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CREB-binding Protein/p300 Co-activation of Crystallin Gene Expression*

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Although some of the transcription factors that are required for expression of crystallins during lens development have been identified, the molecular interactions that contribute to enhanced crystallin expression are not yet well defined. In this study, we designed experiments to test whether the co-activators CREB-binding protein (CBP) and/or p300 interact with c-Maf, Prox-1, or Sox-1 to enhance transcription of crystallin genes. Promoter regions from the mouse αA-, βB2-, and γF-crystallin genes were linked to a luciferase reporter. Expression of c-Maf transactivated each of these promoters. Of particular interest, co-expression of CBP or p300 with c-Maf was found to synergistically co-activate each promoter. CBP and p300 were less effective or ineffective at co-activation with Prox-1 or Sox-1. Co-immunoprecipitation and mammalian two-hybrid experiments revealed that CBP and p300 bind to c-Maf and Prox-1 but not to Sox-1. The co-activation of c-Maf by CBP/p300 requires histone acetyltransferase activity. Our results suggest that c-Maf recruits CBP and/or p300 to crystallin promoters leading to up-regulation of crystallin gene expression through localized histone acetylation and consequent chromatin re-modeling. In a promoter-specific fashion, co-activation can be modulated by Prox-1 and/or Sox-1. This modulation may help to specify the endogenous levels of crystallin gene expression.

During embryonic development, the ocular lens is formed as an invagination of the surface ectoderm. As differentiation proceeds, the lens forms a hollow sphere of epithelial cells termed the lens vesicle (1). The cells located at the posterior surface of the lens vesicle are induced to differentiate into the primary lens fiber cells that elongate to fill the lens vesicle. The lens subsequently is comprised of a monolayer of proliferative cuboidal epithelial stem cells on the anterior surface overlying a core of elongated fiber cells (2, 3). Lens induction is accompanied by localized expression of several different transcription factors including Pax-6, Six-3, Sox-2, c-Maf, Prox-1, and Sox-1 (4). Within the developing and the mature lens there is a distinctive pattern of crystallin gene expression. The α-crystallins are expressed at the lens vesicle stage, and are produced by both lens epithelial and fiber cells. The β- and γ-crystallins are synthesized by the fiber cells, with the expression of most β-crystallins preceding the expression of the γ-crystallins (5, 6). Altogether, the crystallins constitute more than 90% of the soluble lens proteins (7, 8).

Gene targeting studies have shown that c-Maf, Prox1, and Sox1 are required for lens fiber cell differentiation and crystallin gene expression (9–11). c-Maf is required for expression of all three major crystallin gene families. Prox-1 and Sox-1 are specifically required for the expression of γ-crystallins (10–12). Maf consensus binding sequences (MAREs)[1] have been identified in mouse αA-, βB2-, and γF-promoter regions (9, 10, 13). Prox-1 and Sox-1 responsive elements are also present in the γF-crystallin promoter region (9, 12). Recently, it has been reported that Prox-1 and Six-3 act antagonistically to regulate the γF-crystallin promoter (12). It is not yet known how c-Maf, Prox-1, and Sox-1 interact with each other to regulate crystallin gene expression. It is also not known how these transcription factors specify the remarkably high levels of crystallin gene expression seen in lens cells.

CBP (CREB-binding protein) and p300 are well known transcriptional co-activators that have histone acetyltransferase (HAT) activity (14, 15). They interact with many transcription factors and are required for cell differentiation and tissue development (16). CBP/p300 can be recruited to promoters by direct interaction with DNA-binding transcription factors or as components of large complexes containing other cofactors such as P/CAF, SRC-1, and the ACTR/p/CIP group of proteins (17, 18). In previous studies we showed that inhibition of CBP/p300 activity *in vivo* (by expression of adenoviral E1A proteins in the lens of transgenic mice) resulted in inhibition of fiber cell differentiation and loss of β- and γ-crystallin gene expression (19). In the present study, we used a cell culture system to investigate the possibility that CBP and/or p300 may interact directly with the transcription factors that regulate crystallin gene expression.

We generated three crystallin promoter-luciferase reporters (αA, βB2, and γF), as well as a MARE-TK-luciferase reporter, and transfected COS-1 and human lens epithelial cells (HLEC-B3) with each reporter and with plasmids encoding c-Maf, Prox-1, and/or Sox-1 in the presence or absence of CBP or p300. Our results indicate that the crystallin promoters are synergistically co-activated by c-Maf·CBP and c-Maf·p300 complexes. CBP and p300 bind directly to c-Maf and co-activation is dependent upon HAT activity. We also demonstrate that Prox-1 can enhance, and Sox-1 can inhibit, this co-activation. Thus, lens-specific transcription factors interact with CBP and/or p300 to provide differential regulation of individual crystallin gene expression.

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[1] The abbreviations used are: MARE, Maf response element; CBP, CREB-binding protein; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; HAT, histone acetyltransferase; Luc, luciferase; β-gal, β-galactosidase; TK, thymidine kinase.

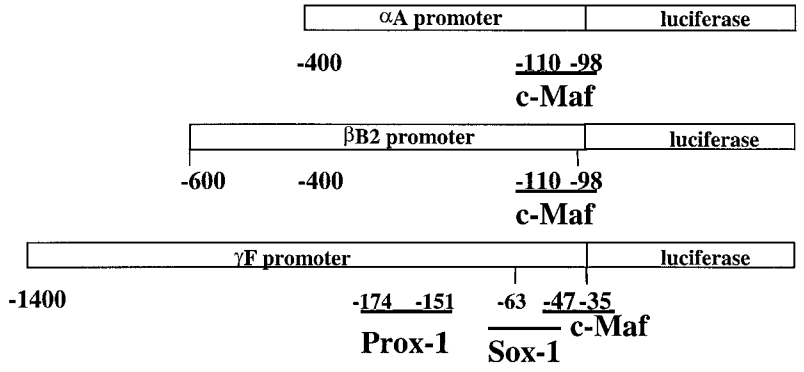

FIG. 1. **Crystallin promoter reporters.** The constructs with the αA-, βB2-, and γF-crystallin promoters linked to a luciferase reporter are depicted. Potential c-Maf recognition sites are located at −110 to −98 in the αA and βB2 promoters, and at −47 to −35 in the γF promoter (30–32). The γF promoter also contains a potential Prox-1 (−174 to −151) and a Sox-1 (−63 to −47)-binding site (9, 12).

EXPERIMENTAL PROCEDURES

Plasmids for Transfection

Reporter Plasmids—A 624-bp βB2-crystallin promoter (−614/+10) and a 1367-bp (−1357/+10) γF-crystallin promoter were amplified from mouse genomic DNA using primers 5′-ATAGAACCCAGGACCACCAG-/5′-GAGTGCCGTGAAGCCAGGCT for βB2-crystallin and 5′-GCCCCA-CCTGCAACAAACAAC/5′-GGCAGGTCAGATGGGATGGTG for γF-crystallin (20, 21). The PCR products were inserted upstream of the luciferase reporter in the pGL3 vector (Promega). The αA-crystallin promoter was isolated from CPV2 (22) by digestion with *Kpn*I and *Bam*HI. For the MARE-TK construct, the herpes simplex virus TK promoter (−109 to +18) was cut out of pTK3-Luc[2] via the *Bgl*II and *Hin*dIII sites and inserted into the *Bgl*II/*Hin*dIII sites of pGL3 to create pGL3-TK-Luc. The Maf response element, MARE, was generated by annealing the oligonucleotides 5′-GTACCTGCTGACTCAGCAACATT-GTTGCTGACTCAGCACG and 5′-CTAGCGTGCTGAGTCAGCAA-CAATGTTGCTGAGTCAGCAG, followed by phosphorylation with T4 polynucleotide kinase and ligation upstream of the TK promoter in pGL3-TK-Luc. The G5E1b-Luc plasmid contains 5 copies of a Gal4-binding site (G5) linked to an E1b minimal promoter driving the luciferase reporter, as described previously (23).

Plasmids for Expressing Transcription Factors—The plasmids pCMX-D, pCMX-VP16-N, and pCMX-Gal4-N have been described elsewhere (23–25). Full-length c-Maf, Prox-1, Sox-1, CBP, p300, E1AΔN (26), and E1AΔCR2 (26) cDNAs were cloned into the pCMX-D expression vector downstream from the cytomegalovirus promoter. The p300 mut1504 construct has a point mutation at amino acid 1504 of human p300 (alanine codon substituted for a tyrosine codon). The p300 Δ1430–1504 clone has amino acids 1430 to 1504 deleted from the human p300. The p300 mut1504 and Δ1430–1504 constructs were made by PCR amplification with Pfu DNA polymerase as described (27), with both constructs being confirmed by sequencing. For the VP16-cMaf, VP16-Prox1, and VP16-Sox1 constructs, primers for *c-maf* (5′-GTAGGTACC-GCTTCAGAACTGGCAATGAAC and 5′-GTAGCTAGCTGGGATCGC-GTGTCACACTCA), *Prox-1* (5′-GATGGTACCCCTGACCATGACAGCA-CAGCC and 5′-CCAAGGGGGAATTCTTCTATCCATACATTC), and *Sox-1* (5′-GCGGGTACCTACAGCATGATGATGGAGACC and 5′-GCG-GAATTCCTAGATGTGCGTCAGGGGCAC) were used to amplify the entire coding regions (except for the ATG) from cDNA clones. The PCR products were digested with *Asp*718/*Nhe*I and cloned in-frame into the pCMX-VP16-N vector. For the mammalian two-hybrid assays, full-length human p300 or domains of p300 were cloned into pCMX-Gal4-N using restriction digests or PCR amplification with Pfu DNA polymerase to generate the required DNA fragments. All constructs were confirmed by sequencing. For the c-Maf-HAT construct, primers 5′-TTAGGTACCGCTTCAGAACTGGCAATGAAC and 5′-GCGGAATT-CCATGAAAAATTCGGGAGAGG were used to amplify the c-*maf* coding sequence without the stop codon. The PCR product was digested with *Asp*718/*Eco*RI and cloned into the pCMX-D vector. The p300 HAT domain (amino acids 1190–1970) was amplified using primers 5′-GC-GGAATTCACTTATTACAGTTACCAGAACAGG AND 5′-CTCGAGTCG-ACCAGGTGGGTTCATACCCATGGG. The PCR product was digested with *Eco*RI/*Sal*I and ligated in-frame to the 3′ end of c-*maf*.

[2] D. D. Moore, unpublished data.

Plasmids for Immunoprecipitation—A start codon followed by two "Myc" tag sequences (EQKLISEEDL) was placed in-frame and upstream from c-Maf, Prox-1, or Sox-1 coding sequences in the pSG5 (Stratagene) vector. For the vector pSG5-p300-F2, two "FLAG" tag sequences (DYKDDDDK) followed by a stop codon were placed in-frame and downstream from the human p300 cDNA sequence corresponding to amino acids 1–2378.

Control Plasmids—The control plasmids pCMX-β-gal and TK-growth hormone have been described elsewhere (23–25).

Cell Culture, Transient Transfections, and Mammalian Two-hybrid Assays

COS-1 and HLEC-B3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum overnight at 37 °C before transfection. For transient transfections, 200 ng of a luciferase reporter were co-transfected with 50 ng of pCMX-β-gal control plasmid plus 20–320 ng of one or more of the transcription factor plasmids per well of a 24-well tissue culture dish unless otherwise indicated. For the mammalian two-hybrid assays, 200 ng of pG5E1b-Luc reporter plasmid, 50 ng of TK-growth hormone control plasmid, and 100 ng each of the pCMX-Gal4-N and pCMX-VP16-N chimeric expression plasmids were used per well of a 24-well tissue culture dish. Plasmid DNAs were mixed with 2.5 M $CaCl_2$ and then added to BES-buffered saline. The DNA mixtures were added to the cells in 1 ml of fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After incubation for 8–12 h, the cells were washed with phosphate-buffered saline, then incubated with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 24–36 h prior to harvesting and assaying for luciferase activity on a Dynex MLX luminometer as described (27). The β-gal activity was assayed using the Tropix β-galactosidase kit according to the manufacturer's instructions (Tropix). Growth hormone secretion was assayed using the human growth hormone transient gene expression kit according to the manufacturer's instructions (Nichols Institute). Luciferase expression was normalized to the β-gal or growth hormone control. Transfections were done in triplicate and were replicated with similar results in at least three independent experiments. Results are the mean ± S.D. from three individual experiments. All statistical analyses were performed with two-tailed Student's *t* tests using Microsoft Excel software. Data were considered to be significantly different for $p < 0.05$.

Co-immunoprecipitation

COS-1 cells in 10-cm plates were transfected with Myc-tagged c-Maf, Prox-1, or Sox-1 plus FLAG-tagged p300 and/or full-length CBP in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were collected 36 h after transfection. Nuclear extracts were prepared following the protocol of Schrieber *et al.* (28). In addition, lenses from newborn FVB mice were collected and sonicated in protein lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitors (Mixture Tablets, from Roche Molecular Biochemicals, one tablet in 15–20 ml solution), 5 mM EDTA, pH 8.0, 10 mM EGTA, pH 8.0), then centrifuged to harvest the supernatant. The lens lysates (2 mg) and the transfected COS-1 cell nuclear extracts (1 mg) were precleared by addition of 50 μl of protein G-agarose beads (1:1 dilution in RIPA wash buffer containing 50 mM Tris-HCl, 0.1% Nonidet P-40, 1 mM EDTA, 67

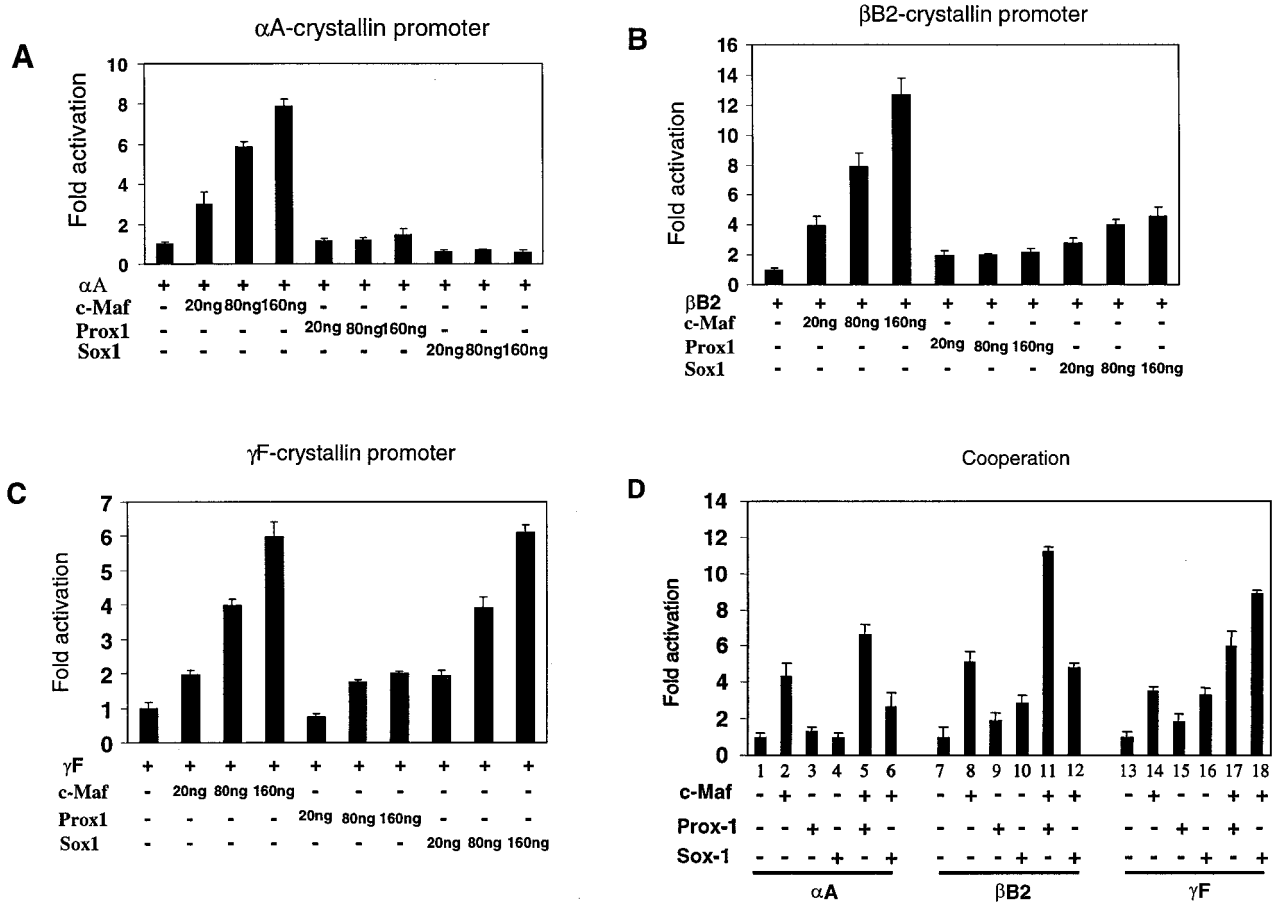

FIG. 2. **Transactivation of crystallin promoters by c-Maf, Prox-1, and Sox-1.** *A–C*, COS-1 cells were transfected with αA-, βB2-, or γF-crystallin reporters and plasmids encoding c-Maf, Prox-1, or Sox-1. Expression of c-Maf activated all three promoters. There was no significant αA promoter transactivation by Prox-1 or Sox-1 ($p > 0.05$) (*A*). Prox-1 activated βB2- and γF-crystallin promoters about 2-fold ($p < 0.01$) (*B* and *C*). Sox-1 significantly transactivated the γF reporter in a dose-dependent manner (*C*), and also activated the βB2 promoter ($p < 0.05$) (*B*). *D*, COS-1 cells were co-transfected with each crystallin reporter and 60 ng of the plasmids encoding c-Maf, Prox-1, and Sox-1 as indicated. Co-expression of Prox-1 and c-Maf enhanced transactivation of the three promoters (*lanes 5*, *11*, and *17*) ($p < 0.05$, $p < 0.001$, and $p < 0.05$), especially βB2 (*lane 11*). Similarly, co-expression of Sox-1 with c-Maf significantly increased γF promoter activation (*lane 18*) ($p < 0.001$), but repressed αA promoter activity (*lane 6*) ($p < 0.01$). Fold activation is expressed relative to the Luc/β-gal ratio seen after co-transfection of each specific reporter (αA, βB2, or γF) with the empty pCMX-D expression vector, which was arbitrarily set at 1.

mM NaCl). After centrifugation, supernatants were collected and incubated with mouse monoclonal anti-p300 antibody (Sigma), anti-CBP antibody (Santa Cruz, A-22), anti-FLAG M2 monoclonal antibody (Sigma), or anti-Myc monoclonal antibody (Roche Molecular Biochemicals). After a 3-h incubation at room temperature, 50 μl of protein A- or G-agarose beads (1:1 dilution in RIPA buffer) were added and incubated overnight at 4 °C. After washing three times in RIPA buffer, bound proteins were eluted with 2 × SDS sample buffer. Proteins were separated by SDS-PAGE (4–15% Tris-HCl), and transferred onto polyvinylidene difluoride membranes (Invitrogen), followed by immunoblotting with anti-c-Maf (Santa Cruz), anti-CBP, anti-FLAG, or anti-Myc antibodies (1:1000 dilution) and detection by enhanced chemiluminesence (ECL) (Amersham Pharmacia Biotech). After SDS-PAGE separation, the proteins were also visualized by Coomassie Brilliant Blue staining.

RESULTS

Transactivation of Crystallin Promoters by c-Maf, Prox-1, and Sox-1—Our previous transgenic studies showed that lens-specific expression of adenoviral E1A proteins that bind to CBP/p300 causes inhibition of lens fiber cell differentiation and nearly complete loss of crystallin expression (19). Although the lens phenotypes resembled the c-*maf* knockout mice (9), the expression of c-*maf* in lens fiber cells expressing the E1A transgene was not affected (19), suggesting that c-Maf-mediated transactivation of crystallin genes may require CBP/p300 co-activation. Previous studies have shown that *Prox-1* and *Sox-1*, like c-*maf*, are expressed at early stages of lens development, and are required for specific crystallin (γ-crystallin) expression (10, 11). We therefore postulated that expression of lens-specific crystallin genes might require interactions of ocular transcription factors with the co-activators CBP and p300.

To test this prediction, we designed experiments to compare the abilities of c-Maf, Prox-1, and Sox-1 to transactivate crystallin promoters. c-Maf has previously been shown to transactivate the γF-crystallin promoter in a cell culture system (29). An electrophoretic mobility shift assay showed that c-Maf could also bind to DNA fragments from the mouse βB2- and αA-crystallin promoters (9). Putative MAREs have been identified in the mouse αA- (position −110/−98), βB2- (position −110/−98), and γF- (position −47/−35) crystallin promoters (9, 30–32). Based on these studies, we subcloned mouse αA, βB2, and γF promoter DNA fragments into the pGL3 basic luciferase vector (Fig. 1) for transient transfection studies. Monkey kidney cells (COS-1) were co-transfected with each crystallin reporter (Fig. 1) and with plasmids encoding the different transcription factors. As predicted, c-Maf transactivated the three promoters in a dose-dependent fashion (Fig. 2, *A–C*). Sox-1 transactivated the γF-crystallin promoter in a dose-dependent fashion, similar to c-Maf (Fig. 2*C*). Sox-1 also stimulated the βB2 promoter, but less strongly than c-Maf (Fig. 2*B*). Prox-1 (80 ng) stimulated the βB2- and γF-crystallin promoters only about 2-fold (Fig. 2, *B* and *C*). To determine whether these

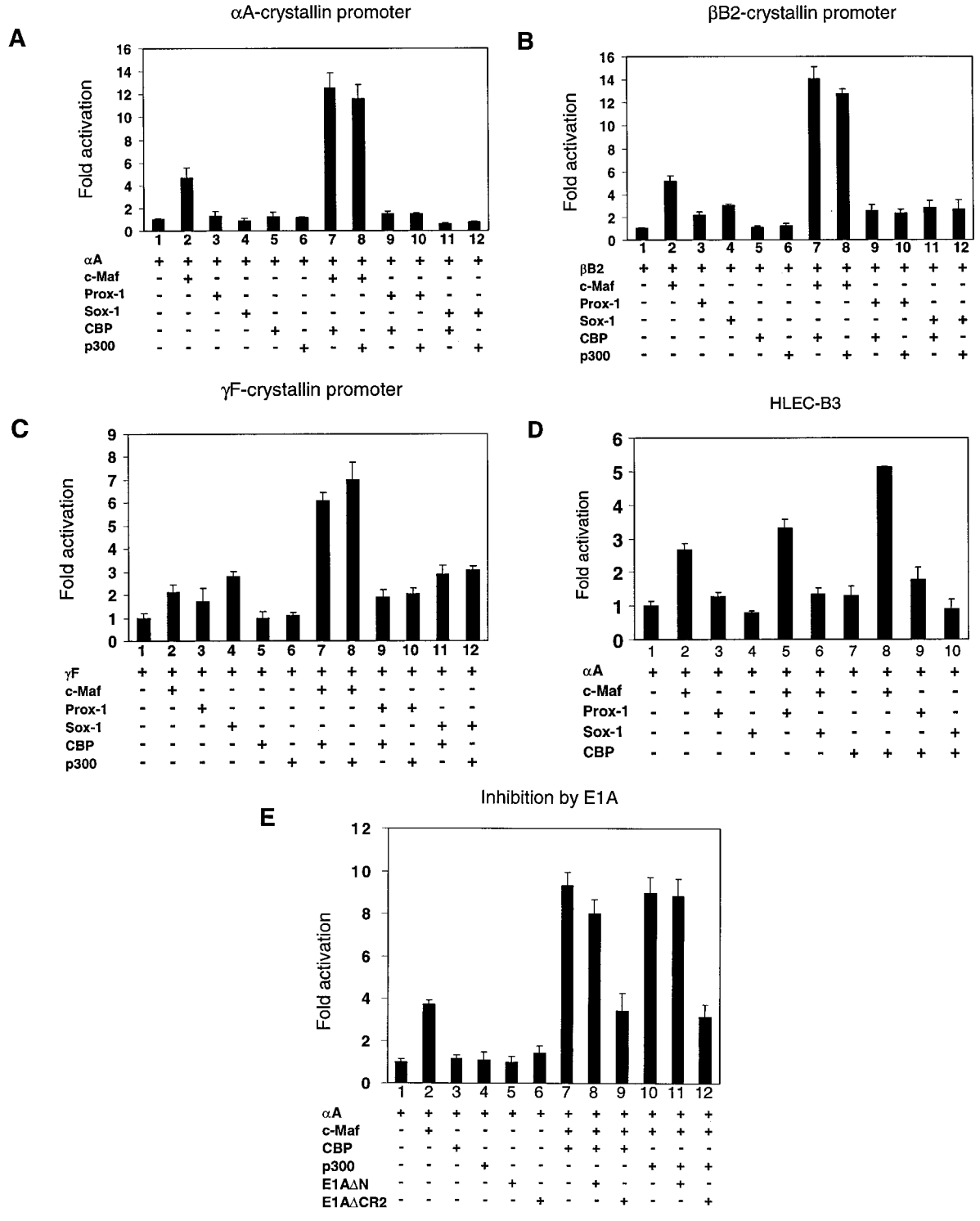

FIG. 3. **CBP/p300 co-activation.** *A–C*, COS-1 cells were co-transfected with each crystallin reporter and 60 ng each of the plasmids encoding c-Maf, Prox-1, or Sox-1 in the presence or absence of CBP or p300. Expression of CBP or p300 significantly increased (*i.e.* co-activated) c-Maf-dependent activation of each crystallin promoter (about 3-fold) (*lanes 7* and *8* in *A–C*). None of the other transcription factors gave more than a 2-fold increase in promoter activity, even in the presence of CBP or p300. *D*, human lens epithelial cells (B3) were co-transfected with the αA-crystallin reporter and 60 ng each of the plasmids encoding c-Maf, Prox-1, or Sox-1 in the presence or absence of CBP. We observed stimulation of the αA promoter by c-Maf (3-fold) (*lane 2*). Co-activation was seen with Prox-1 plus c-Maf and with CBP plus c-Maf co-transfections (*lanes 5* and *8*) ($p < 0.05$). CBP did not significantly co-activate Prox-1 or Sox-1 activity ($p > 0.05$) (*lanes 9* and *10*). Co-expression of Sox-1 with c-Maf repressed the c-Maf-mediated αA promoter activation ($p < 0.001$) (*lane 6*). *E*, COS-1 cells were co-transfected with αA reporter and c-Maf, CBP, or p300, plus or minus two E1A constructs (E1AΔN or E1AΔCR2). Co-expression of E1AΔCR2, which binds to CBP/p300, blocks CBP or p300 co-activation of the αA promoter ($p < 0.001$) (*lanes 9* and *12*). Fold activation is expressed relative to the Luc/β-gal ratio obtained after co-transfection of the specific reporter (αA, βB2, or γF) with the empty pCMX-D expression vector, which is arbitrarily set at 1.

transcription factors can collaborate to transactivate crystallin promoters, co-transfections were done using 60 ng of each transcription factor. We observed cooperative transactivation using Prox-1 plus c-Maf, especially for the βB2 reporter (Fig. 2*D*, *lanes 5*, *11*, and *17*) ($p < 0.05$). Co-expression of Sox-1 with c-Maf significantly increased γF promoter activation (Fig. 2*D*,

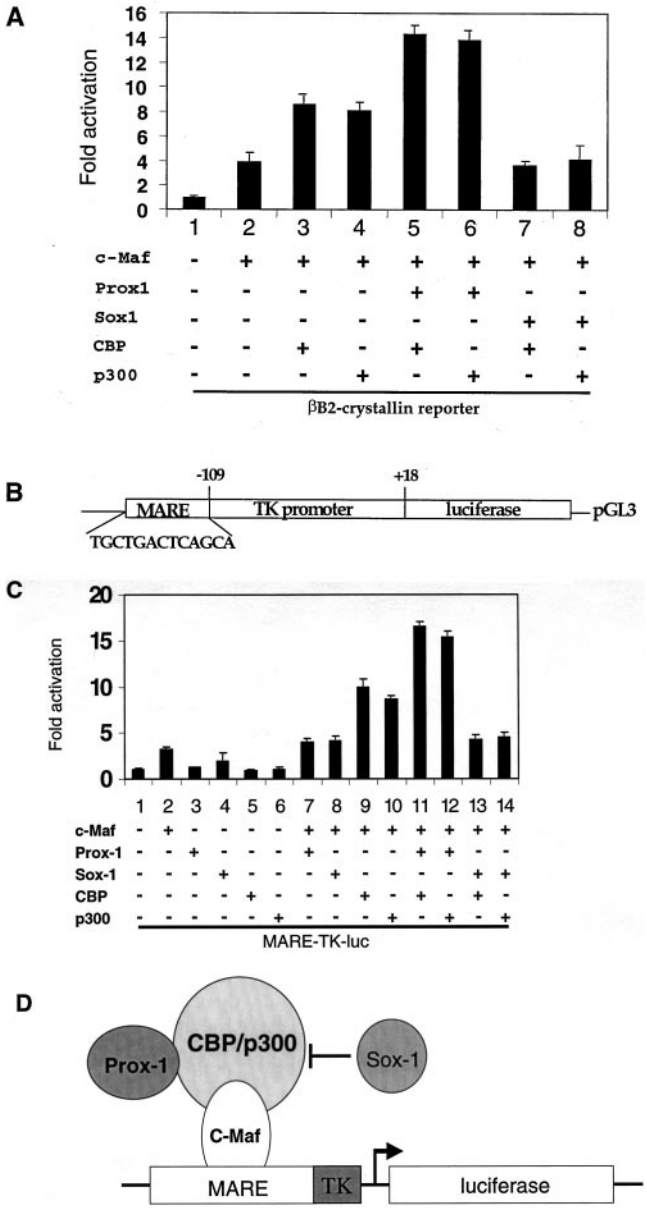

FIG. 4. **Interactions between Prox-1 or Sox-1 and c-Maf·CBP/p300.** *A*, COS-1 cells were co-transfected with the βB2 reporter and 60 ng each of the plasmids encoding c-Maf, Prox-1, Sox-1, CBP, and/or p300. Expression of Prox-1 enhanced CBP/p300-mediated co-activation ($p < 0.01$) (*lanes 5* and *6*), whereas co-expression of Sox-1 repressed the co-activation ($p < 0.01$) (*lanes 7* and *8*). *B*, diagram of the MARE-TK-Luc construct. *C*, COS-1 cells were co-transfected with this reporter and 60 ng each of the plasmids encoding c-Maf, Prox-1, or Sox-1 in the presence or absence of CBP or p300. Prox-1 was found to increase co-activation by c-Maf·CBP ($p < 0.05$) (*lane 11*) or c-Maf·p300 ($p < 0.05$) (*lane 12*). In contrast, Sox-1 inhibited co-activation ($p < 0.01$) (*lanes 13* and *14*). Fold activation is expressed relative to the Luc/β-gal ratio obtained after co-transfection of the specific reporter (βB2 or MARE-TK) with the empty pCMX-D expression vector, which is arbitrarily set at 1. *D*, diagrammatic representation of MARE-TK-Luc regulation.

lane 18) ($p < 0.001$), but Sox-1 repressed c-Maf stimulation of the αA promoter (*lane 6*, $p < 0.01$). Except for activation of βB2 by c-Maf and Prox-1, the co-activation did not significantly exceed the sum of the activations by each individual transcription factor, suggesting a general absence of synergistic interaction.

CBP and p300 Enhance c-Maf-mediated Transactivation of Crystallin Genes—To assay for CBP/p300 co-activation of crystallin promoter activity, COS-1 cells were co-transfected with each of the three crystallin reporters and plasmids encoding c-Maf, Prox-1, or Sox-1 in the presence or absence of CBP or p300. Although CBP or p300 alone did not stimulate any of the crystallin promoters (Fig. 3, *A–C*, *lanes 5* and *6*, $p > 0.05$), both proteins enhanced (or co-activated) c-Maf-dependent crystallin promoter activity for all three promoters (*lanes 7* and *8*, $p < 0.01$). In contrast, expression of CBP or p300 did not significantly alter the promoter activation induced by Prox-1 or Sox-1 ($p > 0.05$) (Fig. 3, *A–C*). Human lens epithelial cell line B3 (HLEC-B3) was also used for transient transfection assays (Fig. 3*D*). Synergistic co-activation of the αA-crystallin promoter was seen upon transfection with CBP plus c-*maf* ($p < 0.01$, *lane 8*). Stimulation of the promoter also occurred with c-Maf plus Prox-1 ($p < 0.05$, *lane 5*). Co-activation was minimal for the other combinations of factors. In addition, COS-1 cells were co-transfected with the αA-crystallin promoter and c-*maf*, CBP, or p300, plus two versions of the E1A gene of adenovirus: E1AΔN (which does not bind to CBP/p300) and E1AΔCR2 (which binds to and inhibits both CBP and p300) (19). Expression of E1AΔCR2 completely blocked co-activation of the αA-crystallin promoter ($p < 0.001$, Fig. 3*E*, *lanes 9* and *12*).

Prox-1 Enhances CBP/p300 Co-activation of the βB2 Promoter, whereas Sox-1 Represses Co-activation—Since Prox-1 and c-Maf can collaborate to stimulate the βB2 promoter (Figs. 2*D* and 3*D*), we tested the effects of Prox-1 and Sox-1 on c-Maf/CBP/p300 co-activation of this promoter (Fig. 4). Prox-1 stimulated co-activation ($p < 0.01$, compare *lanes 5* and *6* to *lanes 3* and *4* in Fig. 4*A*), whereas Sox-1 significantly inhibited CBP/p300 co-activation of c-Maf ($p < 0.001$) (*lanes 7* and *8*). To test whether these interactions required Prox-1- or Sox-1-binding sites, an artificial luciferase reporter (MARE-TK-Luc) was generated by inserting a c-Maf-binding site (TGCTGACTCAGCA) upstream of the TK promoter (Fig. 4*B*). When COS-1 cells were co-transfected with the MARE-TK-Luc reporter and plasmids encoding c-Maf, Prox-1, Sox-1, and CBP or p300, we again observed stimulation of co-activation by Prox-1 ($p < 0.05$, Fig. 4*C*, compare *lanes 11* and *12* to *lanes 9* and *10*), but inhibition by Sox-1 ($p < 0.001$, *lanes 13* and *14*). Prox-1 alone did not activate this reporter (Fig. 4*C*, *lane 3*), indicating that Prox-1 does not co-activate by binding directly to the MARE. Instead, our data suggest that Prox-1 can be indirectly recruited to MARE by the c-Maf·CBP/p300 complex, as shown in Fig. 4*D*. These results indicate, but do not prove, that c-Maf and Prox-1 may bind to CBP/p300 simultaneously.

Binding of CBP/p300 to c-Maf and Prox-1—Since CBP and p300 have been shown to bind to numerous sequence-specific transcription factors (16), we tested whether c-Maf, Prox-1, or Sox-1 can bind directly to CBP/p300 by co-immunoprecipitation. When COS-1 cells were co-transfected with Myc-tagged c-Maf and FLAG-tagged p300, antibodies to either Myc or FLAG were able to co-precipitate the two proteins (Fig. 5*A*, *lane 4*). Similar results were obtained when COS-1 cells were co-transfected with Myc-tagged c-Maf and full-length CBP (Fig. 5*B*). When COS-1 cells were co-transfected with Myc-tagged Prox-1 or Myc-tagged Sox-1 plus FLAG-tagged p300, the anti-FLAG antibody was able to co-precipitate Prox-1 but not Sox-1 (Fig. 5*C*, *bottom panel*, *lanes 6* and *5*, respectively). To investigate whether c-Maf normally is bound to CBP/p300 in the lens, co-immunoprecipitations were performed using newborn mouse lens lysates. As shown in Fig. 5*D*, anti-p300 antibody precipitated a c-Maf protein.

To further analyze the binding of c-Maf and Prox-1 to p300, a mammalian two-hybrid assay was established (Fig. 6). The DNA-binding domain of Gal4 was fused to full-length p300 and to several subregions of p300. At the same time, full-length c-Maf, Prox-1, and Sox-1 were linked to the transactivation domain of the herpes simplex virus protein VP16. The reporter construct (G5E1b-Luc) contains an E1b promoter cloned down-

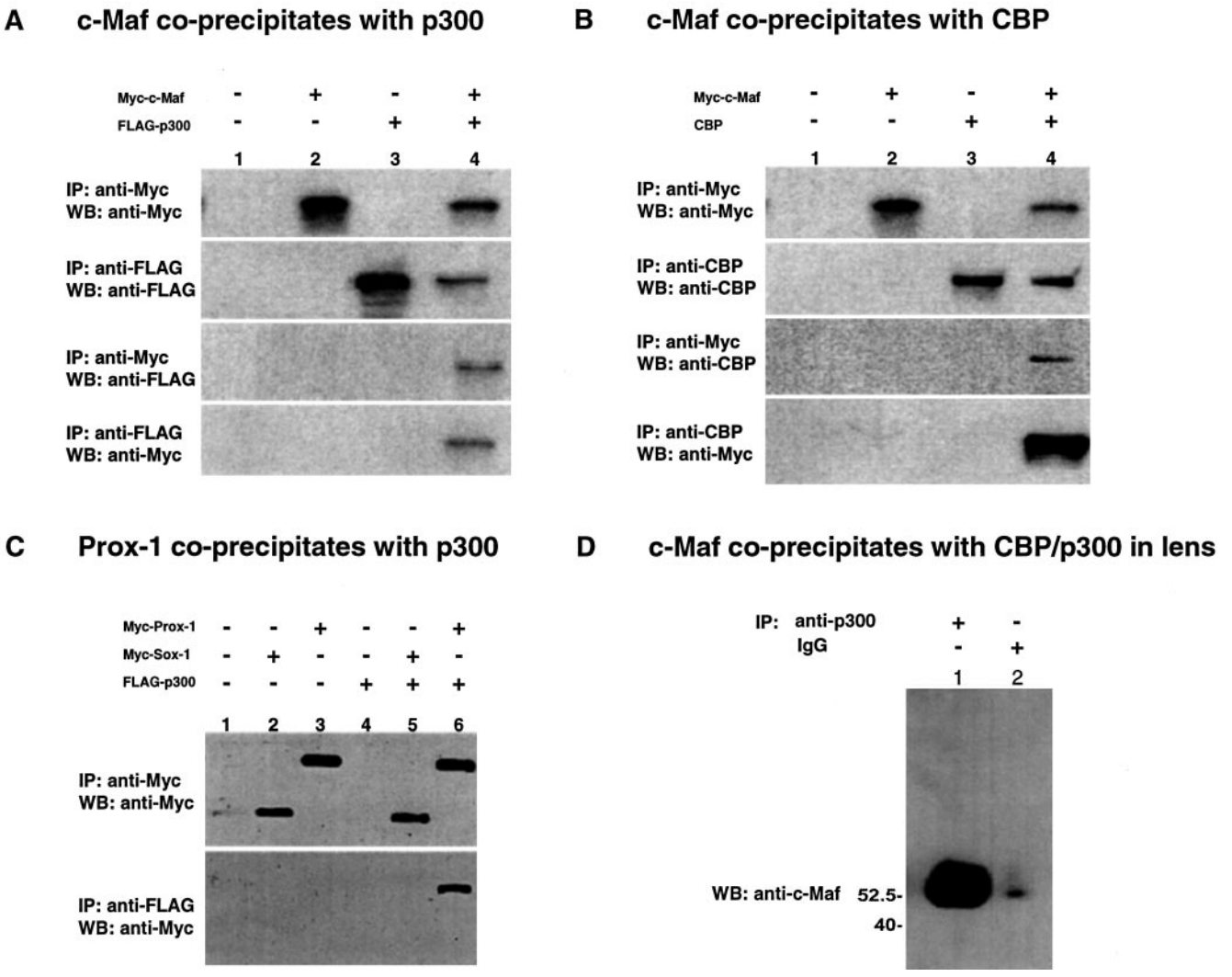

FIG. 5. **c-Maf and Prox-1 bind to CBP/p300.** *A*, co-immunoprecipitation of c-Maf with p300. *Lane 1*, untransfected COS-1 cell nuclear extract as a negative control; *lane 2*, cells were transfected with Myc-tagged c-Maf; *lane 3*, cells were transfected with FLAG-tagged p300; *lane 4*, cells were co-transfected with Myc-tagged c-Maf and FLAG-tagged p300. Nuclear extracts were immunoprecipitated (*IP*) with anti-Myc or anti-FLAG antibody, the complexes were analyzed by SDS-PAGE and Western blotting (*WB*) using anti-Myc or anti-FLAG antibodies as denoted in the figure. *B*, co-immunoprecipitation of c-Maf with CBP. *Lane 1*, untransfected COS-1 cell nuclear extract; *lane 2*, cells were transfected with Myc-tagged c-Maf; *lane 3*, cells were transfected with full-length CBP; *lane 4*, cells were co-transfected with Myc-tagged c-Maf and full-length CBP. Nuclear extracts were immunoprecipitated (*IP*) with anti-Myc or anti-CBP antibodies and immunocomplexes were separated by SDS-PAGE. Western blots (*WB*) were detected with anti-Myc or anti-CBP antibodies as denoted in the figure. *C*, co-immunoprecipitation of Prox-1 but not Sox-1 with p300. *Lane 1*, untransfected COS-1 cell nuclear extract; *lane 2*, cells were transfected with Myc-tagged Sox-1; *lane 3*, cells were transfected with Myc-tagged Prox-1; *lane 4*, cells were transfected with FLAG-tagged p300; *lane 5*, cells were co-transfected with Myc-tagged Sox-1 and FLAG-tagged p300; *lane 6*, cells were co-transfected with Myc-tagged Prox-1 and FLAG-tagged p300. Nuclear extracts were immunoprecipitated (*IP*) with anti-Myc or anti-FLAG antibodies. Western blots were detected with anti-Myc antibody. *D*, co-immunoprecipitation of c-Maf with CBP/p300 in mouse lens tissue. *Lane 1*, lens lysate (2 mg) from newborn FVB mice was immunoprecipitated with anti-p300 antibody (Sigma, also recognizes CBP protein); *lane 2*, lens lysate (2 mg) from newborn FVB mice was immunoprecipitated with mouse IgG as a control. Immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-c-Maf antibody.

stream of five copies of the Gal4-binding site and upstream from a luciferase reporter (Fig. 6*A*). Since p300 alone does not transactivate this promoter, expression of the reporter requires interaction between p300 and c-Maf, Prox-1, or Sox-1 to recruit the VP16 transactivator to the vicinity of the promoter. As shown in Fig. 6*B*, Gal4-p300 plus VP16 (without c-Maf) slightly induced transcription (*lane 3*). When Gal4-p300 was co-expressed with VP16-c-Maf (*lane 7*), a dramatic increase in luciferase expression was obtained. Co-expression of VP16-c-Maf with Gal4-p300 (1–1235), which has the amino-terminal half of p300 fused to Gal4, was also able to increase transcriptional activity (*lane 8*). Co-expression of Gal4-p300 (1256–2415) with VP16-c-Maf showed minimal activation (*lane 9*). Subregions of p300 containing amino acids 1–150, 180–662, and 595–1068 were therefore linked to Gal4 and tested (Fig. 6*C*). When Gal4-p300 (180–662) was co-expressed with VP16-c-Maf (*lane 8*), we observed a dramatic increase in transcriptional activity. Thus, c-Maf binds to the region of p300 that contains the first zinc finger and the CREB-binding domain (Fig. 6*D*).

By comparison, when COS-1 cells were co-transfected with VP16-Prox-1 or VP16-Sox-1 plus Gal4-p300 (full-length), expression of Prox-1 (Fig. 7, *lane 8*) but not Sox-1 (Fig. 7, *lane 9*) dramatically increased promoter activation, confirming that Prox-1 but not Sox-1 can directly bind to full-length p300. When the cells were co-transfected with VP16-Prox-1 and various subregions of p300 (1–1235, 1256–2415, and 1190–1966) fused to Gal4, we did not observe significant changes in promoter activation (Fig. 7, *lanes 13–15*), suggesting that the binding of Prox-1 to p300 may be dependent upon the configuration of full-length p300.

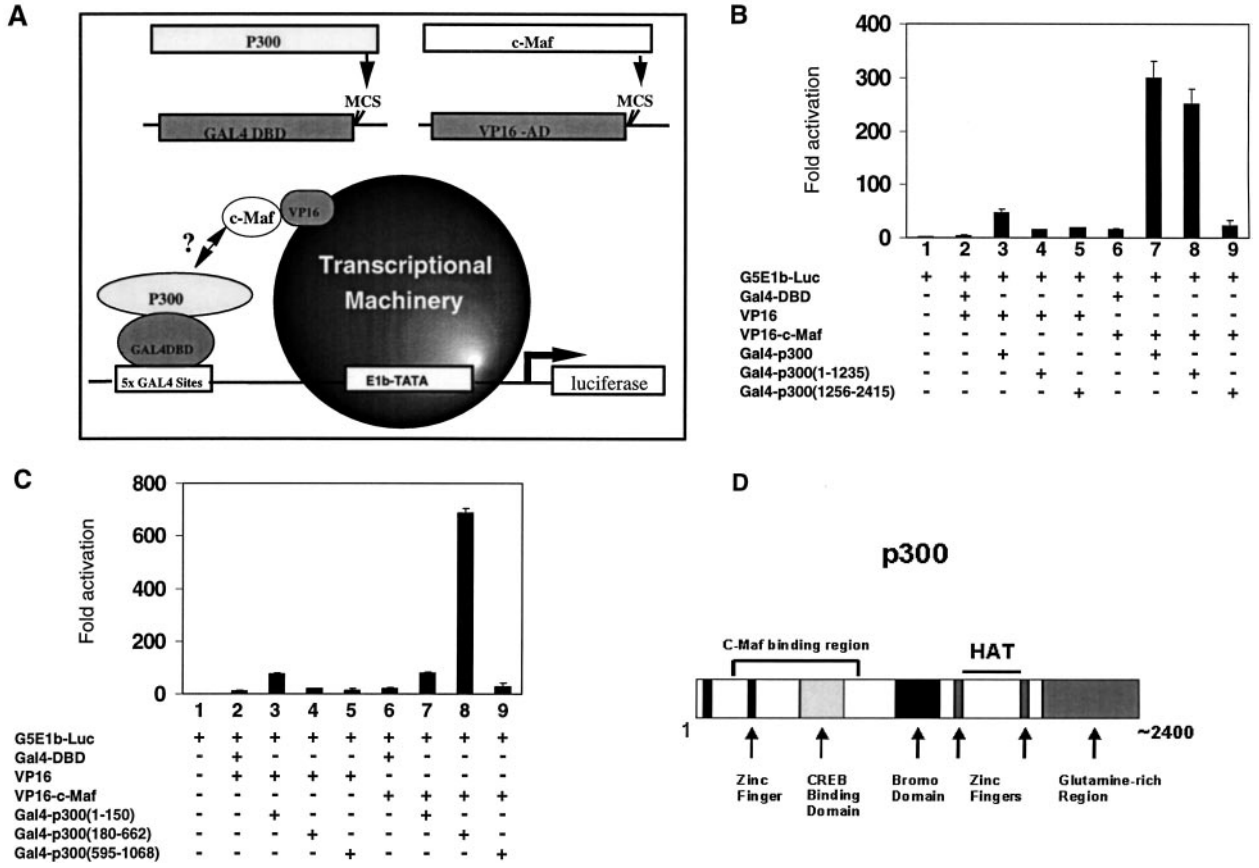

FIG. 6. **Binding of c-Maf to a specific region of p300.** *A*, diagrammatic representation of our mammalian two-hybrid assay. COS-1 cells were co-transfected with a pG5E1b-luciferase reporter plus plasmids encoding the Gal4 DNA-binding domain (*DBD*) either alone or fused to various regions of p300, and with a plasmid expressing the VP16 acidic activation domain (*AD*) either alone or fused to c-Maf. Expression of the luciferase reporter is dependent upon interaction between Gal4-p300 and VP16-c-Maf chimeras to recruit VP16 to the E1b-TATA promoter to initiate transcription. *B*, co-expression of full-length Gal4-p300 (*lane 7*) or the amino terminus of p300 (amino acids 1–1235) (*lane 8*) together with VP16-c-Maf significantly increased luciferase activity. *C*, VP16-c-Maf interacts specifically with Gal4-p300-(180–662) (*lane 8*), which includes the first zinc finger domain and the CREB-binding domain of p300 (shown in *D*). Fold activation is expressed relative to luciferase/growth hormone activity obtained after co-transfection of the specific reporter (G5E1b reporter) and an empty expression vector. *D*, schematic drawing of p300.

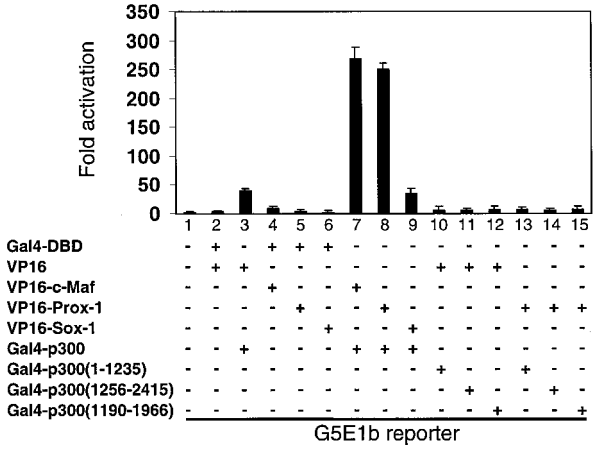

FIG. 7. **Binding of Prox-1 to p300.** Similar to c-Maf (*lane 7*), co-expression of VP16-Prox-1 but not VP16-Sox-1 together with full-length Gal4-p300 dramatically increased the luciferase activity in the mammalian two-hybrid assay (*lanes 8* and *9*). However, co-expression of VP16-Prox-1 with various subregions of p300 (1–1235, 1256–2415, and 1189–1966) fused to Gal4 did not activate the reporter (*lanes 13–15*). Fold activation is expressed relative to luciferase/growth hormone activity obtained after co-transfection of the specific reporter (G5E1b reporter) and empty expression vector.

CBP/p300 Co-activation Requires HAT Activity—To ascertain whether the HAT activity of CBP/p300 is essential for co-activation of the crystallin genes, we constructed plasmids encoding two different mutations of p300. The p300 mut1504 clone has a point mutation in the HAT domain (alanine codon substituted for a tyrosine codon at amino acid 1504), whereas Δ1430–1504 has a deletion in the HAT domain (from amino acids 1430 to 1504). Mut1504 corresponds to a point mutation in CBP (mutation 1541) found to eliminate HAT activity (33). The Δ1430–1504 deletion removes several important domains of p300 that correspond to motifs required for CBP HAT activity (33). Co-expression of c-Maf with either p300 mut1504 or Δ1430–1504 produced no co-activation of the γF-crystallin promoter or the MARE-TK promoter (Fig. 8, *A* and *B*, *last two lanes*). To test whether the HAT activity of CBP/p300 is sufficient to co-activate c-Maf, we constructed a plasmid encoding a c-Maf-HAT fusion protein (c-*maf* coding region fused with the HAT domain of p300). We found that expression of the c-Maf-HAT fusion protein activated the γF-crystallin and MARE-TK promoters to the same extent as c-Maf plus p300 (Fig. 8, *C* and *D*, *two lanes* on the *right*), indicating that histone acetylation is sufficient to enhance c-Maf activation of crystallin gene expression.

DISCUSSION

We have used a cell culture system to begin to study the mechanism(s) by which CBP and p300 become involved in crystallin promoter activation. We demonstrate that representative crystallin promoters are transactivated by the lens-specific c-Maf transcription factor, and that the promoter activity can be significantly enhanced by the co-activators CBP and/or

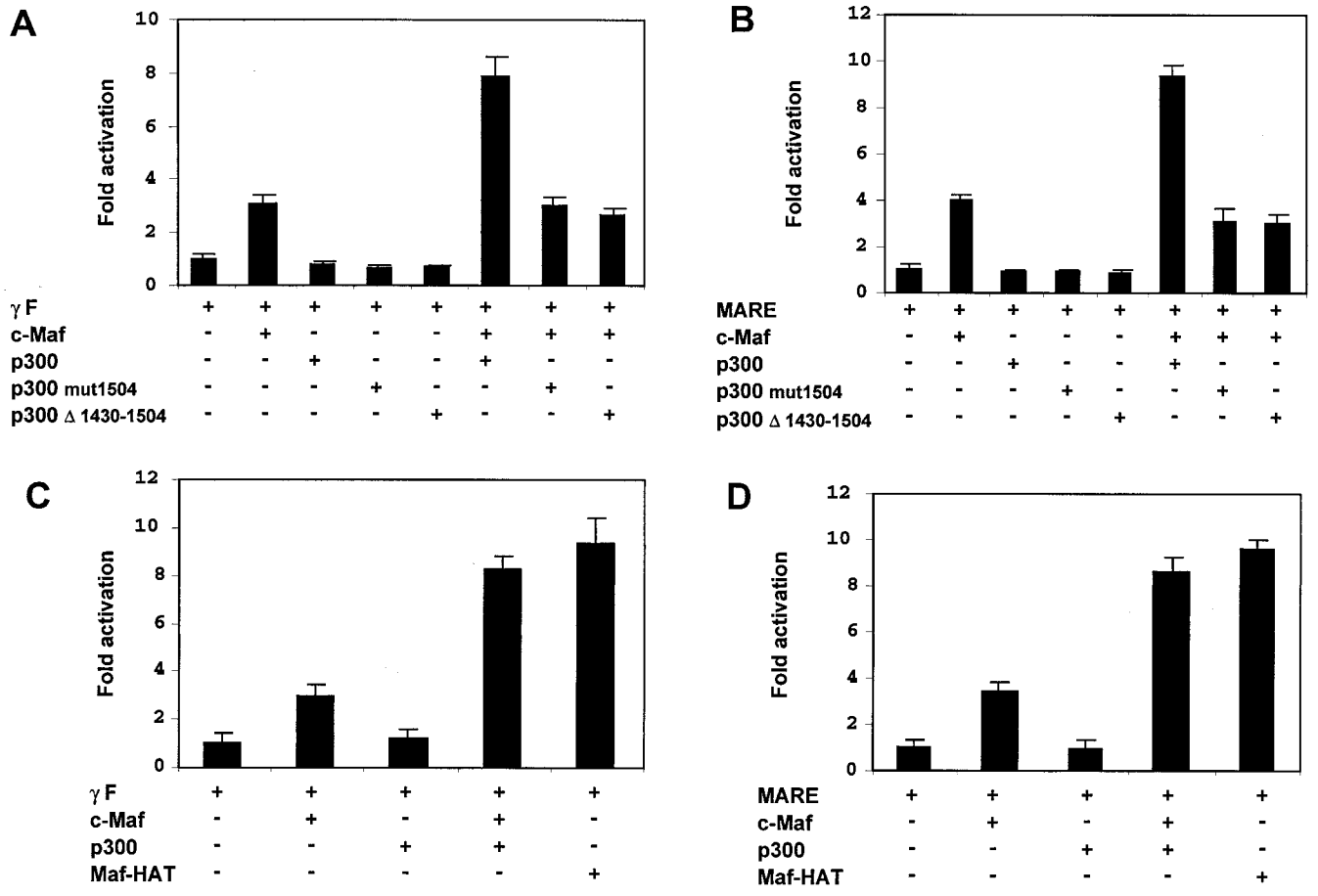

FIG. 8. **CBP/p300 co-activation requires HAT activity.** *A* and *B*, COS-1 cells were co-transfected with the γF-crystallin reporter or the MARE-TK-luc reporter plus plasmids encoding c-Maf, full-length p300, p300 with a point mutation (*mut1504*), or p300 with a deletion (Δ*1430–1504*) in the HAT domain. The p300 mutants did not co-activate either reporter. *C* and *D*, COS-1 cells were co-transfected with the γF-crystallin reporter or the MARE-TK-luc reporter and plasmids encoding c-Maf, full-length p300, or c-Maf-HAT (encoding c-Maf fused with the HAT domain of p300). Expression of the c-Maf-HAT fusion protein was sufficient to co-activate both reporters ($p < 0.001$). Fold activation is expressed relative to luc/βgal activity obtained after co-transfection of the specific reporter (γF or MARE-TK) and an empty expression vector.

p300. Co-activation is mediated by binding of c-Maf to a specific domain of p300 (and presumably also of CBP). In addition, co-activation involves histone acetyltransferase activity and can be influenced by Prox-1 and Sox-1. Our results indicate that high levels of crystallin gene expression in the lens are dependent upon binding of c-Maf plus CBP and/or p300 to specific crystallin promoters, followed by localized histone acetylation and, we predict, consequent chromatin remodeling.

Regulation of αA-Crystallin—In the mouse, α-crystallins are first expressed at the lens vesicle stage. Both αA- and αB-crystallins are synthesized by lens epithelial and early fiber cells. At later stages αA-crystallin continues to be expressed in both cell types (although more strongly in early fiber cells), but αB-crystallin persists only in epithelial cells (34). Previous studies indicated that transcriptional regulation of the mouse αA-crystallin gene is dependent upon a cAMP-responsive element (DE1/CRE) and a Pax-6-binding site (30), indicating that Pax-6 plays a critical role in transactivation of the αA-crystallin gene. More recently, c-*maf* null mice were found to have defects of αA-crystallin expression (9), and a Maf responsive element (13) was found to be present in the αA-crystallin promoter region. Consistent with these later findings, our results indicate that expression of c-Maf significantly transactivates the αA-crystallin promoter. We also found that c-Maf can recruit the co-activators CBP/p300 to the αA-crystallin promoter. Although Pax-6 is required for αA-crystallin expression (30), the requirement may be indirect since recent evidence shows that c-*maf* expression can be up-regulated by Pax-6 (35). In our tissue culture system, we co-expressed Pax-6 plus c-Maf and CBP or p300 but did not see a significant further increase in promoter activity (data not shown). Therefore, we predict that Pax-6 does not directly interact with the c-Maf·CBP/p300 complex. Currently, there is no evidence that the 400-bp αA-crystallin promoter that we tested here contains Prox-1- or Sox-1-binding sites. We did not observe significant transactivation of the αA-crystallin promoter by Prox-1 or Sox-1. Interestingly, expression of Sox-1 significantly repressed c-Maf transactivation and c-Maf·CBP/p300 co-activation of the αA promoter ($p < 0.001$, Figs. 2*D* and 3*D*). It is unknown whether Sox-1 competes with c-Maf for binding to the αA-crystallin promoter.

Regulation of βB2-crystallin—The β-crystallins can be divided into βA- (A1, A2, A3, and A4) and βB- (B1, B2, and B3) crystallins, and are considered as early markers of lens fiber cell differentiation (7). Similar to the αA-crystallin promoter, a putative MARE has been identified in the βB2-crystallin promoter in both mouse and rat (9, 36). A recent study (37) indicates that c-Maf is not essential for the activity of the βB2-crystallin promoter in the rat lens. Instead, a putative Sox-binding site at −164/−159 and a positive element at −14/−7 seem to be the primary regulatory elements (37). In our transfection study, we observed weaker stimulation of the βB2 promoter by Sox-1 than by c-Maf. In fact, Sox-1 was found to block c-Maf·CBP/p300 mediated co-activation, suggesting that Sox-1 may not be a significant positive regulator of the mouse βB2-crystallin gene. We did find that c-Maf-mediated transactivation of the mouse βB2-crystallin promoter activity can be en-

hanced by Prox-1 (Fig. 2*D*, *lane 11*). Since this cooperative stimulation was significant with the MARE-TK-luc reporter ($p < 0.05$, Fig. 4*C*, *lane 7*), which does not have a Prox-1-binding site, and since Prox-1 can bind to CBP/p300, it suggests that Prox-1 may be recruited indirectly to the mouse βB2-crystallin promoter. Developmental regulation of the βB2-crystallin promoter may involve an additional level of complexity that is not reflected by our tissue culture assays. Previous studies have shown that expression of mouse βB2-crystallin mRNA is undectable at embryonic day 11.5 (E11.5) (9), and that mouse (and rat) βB2-crystallin protein levels are low prior to birth, but are up-regulated postnatally (38). It is not yet known whether protein levels reflect transcriptional or post-transcriptional changes during development. Although our current and previous studies (9, 19) indicate that c-Maf and CBP/p300 are necessary for high level mouse βB2 promoter activity, it is important to remember that the endogenous gene is likely to have additional important regulatory domain(s) that are not present in the promoter region that we tested here.

Regulation of γF-crystallin—The γ-crystallins are encoded by a cluster of six genes (γA-γF) (7). In the mouse, expression of γ-crystallins begins around embryonic day 13 (E13), and is restricted to maturing fiber cells in the lens (39). Previous loss-of-function studies showed that mice lacking Sox-1 have an almost complete absence of γ-crystallin expression (10). c-*maf* and *Prox-1* null mice also showed reduced expression of γ-crystallins (9, 11). A MARE and a Sox-1-binding site are present in the γF-crystallin promoter region (10, 29). Our experimental results show that either c-Maf or Sox-1 can significantly transactivate the γF-crystallin promoter, which is consistent with previous findings. In addition, we found that c-Maf and Sox-1 can have a cooperative interaction at this promoter (Fig. 2*D*). A recent study indicates that the γF promoter also contains a Prox-1 responsive element, and that expression of Prox-1 in CD5A (human lens epithelial) cells significantly enhances promoter activation (12). In our transfection studies using COS-1 and HLEC-B3 cell lines, we observed a slight, but significant ($p < 0.05$), transactivation of the γF-crystallin promoter by Prox-1. We did not observe CBP/p300 co-activation with either Prox-1 or Sox-1 (Fig. 3*C*), suggesting that Prox-1- or Sox-1-mediated transactivation of the γF-crystallin gene might use an alternative co-activator system. Given the fact that Sox-1 cooperates with c-Maf to enhance γF-crystallin but repress αA-crystallin promoter activity, our studies suggest that the patterns of crystallin gene expression in the lens are modulated by interactions between the lens-specific transcription factors and their co-activators.

CBP/p300 Co-activation—CBP and p300 are transcriptional co-activators that are often required for cell differentiation and tissue development. Since CBP and p300 have HAT activity (14, 15, 17), histone acetylation and consequent chromatin remodeling are considered to be important for gene activation (40, 41). Our previous transgenic studies have shown that c-Maf expression in the lens is not sufficient to activate β- and γ-crystallin expression in the absence of CBP/p300 function (19). Our current observations provide an explicit molecular model for the role of CBP/p300 in crystallin gene regulation, which is consistent with previous models for CBP/p300 function. Specifically, crystallin gene expression appears to be activated by sequence-specific binding by the c-Maf transcription factor, recruitment of the co-activators CBP/p300, and subsequent localized histone acetylation. Prox-1 and Sox-1 probably act to differentially modulate this activity for specific crystallin genes. Our studies do not establish the mechanism(s) by which Sox-1 can repress CBP/p300 co-activation. One possible explanation is that Sox-1 can recruit repressors to block c-Maf·CBP/p300 binding to the crystallin promoters. Another possible explanation is that Sox-1 may indirectly inhibit the HAT activity of CBP/p300. Although additional features of crystallin gene regulation surely exist, our studies pinpoint the interaction between c-Maf and CBP/p300 as being essential for the high levels of crystallin gene expression that are seen in the fiber cells of the ocular lens.

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CREB-binding Protein/p300 Co-activation of Crystallin Gene Expression

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