Molecular Vision 2003; 9:288-94 <http://www.molvis.org/molvis/v9/a40>
Received 31 October 2002 | Accepted 24 June 2003 | Published 30 June 2003

Analysis of non-crystallin lens fiber cell gene expression in c-Maf -/- mice

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Purpose: To examine the role of the c-Maf transcription factor in the regulation of non-crystallin lens fiber cell specific gene expression.
Methods: Expression of lens fiber cell genes in c-Maf null mice was analyzed by immunohistochemical and RT-PCR methods. In addition, the effect of c-Maf on fiber cell gene promoter fragments was examined in co-transfection experiments.
Results: Examination of protein expression in c-Maf homozygous mutant mice revealed undetectable levels of CP49, CP115 and MIP protein. mRNA transcripts for these non-crystallin genes were observed at reduced levels in subsequent RT-PCR experiments. The co-transfection of a c-Maf expression construct with reporter constructs containing the proximal promoters of the CP49 and CP115 genes, the structural components of the lens fiber cell beaded filament, did not result in activation of reporter gene expression, as was observed with a crystallin promoter.
Conclusions: While c-Maf has been shown to play a critical role in lens specific expression of the numerous crystallin genes, it does not appear to regulate some non-crystallin fiber cell genes in the same manner. In contrast to most crystallin genes, detectable levels of CP49, CP115, and MIP transcripts are achieved in the absence of c-Maf. In co-transfection experiments, the CP49 and CP115 proximal promoter sequences were not activated by c-Maf. This suggests that increases in transcription of non-crystallin lens fiber cell genes can be enacted in a c-Maf independent fashion.

The later stages of lens development involve the continuous differentiation of epithelial cells at the equator of the lens. The differentiation process results in a marked increase in cell length and changes in cell architecture, resulting in the degradation of the nucleus and other membrane bound organelles. A small number of genes are upregulated during differentiation. These include the crystallin gene family that is comprised of a number of highly soluble proteins recruited for high-level expression within the ocular lens. Differentiated fiber cells also possess a novel cytoskeletal network formed by association of CP49 and CP115 proteins which are fiber cell specific. Finally, MIP (major intrinsic protein), also lens fiber cell specific, represents the most abundant integral membrane protein within lens fiber cells [1].

The members of the α-, β-, and γ-crystallin gene families are spatially and temporally regulated, yet these genes share a number of regulatory elements within their promoter/enhancer regions. Maf response elements have been identified in a number of crystallin promoter regions shown to be necessary for transcriptional activation of these genes [2-7]. Further investigation into the expression of Maf family members within the mouse lens indicated that the c-Maf transcription factor was most likely responsible for upregulation of crystallin gene expression within lens fiber cells [8,9]. The onset of c-Maf expression is detected in the head surface ectoderm, at E9, prior to placode formation and continues throughout lens development into differentiated lens fiber cells. In vitro studies have determined that the expression of c-Maf can stimulate co-transfected crystallin promoters in a non-lens cell environment [2,10]. Ectopic expression of L-Maf, present in the chicken lens, was able to induce the expression of several different lens fiber cell genes, including CP115, when electroporated into chicken embryos and examined after 5 days via RT-PCR [2]. This evidence suggests that c-Maf is a driving force in the expression of lens fiber cell crystallin genes.

The role of c-Maf in lens development has been addressed through the targeted mutation of the c-Maf gene [9-11]. Mutation to both alleles of the c-Maf gene results in lethality, with few homozygous knockout mice surviving beyond birth. Homozygous knockout animals exhibit abnormal lens development that includes incomplete elongation of primary fiber cells, and no secondary lens fiber cell formation, leaving the lumen of the lens vesicle vacant. Characterization of these knockout animals has focused on crystallin gene expression in the absence of c-Maf. β- and γ-crystallin transcripts are significantly reduced or non-detectable in knockout animals. The mRNA and protein levels of the α-crystallins, expressed within both lens epithelial and lens fiber cells, are reduced within the lenses of the knockout animals.

Little is known about the regulatory mechanism(s) that establish the cell type specific expression pattern of the non-crystallin lens fiber cell specific genes. The 5' flanking DNA sequences of the human and/or mouse CP49, CP115, and MIP genes demonstrate lens preferential activation of reporter gene expression in cell culture transfection experiments. Examina-

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tion of these proximal promoter sequences provides little indication that c-Maf is involved in the transcriptional regulation of these genes [12-14]. At this point it is not known if c-Maf is involved in the regulation of these genes in vivo. In this study, CP49, CP115, and MIP mRNA and protein levels were analyzed in c-Maf knockout mice. Co-transfections of CP49 and CP115 promoter constructs with a c-Maf expression vector were performed to reveal any direct effect on the activity of the beaded filament proximal promoter sequences.

METHODS

Analysis of c-Maf knockout animals: The offspring of heterozygote mutant c-Maf mice, generously provided by Dr. Glimcher(10) were genotyped using PCR. Genomic DNA was prepared using the DNeasy Kit (Qiagen, Valencia, CA). PCR on genomic DNA isolated from tail clippings utilized primers complementary to the c-Maf coding sequence flanking the insertion point, a *Not* I site, of the neomycin resistance gene. The sequence of the downstream sense c-Maf primer (5'-GCT CCA GGG TGG CTT CGA TGG CT-3') lies upstream of the *Not* I site at base 510 of the coding sequence and spans bases 369-391. The upstream antisense primer (5'-CGT GGT GGT GGT GGT GAT GGT AGT-3') lies downstream of the *Not* I site spanning bases 539-562 of the coding sequence. PCR was performed using the Advantage GC Genomic PCR Kit (Clontech, Palo Alto, CA) as recommended by the manufacturers. The 2-step PCR profile included a 30 s denaturation at 94 °C and a 3 min elongation at 68 °C and was cycled 35 times. A wildtype c-Maf allele results in a 200 bp PCR product while the mutated c-Maf allele that possesses the inserted neomycin yields a 1,700 bp PCR product. The profile for a wild type mouse will consist of a single band of 200 bp. Heterozygote animals, possessing a wild type and a mutated allele, will yield both the 200 bp and 1,700 bp bands. The homozygote knockout mouse gives rise to only the 1,700 bp band.

Mice were also genotyped for a mutation within the CP49 gene inherent to the mouse 129 strain, as the embryonic stem cells utilized to generate the targeted c-Maf mutation were of this strain. To detect the mutated 129 allele, PCR was performed on genomic DNA prepared from tissue samples using the DNeasy Kit(Qiagen, Valencia, CA). The primers used in the PCR screen flank the deleted region of 6,303 bp extending from intron B into exon 2 of the CP49 gene. The sequence of the downstream sense primer (5'-TGG GGT TGG GCT AGA AAT CTC AGA-3') lies within intron B of the CP49 gene. The upstream antisense primer (5'-AGC CCC TAC GAC CTG ATT TTT GAG-3') anneals to complementary DNA within intron C of the CP49 gene. PCR was performed using recombinant Taq DNA polymerase(Invitrogen, Carlsbad, CA) as suggested in the product manual. The 3 step PCR profile included an initial 1 min denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s. The mutated 129 allele gives rise to a 400 bp PCR product.

Immunohistochemistry: Embryos from c-Maf heterozygote knockout matings were collected at different embryonic stages in order to analyze lens fiber cell gene expression. The embryos were fixed in a 4% paraformaldehyde, 0.1 M phosphate buffer at 4 °C overnight, and subsequently embedded in paraffin. Six μm sections from the paraffin blocks were collected for immunostaining. The sections were deparaffinized, rehydrated and subjected to the immunostaining protocol. Briefly, the sections were treated with pepsin (Sigma, St. Louis, MO) at 1 mg/ml for 20 min, rinsed in distilled H_2O, and incubated for 15 min in a 3% H_2O_2 in methanol solution. The sections were blocked with 10% normal goat serum in 1X PBS for 30 min. Antiserum was produced by the injection of recombinant mouse CP49 protein, recombinant mouse CP115 protein, or purified mouse MIP into rabbits and subsequent serum collection [15,16]. Preimmune serum was collected and used as a negative control. Rabbit antisera to CP115 was used at 1:200 dilution, antisera to CP49 at 1:250, and antisera to MIP at 1:200 overlaid onto tissue sections for 90 min at room temperature. After rinsing in 1X PBS the secondary antibody solution, a biotin goat anti rabbit antibody (Zymed, Orange, CA), was placed on the sections for 15 min, followed by a 15 min incubation with streptavidin/peroxidase (Zymed, Orange, CA). 3,3-Diaminobenzidine (DAB, Sigma, St. Louis, MO) was used as the chromagen to identify the regions of reactivity. A 3 min incubation in the DAB solution was followed by counterstaining in hematoxylin (Sigma St. Louis, MO) for 3 min. The sections were then dehydrated and coverslipped.

Total RNA isolation and RT-PCR on wildtype and homozygous c-Maf knockout animals: Mouse embryo heads were isolated and placed in 200 μl of RNA Later solution (Qiagen, Valencia, CA). Tissue samples were collected for genotyping with respect to both the c-Maf and CP49 loci. Total RNA from the eye region of knockout and wildtype mice was extracted using the Rneasy kit (Qiagen, Valencia, CA). Aliquots of RNA were treated with amplification grade Dnase I (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA. First strand cDNA was synthesized using one microgram of total lens RNA, an oligo dT primer (Invitrogen, Carlsbad, CA) and the Superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA) as described in the product protocol. Upon completion of first strand cDNA synthesis the reaction was treated with RnaseH (Invitrogen, Carlsbad, CA) for 20 min at 37 °C. Aliquots of the resultant first strand cDNA were diluted by a factor of 10, 25, 100, and 200. The cDNA was then utilized in PCR reactions with primers specific to the αA-crystallin, γB-crystallin, CP49, CP115, MIP, and GAPDH genes. Each primer pair used in PCR amplification flanked intron sequences in order to distinguish between products generated from the amplification of genomic templates or cDNA. First strand cDNA (2 μl) generated from wildtype or knockout mice served as template for amplification in 25 μl PCR reactions using recombinant Taq DNA polymerase(Invitrogen, Carlsbad, CA). Negative controls were included in which the RNA was not subjected to first strand synthesis. The reactions were initially denatured for 1 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final incubation at 72 °C for 5 min in a PTC-200 thermal cycler (Perkin Elmer, Wellesley, MA). The reactions (10 μl each) were run on a 3% gel composed of a 3:1 ratio of agarose and Seakem (FMC, Houston, TX). The use of animals was in ac-

cordance with the care and treatment protocols for the UC Davis Veterinary Medicine Facility.

Transfection constructs: The -3500/+100 portion of the human CP115 promoter, with respect to the transcriptional start site, was cloned by ligating two DNA fragments. A 2.8 kb blunt ended *Eco*R I/*Nco* I fragment that encompassed the -3500/-700 upstream region and an 800 bp *Nco* I/*Xho* I that spans the -700/+100 sequence were inserted into the pGL2-Basic vector (Promega, Madison, WI) cut with *Sma* I and *Xho* I. The -700/+100 fragment was amplified by PCR with a primer that introduced an *Xho* I site at the end proximal to the coding region. The integrity of the -700/+100 sequence was confirmed by sequencing at the UC Davis DBS Sequencing facility.

The mouse CP115 promoter construct was created by restriction digestion of a P1 clone, from Incyte Genomics (Palo Alto, CA), that contained greater than 6 kb of 5' flanking DNA sequence extending into the coding region of the CP115 gene. The -1284/+45 portion of the 5'-flanking sequence was obtained by digestion with *Eco*R I and *Eag* I. The 1.3 kb fragment of interest was blunt ended and cloned into *Sma* I digested pGL2-Basic. Construction of the human CP49, mouse CP49, and mouse αA-crystallin constructs was detailed in a previous report[14].

Tissue culture and transfection: The mouse embryonic fibroblast cell line, NIH3T3, was cultured in Dulbecco's Minimal Essential Media (DMEM, Invitrogen, Carlsbad, CA), supplemented with 10% bovine calf serum (Omega Scientific, Tarzana, CA), 25 mM Hepes (Invitrogen, Carlsbad, CA), and penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a humidified 6% CO_2 environment. Cells were trypsinized, seeded in 12 well plates, and cultured overnight until 50-80% confluent. The cells were transfected with 250 ng of the pMex construct, containing no insert, or the pMex Neo/cMaf construct, an expression vector containing the c-Maf cDNA sequence, along with 250 ng of a given lens promoter reporter construct. The pMex and pMex Neo/cMaf constructs were provided by Dr. J. Kim [10]. An internal standard vector, pRL-TK (100 ng, Promega, Madison, WI), was included in each of

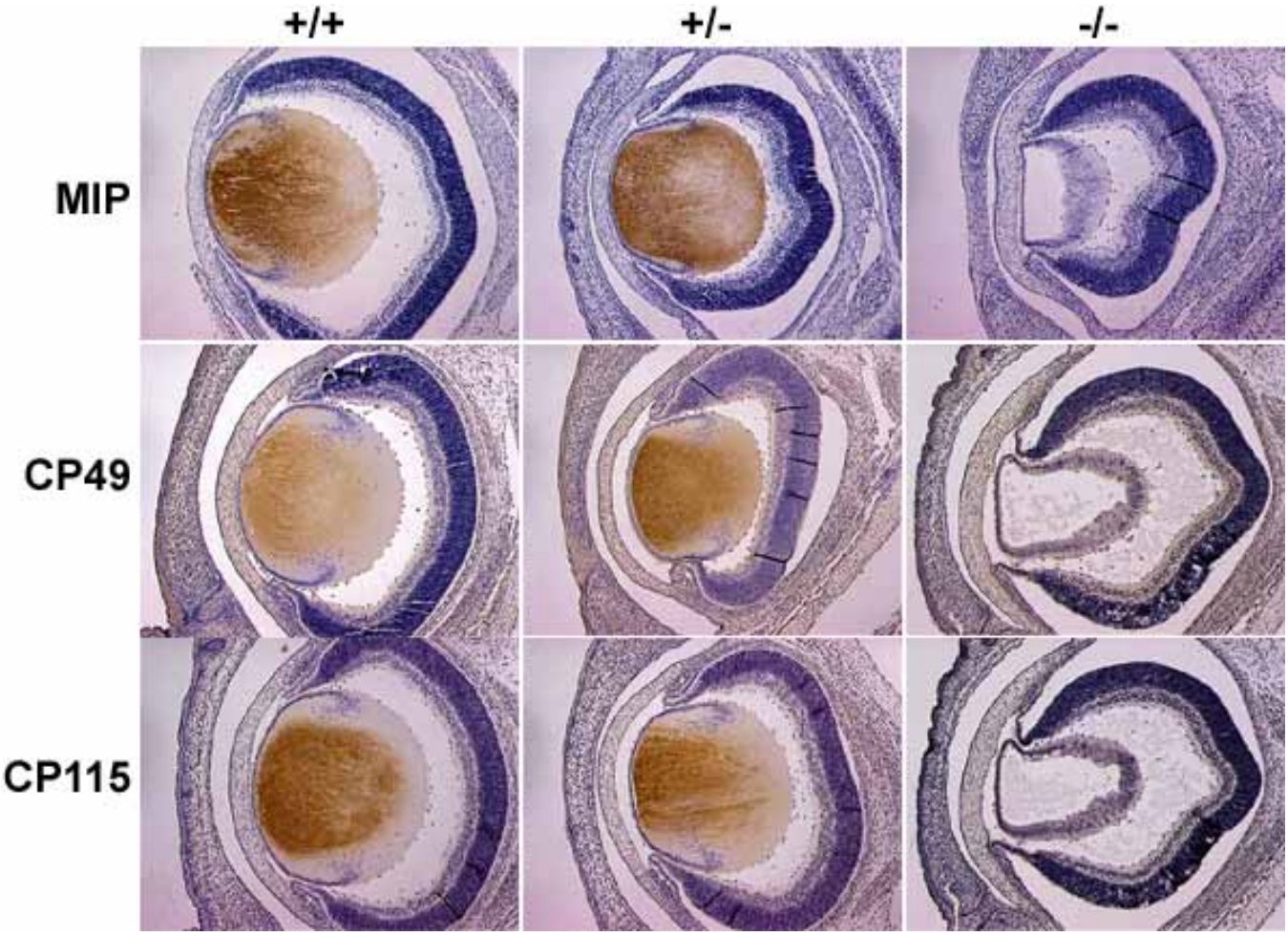

Figure 1. Immunocytochemical localization of MIP, CP49, and CP115 expression. Immunocytochemical localization of MIP, CP49, and CP115 expression in wildtype (+/+), heterozygous mutant c-Maf (+/-), and homozygous mutant c-Maf (-/-) mice. Paraffin sections of E16 to E19 mouse embryonic head were used in immunohistological studies to analyze gene expression. The dark brown color denotes reactivity to the protein of interest. While the lenses of the wildtype and heterozygous mutant c-Maf mice exhibit normal lens development, homozygous c-Maf mutant show no signs of secondary fiber cell formation.

the transfections. The transfection was performed using lipofectAMINE Plus (Invitrogen, Carlsbad, CA) as suggested in the product protocol. LipofectAMINE (5 μl) and 5.0 μl of Plus reagent were utilized in each transfection. The transfected cells were harvested at 72 h post transfection and assayed for luciferase activity. Cell extract (20 μl), prepared using the Dual Luciferase Assay Kit (Promega, Madison, WI), was assayed on a ML3000 Dynatech Luminometer (Thermo Labsystems, Chantilly, VA) over a 10 s interval for both the experimental and internal standard values. The renilla luciferase activity from the internal standard vector was used to normalize the experimental firefly luciferase activities. Each construct within an experiment was transfected in triplicate and the summarized data is the result of at least three separate transfection experiments.

RESULTS

Genotyping: PCR was used to genotype progeny from c-Maf heterozygote matings at both the c-Maf and CP49 loci. The mouse 129 embryonic stem cells used in the generation of the c-Maf targeted mutation harbor a deletion within the CP49 gene. The deletion results in markedly decreased expression of a mutant CP49 where exon 2 is excluded from the mRNA transcript. The reduction of CP49 gene expression results in a coincidental decrease in CP115 protein levels. All animals utilized in the immunohistochemical and RT-PCR studies did not possess the mutated CP49 allele from the 129 mouse strain, because of outbreeding.

Immunostaining: Figure 1 summarizes the immunohistochemical analyses utilized to determine the presence and distribution of CP49, CP115, and MIP protein within the lenses of wild type (+/+), c-Maf heterozygote (+/-), and c-Maf homozygote knockout (-/-) mice. Several mice of each genotype from different litters and gestational stages were examined for non-crystallin, lens fiber cell gene expression. A total of twenty-five mice from six different litters were examined histologically. The age of the litters examined ranged from embryonic day 16 to 19. At these stages of development, wildtype mice lenses have completed primary lens fiber cell elongation and secondary fiber cell formation is ongoing. Five wild type animals, each from a separate litter, seven homozygous knockouts, from four different litters, and thirteen heterozygous animals, from five litters comprise the set of animals used in this study.

In wildtype animals, both CP49 and CP115 were readily detected within the terminally differentiated lens fiber cells with no detectable reactivity in lens epithelial cells. As seen in previous studies, the majority of the staining was located within the anterior half of the fiber cells and distributed throughout the cytoplasm. Reactivity in lens fiber cells for CP49 and CP115 did not appear to reach a maximum until cells cleared the transitional zone and nearly completed elongation. The staining pattern observed for wildtype animals in these studies was identical regardless of age or litter.

The analysis of c-Maf heterozygote animals for beaded filament proteins showed that both CP49 and CP115 gene products are present. The level of protein expression in the heterozygote animals varied between animals within and across litters. The magnitude of reactivity registered within the set of heterozygotes ranged from levels comparable to that detected in wild type animals to significantly diminished levels of expression for both genes. As with the wild type animals, there was no staining within the lens epithelial cells and the staining was not appreciable until the cells had elongated and cleared the transitional zone.

As documented in previous studies, lens development in c-Maf homozygous knockout mice was significantly compromised. Lens development in null mice progressed up to the formation of the lens vesicle. Partial elongation of primary lens fibers cells was observed. Secondary fiber cell formation was absent, leaving the lumen of the lens vacant. We observed this same phenotype for individual knockout animals, each appearing to have arrested development at the lens vesicle

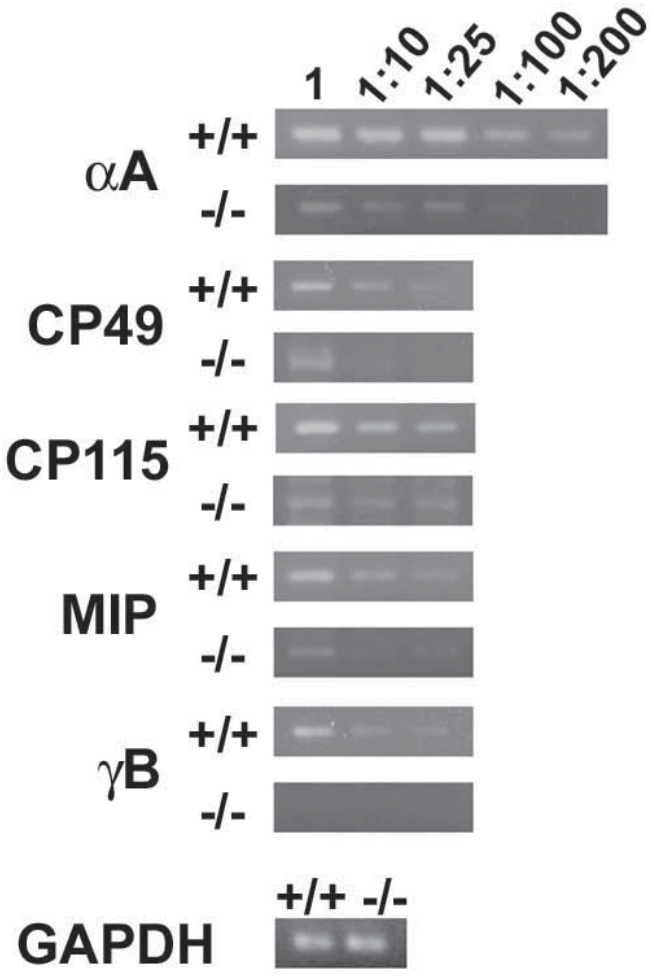

Figure 2. RT-PCR analysis of wildtype mutant mice. RT-PCR analysis of wildtype (+/+) and homozygous mutant c-Maf (-/-) mice for the expression of lens fiber cell genes. The eye regions of E17 mice were isolated and pooled according to genotype. Total RNA was isolated and first strand cDNA was produced using an oligo dT primer. PCR with gene specific primers that flanked intron sequences were utilized to assay for the presence of mRNA transcripts.

stage. In some cases the anterior portion of the lens remained attached to the surface ectoderm that later develops into the cornea. All homozygous animals analyzed lacked immunoreactivity to either the CP49 or CP115 gene products. Analysis of the knockout animals for vimentin expression showed that the expression of vimentin is not affected by the absence of c-Maf (data not shown).

In wildtype mice an immunological signal corresponding to MIP protein expression was observed at the perimeter of fiber cells, as expected for an integral membrane protein. MIP protein was present in elongated fiber cells as well as cells at the initial stages of elongation near the bow or equator region. The majority of MIP protein appeared to be located within the anterior half of fiber cells, similar to the distribution of signal for CP49 and CP115. The registered immunoreactivity confirmed the fiber cell specificity of MIP expression, as there was a complete lack of signal in the anterior epithelium. Analysis of the heterozygous c-Maf mice show that MIP was expressed at, or near, the magnitude observed for wildtype mice. MIP gene expression, at the protein level, was not detected in c-Maf homozygous mice as no reactivity was identified within the lens or any other tissue.

RT-PCR on wildtype and c-Maf knockout mice: To determine if lens fiber cell specific genes are actively transcribed in the absence of the c-Maf transcription factor, lenses of wildtype and c-Maf knockout embryonic mice were collected and total RNA prepared for RT-PCR, (Figure 2). Previous characterization of c-Maf null mice has revealed a significant reduction of mRNA levels for the majority of crystallin genes. Similar to results in previous reports, RT-PCR experiments performed in these studies confirm the presence of αA-crystallin mRNA transcripts in both wildtype (+/+) and knockout (-/-) lenses, as well as a decrease in γB-crystallin transcripts in c-Maf null mice versus wildtype mice [9-11]. RT-PCR reactions for these two crystallin genes were included in this set of experiments to provide an established positive and negative control. Transcripts for each of the non-crystallin members of lens fiber cell genes, CP49, CP115 and MIP, were detected in both the wildtype and knockout mice revealing that the absence of c-Maf does not abolish transcription of these genes. While detectable, the mRNA levels of non-crystallin genes are reduced compared to wildtype. Amplification of the glyceraldehyde-3-phosphate dehydrogenase gene was used as a control for RNA integrity and confirmation of successful reverse transcription.

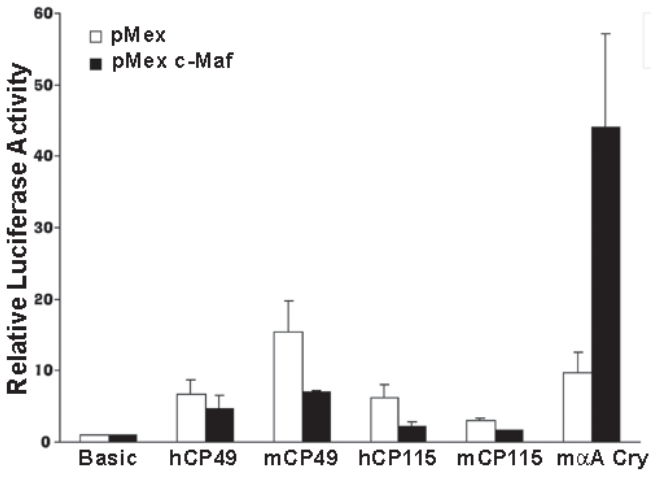

Figure 3. Transactivation potential of the c-Maf transcription factor. Transactivation potential of the c-Maf transcription factor in co-transfection experiments. Mouse (m) and human (h) lens fiber cell promoter constructs were transfected with the c-Maf expression vector, pMex/Neo c-Maf or the pMex/Neo vector containing no insert. The luciferase activities are relative to the promoter-less and enhancer-less pGL2-Basic vector. Each construct within an experiment was transfected in triplicate and summarized data is the result of at least three separate transfection experiments. All errors are expressed as standard deviation.

Co-transfection of c-Maf expression vector and lens promoter fragments: 5' flanking DNA sequences from the mouse and human CP49 and CP115 genes were utilized in co-transfection experiments to examine promoter activity modulation in the presence of the c-Maf transcription factor. As seen in Figure 3, the co-transfection of the human and mouse CP49 and CP115 gene promoters with the c-Maf expression construct showed no increase in reporter gene expression when compared to transfection with the pMex vector. This suggests that no direct interaction occurs between c-Maf and the proximal promoter sequences of the beaded filament genes. All luciferase values were expressed as fold induction over the activity observed for the pGL2-Basic vector, which does not possess promoter or enhancer elements. The human and mouse CP49 promoter constructs displayed 6.7 and 15.4 fold luciferase activities, respectively, in co-transfections with the control pMEX plasmid. Co-transfection with the c-Maf expression plasmid resulted in relative increases in luciferase activities of 4.6 for the human CP49 promoter and 6.9 for the mouse CP49 promoter. The human CP115 promoter construct displayed an activity of 6.15 fold compared to pGL2-Basic when co-transfected with pMEX. The activity of this promoter in co-transfections with the pMEX c-Maf plasmid was 2.1. Finally, the mouse CP115 promoter showed 2.9 and 1.7 fold relative luciferase activities when co-transfected with pMEX and pMEX c-Maf respectively. Rather than activating reporter gene expression, the CP49 and CP115 constructs displayed slightly lower activities when co-transfected with the c-Maf expression vector, revealing a potential inhibitory effect of c-Maf on promoter activity in a non-lens cell context.

Binding sites for c-Maf have been identified within the proximal promoters of several crystallin genes, including the mouse αA-crystallin promoter. A segment of this promoter, -364/+45, was included in these experiments as a positive control for activation by c-Maf. As seen in Figure 3, co-transfections of the mouse αA-crystallin promoter in conjunction with the c-Maf expression vector, pMex/c-Maf, correlates to a 44.03 fold increase in relative luciferase activity. When co-transfected with the control pMEX vector, the crystallin promoter registered a relative luciferase activity of 9.7. The 4.53 fold increase in luciferase activity demonstrates the ability of c-Maf to upregulate reporter gene expression levels in a direct fash-

ion through interaction with sequence elements of the promoter sequence.

DISCUSSION

To better understand the role of c-Maf in lens development and differentiation, expression of CP49, CP115, and MIP was examined in c-Maf knockout mice. Immunohistochemical studies revealed the expression of beaded filament proteins in the anterior portion of elongated fiber cells of wildtype mice, while MIP exhibited localization to the cell periphery. Heterozygous c-Maf mouse lenses developed normally and expression of the CP49, CP115, and MIP genes was observed at variable levels. Mutation to both c-Maf alleles, in knockout mice, yielded incomplete primary fiber cell formation and a lack secondary fiber cell differentiation. Protein levels for CP49, CP115, and MIP in knockout mice fell below the detectable range in immunohistochemical experiments, suggesting that they were absent,or at greatly reduced levels.

The more sensitive RT-PCR method was employed to investigate if transcription of non-crystallin genes is observed independently of c-Maf. The amplification of cDNA, generated from the total RNA of wildtype and knockout eyes, established the presence of CP49, CP115, and MIP mRNA transcripts in the absence of the c-Maf transcription factor. The signal for each non-crystallin gene in knockout animals was lower relative to the wildtype mouse. As observed in previous studies, transcription of the αA-crystallin gene was apparent in knockout lenses, while transcripts for the lens fiber cell specific γB-crystallin were not detected [9-11]. While c-Maf has been shown to impact αA-crystallin gene expression, detectable mRNA levels are observed in knockout mice. The remaining signal can be attributed to expression within lens epithelial cells supported, in part, by the Pax6 transcription factor.

These results suggest that c-Maf is not absolutely required for detectable promoter activity of these non-crystallin genes. We can only speculate as to why transcripts of non-crystallin genes are present without detectable protein levels. There is a greater sensitivity attributed to PCR techniques compared to immunohistochemical methods, which contribute to the results obtained in these experiments. In the case of the βB2-crystallin gene, mRNA levels can be detected well before a significant amount of protein is accumulated [17]. Transgenic experiments involving the βB1-crystallin promoter have shown that a component of the regulation occurs at the translational level [18]. Translational regulation offers a potential explanation for lack of signal in immunostaining experiments. In addition, lens fiber cell proteins may also be destabilized in the absence of crystallins, which have been shown to interact with beaded filament proteins.

It is not clear whether fiber cell specific genes such as the γ-crystallins, predominantly expressed in secondary fiber cells, are absent in c-Maf null mice due to a lack of secondary fiber cell formation or to the loss of c-Maf. The expression of these genes may also be dependent upon the elongation and/or other features that accompany secondary fiber cell formation. Of note, the disruption of the Prox1 gene, responsible for withdrawal of epithelial cells from the cell cycle and the initiation of differentiation, results in a defective fiber cell differentiation similar to the c-Maf null mouse [19]. Analysis of gene expression in the Prox1 mutant revealed the presence of mRNA transcripts for crystallin genes including βB1-crystallin, and the fiber cell specific γA-, C-, E-, and F-crystallin genes. This suggests that fiber cell gene expression can be established without completion of the differentiation process, and the absence of crystallin expression is most likely due to the lack of c-Maf rather than defective fiber cell differentiation. This highlights an apparent difference in gene regulation between the majority of the crystallin genes and the CP49, CP115, and MIP genes as suggested by the evidence gathered in this study and previous promoter characterizations.

The direct effect of c-Maf on non-crystallin gene promoter activity was examined in co-transfection experiments. Both the human and mouse CP49 and CP115 proximal promoter sequences were utilized in these studies. The presence of the c-Maf expression vector did not result in an upregulation of reporter gene expression. Positive control experiments with the mouse αA-crystallin promoter, which possesses a Maf response element within the proximal 5' flanking DNA sequence, displayed a 4.53 fold increase in luciferase activity consistent with previous findings utilizing the chicken α-crystallin and the mouse γF-crystallin promoters [2,10]. The 5' flanking regions of the CP49 and CP115 were able to drive lens preferred reporter gene expression in transfection and transgenic experiments. The inability of c-Maf to enhance reporter gene expression driven by beaded filament promoters suggests that cell type specific expression was achieved without the direct involvement of c-Maf [12,14]. Activation of transcription by c-Maf may require the presence of additional lens transcription factors that are not expressed in the NIH3T3 cell line. Recently it has been established that c-Maf can act as a negative regulator of gene expression through binding to anti-oxidant response elements (ARE) [20]. The CP49 and CP115 gene promoters were inspected for the core ARE sequence, TGACNNNGC. Both the human and mouse CP49 promoters possess ARE sequences at non-conserved locations, while neither of the CP115 proximal promoter regions harbor ARE sequence elements. While it is possible that c-Maf could influence CP49 expression in a negative manner, this does not address the consistent lowered activity observed with the CP115 promoter constructs.

Sequence analysis of the 5' flanking region for the human and mouse CP49, CP115, and MIP genes identified a single c-Maf binding motif within the proximal promoter of the mouse CP115 gene [12]. This sequence element is not conserved within the human CP115 proximal promoter and does not appear to be integral to activity of the mouse CP115 proximal promoter, as binding by nuclear proteins was not evident in gel shift experiments [12]. It is possible that a c-Maf consensus site exists at an alternate location other than the 5' flanking DNA, as in the δ1-crystallin enhancer, which resides within the third intron of the gene [3]. Preliminary analysis of the human CP49 and CP115 intron sequences has not revealed any such element. The characterization of the proximal pro-

moters of the CP49, CP115, and MIP genes have revealed the presence of putative binding sites for transcription factors such as AP-1, AP-2, SP-1, and SP-3 [12,21,22]. The absence of conserved Sox, Pax, or Maf sequence elements in non-crystallin lens fiber cell gene promoters indicates regulation by a separate array of factors compared to crystallin expression.

The use of alternate transcriptional regulators may be required to achieve the different levels of gene expression characteristic of crystallin and non-crystallin genes. Crystallin gene expression far exceeds the appreciable level of other lens fiber cell-specific genes such as CP49, CP115, and MIP. Recruitment of crystallin gene expression to the lens is often accomplished through c-Maf response elements within crystallin promoter/enhancer regions that contribute to high-level expression, as illustrated through transfection and transgenic analyses [6,23]. The findings reported here do not eliminate c-Maf as a regulator of non-crystallin gene expression within lens fiber cells, but its effect is most likely to be indirect. The possibility exists that c-Maf may affect non-crystallin lens fiber cell gene expression in concert with other transcriptional regulators or through intermediates that act at either the transcriptional or translational level. Candidate targets of c-Maf! would include factors such as AP-1 and SP-1 that have been implicated in proximal promoter activity of the CP115 and MIP genes.

ACKNOWLEDGEMENTS

We thank Dr. Laurie Glimcher and Dr. James Kim for the breeding pair of c-Maf heterozygous mutant mice and the c-Maf expression construct. This work was supported by grants from the National Eye Institute to PGF (RO1EY08747 and P30EY12576).

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The print version of this article was created on 30 Jun 2003. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.