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The *maf* Proto-oncogene Stimulates Transcription from Multiple Sites in a Promoter That Directs Purkinje Neuron-Specific Gene Expression

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***L7* is expressed in all adult cerebellar Purkinje cells, although during development it appears in a stereotyped spatial and temporal pattern that is manifested as parasagittal domains of neurons. Mutations of the *L7* promoter in transgenic mice have established that these domains represent functional compartments of Purkinje neurons. Therefore, it is hoped that by defining the transcriptional control of the *L7* gene insights into the mechanisms that control functional fate and organization in the nervous system can be gained. Fragments of the *L7* promoter were introduced into a selectable reporter gene in *Saccharomyces cerevisiae*, and these strains were used to select for cerebellar cDNAs encoding proteins that can bind to, and activate transcription from, these elements. This assay identified the c-Maf proto-oncogene as activating transcription from two sites in the *L7* promoter. We did a functional domain analysis of vertebrate c-Maf based upon transcriptional activation in *S. cerevisiae* and showed the requirement for a transactivation domain, leucine zipper, and DNA-binding region in c-Maf. The c-Maf interaction site was mapped to the sequence G/TGG/CNG/TNCT CAGNN in the *L7* promoter, which represents an atypical 12-*O*-tetradecanoate-13-acetate-responsive element-type Maf-responsive element. However, neither Fos nor Jun, either alone or in combination with each other or c-Maf, altered transcription from this element. In contrast, a Maf-related protein, Nrl, completely mimicked c-Maf actions. These data suggest that Maf may interact with additional basic-zipper proteins that determine a subtype of Maf-responsive element binding.**

A fundamental issue in developmental neurobiology is to identify the molecular and cellular mechanisms that orchestrate the genesis of functional networks of neurons from relatively homogeneous populations of precursors. One approach to this problem is to investigate the regulation of genes that define functional compartments in the nervous system. The *L7* gene is expressed specifically in cerebellar Purkinje cells and retinal rod bipolar neurons (2, 17). Within the cerebellum, *L7* is expressed in a dynamic temporal and spatial pattern that is manifested as parasagittal bands, or domains, of Purkinje cells (20). At least some of these bands represent functional compartments of Purkinje cells that project to specific cerebellar nuclei (16). Therefore, it is hoped that by identifying the transcription factors involved in conveying this spatial representation to *L7* expression some of the principles that underlie the development of functional organization in the nervous system in general can be elucidated.

Previously it was shown that truncations and point mutations of the *L7* promoter in the context of *L7-lacZ* fusion genes led to perturbations of the cerebellar banding pattern in transgenic mice (16). This established that the *L7* promoter is sensitive to spatial cues and must contain some representation of the banding map. While some promoter mutations pointed to particular classes of transcription factors as playing a role in this process, many of the DNA elements identified in the analysis did not conform to any known consensus binding sequence. Therefore, we wished to devise a strategy that would permit us to clone transcription factors from the cerebellum by using a functional assay that required prior knowledge of neither the general structure of the proteins involved nor their precise DNA-binding sites. From the practical standpoint, the screen also needed to accommodate relatively large DNA fragments to permit analysis of a significant fraction of the *L7* promoter. In addition, it was reasoned that if a single copy of the *L7*-promoter-reporter system was stably integrated into the host cell genome, it would be organized into chromatin, thereby providing an environment that might favor relevant protein-DNA interactions over less specific ones.

To isolate *L7* promoter-binding proteins, we used a one-hybrid yeast expression screening paradigm that is similar to the two-hybrid system (4). In this system, reporter yeast strains have iso-1-cytochrome *c* (*cyc1*) promoter-*lacZ* gene fusion constructs integrated into the genome. The *cyc1* promoter contains an upstream activating sequence (UAS), and binding of transcriptional activators to this site enhances gene expression about 50-fold (6). Replacement of the UAS with *L7* promoter fragments makes it possible to select for binding proteins by measuring the activation of the *cyc1-lacZ* gene after transformation with a library of cDNA-transcriptional activator hybrids; this library is composed of cerebellar cDNAs that have the transactivation domain of herpes simplex virus protein VP16 fused to their 5′ ends (Fig. 1). Since this method selects for DNA binding by a functional assay in a living cell, it also permits structure-function analysis of putative transcription factors. In principle, a similar approach has been used by others to clone Olf-1, a transcriptional activator that binds to the regulatory sequences of several olfactory cell-specific genes (22).

Here we report the isolation and functional characterization of a basic-zipper superfamily member, c-Maf, that transactivates reporter gene expression by an interaction that requires nucleotides −200 to −210 and a second sequence lying between nucleotides −209 and −318 of the *L7* promoter. This transactivation capacity is not shared by two other basic-zipper superfamily members, c-Fos and c-Jun, either singly or in com-

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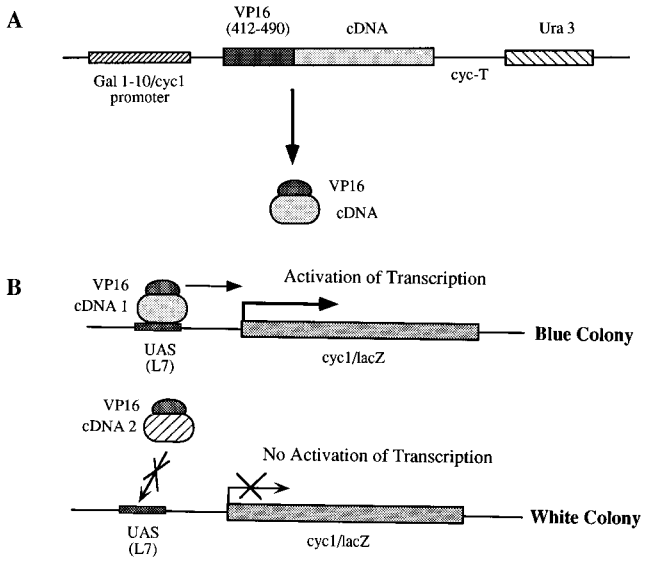

FIG. 1. Screening for *L7* promoter-binding proteins. (A) A mouse cerebellum cDNA library was constructed in which the cDNA was inserted downstream of codons 412 to 490 of herpes simplex virus transcriptional activator protein VP16. The library vector is a yeast expression plasmid (pSD.10a; reference 3) with the *URA3* gene for positive selection of yeast transformants. Expression of the VP16-cDNA fusion construct is under the control of a *gal1-10–cyc1* hybrid promoter, making it galactose inducible. The *cyc1* terminator region (cyc-T) is downstream of the VP16-cDNA fusion. (B) After transformation of yeast strains that contain *L7* promoter fragments in the UAS locus of the *cyc1-lacZ* reporter gene with the VP16-cDNA fusion library, reporter gene expression is activated, if the protein encoded by the cDNA binds to the *L7* promoter sequence. Colonies of the corresponding yeast transformants turn blue in a colony color β-galactosidase activity assay. In contrast, *S. cerevisiae* transformed with plasmids that do not encode *L7* promoter-binding proteins forms colonies that are white.

bination. This study established that this screening method is capable of identifying relevant transcription factors without prior knowledge of the class of protein involved. Furthermore, it revealed an unexpected degree of binding specificity among basic-zipper superfamily members for their target sequence that would not have been predicted from transient transfection or gel shift analyses.

MATERIALS AND METHODS

Construction of mouse cerebellum cDNA libraries. Total RNA was prepared from 29 cerebella of adult female BALB/c mice (1.5 g of tissue) with RNAzol B (Biotex Laboratories, Inc.) in accordance with the supplier's recommendations. cDNA synthesis was carried out as previously described (7), with a total of 12 μg of polyadenylated RNA and oligo(dT), as well as random hexamer primers. Half of the resulting cDNA was ligated to *Bst*XI linkers and inserted into the backbone of plasmid pSD.10a, a shuttle vector containing the *URA3*$^+$ yeast selection marker and herpes simplex virus VP16 codons 412 to 490 5′ of the *Bst*XI cloning site (3). The ligated DNA was transformed into *Escherichia coli* ElectroMAX DH10 cells (Gibco BRL) by electroporation with a Bio-Rad electroporator, and plasmid DNA was prepared from transformants as previously described (3). The final plasmid library contained 5.3×10^6 clones. The other half of the cDNA was ligated to *Eco*RI adaptors and inserted into phage lambda ZAP II (Stratagene). The final phage library comprised 3.75×10^6 PFU.

Yeast growth, transformation, and plasmid DNA preparation. Growth and maintainence of *Saccharomyces cerevisiae* were done as described previously (8).

Yeast transformations were performed by the lithium acetate method of Ito et al. (11) with the following modifications. For transformations with single plasmids, exponentially growing cells were collected by centrifugation, washed once in water and once in TEL (10 mM Tris-HCl [pH 8.0], 1 mM EDTA-NaOH, 100 mM lithium acetate dihydrate), resuspended in TEL at 10^9/ml, and incubated for 30 min at 30°C. A 100-μl volume of of cells was combined with 1 μg of plasmid DNA in 50 μl of TE in a microtube and incubated for 30 min at 30°C. A 700-μl volume of 40% polyethylene glycol 4000 in TEL was added, and incubation was continued for 60 min. After a 42°C heat shock for 5 min, cells were set on ice for 5 to 30 min and then centrifuged for 10 s in a microcentrifuge, washed twice in water, resuspended in water, and plated on selective plates. For transformations with the cDNA plasmid library, 10^{10} cells were transformed with 150 μg of library DNA and 1 mg of calf thymus DNA as a carrier. Reaction volumes were scaled up accordingly.

Yeast plasmid DNA was prepared as previously described (9) and recovered by transformation of *E. coli* ElectroMAX DH10 cells (Gibco BRL) by electroporation.

Yeast reporter strains. Construction of yeast reporter strains was performed as described by Dalton and Treisman (3). Briefly, *L7* promoter fragments or derivatives were inserted into the *Xho*I site of plasmid pLGΔ-178, about 200 bp 5′ to the TATA box of the *cyc1-lacZ* gene, which lacks a UAS. *L7-cyc1-lacZ* reporter genes were then embedded in the *S. cerevisiae URA3* gene, disrupting the *URA3* coding region. This construct was transformed into *S. cerevisiae* S50, and transformants were selected for integration of the reporter gene into the *URA3* locus by homologous recombination, which makes the yeast Trp$^-$ Ura$^-$. Yeast reporter strains CK17 and CK19, containing one and three AP-1 sites, respectively, in the *cyc1-lacZ* UAS locus, were generated by using the oligonucleotide CGTGACTCATCGC, which includes a 12-*O*-tetradecanoate-13-acetate-responsive element (TRE), or a three-times-reiterated version of it.

Screening of the plasmid library in *S. cerevisiae*. Reporter yeast strains were transformed with the library and plated on Amersham Hybond-N filters laid on Ura$^-$ selective plates (24.5 by 24.5 cm) containing 2% glucose as a carbon source at a density of 1×10^4 to 2×10^4 colonies per plate. After incubation at 30°C for 44 to 68 h, filters were transferred to plates containing 2% galactose instead of glucose and incubated for 18 h to induce expression of the VP16-cDNA fusion proteins encoded by the library plasmids. Expression of the reporter gene was detected by a qualitative colony color β-galactosidase activity assay carried out as described by Dalton and Treisman (3). Positive (blue) colonies were streaked on Ura$^-$ glucose plates and grown at 30°C for 2 days to obtain single colonies. The colonies were replicated onto Hybond-N filters, grown on galactose-containing medium for 1 day, and retested for β-galactosidase activity. Positive colonies were streaked on fresh Ura$^-$ glucose plates, and their plasmids were retrieved by transformation of *E. coli* with total yeast DNA.

Screening of the phage library. As a probe for screening in the lambda ZAP II cDNA library, a 490-bp PCR fragment, amplified from the 3′ end of the pNo8 cDNA insert, was used. The first screening was performed precisely as recommended by the lambda ZAP II supplier (Stratagene). Instead of a second screening in lambda, an *E. coli* colony screening was done with bacterial colonies obtained after in vivo excision (with the Stratagene ExAssist/SOLR system) of plasmid pBluescript SK(−) (with the inserted cDNA) from the first-screening lambda plaques. Bacterial colonies were grown on 10-cm-diameter Luria broth-ampicillin (50 μg/ml) plates at a density of 500 to 1,000 single colonies per plate and then lifted onto Colony/Plaque Screen Hybridization Transfer Membranes (Biotechnology Systems NEN Research Products, Du Pont). Filters were soaked in 2 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5% sodium dodecyl sulfate (SDS) for 2 min, subsequently baked in a microwave oven for 3 min, and then dipped into hot (60 to 65°C) hybridization solution (1% crystalline grade bovine serum albumin, 1 mM EDTA, 0.25 M sodium phosphate buffer [pH 7.2], 7% SDS). They were transferred to hybridization tubes containing hybridization solution (65°C) and the probe and hybridized for 1 to 2 h at 65°C. Washes were carried out once in 2× SSC–1% SDS for 10 min at room temperature and once in 0.1× SSC–0.1% SDS for 20 min at 65°C. Times of exposure to Kodak X-Omat AR autoradiography films were according to counts per minute, typically 30 min. Bacterial colonies on the original plates that could be aligned with dark spots on the autoradiograph were streaked on fresh plates.

Truncations and mutations of c-Maf; c-Fos and c-Jun constructs. Truncated c-Maf protein was generated by PCR amplification of the corresponding DNA fragments from the cDNA. The PCR primers were chosen in such a way that they added *Eco*RI and *Bam*HI restriction sites to the 5′ ends of the PCR products and a stop codon and an *Spe*I restriction site to the 3′ ends. To create mutant c-Maf, point mutations were introduced into the amplified cDNA by using mismatched primers in the PCR. The resulting DNA sequences were inserted into plasmid pSD.10a (3), previously cut with *Eco*RI and *Spe*I (for VP16 fusion constructs) or *Bam*HI and *Spe*I (for constructs without the VP16 domain). The same procedure was used to clone full-length rat c-Fos and mouse c-Jun cDNAs into pSD.10a.

For use in simultaneous transformations of *S. cerevisiae* with c-Maf or c-Fos and other proteins, c-*maf* and c-*fos* cDNAs, respectively, were inserted as a *Bam*HI-*Spe*I fragment into plasmid pSD.04a, a shuttle vector containing the *TRP*$^+$ yeast selection marker (3).

Other methods. Standard DNA manipulations and preparations were performed as described by Sambrook et al. (18). DNA sequencing was performed on an Applied Biosystems 373A DNA Sequencer. To resolve sequences with high G+C content, 5 to 10% dimethyl sulfoxide was included in the sequencing reaction. Analysis of the sequencing data was performed with the GCG Sequence Analysis Software Package (version 7, April 1991) of the Genetics Computer Group, Madison, Wis.

RESULTS

Studies with an *L7-lacZ* transgene have established that as little as 250 bp of the *L7* promoter flanking the transcriptional start site at its 5′ end are sufficient to direct specific *L7* expres-

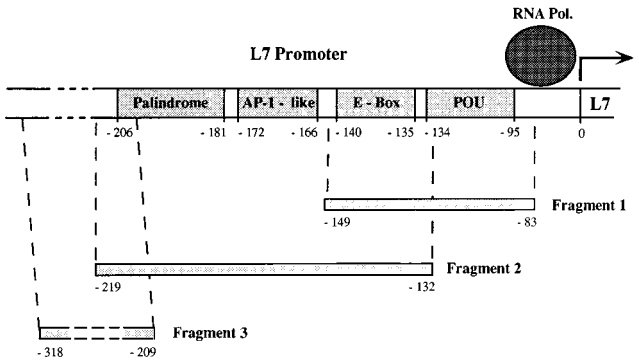

FIG. 2. Organization of the *L7* promoter; *L7* promoter fragments chosen for screening. The *L7* promoter contains at least four motifs that are potential binding sites for transcription factors: an AT-rich region with three POU-binding domains, an E box, an AP-1-like site, and a long, imperfect palindrome. Three fragments of this *L7* promoter region were chosen for initial screening experiments. Numbers indicate the positions of motifs and fragments in base pairs relative to the transcriptional start site of *L7*. Pol., polymerase.

sion to Purkinje cells (16). This region of the gene contains at least four motifs that are protected in DNase I footprinting analyses and may serve as target sites for proteins involved in the regulation of *L7* transcription (Fig. 2) (16): First, between nucleotides −95 and −134 (relative to the transcriptional start site) lies an AT-rich region that includes three potential POU-binding domains (TAATT). Point mutations in the POU region result in marked alterations in the spatial pattern of *L7-lacZ* transgene expression (16). Directly adjacent to this region is an E box (CACCTG). The third motif is an AP-1-like site located between nucleotides −166 and −172 (CTGACTC) that may interact with proteins of the basic-zipper superfamily, such as c-Fos and c-Jun. The fourth motif is a large region containing a long, although imperfect, inverted repeat between nucleotides −181 and −206. Further circumstantial evidence pointed to this region as being important, since a similar inverted repeat was noted in approximately the same position of the gene (p*RIMB19*) encoding PEP-19, which is also expressed in cerebellar Purkinje cells (19). Therefore, we have established a method in which a fragment of the *L7* promoter containing three of these motifs was used to isolate transcription factors encoded in a cerebellar cDNA library.

Construction of *L7-cyc1-lacZ* reporter genes and yeast strains. For the initial screening, a fragment of the *L7* 5′-flanking sequence (fragment 2) was integrated into the promoter of the *cyc1-lacZ* hybrid gene, where it replaced the *cyc1* UAS. This fragment spanned nucleotides −219 to −132 of the *L7* promoter and encompassed the E box, the AP-1-like site, and the inverted repeat. To establish specificity and detect potential multiple binding sites for the same factor, two additional fragments that spanned nucleotides −149 to −83 (fragment 1) and −318 to −209 (fragment 3), respectively, of the *L7* promoter were integrated into the UAS. Fragment 1 contained three POU-binding sites and the E box, while fragment 3 was included to complete the coverage of the 250-bp promoter region that directs specific *L7* expression to the cerebellum. The yeast strains generated by integration of these constructs are referred to as CK1a, CK2, and CK4, respectively (Table 1). Subsequently, we constructed further yeast strains that harbored subregions or mutations of fragment 2 and were designated CK5a to CK10 and CK13a to CK30. In addition, as controls for binding of c-Fos and c-Jun, we developed yeast strains CK17 and CK19, which contain one and three AP-1 sites, respectively (Table 1).

TABLE 1. Yeast strains used in this study[a]

Yeast strain	UAS	*L7* promoter region
CK1a	L7 nucleotides −149 to −83	Fragment 1
CK2	L7 nucleotides −219 to −132	Fragment 2
CK4	L7 nucleotides −318 to −209	Fragment 3
CK5a	L7 nucleotides −219 to −198	Fragment 2 subfragments
CK6	L7 nucleotides −197 to −176	Fragment 2 subfragments
CK7	L7 nucleotides −175 to −154	Fragment 2 subfragments
CK8	L7 nucleotides −153 to −132	Fragment 2 subfragments
CK10	L7 nucleotides −144 to −132	Fragment 2 subfragments
CK13a–CK16	Mutations of CK2 or CK5 UAS	Fragment 2 mutations
CK21a–CK30	Mutations of CK2 or CK5a UAS	Fragment 2 mutations
CK17	1 AP-1 site	
CK19	3 AP-1 sites	

[a] Several yeast strains that contain a *cyc1-lacZ* reporter gene were generated. Yeast strains CK1a to CK4 were constructed for initial screening experiments; they carry *L7* promoter fragments 1 to 3 as a UAS in the reporter gene. Strains CK5a to CK16 and CK21a to CK31 harbor truncated or mutated versions of *L7* promoter fragment 2; they were made for delineation of the c-Maf binding site in the *L7* promoter. CK17 and CK19 contain one and three canonical AP-1 sites (TRE), respectively; these strains served as controls for binding of c-Fos and c-Jun.

Screening for *L7* promoter-binding proteins. A mouse cerebellum cDNA library was constructed in which the cDNA was inserted downstream of codons 412 to 490 of herpes simplex virus protein VP16, thereby fusing the proteins encoded by the cDNA to the transcriptional activator domain of VP16. Strain CK2 was transformed with the cDNA library, and about 10^6 transformants from two separate screenings were subjected to a colony color β-galactosidase activity test. Only one colony went dark blue by 60 min of incubation, and its plasmid was purified and subjected to further analysis. Upon retransformation with this clone, yeast strain CK2 was still positive for β-galactosidase while strains CK1a and S144 (a yeast strain without a UAS in the *cyc1-lacZ* gene) were negative.

The approximately 900-bp insert of the clone was sequenced and found to be homologous to v-*maf*, a retroviral oncogene cloned from chicken cells transfected with avian musculoaponeurotic fibrosarcoma virus strain AS42 (15). To obtain a full-length cDNA clone of murine c-*maf*, a mouse cerebellum cDNA library in phage lambda was screened with a 490-bp PCR product derived from the 3′ end of the insert. We isolated two independent clones, Φ2-1 and Φ3-1, that had cDNA inserts of about 2,700 and 2,300 bp, respectively.

Sequence analysis of c-*maf*. DNA sequencing revealed that both Φ2-1 and Φ3-1 contained cDNA inserts that included the complete coding region for c-*maf*. Figure 3A shows the sequence of the Φ2-1 cDNA insert. All three c-*maf* cDNA clones have the same nucleic acid sequence in regions of overlap. The fact that the Φ2-1 and Φ3-1 sequences include stop codons in all three reading frames between bp −931 and −1 led us to conclude that the ATG in positions 1 to 3 is the start codon of c-*maf*, which is in agreement with the data published for v-Maf by Nishizawa and colleagues (15).

The N-terminal amino acid sequences of v-Maf and c-Maf are highly conserved (87% identical) over a stretch of 187 residues (Fig. 3B). Likewise, the C-terminal 120 amino acids are highly related, except that the viral protein is extended by 10 amino acids. The sequence between amino acids 249 and 370 of c-Maf contains the basic region (amino acids 284 to 312)

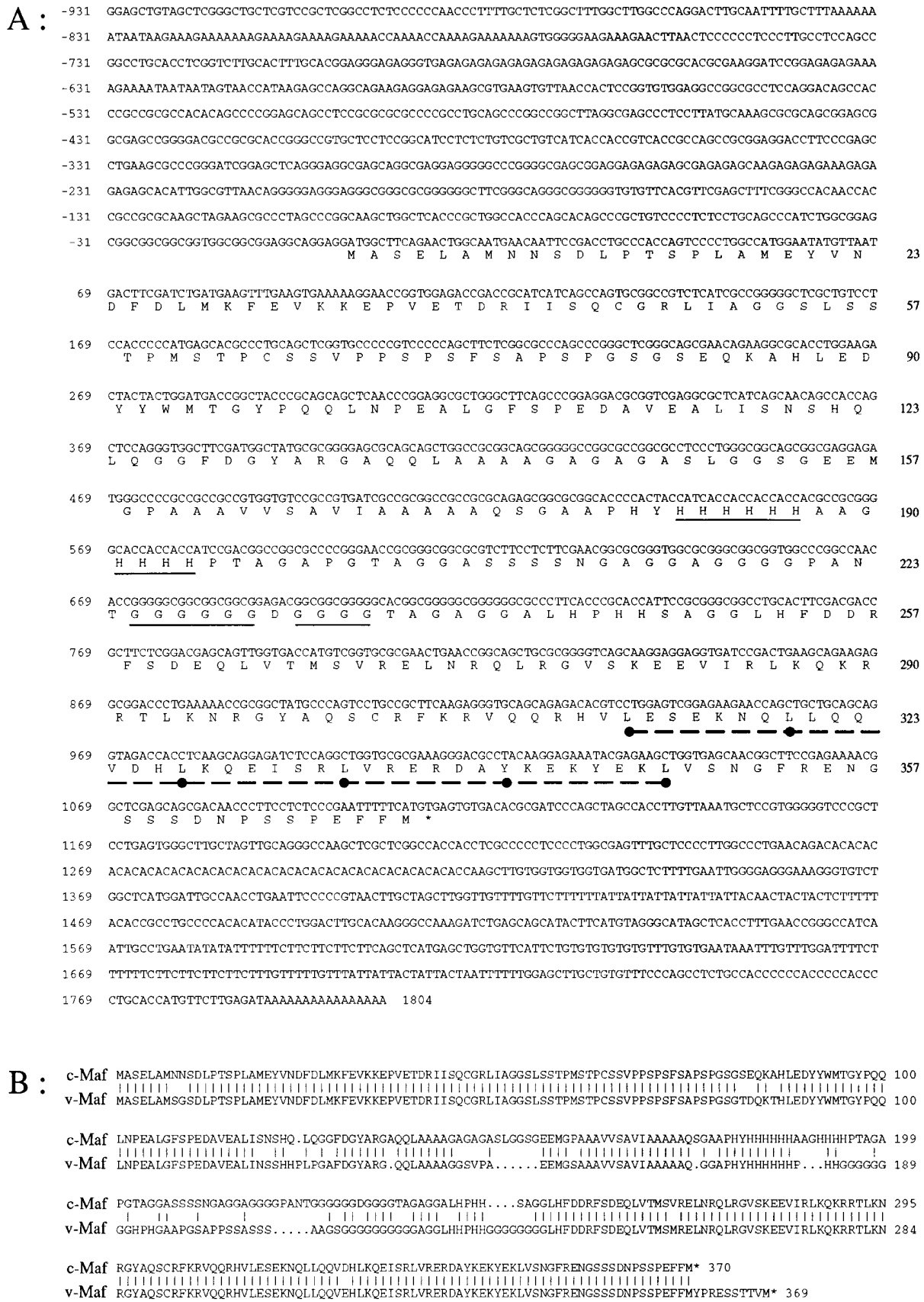

FIG. 3. Nucleic acid and amino acid sequences of c-Maf (Φ2-1 cDNA). (A) Numbers on the left indicate the positions of nucleotides relative to the translational start site, while numbers on the right refer to amino acids. Polyhistidine and polyglycine sequences in the protein are underlined. The leucine zipper motif is marked by a dashed line, and the amino acids forming the leucine zipper structure are labeled with dots. (B) Comparison of c-Maf and v-Maf amino acid sequences. Vertical lines show identity of amino acids. Dots indicate gaps introduced to achieve optimal alignment. Numbers on the right refer to amino acids.

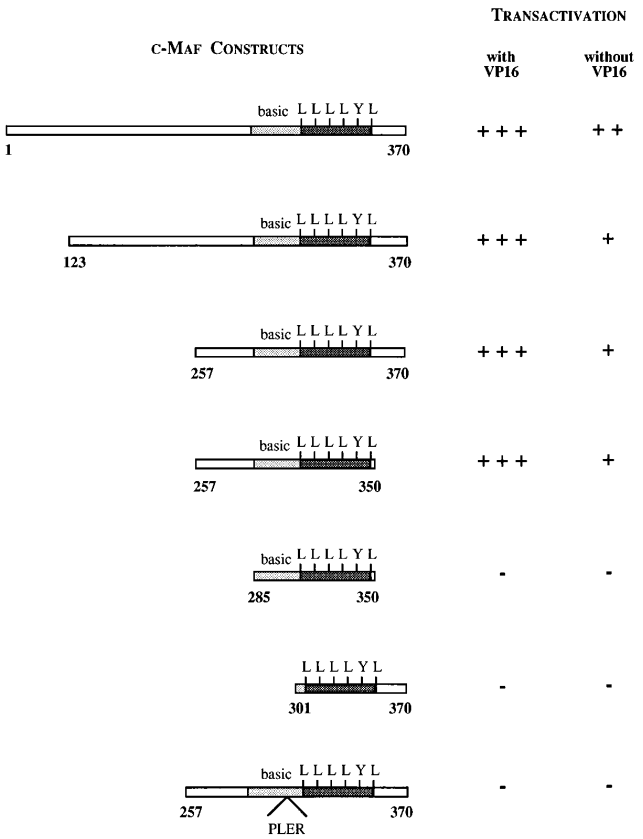

FIG. 4. Truncation analysis of c-Maf. Truncated forms of c-Maf, as well as a form of c-Maf in which the basic region was disrupted by the insertion of four amino acids (PLER), were tested for transactivational activity in strain CK2. All constructs were made both with and without N-terminal fusion of the VP16 transactivation domain. Activation of the reporter gene was tested in the colony color β-galactosidase activity assay. +++, blue colonies after 20 min of incubation; ++, blue colonies after 45 min; +, blue colonies after 60 min; −, no blue colonies. The basic region of c-Maf is indicated by light shading, while the leucine zipper motif is shown in dark shading. Letters above the darkly shaded region indicate the amino acids in the heptad repeat position of the zipper. Numbers refer to the first and last c-Maf amino acids included in the respective constructs.

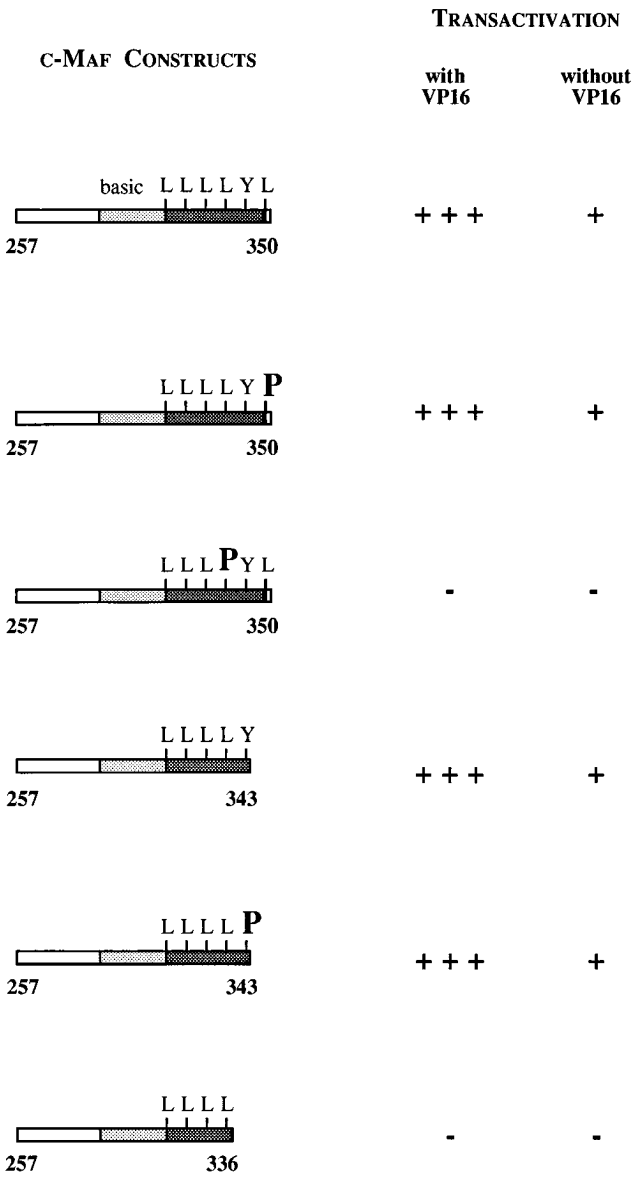

FIG. 5. Mutation analysis of the c-Maf leucine zipper motif. c-Maf constructs (with and without an N-terminal VP16 fusion) with a mutated or truncated leucine zipper motif were introduced into strain CK2. Transformants were tested in the colony color β-galactosidase activity assay for transactivation of the *L7-cyc1-lacZ* reporter gene. +++, blue colonies after 20 min of incubation; +, blue colonies after 60 min; −, no blue colonies. Shading and numbering are as in Fig. 4. P, amino acid substitution with proline.

and the leucine zipper motif (amino acids 313 to 348) and is 98% identical to the v-Maf sequence. The leucine zippers of both proteins are configured similarly (and somewhat atypically for the basic-zipper superfamily) in that five of the six residues in the heptad repeat of hydrophobic amino acids forming this protein dimerization motif are leucines, while the fifth position is a tyrosine. The central regions of the proteins are very divergent and show almost no homology to one another. While there are short polyhistidine and polyglycine repeats in the centers of c-Maf and v-Maf, only the most N-terminal polyhistidine sequence is homologous in the two proteins.

Truncation and mutation analyses of c-Maf. To determine which regions of c-Maf are necessary for activation of *L7-cyc1-lacZ* gene transcription in CK2, several truncated and mutated derivatives were constructed. Since longer c-Maf constructs had considerable transcriptional activity even when no VP16 sequences were present, all c-Maf variants were made both with and without the VP16 transactivator domain. Figures 4 and 5 depict the various constructs.

Deletion of the amino acids lying between the end of the leucine zipper and the C terminus of the protein did not interfere with c-Maf-dependent transcription, irrespective of whether VP16 sequences were present or not. On the other hand, while deletion of the first 256 amino acids from the N terminus of c-Maf had no significant effect on the activity of the Maf-VP16 fusion protein, it markedly reduced the intrinsic activity of c-Maf. In fact, removal of the N-terminal 122 amino acids of c-Maf attenuated its transcriptional activity, indicating that this region must contribute to activation.

An intact basic region was required for activity, since deletion of this region or insertion of four amino acids into this motif (PLER between amino acids 306 and 307) abolished activity. Besides the basic region, 28 amino acids preceding it (amino acids 256 to 284) were also essential, presumably because they too were involved in DNA binding (rather than

transactivation). This was confirmed by the observation that the construct comprising c-Maf amino acids 285 to 350 (basic-zipper motif lacking the 28 amino acids) was unable to induce reporter gene expression even when fused to the VP16 domain. I.e., replacement of the 28 amino acids with another transcriptional activator domain did not restore function.

Given the atypical configuration of the leucine zipper in c-Maf, a number of mutations were tested to determine how much of the domain is essential for activity. Deletion of the last two (the fourth and fifth) zipper repeats completely abolished the biological activity of c-Maf and the c-Maf–VP16 fusion protein. However, conversion of the last leucine of the heptad repeat structure to a proline had no effect upon Maf's transactivation potential, indicating that the final zipper repeat might not be essential in this type of assay. This was confirmed by the finding that complete elimination of the fifth repeat had no effect on activity. In addition, the same deletion when combined with a conversion of the tyrosine at the fifth position of the zipper repeat to a proline also had no significant effect on Maf activity. In contrast, conversion of leucine 4 to a proline destroyed transactivational activity, indicating that this part of the zipper was essential. From these data, we conclude that the last (fifth) zipper repeat and the penultimate position in the heptad repeat of hydrophobic amino acids are not essential in this yeast transactivation assay.

Identification of the c-Maf target site in the *L7* promoter. To delineate the target DNA site for c-Maf in the *L7* promoter, we generated four new yeast stains, CK5a to CK8, in which the UAS of the *L7-cyc1-lacZ* reporter genes consisted of subregions of fragment 2 (Table 1). After transformation with the plasmids encoding full-length (amino acids 1 to 370) or truncated (amino acids 123 to 370 or 257 to 370) c-Maf, only CK5a was positive in the colony color assay (Fig. 6). From this result, it was concluded that the target site of the protein lies between nucleotides −219 and −198 of the *L7* gene. Subsequently, several point mutations of this region were made and tested in the context of both the long (nucleotides −219 to −132; as in CK2) and short (nucleotides −219 to −198; as in CK5a) *L7* fragments. It was found that the long and short fragments containing the same mutation behaved the same (Fig. 6). This excluded the possibility that it was simply the spacing between the transcriptional start site of the *lacZ* gene and the *L7* sequence that determined the activity of c-Maf.

None of the single or double point mutations in the putative c-Maf target sequence completely blocked transactivation. However, reduced transcriptional activity was evident in some of the new yeast strains, thereby permitting further delineation of the c-Maf binding site (Fig. 6). Almost all point mutations within the sequence GAGGCTCAGAC (nucleotides −210 to −200) resulted in reduced transcriptional activity, whereas mutations outside of this domain had no effect, suggesting that this region is necessary for Maf action. Recently, a v-Maf DNA-binding consensus sequence was published (13) and within the region conferring Maf sensitivity (bases −200 to −210 of the *L7* promoter) there is some similarity to the Maf site (Fig. 6). Therefore, the Maf binding site in the CK2 fragment appeared to be encompassed by the sequence GAGG CTCAGAC. One final yeast strain, CK10, that contained only the putative Maf site in its UAS was constructed (Fig. 6). This construct was also activated by c-Maf, showing that the active sequence was contained in the element. Furthermore, the most 5′ G residue that was common to the canonical Maf site and the CK2 site (Fig. 6) was eliminated in the CK10 construct, indicating that it was not essential in this assay.

To determine whether there were additional Maf sites within the *L7* promoter, fragments 1 and 3 were examined for

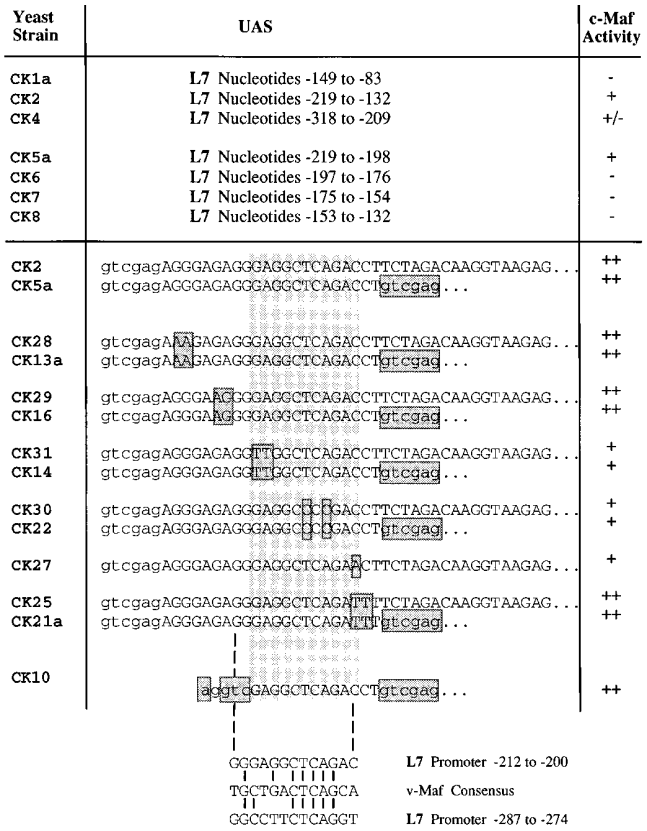

FIG. 6. Identification of the c-Maf target site in the *L7* promoter. *L7* promoter fragments 1, 2, and 3, as well as four subfragments of fragment 2, were inserted into the UAS locus of the *cyc1-lacZ* reporter gene and integrated into the yeast genome, generating yeast strains CK1a, CK2, CK4, and CK5a to CK8. Furthermore, mutated versions of the *L7* promoter fragments present in yeast strains CK2 and CK5a (nucleotides −219 to −132 and −219 to −198, respectively) were made and used to generate strains CK10 to CK30. Subsequently, the yeasts were transformed with a plasmid encoding c-Maf amino acids 123 to 370 (yeast strains CK2 and CK5a to CK8; upper section) or full-length c-Maf (yeast strains CK2, CK5a, and CK10 to CK30; lower section). Transformants were tested for c-Maf-dependent reporter gene activity in the colony color assay. ++, blue colonies after 45 min of incubation; +, blue colonies after 60 min; +/−, blue colonies after 90 min; −, no blue colonies. *L7* sequences are in uppercase, and vector sequences are in lowercase. Darkly shaded boxes indicate the locations of mutations of the original *L7* sequence in the UAS, as well as differences between the vector sequence in the short *L7* constructs and the original longer *L7* sequence present in yeast strain CK2. The large, lightly shaded box shows the *L7* sequence in which mutations affect the ability of c-Maf to transactivate the reporter gene. At the bottom, this region of the *L7* promoter is compared to the TRE-type MARE, one of the two consensus target sites described for v-Maf (13). In addition, a related sequence in *L7* promoter fragment 3 is also shown for comparison.

transactivation by c-Maf. Fragment 1 (CK1a) was not influenced by Maf, whereas fragment 3 (CK4) was activated, albeit not quite as robustly as the CK2 fragment (Fig. 6). While we did not attempt to map the Maf site in CK4, inspection of its sequence revealed a stretch of nucleic acids that had similarity to both CK2 and the Maf consensus (residues −287 to −274; AGCCTTCTCAGGT). Within this region of similarity, there is a core sequence, CTCAG, that is completely conserved in CK2, CK4, and the Maf consensus site (Fig. 6).

The conserved core sequence contains an AP-1 half site (TGACTCA). Since c-Maf may interact with other basic-zipper superfamily members and AP-1 complexes might conceivably substitute for c-Maf, we determined whether Fos and Jun could influence transcription from the CK2 promoter frag-

TABLE 2. Binding of c-Maf, c-Fos, and c-Jun to different target sequences[a]

Protein	Colony color in yeast strain:	
	S144	CK2
None	−	−
c-Maf	−	+
c-Maf–VP16	−	+++
c-Fos	−	−
c-Fos–VP16	−	−
c-Jun	−	−
c-Jun–VP16	−	−
c-Maf + c-Fos	−	+
c-Maf + c-Fos–VP16	−	+
c-Maf + c-Jun	−	+
c-Maf + c-Jun–VP16	−	+
c-Fos + c-Jun	−	−
c-Fos–VP16 + c-Jun	−	−
c-Fos + c-Jun–VP16	−	−

[a] Yeast strains S144 (no UAS in the *cyc1-lacZ* reporter gene) and CK2 (UAS, *L7* nucleotides −219 to −132, containing the c-Maf target site and an AP-1-like motif) were transformed with either c-Maf, c-Fos, or c-Jun alone (with or without N-terminal fusion of the VP16 transactivation domain) or with a combination of two proteins. Transformants were tested in the colony color β-galactosidase activity assay for transactivation of the *L7-cyc1-lacZ* reporter gene. +++, blue colonies after 20 min of incubation; +, blue colonies after 60 min; −, no blue colonies.

ment. As shown in Table 2, neither Fos nor Jun, either alone or in combination, transactivated the CK2 fragment. Thus, neither the Maf site nor the AP-1-like site located at −166 to −172 is functional in the context of this assay system. Furthermore, neither Fos nor Jun influenced (either positively or negatively) Maf's ability to transactivate the CK2 UAS. This result was somewhat surprising, since leucine zipper interactions among Maf, Fos, and Jun have been documented (13, 14). Therefore, the Maf site in the *L7* promoter cannot be permissive for either Fos-Maf or Jun-Maf heterodimer binding. This was true for constructs both with and without a VP16 fusion, proving that the lack of activity was not due to the fact that the rodent-derived proteins were transcriptionally inactive in *S. cerevisiae*. As a final control, we generated two additional yeast strains that contained one (CK17) and three (CK19) canonical AP-1 sites, respectively, in the UAS. While both of these strains showed a high basal level of transcription from these reporter genes, this could be augmented by Fos and Jun (and Maf), indicating that the expression constructs did produce active proteins (data not shown). Thus, there appear to be at least two sites in the *L7* promoter that are activated by c-Maf either as a homodimer or in combination with an unidentified yeast protein(s).

DISCUSSION

Studies aimed at characterizing promoter elements within the *L7* gene that are responsive to the factors that coordinate the spatiotemporal organization of the mammalian cerebellum identified a series of regions that are critical in this regard. To isolate the transcription factors that interact with these sites, we turned to a yeast-based assay that has identified the *L7* gene as a potential target for basic-zipper transcription factor c-Maf.

Recently it has been established that c-*maf* is a member of a gene family that includes, *mafF*, *mafG*, *mafK*, and *nrl* (5, 21). Nrl is related by sequence and general domain structure to Maf. However, the additional Mafs are smaller proteins that lack the large N-terminal domain that is necessary for transactivation in v-Maf (5) and c-Maf (Fig. 4). Like the Fos and Jun families of transcription factors, these Maf-related products can also form various homo- and heterodimeric complexes among themselves, as well as with other members of the basic-zipper superfamily. For example, MafK is believed to be the small (p18) component of hemopoiesis-specific transcription factor NF-E2, where it is in complex with the basic-zipper protein NF-E2 p45 (1). Since they lack a putative transactivation domain, the small Mafs behave as transcriptional repressors when they dimerize among themselves (10). However, they seem to serve as transcriptional activators by dimerizing with other (usually larger) basic-zipper proteins and recruiting them to specific DNA-binding sites. These properties raise a number of issues regarding the role Maf might play in *L7* expression.

Expression of the *maf* family members shows various degrees of cell and tissue specificity. For example, *mafK* is ubiquitously distributed in vivo, while c-Maf is differentially expressed, being most abundant in the kidneys, gut, lungs, and brain (5). At the other extreme, *nrl* is expressed exclusively in the neural retina and fetal brain (21). The latter finding is striking in terms of the results presented here, since *L7* is expressed only in cerebellar Purkinje cells and retinal rod bipolar neurons (2, 17). Therefore, we have also examined whether Nrl can substitute for c-Maf. To our surprise, Nrl not only substituted for c-Maf in being able to support transcription from fragment 2 but also showed the same DNA sequence requirement in the various promoter mutations (data not shown). In contrast, Fos and Jun, either alone or in combination, had no effect on this fragment (Table 2). Thus, the same DNA element in *L7* may be involved in its regulation in the cerebellum and retina, although distinct transcription factors may interact with the site. In addition, it is conceivable that tissue-specific regulation of *L7* can be effected through the interaction of fairly ubiquitous transcription factors, such as c-Maf, with proteins having a more restricted distribution, such as Nrl and NF-E2 p45.

The latter finding raises the second issue that must be addressed, namely, the composition of the Maf-Nrl complex that interacts with the *L7* promoter. Both Maf and Nrl can form homodimeric complexes; therefore, they could function alone to regulate *L7* expression in Purkinje cells and retinal bipolar neurons, respectively. However, both Maf and Nrl can also interact with other basic-zipper proteins (13, 14). Therefore, in the yeast assay, Maf and Nrl could function as either homodimers or heterodimers in combination with an endogenous yeast protein(s) that is recruited to the *L7* promoter. While the DNA site that is required for transcriptional activation by Maf and Nrl in the yeast assay is part of the canonical Maf binding site identified by gel shift analysis (13), it appears to only be a half site. In fact, Kataoka et al. have reported two types of DNA consensus sequences that are bound by v-Maf homodimers, a 13-bp 12-*O*-tetradecanoate-13-acetate-responsive element (TRE)-type Maf-responsive element (MARE) and a 14-bp cyclic AMP-responsive element-type MARE (13). The former MARE contains an AP-1 (TGCTGACTCAGCA)-binding site, while the latter harbors a cyclic AMP-responsive element (TGCTGACGTCAGCA) site. The c-Maf target sequence identified in the *L7* promoter contains five of seven nucleotides that constitute the AP-1 consensus, including the four nucleotides that make up the 3′ half site. Overall, the site in *L7* is closest to a TRE-type MARE and has five mismatches with the canonical sequence.

Unlike AP-1 and cyclic AMP-responsive element sites, the MARE consensus is much less rigid and none of the nucleotides are completely conserved among the various oligonucleotides that bind to v-Maf homodimers. Thus, sequences con-

taining up to five mismatches with the TRE-type MARE can still bind v-Maf (13) and therefore the site in *L7* could potentially bind c-Maf homodimers. However, it is currently thought that the specificity of Maf binding is provided by its dimerization with other basic-zipper proteins. For example, heterodimers of v-Maf with Jun or Fos have DNA target specificities different from those of Maf or Jun homodimers (14). Furthermore, we have been unable to show a robust gel shift of the *L7* site by using in vitro-translated c-Maf protein (data not shown). Therefore, we leave open the possibility that Maf dimerizes with a yeast basic-zipper protein that provides a further level of sequence-specific binding. We must also emphasize that in the cerebellum, it may not be c-Maf per se but some Maf-related protein(s) with a similar profile of binding specificity that regulates the tissue-specific expression of *L7*.

Despite the fact that Fos and Jun can heterodimerize with v-Maf (and c-Maf [data not shown]), neither of these proteins alters c-Maf activity in *S. cerevisiae* (Table 2). Therefore, we conclude that the MARE in *L7* is not permissive for either Maf-Fos or Maf-Jun dimer binding, although it is conceivable that Fos- and Jun-like yeast proteins (like GCN4) saturate the binding sites of c-Maf and obscured our analysis of Fos and Jun interactions with Maf on the *L7* promoter. However, we can state that the *L7* MARE is not a functional AP-1 site since Fos-Jun heterodimers are inactive on this element (Table 2).

Surprisingly, neither the properties nor the sequence of full-length c-Maf has been reported. Since we have obtained full-length c-Maf, we have been able to define a number of the functional domains of c-Maf by using the in vivo yeast transcription assay. Furthermore, full-length c-Maf can activate transcription in *S. cerevisiae* even without a VP16 domain, permitting us to study this aspect of function more precisely. In agreement with data from v-Maf, the transcriptional activation domain of c-Maf resides in the N-terminal region of c-Maf. Thus, truncation of 122 amino acids from the N terminus compromises its intrinsic transcriptional capacity. Truncations beyond residue 122 still retain some transcriptional activity; however, truncations that eliminate residue 257 and beyond completely abolish transcription. Thus, we can identify a region of approximately 28 amino acids in c-Maf that is necessary for transcriptional activation. However, this must be attributable to a loss of DNA binding since even when this truncation bears a VP16 domain it is transcriptionally inactive. Thus, despite the fact that this is not classically part of the basic domain, it must contribute to DNA binding in some manner. Indeed, Kataoka et al. have shown that deletions that eliminate the equivalent region in v-Maf result in a loss of DNA binding and transforming activity (13). The fact that forms of Maf that are truncated beyond residue 122 retain a relatively constant level of transcriptional activity may be a reflection of a yeast protein dimerizing with Maf. This interpretation is also consistent with the fact that truncations or point mutations that compromise Maf DNA binding or dimerization result in complete loss of activity.

As in v-Maf, the leucine zipper domain of c-Maf is somewhat atypical for basic-zipper proteins since position 5 in the heptad repeat of hydrophobic residues is occupied by a tyrosine rather than a leucine. Previously, Kataoka and colleagues established that deletion of the most C-terminal leucine repeat of the zipper had no effect on the dimer-forming ability or transforming activity of v-Maf. However, deletion of the last three zipper repeats resulted in a loss of protein-protein association and biological activity (12). In agreement with those data, we show that a deletion at the C terminus of c-Maf, including the last leucine of the zipper, does not interfere with transcriptional activity. In addition, we demonstrate that at least four zipper repeats have to be present to ensure function of the protein since a truncated c-Maf with only the three most N-terminal repeats of the zipper does not transactivate reporter gene expression. Kataoka et al. reported that simultaneous replacement of leucines 2 and 4 proline residues prevented dimer formation, while substitution at position 2 alone resulted in competent dimer formation. We extend this analysis by establishing that a single proline substitution at leucine 4 destroys c-Maf activity, implying that this is a key and essential position in the dimerization domain. This result brings into question Kataoka's conclusion that no single substitution of leucines in the repeat structure destroys the dimer-forming ability of Maf (12).

As noted above, the C-terminal regions of Nrl and c-Maf show extensive homology. Indeed, the primary sequences of the leucine zippers are 50% identical while the basic regions share 78% identity. In addition, the 28 amino acids preceding the basic domain of c-Maf that were identified in this study are 68% conserved in Nrl. This similarity in the DNA-binding domains may account for the fact that the two proteins recognize the same DNA target site. However, the proteins have very dissimilar N termini, which we established as the region that carries the transactivating capacity of c-Maf. This may indicate that there are distinct ancillary factors or components of the basal transcriptional complex in the retina and cerebellum that interact with Nrl and Maf to transmit their transcriptional potential.

Even though c-Maf is not specific for the cerebellum, it may still contribute to the regulation of tissue-specific expression of *L7* by recruiting other transcription factors to the *L7* promoter. Such proteins may have a much greater degree of tissue specificity. Now that c-Maf and Nrl have been identified as being able to transactivate the *L7* promoter, it is possible to convert the yeast screen to the two-hybrid version by using truncated Maf or Nrl as the bait and rescreening the same cerebellar VP16 fusion library we used in this study. This will permit us to sequentially build up mammalian transcriptional complexes in *S. cerevisiae* that can be subsequently reexamined in the murine cerebellum.

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