probably sterically hinder the binding of a second complex to the right motif. The hMAF homodimer (M/M) has an interference pattern that closely resembles that of the hMAF/Nrf1 heterodimers (Fig. 5A, lanes 1 and 2), confirming a previous observation in which the small subunit of the p18-NFE2 heterodimer drives binding site specificity (40).

Tissue Expression of hMAF—Northern blot analysis showed a complex pattern of hybridization with multiple bands ranging from reticulocyte lysates. In panel D nuclear extracts from K562 were compared with in vitro translated protein products. In the panels the transcription factors assayed were the products of the in vitro translation reactions from reticulocyte lysates. In panel D nuclear extracts from K562 were compared with in vitro translated protein products. hNFE2 and hNFE2 are the protein products of the two splicing variants of the NFE2 gene. The Δ symbol is used to indicate truncated proteins. All probes were derived from the NFE2/AP1 tandem repeat of HS2, which is schematically indicated on the top of each panel by two linked open rectangles (a broken line rectangle is used to indicate the absence of a single repeat). Unlabeled oligonucleotides used in competition are preceded by opposing arrows (>) and derive from the left repeat (NFE2), the right repeat (AP1), and a mutant left repeat (NFM) carrying a nucleotide substitution that selectively abolishes NFE2 binding. In the lanes marked F the band-shift reactions did not contain any protein extract, whereas in the lanes L or Lys they contained extracts of reticulocyte lysates incubated in the absence of DNA.

Fig. 3. Band-shift analysis. In panels A, B, C, and E the transcription factors assayed were the products of the in vitro translation reactions from reticulocyte lysates. In panel D nuclear extracts from K562 were compared with in vitro translated protein products. hNFE2 and hNFE2 are the protein products of the two splicing variants of the NFE2 gene. The Δ symbol is used to indicate truncated proteins. All probes were derived from the NFE2/AP1 tandem repeat of HS2, which is schematically indicated on the top of each panel by two linked open rectangles (a broken line rectangle is used to indicate the absence of a single repeat). Unlabeled oligonucleotides used in competition are preceded by opposing arrows (>) and derive from the left repeat (NFE2), the right repeat (AP1), and a mutant left repeat (NFM) carrying a nucleotide substitution that selectively abolishes NFE2 binding. In the lanes marked F the band-shift reactions did not contain any protein extract, whereas in the lanes L or Lys they contained extracts of reticulocyte lysates incubated in the absence of DNA.

Fig. 4. Glutathione S-transferase assay. Denaturing SDS-PAGE of the [35S]methionine-labeled proteins specifically and not specifically retained by the beads of glutathione-Sepharose primed with the GST/hMAF fusion or with the GST protein, respectively, is shown. The same amount of labeled protein assayed in the binding reactions was loaded in the input lanes as a size reference marker.
In this paper we have reported the cloning of hMAF, a new member of the small Maf family of bZip transcription factors. hMAF is highly related to the other small Maf proteins and is the human homologue of the cMAFG gene recently cloned from chicken. Although similar, hMAF shows obvious differences when compared with p18-Maf, the specific partner of p45-NFE2. Structurally, the two proteins are very similar especially in the DNA binding domain and in the leucine zipper. But beyond the leucine zipper, the carboxyl terminus of hMAF differs from p18 by the presence of a 14-amino acid insertion after the leucine zipper and a premature termination at the carboxyl end. The latter differences are not responsible for the differential partnership as preferential association with Nrf1 is maintained in hMAFA, which lacks the carboxyl terminus of the protein starting from the last leucine heptad. Functionally the two proteins differ in the partner they associate with, as p18 prefers NFE2, whereas hMAF interacts only with Nrf1 and Nrf2 and not with NFE2. This difference occurs despite a very similar leucine zipper domain in which the most dramatic amino acid variation (Arg→Ser) occurs in the predicted coiled coil at residue 95 in the Phe position, which is opposite to the surface of dimerization and hence does not participate in the dimer interaction. The other few amino acid substitutions that do occur are conservative substitutions. Thus subtle changes in the polarity of the substituting amino acid must account for the observed shift in the preferential partnership. Another feature that differentiates hMAF from p18 is the ability to form homodimers easily. In fact, p18 did not bind or barely bound the NFE2 recognition site (GTCTGAGTCAGCA) whereas hMAF readily formed homodimers both in the absence of DNA binding, as shown in the GST fusion assay (Fig. 4) and in the presence of DNA binding as shown in the band-shift assays with the tandem repeat of HS2. The propensity of hMAF to form homodimers suggests a physiological role independent of the association with members of the CNC-bZip family. Even though hMAF is able to bind as a homodimer, judging from the relative lower intensity of the homodimer compared with the heterodimer bands in band-shift assays in which the two partner proteins were allowed to interact in equimolar amounts, the homodimer is either less stable or has lower affinity for the
DNA sites than the heterodimers hMAF/Nrf1 and hMAF/Nrf2 (compare Fig. 3A, lane 7, band c with band a and Fig. 3C, lane 13 or Fig. 3E, lane 4, band c with band b). Such an increase in the DNA binding affinity following the shift from homo- to heterodimers has recently been observed for the interaction between the chicken MafG and TCF11, a splicing isoform of Nrf1 (60). We have also shown that shifting from the homodimer to the heterodimer concomitantly modifies the specificity for subsets of NFE2 binding sites, promoting higher affinity for the sequence that most perfectly matches the consensus for NFE2 (Fig. 3C). If we now consider that hMAF does not have an activation domain, only heterodimer assembling over an NFE2 binding site will be able to activate transcription, whereas homodimer assembly on the same site will probably be functionally inert or detrimental. In addition, homodimerization in the absence of DNA binding may sequester hMAF away from dimerization with Nrf1 and Nrf2. Thus heterodimerization or homodimerization has the potential to produce functional changes that allow enhancement or repression of gene expression.

Both the homodimer hMAF and the heterodimer hMAF/Nrf1 mimic the heterodimer p45-NFE2 in their ability to bind NFE2 recognition sites, which can be competed by cold NFE2 and AP1 but not by NPM oligonucleotides. Thus the latter oligonucleotide should no longer be considered discriminatory and specific for NFE2 binding.

In band-shift assays with the NFE2 repeat probe the complexes formed with K562 crude nuclear extracts (Fig. 3D) show the same electrophoretic mobility as the complexes formed with reticulocyte lysate translates of hMAF and Nrf1. As we have previously shown in K562 expression studies that mutants of the NFE2 repeat, which impair formation of these complexes, dramatically reduce the activity of the HS2 enhancer (5), the heterodimer hMAF/Nrf1 may participate in the enhancer function of HS2.

Previous expression studies in NIH3T3 and QT6 cells had shown that the small Maf proteins can form heterodimers with p45-NFE2 and activate or suppress a reporter gene in dependence of their relative equilibrium concentrations (44). In our transfection studies we wanted to confirm these results and to analyze if they hold true for the new p45-NFE2 related members Nrf1 and Nrf2 in a more physiological context such as the one offered by the human erythroid cell line K562. We used plasmids expressing either hMAF, Nrf1, Nrf2, and p45-NFE2 individually or hMAF in increasing molar ratios (from 0.1 to 2) against each of the NFE2 family members. In all of the transfection experiments we observed an apparent small increase in the reporter activity at lower hMAF ratios followed by a substantial inhibition at the higher ratios. The lack of a clear activation from all members of the NFE2 family in the absence or at lower ratios of hMAF is probably dependent on the expression system we have used. The erythroid cell line K562 may contain endogenous activators in near-saturation amounts, and the 350-base pair γ-globin promoter may be less prone to be further stimulated than the minimal TATA β-globin promoters used in the other studies. The observation of similar pattern of suppression by hMAF on p45-NFE2, in the absence of any demonstrable heterodimerization between hMAF and p45-NFE2, can be due to the displacement of the endogenous complexes p45-NFE2 from their NFE2 binding sites in the HS2 enhancer by the hMAF homodimers. Alternatively erythroid specific post-translational modifications of NFE2 may allow NFE2-hMAF interactions.

Although the expression studies support a role for hMAF in the regulation of the globin genes, other observations such as the absence of hMAF mRNA variation in response to hemin induction in K562 cells or the comparable levels of expression in the fetal liver and in the bone marrow (data not shown), do not support a role of hMAF in the regulation or in the switching of the globin genes. The strong suppression on the activity of the γ-globin promoter may also imply that in another context and with different genes hMAF may display an oncogenic potential with the same final result although with opposite mechanism to the one observed with the larger v-MAF gene.

More complex genetics studies such as the hMAF gene knockout in mice will be required to define hMAF roles in the mouse erythroid cells and in other tissues that express the gene. In this respect the lack of any phenotype in the recently published inactivation of the p18 gene in mice (61) may suggest that other small Maf proteins may compensate for that gene defect and that multiple gene knockouts may be necessary to produce a defective phenotype.

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