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Transcriptional Regulation of the Mouse αA-Crystallin Gene: Activation Dependent on a Cyclic AMP-Responsive Element (DE1/CRE) and a Pax-6-Binding Site

ALEŠ CVEKL,[1] FATAH KASHANCHI,[2] CHRISTINA M. SAX,[1] JOHN N. BRADY,[2] AND JORAM PIATIGORSKY[1]*

Laboratory of Molecular and Developmental Biology, National Eye Institute,[1] and Laboratory of Molecular Virology,[2] National Cancer Institute, Bethesda, Maryland 20892-2730

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Two *cis*-acting promoter elements (−108 to −100 and −49 to −33) of the mouse αA-crystallin gene, which is highly expressed in the ocular lens, were studied. Here we show that DE1 (−108 to −100; 5′TGACGGTG3′), which resembles the consensus cyclic AMP (cAMP)-responsive element sequence (CRE; 5′TGACGT[A/C][A/G]3′), behaves like a functional CRE site. Transfection experiments and electrophoretic mobility shift assays (EMSAs) using site-specific mutations correlated a loss of function with deviations from the CRE consensus sequence. Results of EMSAs in the presence of antisera against CREB, ΔCREB, and CREM were consistent with the binding of CREB-like proteins to the DE1 sequence. Stimulation of αA-crystallin promoter activity via 8-bromo-cAMP, forskolin, or human T-cell leukemia virus type I Tax_1 in transfections and reduction of activity of this site in cell-free transcription tests by competition with the somatostatin CRE supported the idea that DE1 is a functional CRE. Finally, Pax-6, a member of the paired-box family of transcription factors, activated the mouse αA-crystallin promoter in cotransfected COP-8 fibroblasts and bound to the −59 to −29 promoter sequence in EMSAs. These data provide evidence for a synergistic role of Pax-6 and CREB-like proteins for high expression of the mouse αA-crystallin gene in the lens.

Crystallins are defined as the major soluble proteins in the ocular lens (74). The α- and βγ-crystallins are present in all vertebrate lenses. The α-crystallins are encoded in two genes, αA and αB, which arose by duplication early in evolution. In the mouse, αA-crystallin constitutes about 25% of the total soluble proteins of the lens. Although the αA- and αB-crystallin polypeptides are closely related, their corresponding genes differ in their transcription regulatory elements. αA-crystallin is found almost exclusively in the lens, while αB-crystallin is also expressed in many nonlens tissues (e.g., heart, lung, brain, kidney, skeletal muscle, and other tissues) (5, 21, 63). Although both of the α-crystallin polypeptides are related to the small heat shock proteins (17, 37), only αB-crystallin can be induced by heat and other stresses (14, 37, 43). αA and αB have also been shown to be molecular chaperones (33) and to possess autokinase activity (40).

The molecular mechanisms regulating high lens expression of the crystallin genes are not well understood. Pax-6, a paired-domain- and homeodomain-containing protein, has been associated with eye development (65, 70). Mutations of Pax-6 result in eye abnormalities in different species; these include *small eye* in mice (31), aniridia in humans (26, 27, 47, 65), and *eyeless* in *Drosophila melanogaster* (57). Recently, we have found that Pax-6 is a critical transcription factor of the chicken αA-crystallin gene (11).

This report concerns the molecular basis for the expression of the mouse αA-crystallin gene (see reference 63 for a review). Initial transfection (10) and transgenic mouse (53, 71) experiments using the chloramphenicol acetyltransferase (CAT) reporter gene demonstrated lens-specific activity of the −111 to +46 promoter fragment of the mouse αA-crystallin gene. In contrast, the chicken αA-crystallin gene requires a larger promoter fragment (−162 to +77) for expression in the lens (42). In vivo and in vitro footprinting analysis revealed at least five protein binding sites within the −111 to +46 promoter fragment and suggested that the activity of the αA-crystallin gene is determined by the interactions of multiple factors (39). Mutagenesis experiments of the mouse αA-crystallin promoter revealed regulatory sequences located at −111 to −106 (called DE1) and −69 to −40. A zinc finger protein (αA-CRYBP1) binds to the αA-CRYBP1 site (−66 to −57) and appears to be differentially processed in a tissue-specific fashion (38, 51). The Pax-6 regulatory site at positions −57 to −40 in the chicken αA-crystallin gene and the apparent conservation of this region in the mouse and chicken αA-crystallin genes have raised the possibility that Pax-6 is used to express the αA-crystallin in both chickens and mice.

Sequence analysis of the mouse αA-crystallin promoter fragment also revealed that the DE1 regulatory element (−107 to −100; 5′CTGACGGTG3′) resembles the consensus sequence (5′GTGACGT[A/C][A/C]3′) for a cyclic AMP (cAMP)-responsive element (CRE), suggesting that promoter activity may be connected with a cAMP transduction pathway (16, 28, 29, 48). A considerable number of proteins forming the ATF/CREB subfamily of the basic domain-leucine zipper family of transcription factors (for recent reviews, see references 16 and 48) were found by their ability to interact with CREs. A role for CREB, ATF1, and CREM as fourth messengers in the cascade involving cAMP has been established (16, 29, 48, 49).

In this study, we tested a model for CRE- and Pax-6-binding sites in the mouse αA-crystallin promoter. Our data are consistent with such a model and implicate general mechanisms for high lens expression of crystallin genes.

* Corresponding author. Mailing address: LMDB, NEI, NIH, Bldg. 6, Rm. 201, Bethesda, MD 20892-2730. Phone: (301) 402-4343. Fax: (301) 402-0781.

MATERIALS AND METHODS

Plasmid constructions. Plasmids p−111/+46-CAT and p−88/+46-CAT were described previously under the names pαA111$_a$CAT and pαA88$_a$CAT, respectively (10). Plasmids containing point mutations within the −108 to −100 sequence were generated by PCR using mutated primers including *Hin*dIII flanking sites and −111/+46-CAT as a template. The DNA templates used for in vitro transcription assays were prepared by using pLovTATA digested with *Eco*RI and *Bgl*II (41). The subcloned oligonucleotides (top strand, 5′ to 3′) were (−111 to −84)2× (aattCTGCTGACGGTGCAGCCTCTCCCCCGAGCGCTGCTGACGGTGCAGCCTCTCCCCCGAGC), (−111 to −96)2× (aattCTGCTGACGGTGCAGCCTCTCTGCTGACGGTGCAGC), and (E4 ATF)2× (aattAAAATGACGTAACGGTCTAAAAAATGACGTAACGGT) (30). The lowercase letters specify the cloning sites. The internal control pMLCas190 has been described elsewhere (41).

Cell cultures, transfections, and CAT assays. The N/N1003A rabbit lens cell line (58) and COP-8 mouse fibroblasts (67) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 60 μg of gentamicin per ml. Cells were transfected by the calcium phosphate method, using 7.5 μg of the specified CAT construct and 2 μg of pCH110, which encodes β-galactosidase (Pharmacia, Piscataway, N.J.), per 60-mm-diameter dish (62). In the cotransfection experiments, pTax$_1$ (6, 52) or pKW10-Pax-6 (13) was present in amounts given in the figure legends. For Pax-6 cotransfection, mouse COP-8 fibroblasts were transfected with p−111/+46-CAT, pKW10-Pax-6, and pCMVβ (Clontech). Thin-layer chromatography CAT assays and β-galactosidase assays were performed as described elsewhere (10, 62). Acetylation reactions were performed in the range of 1 to 20% conversion of the substrate. CAT assays were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Transfections were performed in triplicate, and the average values from at least two experiments are shown. For the experiments with 8-bromo-cAMP (8-BrcAMP) or forskolin (Sigma, St. Louis, Mo.), transfected N/N1003A cells were grown for 36 h in medium containing 0.5% fetal calf serum in the presence of either 1 or 2 mM 8-BrcAMP or 20 μM forskolin (3).

Nuclear extracts, proteins, and EMSA. Nuclear extracts from 20 g of αTN4-1 mouse lens cells (75), 50 liters of HeLa cells (Cellex Biosciences, Minneapolis, Minn.), or ca. 5,000 newborn mouse lenses were prepared as described by Shapiro et al. (64). This method for preparing nuclear extracts was absolutely required to obtain preparations from αTN4-1 cells as transcriptionally active as those derived from HeLa cells. Whole-cell extract from 2.5 g of N/N1003A rabbit lens cells was prepared as described by Manley et al. (46). Pax-6-containing COP-8 whole-cell extract was prepared as described elsewhere (13). Purified human ΔCREB (72) was a gift from O. M. Andrisani (Purdue University), CREMτ was purchased from Santa Cruz Biotechnology, and the CREM isoform encoded by six BCEFHbZIPII exons (48) was a gift from J. Bodor (Howard Hughes Medical Institute and Massachusetts General Hospital). Electrophoretic mobility shift assays (EMSAs) were performed as described previously (11), using 24 μg of N/N1003A whole-cell extract, 8 μg of αTN4-1 or L929 nuclear extract, and 500 ng of poly(dI-dC). The experimental preparations containing mouse lens nuclear extract were incubated only on ice because of an endogenous DNase activity.

In vitro transcription. In vitro transcription assays were performed at 28°C for 60 min in 15 μl of standard buffer (12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.9], 60 mM KCl, 7.5 mM $MgCl_2$, 5 mM dithiothreitol, 5% [wt/vol] polyethylene glycol), 0.6 mM ATP and CTP, 12.5 μM UTP, 10 μCi of [α-^{32}P]UTP (3,000 Ci/mmol), 0.1 mM 3′-*O*-CH_3GTP (Pharmacia), and 15 U of RNase T_1 (Gibco-BRL). Two hundred fifty nanograms of plasmid templates derived from pLovTATA and 125 ng of pMLCas190 were assayed with 80 μg of protein of αTN4-1 cell extract or 16 μg of protein of HeLa cell nuclear extract. Reactions were stopped with 85 μl of stop solution (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5% sodium dodecyl sulfate, 100 mM LiCl, 300 mM sodium acetate, and 50 μg of tRNA per ml), processed, and analyzed on 5% polyacrylamide–7 M urea gels as described previously (19). The specific RNA products (360 nucleotides [nt]) were quantified with a PhosphorImager, and values were normalized with respect to a 180-nt RNA initiated from pMLCas190, which was used as an internal standard.

RESULTS

EMSAs using DE1 probes. Our previous transgenic mouse (53, 71) and mutagenesis (20, 51) experiments showed that the mouse αA-crystallin −111 to −88 promoter sequence contains at least one positively *cis*-acting element. Both in vivo and in vitro footprinting data for nuclear extracts from the αTN4-1 cells (39) or newborn mouse lenses (data not shown) demonstrated protein binding at the −108 to −97 region. Since the DE1 regulatory element (−108 to −100; 5′CTGACGGTG3′) of the mouse αA-crystallin gene resembles a CRE consensus sequence (5′GTGACGT[A/C][A/G]3′) (44, 50), EMSAs were performed to examine the possibility that it interacts with proteins from the ATF/CREB family. Oligonucleotides corresponding to positions −113 to −84 and −113 to −93 of the mouse αA-crystallin gene were incubated with extracts prepared from αTN4-1 lens cells (Fig. 1). Depending on the 3′ end of the oligonucleotide, four (A1 to A4, probe −113 to −84; Fig. 1B, lane 2) or two (A2 and A3, probe −113 to −93; lane 11) complexes were detected. The control somatostatin CRE probe (Fig. 1A) incubated with αTN4-1 or N/N1003A lens extracts formed complexes of similar mobility (data not shown). The specificity of A2 and A3 was confirmed in EMSAs with self-competitors (−113 to −84 or −113 to −93; Fig. 1B, lanes 3, 4, 12, and 13), oligonucleotide −97 to −84 (lanes 5 and 14), or a set of four different published CREs (Fig. 1A; Fig. 1B, lanes 6, 8 to 10, 15, and 17 to 19). The results of the cross-competition experiments using both probes suggested that A2 and A3 contain similar or identical proteins bound to the −113 to −84 and −113 to −93 probes (Fig. 1B, lanes 4 and 13). In contrast, the mutated somatostatin CRE (2) (Fig. 1A), as expected, did not compete for complex formation (Fig. 1B, lanes 7 and 16). Additional experiments showed that N/N1003A lens extracts also formed specific complexes A2 and A3 (data not shown).

Transfection and EMSAs using constructs mutated at the −107 to −100 sequence of the mouse αA-crystallin promoter fragment. Nine different point mutations were introduced into the −107 to −100 sequence within the −111 to +46 promoter fragment of the αA-crystallin gene; these mutated constructs were fused to the CAT reporter gene and tested for promoter activity in transfected N/N1003A lens cells. Four mutated nucleotides created versions of a perfect CRE consensus (GT GACGT[A/C][A/G]; mutants I to IV), while five mutations introduced differences from a consensus CRE (mutants V to IX). The sequences and functional data from the transfection tests are summarized in Table 1. The mutants maintaining the CRE consensus sequence had little effect (I and II) or some positive effect (III and IV) on CAT activities generated by the corresponding reporter constructs. Except for mutation IX, four mutations (V to VIII) which disrupted the CRE consensus sequence resulted in a loss of promoter activity in the transfected cells. Mutation IX resulted in a marginal positive effect on the CAT activity. Mutations which had little effect (IX) or increased (I and II) promoter activity resulted in oligonucleotides that competed efficiently with the wild-type oligonucleotide for formation of complexes A2 and A3 (Fig. 2, lanes 4, 5, and 10), while mutants which had decreased promoter activity (V to VIII) were poor competitors (lanes 6 to 9). Together, these experiments support the idea that one or more CRE-binding proteins interact with the DE1 regulatory sequence of the mouse αA-crystallin gene.

Antisera against CREB and/or CREM eliminate complexes A2 and A3. We next attempted to identify the proteins forming complexes A2 and A3. Positive identification of ATF/CREB transcription factors is complicated by the fact that they are encoded by structurally related genes encoding numerous isoforms generated by alternative RNA splicing (16, 48). Two major CREB activator isoforms, αCREB and ΔCREB (3, 32), and at least seven CREM isoforms are encoded by two separate genes. Isoforms CREMτ, CREMτ2, and CREMτ1 are activators, while isoforms CREMγ, CREMβ, CREMα, and S-CREM are repressors (16, 24, 25). In addition, CREB, CREM, and ATF1 bind DNA as homo- or heterodimers. We approached this problem by using different antisera raised against various epitopes in a series of EMSAs and in Western blots (immunoblots); known purified proteins were used as positive controls.

Initially, antisera against CREB and ATF1 were used in

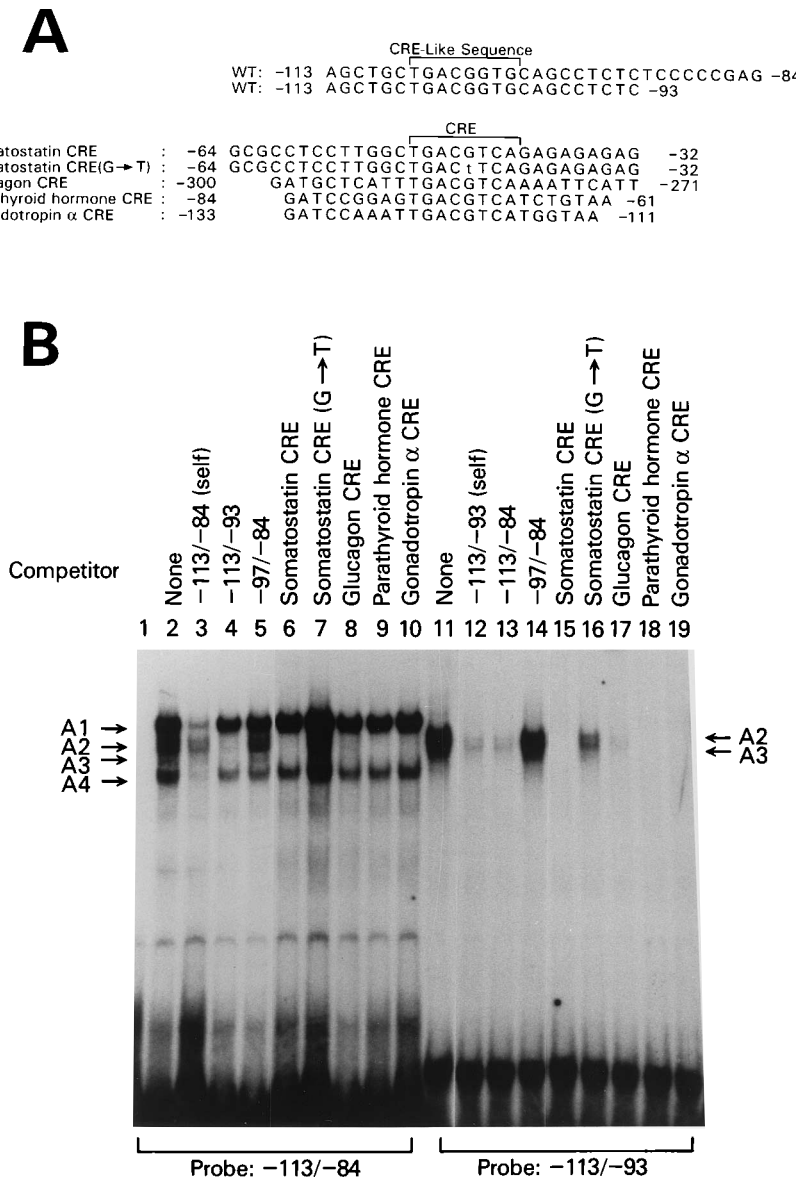

FIG. 1. EMSA using nuclear extract from αTN4-1 mouse lens cells and radioactively labeled probes −113 to −84 and −113 to −93 in the presence of nonlabeled oligonucleotide competitors. (A) Sequences of the wild-type (WT) oligonucleotide −113 to −84 and various CREs (2, 18). Competitors were present at a 50-fold molar excess over the probe. (B) Four major specific retarded complexes were detected with the −113 to −84 probe and labeled A1, A2, A3, and A4. Two major specific retarded complexes, A2 and A3, were detected with the −113 to −93 probe.

EMSAs using a nuclear extract from N/N1003A cells and probe −113 to −93 (Fig. 3A). An anti-CREB antiserum [anti-CREB (C-end)] raised against a synthetic peptide corresponding to the carboxy-terminal 10 amino acid residues of CREB (35) (lane 3) and an antiserum raised against purified ΔCREB (lane 4) (CREB 327; provided by O. M. Andrisani) (lane 4) reduced complexes A2 and A3. In contrast, an antiserum recognizing the α peptide (3) of αCREB (CREB 341) (lane 5) and an antiserum against purified ATF1 (36) (lane 6) did not significantly reduce the amount of complexes A2 and A3. Incubations using preimmune sera (lanes 8 to 10) and assays in the absence of extracts (lanes 12 to 16) are shown for comparison. Similar results were obtained with probe −113 to −84 and αTN4-1 nuclear or N/N1003A whole-cell extracts (Fig. 3B). Interestingly, anti-CREB (C-end) and anti-ΔCREB antisera not only inhibited complexes A2 and A3 formed by lens nuclear extracts derived from newborn mice but also produced supershifted complexes (Fig. 3C, lanes 3 and 4). These experiments are consistent with CREB-like proteins being involved in the formation of complexes A2 and A3.

Additional experiments were performed with purified ΔCREB, CREMτ (full-length CREM, an activator), and bacterial lysate containing CREM (isoform encoded by exons BCEFHbZIPII) with probe −113 to −93 (Fig. 4A, lanes 4 to 6). Each protein formed a different specific complex, called B, C, or D, which could be competed for by an excess of self oligonucleotide (data not shown). The mobilities of these complexes were similar to that of A2 and A3 formed with the N/N1003A extracts. Antiserum against purified CREM isoform (exons BCEFHbZIPII, antiserum CREMS4, provided by J. Habener) reduced complexes A2 and A3 in EMSAs with N/N1003A whole-cell extracts (Fig. 4B, lanes 4 and 5). Anti-

TABLE 1. Transfection of N/N1003A lens cells, using p−111/+46-CAT and the specified mutants

Plasmid	Sequence (−108/−100)	Relative CAT activity (avg ± SD)[a]
p−111/+46-CAT	CTGACGGTG	1.00 ± 0.05
Mutants		
I	TCA	1.61 ± 0.20
II	G TAA	1.55 ± 0.14
III	G TC	3.71 ± 0.55
IV	G TCA	3.25 ± 0.16
V	A	0.30 ± 0.04
VI	AT	0.10 ± 0.03
VII	G	0.27 ± 0.05
VIII	C	0.12 ± 0.06
IX	C	1.13 ± 0.11
CRE consensus	GTGACGTAA CG	

[a] Values are from at least two separate transfection tests performed in triplicate. Absolute CAT activities of p−111/+46-CAT corresponded to about 10% of the acetylation by one-fifth of the cellular extract incubated at 37°C for 60 min.

serum against the C terminus of CREB or CREM exhibited lower specificity than antiserum against CREM, which did not recognize ΔCREB in an EMSA (data not shown). Western immunoblotting detected proteins of 45 to 46 kDa (data not shown), corresponding to CREMτ rather than to the 43-kDa CREB (24, 25, 35). Our immunoshift experiments appear to eliminate αCREB in the formation of the complexes with DE1. Although not definitive, our data suggest that the proteins forming complexes A2 and A3 belong to the CREM rather than the CREB subfamily of proteins.

Stimulation of αA-crystallin promoter activity via the DE1 site. We next tested the ability of 8-BrcAMP to affect the promoter strength of p−111/+46-CAT (which has the DE1 site) and p−88/+46-CAT (which lacks the DE1 site) in transfected N/N1003A lens cells. 8-BrcAMP is known to stimulate transcriptional activity by phosphorylation of CRE-binding proteins (4, 18). 8-BrcAMP (Fig. 5) enhanced CAT activity in the cells transfected with p−111/+46-CAT; by contrast, no effect was observed in cells transfected with p−88/+46-CAT. The maximum stimulation, 3.8-fold, was detected with 2 mM 8-BrcAMP. Comparable experiments were performed with forskolin, which activates adenylate cyclase (15). Treatment with 20 μM forskolin doubled the activity in N/N1003A cells transfected with p−111/+46-CAT but had no effect on cells transfected with p−88/+46-CAT (Fig. 5). Finally, human T-cell leukemia virus type I Tax_1 ($p40^{tax}$), which activates the CRE-like element in the viral 21-bp enhancer (45) by interacting with CREB homodimers and ATF1/CREB heterodimers (1, 54, 69), activated p−111/+46-CAT fivefold in cotransfected N/N1003A cells (data not shown). As with the stimulatory effects of 8-BrcAMP (Fig. 5), stimulation with Tax_1 was dependent on the presence of DE1, since the average CAT activities of cells transfected with p−88/+46-CAT were not increased by cotransfection with $pTax_1$.

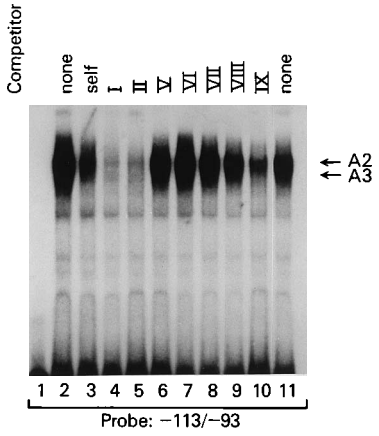

FIG. 2. EMSA using a whole-cell extract from the rabbit N/N1003A lens cells and the −113 to −93 probe in the presence of oligonucleotide competitors (5 ng, 10-fold molar excess) specified in Table 1. Complexes A2 and A3 are indicated.

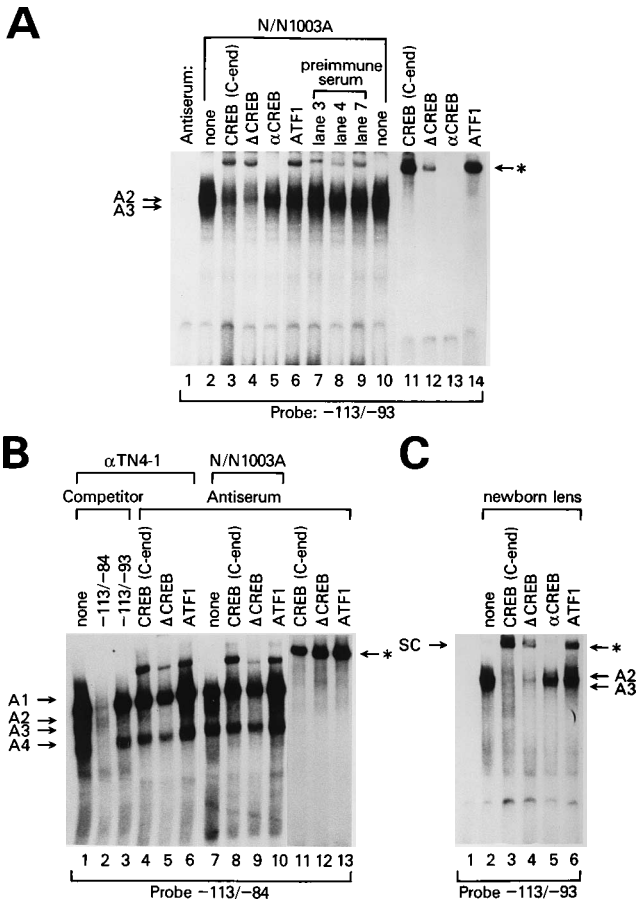

FIG. 3. Effects of specific antisera on complexes A1 to A4 in EMSAs. (A) Probe −113 to −93 was incubated with N/N1003A whole-cell extract in the presence of the indicated antisera or preimmune sera. (B) Probe −113 to −84 was incubated with αTN4-1 extract in the presence of the indicated antisera. (C) Probe −113 to −93 was incubated with newborn mouse lens nuclear extract. A supershifted complex, SC, is indicated. Asterisks denote bands originating from DNA-binding proteins present in both the antisera (A, lanes 11 to 14; B, lanes 11 to 13) and the preimmune sera (A, lanes 7 to 9).

In vitro transcription using G-free templates. Experiments have shown that isolated CREs can transactivate basal in vitro transcription directed by minimal promoters containing TATA boxes (30, 34, 68). We thus tested whether the DE1 site of the αA-crystallin gene can activate in vitro transcription of pLovTATA, a G-free cassette-derived construct containing the chicken ovalbumin TATA box (41). pLovTATA allows one to distinguish between correctly (360-nt RNA) and incorrectly (upstream, 377-nt RNA) initiated RNA transcripts (41) (Fig. 6A). To amplify transcription in these tests, dimers of −111 to −83, −111 to −96, or adenovirus type 2 (Ad2) E4 ATF were cloned in tandem in pLovTATA. The two Ad2 E4 ATF sites were used as a positive control (30). The inserts were intro-

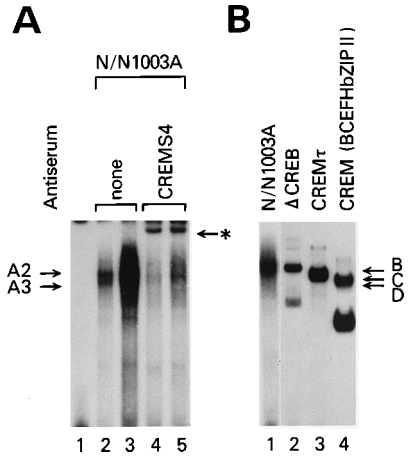

FIG. 4. Effects of anti-CREB/CREM antisera on binding of the labeled −113 to −93 oligonucleotide probe to N/N1003A whole-cell extract (A) or different purified CREB/CREM proteins (B). ΔCREB (complex B), CREMτ (complex C), and CREM (exons BCEFHbZIPII) (complex D) are indicated.

duced to maintain the 10.5-bp periodicity per turn of the DNA α helix (Fig. 6A). This resulted in the center of DE1 or E4 ATF and the TATA box being located on the same side of the DNA helix, an alignment that is similar to the relative positions of DE1 and the TATA box in the mouse αA-crystallin promoter. Representative in vitro transcription data for αTN4-1 and HeLa nuclear extracts are shown in Fig. 6B. The addition of 50 μg of α-amanitin per ml to the reaction mixture eliminated transcription (data not shown). The extent of transcriptional activation was estimated by standardization with an internal control as described in Materials and Methods. The tandem copies of −111 to −83 and −111 to −96 stimulated transcription of correctly initiated 360-nt pLovTATA RNA three- to fourfold, in contrast to an approximate ninefold stimulation by the dimer of Ad2 E4 ATF. In the presence of 50 ng of somatostatin CRE oligonucleotide, the activation effect of the DE1 sequences was suppressed (Fig. 6C), supporting the idea that dimers of DE1 stimulate transcription of pLovTATA by CRE-binding proteins.

Activation of the αA-crystallin promoter by Pax-6. We have recently shown that Pax-6, a paired-box homeodomain transcription factor implicated in eye and lens development (26, 27,

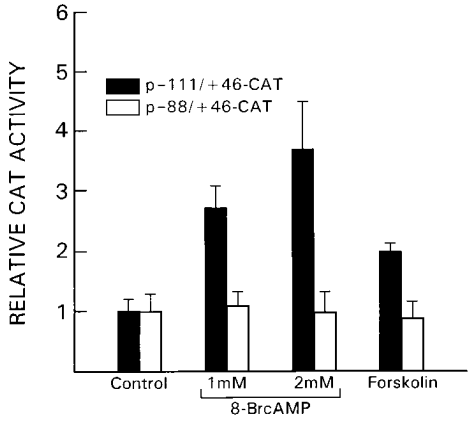

FIG. 5. Effects of 8-BrcAMP and forskolin on CAT activity in rabbit N/N1003A lens cells transfected with p−111/+46-CAT or p−88/+46-CAT. The data are expressed as the ratio of normalized CAT activity in 8-BrcAMP-treated and nontreated cells. The activity of cells transfected with p−88/+46-CAT was 47% ± 4% of that transfected with p−111/+46-CAT.

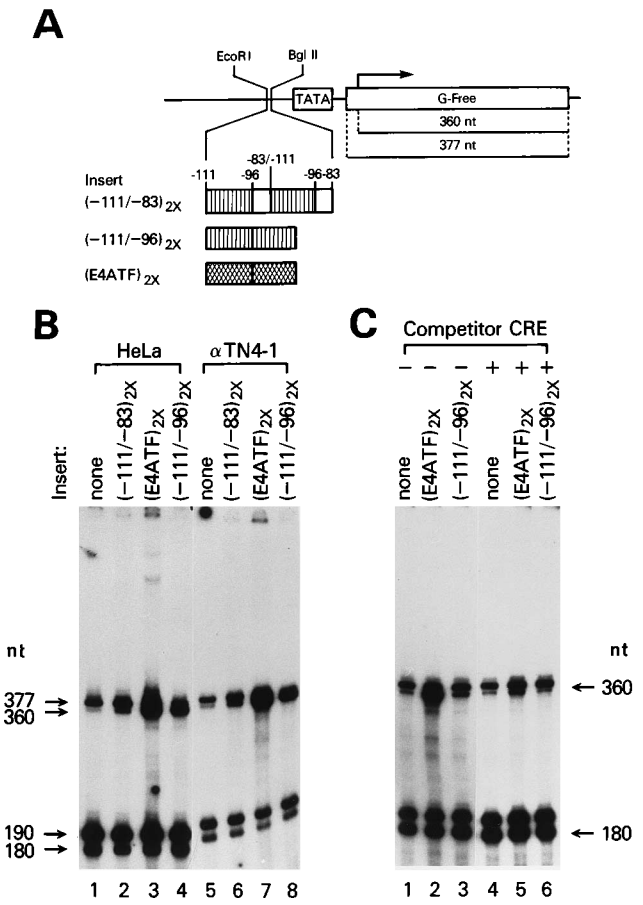

FIG. 6. In vitro transcription assays using a nuclear extract from αTN4-1 cells and G-free templates derived from pLovTATA. (A) Schematic descriptions of templates derived from pLovTATA (41). The −111 to −83, −111 to −96, and E4 ATF sequences are described in Materials and Methods. (B) Autoradiogram showing a representative experiment with nuclear extracts from HeLa or αTN4-1 cells. Correctly initiated RNAs from pLovTATA (360 nt) and from internal control template pMLCas190 (180 nt) are labeled by arrows; 377 and 190 indicate nonspecific transcripts that initiated upstream of the correct initiation site (see panel A). (C) Effect of a 50-fold excess of CRE oligonucleotides (somatostatin CRE and SMS [2]) on transcription assays with HeLa nuclear extracts.

31, 47, 56, 57, 60, 65, 70), can bind to and activate the chicken αA-crystallin gene (11). We thus tested the possibility that Pax-6 also activates the mouse αA-crystallin promoter. The −56 to −33 region (Fig. 7A) was a primary candidate for Pax-6 binding since it resembles the Pax-6 consensus binding site, and the homologous sequence in the chicken αA-crystallin promoter binds Pax-6 (11). In an initial experiment, we determined the activity of p−111/+46(M12)-CAT (51) relative to its wild-type parent p−111/+46-CAT in transfected N/N1003A lens cells. p−111/+46(M12)-CAT contains a mutation at positions −45 to −40 within the presumptive Pax-6 binding site (Fig. 7A and B). These data confirmed the presence of another *cis* element active in N/N1003A cells between the αA-CRYBP1 site (−66 to −57) and the TATA-box (−32 to −26). We next investigated a role for Pax-6 by cotransfecting COP-8 mouse fibroblasts (67), which do not express Pax-6, with p−111/+46-CAT and the mouse Pax-6 cDNA expression vector, pKW10-Pax-6 (13). Increasing amounts of pKW10-Pax-6 stimulated p−111/+46-CAT activity in the cotransfected COP-8 cells (Fig. 7C); the parental vector pKW10 lacking Pax-6 coding sequences had no effect on p−111/+46-CAT (data not shown). We conclude that Pax-6 can activate the

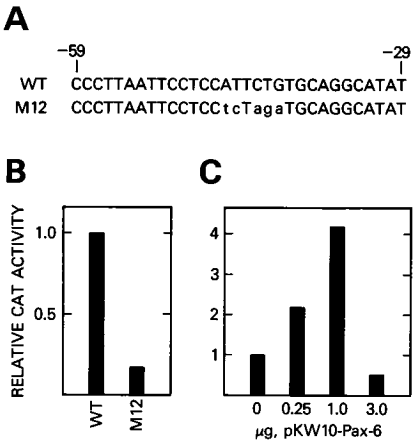

FIG. 7. Pax-6 activation of the mouse αA-crystallin promoter fragment −111 to +46 in transfected rabbit N/N1003A lens cells. (A) Sequence −59 to −29 of the mouse αA-crystallin promoter. Mutation M12 (51) (lowercase letters) is located at a presumptive Pax-6 binding site (11). WT, wild type. (B) Transfection of N/N1003A cells with p−111/+46-CAT (WT) or p−111/+46(M12)-CAT (M12). Average relative CAT activities from two independent transfections (performed in duplicate) are shown. (C) Cotransfection of COP-8 cells with p−111/+46-CAT and pKW10-Pax-6. Experiments were performed as described in Materials and Methods.

mouse αA-crystallin promoter in cells which normally express neither αA-crystallin nor Pax-6 (see below).

Evidence for binding of Pax-6 to the −59 to −29 sequence of the mouse αA-crystallin promoter was obtained by EMSAs using an anti-Pax-6 antiserum recognizing a quail Pax-6 paired domain (7) and extracts from COP-8 cells transfected with pKW10-Pax-6 (13). Two complexes, C1 and C2 (Fig. 8), were obtained when the −59 to −29 oligonucleotide was incubated in the COP-8 whole-cell extract (lane 2). Treatment with an anti-Pax-6 antiserum (lane 3) implicated Pax-6 or a cross-reactive protein involved in complex C1. A similar result was obtained with the Pax-6 paired-domain binding oligonucleotide (22) (lanes 5 and 6). A specific comigrating C1 complex reacting with the anti-Pax-6 antiserum was also obtained with an N/N1003A whole-cell extract (data not shown). C1 was not present when a nuclear extract from L929 fibroblasts was used (data not shown).

Finally, the presence of Pax-6 in extracts of N/N1003A,

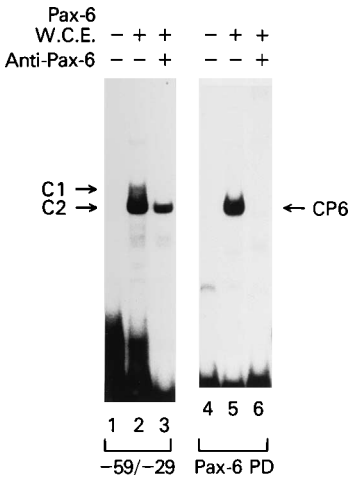

FIG. 8. EMSA of Pax-6 binding to the −59 to −29 sequence of the mouse αA-crystallin promoter. Complex C1 or CP6 was detected with Pax-6-containing COP-8 whole-cell extract (W.C.E.) (see Materials and Methods) and −59 to −29 or Pax-6 paired-domain (PD) binding oligonucleotide (22), respectively.

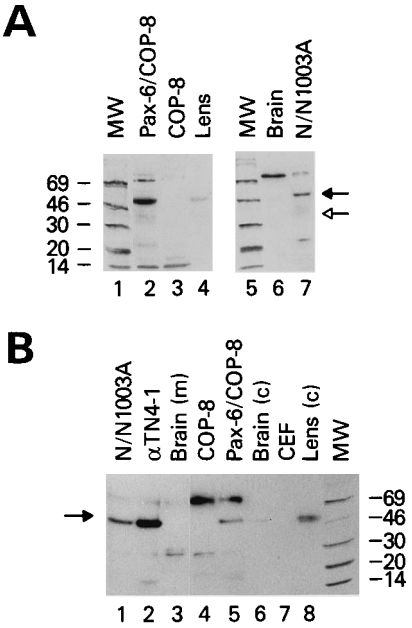

FIG. 9. Western immunoblotting of various extracts, using anti-Pax-6 antiserum. The solid arrow indicates the major 46- to 48-kDa immunoreactive band known to be Pax-6 (7, 11, 23); the open arrow indicates bands near 32 to 33 kDa which are probably alternatively spliced products of the Pax-6 gene (7). The extracts are Pax-6/COP-8 cells (COP-8 cells transfected with pKW10-Pax-6), COP-8 cells, and lens (chicken embryonic lens nuclear extracts), brain (chicken embryonic brain nuclear extracts), and N/N1003A cells. A sodium dodecyl sulfate–4 to 20% gradient polyacrylamide gel (Novex, San Diego, Calif.) was used. (A) Antiserum against quail Pax-6 paired domain (7, 11). (B) Antiserum against the zebra fish C-terminal domain of Pax-6 (48a). The extracts are αTN4-1 cells, brain (m) (whole brain from 3-week-old mice), brain (c) (whole brain from 14-day-old chicken embryos), CEF (chicken primary fibroblasts), lens (c) (chicken embryonic lenses), COP-8 cells, and Pax-6/COP-8 cells (see above).

αTN4-1, mouse lens, and brain cells was examined by Western immunoblotting (Fig. 9). A whole-cell extract of COP-8 cells transfected with pKW10-Pax-6 (13) was used as a positive control (Fig. 9A, lane 2), while an extract from nontransfected COP-8 cells provided a negative control (lane 3). Antiserum against the quail Pax-6 paired domain (7) reacted with both 46- to 48-kDa and 32- to 33-kDa Pax-6 isoforms (7, 11) (Fig. 9A, lanes 2, 4, 6, and 7, arrows); antiserum against the highly conserved C terminus of zebra fish Pax-6 (provided by I. Mikkola) reacted with only the 46- to 48-kDa isoforms in these extracts (Fig. 9B, lanes 1 to 3, 5, 6, and 8). A 46-kDa Pax-6 contains a canonical paired domain, while a 48-kDa isoform contains an insertion of 14 amino acid residues in its paired domain (70). These Pax-6 isoforms exhibit distinct DNA-binding properties (22, 23). The identities of the polypeptides greater than 60 kDa which reacted with the antisera are not known at present.

DISCUSSION

Previous mutagenesis and DNA-protein binding experiments have demonstrated the functional importance of the −111 to −100 (DE1) and −68 to −40 sequences for activity of the mouse αA-crystallin gene (10, 20, 39, 51, 61). The present data provide evidence that DE1 behaves as a functional CRE and that the −59 to −29 sequence interacts with Pax-6. The fact that the −88/+46-CAT transgene, which lacks the DE1 site, is expressed specifically in the lenses of transgenic mice (71) is consistent with the interpretation that the Pax-6 binding site is a target sequence for lens specificity and the DE1/CRE

is an additional regulatory element required for high-level expression of the αA-crystallin gene in the lens. However, double mutation of the DE1/CRE and αA-CRYBP1 (−66 to −55) sites in the −111/+46-CAT transgene eliminated activity of the αA-crystallin promoter in the lens (61), indicating that Pax-6 alone is not sufficient to activate the αA-crystallin promoter and requires a synergistic interaction with at least one different activator. Direct or indirect interaction of Pax-6 and CREB-like proteins is currently being investigated.

What is the possible biological role of DE1? It is likely that the DE1/CRE site contributes to the temporal and spatial control of the expression of the mouse αA-crystallin gene in the lens as does the homologous sequence of the hamster αA-crystallin gene (76). Thus, CREM isoforms may provide a sensitive mechanism for developmental, spatial, and/or temporal control of αA-crystallin gene expression in the mouse lens. The possible use of CREM in the transcriptional control of the αA-crystallin gene would raise the interesting possibility that alternatively spliced isoforms can activate or repress transcription (24, 25).

The present immunoshift, EMSA, and transfection experiments show that Pax-6 activates the mouse αA-crystallin gene by its binding between the αA-CRYBP1 site and the TATA box. The Pax-6 site overlaps with the previously identified *cis*-acting sequence −56 to −39 (51). Evidence indicating that the chicken δ-crystallin lens-specific enhancer contains at least two Pax-6 binding sites has also been obtained (12).

Comparison of known Pax-6 binding sites from the mouse and chicken αA-crystallin genes, the chicken δ1-crystallin gene enhancer (12), the guinea pig ζ-crystallin gene (59), and the promoter region of the neural cell adhesion molecule L1 (9) shows that Pax-6 binding sites are degenerate as previously demonstrated for BSAP/Pax-5 binding sites (13). The 46-kDa Pax-6 binding site alignment (11, 12) suggests a bipartite structure similar to that of Pax-1 (8), BSAP/Pax-5 (13), and Pax-8 binding sites (77) which is related (but not identical) to the Pax-6 paired-domain consensus binding site determined by a PCR selection procedure (22). The identification of several natural Pax-6 binding sites provides a basis for determining the relative contributions of the Pax-6 paired domain, the homeodomain, or other regions for the function of this transcription factor (66, 73).

Finally, a comparison of the regulatory elements of the chicken and mouse αA-crystallin genes shows that sequence similarity per se does not dictate which transcription factor is employed for high-level expression in the lens. For example, despite its similarity to the DE1/CRE mouse sequence, site D (−102 to −93) of the chicken αA-crystallin gene binds the transcription factor USF (11). Moreover, the chicken site D is a negative element, while the mouse DE1/CRE site is a positive regulatory element. CREB-like proteins are, however, used as positive transcription factors in the chicken αA-crystallin gene by binding at site B (−138 to −131). In addition, Pax-6 appears to be an essential transcription factor for lens-specific expression of the αA-crystallin gene in chickens and mice. Thus, the juxtapositioning of *cis* control elements and use of different transcription factors on similar DNA regulatory sequences are associated with differences in the quantitative, temporal, and spatial requirements for expression of the αA-crystallin gene in mice and chickens (55, 63, 78).

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