MOLECULAR AND CELLULAR BIOLOGY, Nov. 1994, p. 7363–7376
0270-7306/94/$04.00+0

Vol. 14, No. 11

A Complex Array of Positive and Negative Elements Regulates the Chicken αA-Crystallin Gene: Involvement of Pax-6, USF, CREB and/or CREM, and AP-1 Proteins

ALEŠ CVEKL,[1] CHRISTINA M. SAX,[1] EMERY H. BRESNICK,[2] AND JORAM PIATIGORSKY[1]*

Laboratory of Molecular and Developmental Biology, National Eye Institute,[1] and Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases,[2] National Institutes of Health, Bethesda, Maryland 20892-2730

Received 20 May 1994/Returned for modification 14 July 1994/Accepted 11 August 1994

The abundance of crystallins (>80% of the soluble protein) in the ocular lens provides advantageous markers for selective gene expression during cellular differentiation. Here we show by functional and protein-DNA binding experiments that the chicken αA-crystallin gene is regulated by at least five control elements located at sites A (−148 to −139), B (−138 to −132), C (−128 to −101), D (−102 to −93), and E (−56 to −41). Factors interacting with these sites were characterized immunologically and by gel mobility shift experiments. The results are interpreted with the following model. Site A binds USF and is part of a composite element with site B. Site B binds CREB and/or CREM to enhance expression in the lens and binds an AP-1 complex including CREB, Fra2 and/or JunD which interacts with USF on site A to repress expression in fibroblasts. Sites C and E (which is conserved across species) bind Pax-6 in the lens to stimulate αA-crystallin promoter activity. These experiments provide the first direct data that Pax-6 contributes to the lens-specific expression of a crystallin gene. Site D (−104 to −93) binds USF and is a negative element. Thus, the data indicate that USF, CREB and/or CREM (or AP-1 factors), and Pax-6 bind a complex array of positive and negative *cis*-acting elements of the chicken αA-crystallin gene to control high expression in the lens and repression in fibroblasts.

The lens and its characteristic soluble proteins, the crystallins, are useful for studying the mechanisms of embryonic determination, cellular differentiation, and tissue-specific gene expression (43, 51). Lens determination is a multistep process that begins in the gastrula and early neurula and is completed when predetermined head ectoderm makes contact with the optic vesicle (21, 39, 58, 68).

Recent studies aimed at understanding the molecular mechanisms that control lens induction have suggested an important role for Pax-6, a member of the paired-box family of proteins (36, 37, 39, 64, 68). Pax-6, like other members of the Pax family (11, 22), acts as a DNA-binding protein and a transcription activator (54). Abnormal ocular phenotypes, i.e., complete absence of eyes, human aniridia, and the mouse *Small eye* (*Sey*) mutation, are associated with defects in the Pax-6 gene (20, 25, 40, 64). The spatial and temporal patterns of *Pax-6* gene expression in mice and chickens suggest that Pax-6 participates in the later stages of lens induction and might be required for growth, differentiation, and maintenance of the lens and cornea (37, 64, 68).

The crystallins are the major soluble proteins which occur in the ocular lens and are required for its proper optical properties (70). The crystallins constitute a diverse group of proteins whose expression is tightly regulated on tissue-specific, temporal, and spatial levels. In addition, the crystallins are distinct markers for lens differentiation (51). The two α-crystallins, αA- and αB-crystallin, are descendants of an ancestral small heat shock family of proteins (29) and have recently been shown to function as molecular chaperones (26). αA-crystallin is highly specialized for lens expression, while αB-crystallin is also expressed in many nonlens tissues (heart, lung, brain, kidney, skeletal muscle, and other tissues) (9, 62).

Numerous experiments, especially those including transgenic mice, suggest that crystallin gene expression is regulated at the level of transcription (52, 53). Several *cis*-acting transcriptional regulatory elements have been described for the chicken αA-crystallin gene (34, 35, 41, 42). The chicken αA-crystallin region from positions −242 to +77 (−242/+77 region) directed the lens-specific expression of a fused reporter gene in transgenic mice (35). A 5′ deletion analysis demonstrated that essential elements required for lens expression are located within the −162/+77 region (35). The −162/−88 region was identified as a lens-specific enhancer (41) and shown to be composed of at least three positively acting elements called DE2A (−144 to −134), DE2B (−128 to −118), and DE1A (−114 to −104) and possibly one negatively acting element called DE1B (−102 to −91) (34). A detailed review of this highly expressed lens gene can be found elsewhere (62). Here we identified transcription factors interacting with these sites and propose a model for regulation of the chicken αA-crystallin gene.

MATERIALS AND METHODS

Cell cultures, transfections, and expression of Pax-6. Cell cultures of embryonic chicken primary lens epithelial cells (PLEs), transfections, and chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as described previously (34). Primary chicken embryo fibroblasts (CEFs) were grown as described elsewhere (61). Transfections were performed in duplicate two to four times, and the average

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

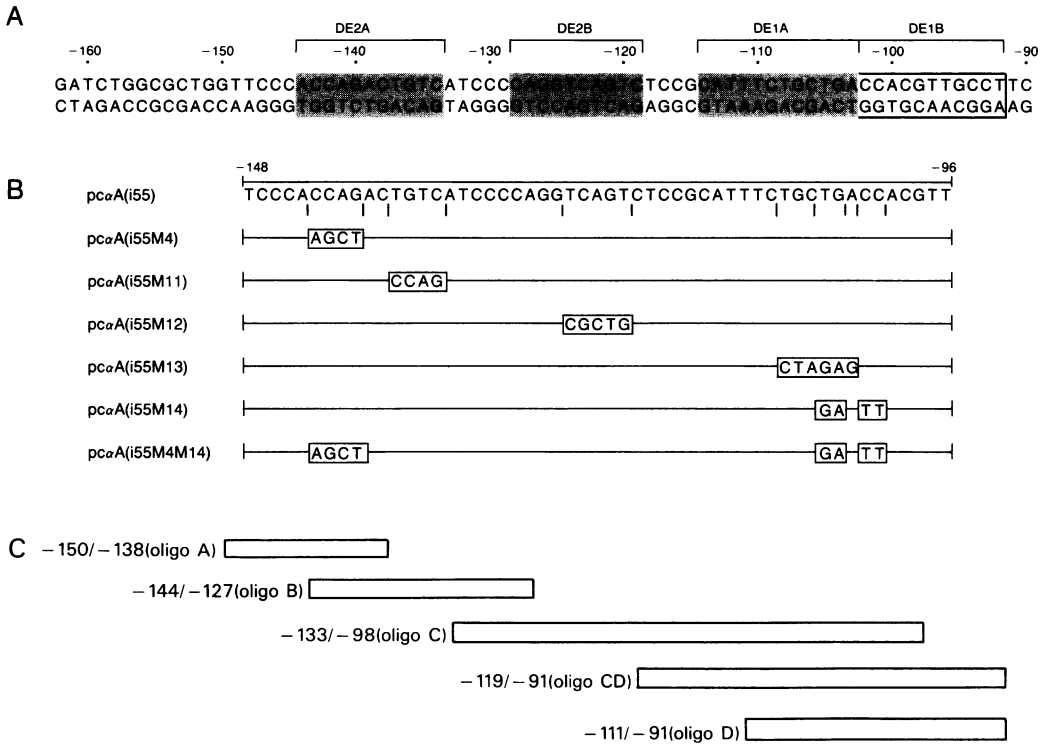

FIG. 1. Diagrammatic summary of *cis* elements found in the −162/−90 chicken αA-crystallin lens-specific enhancer. (A) Positively (DE2A, DE2B, and DE1A) and negatively (DE1B) acting sites (34) are labeled within the shaded and open boxes, respectively. (B) Oligonucleotides used for construction of pcαA(i55) and its six mutant derivatives. Mutated nucleotides are shown within the open boxes. (C) Oligonucleotides (A, B, C, CD, and D) used as probes for EMSA.

values ± standard deviations are shown. pCMVβ (Clontech) was included in each test to control for transfection efficiency. A cloned mouse Pax-6 cDNA (pKW10-Pax-6, kindly provided by M. Busslinger and T. Czerny) was expressed in COP-8 cells (7). CEFs were cotransfected by calcium phosphate precipitation with pcαA−162/+77-CAT (35) and increasing amounts of pKW10-Pax-6 or the parental pKW10 vector, and the cells were harvested 72 h after transfection.

Oligonucleotides and plasmid constructions. Oligonucleotides were made on an Applied Biosystems 380B DNA synthesizer and processed as described previously (34). The following oligonucleotides were described elsewhere: adenovirus type 2 major late transcription factor (40-mer [2]); NF-κB, somatostatin cyclic AMP-responsive element (CRE), and DE1B (oligonucleotide D; Fig. 1C [34]); collagenase AP-1 site (24-mer [57]); and H2B2.2, CD19-1, e5, and H2A2.1 BSAP/Pax-5-binding sites (7). Additional oligonucleotides from the chicken αA-crystallin promoter studied in electrophoretic mobility shift assays (EMSAs) (Fig. 1C) are as follows (lowercase letters indicate nucleotide additions to provide sufficient flanking sequences to ensure proper binding): oligonucleotide A, −150 to −138 (gatcGTTCCCACCAGACTttga); oligonucleotide B, −144 to −127; oligonucleotide C, −133 to −98; oligonucleotide CD, −119 to −91; oligonucleotide E, −60 to −29; and c-*myc* (21-mer, GTGTCCACCACGTGCTGCTTG [33]). Linker-scanning mutants within the −151/−134 sequence of the chicken αA-crystallin gene (Fig. 2A) were generated by PCR using mutated primers containing *Bam*HI or *Hin*dIII site extensions and pcαA−162/+77-CAT (35) as a template. The PCR products were subcloned into p8-CAT (34). Wild-type and mutant −148/−96 oligonucleotides were prepared with *Bam*HI overhangs and cloned into the *Bam*HI site of pcαA−56/+77-CAT (35), yielding the pαA(i55) series of plasmids (Fig. 1B). Wild-type and mutant −106/−86 oligonucleotides (Table 1) were prepared with *Sal*I overhangs and cloned into the *Sal*I site of pαA(i55). All constructs were verified by dideoxy sequencing.

Nuclear extracts, proteins, antisera, EMSA, and DNase I footprinting. Nuclear extracts from 14-day-old chicken embryonic lenses and from CEFs were isolated as described by Shapiro et al. (63). The final protein concentrations were 16 mg/ml (lens extract) and 4.2 mg/ml (CEF extract). Purified human USF was described previously (2), purified ΔCREB (69) was a gift from O. M. Andrisani, c-Jun was obtained from Promega, and CREMτ was obtained from Santa Cruz Biotechnology. The following antisera were used: anti-USF (3); anti-CREB (27); anti-ATF1 (28); anti-Pax-6 (antiserum 11 [4]); and anti-αCREB, anti-c-Jun, anti-JunB, anti-JunD, anti-c-Fos, anti-FosB, and anti-Fra2 (obtained from Santa Cruz Biotechnology [catalog no. SC-58X, 45X, 73X, 74X, 52X, 48X, and 57X, respectively]). EMSAs were performed in a final volume of 12.5 μl containing the standard buffer (12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 60 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 4% [wt/vol], Ficoll 400, 0.2 mg of bovine serum albumin per ml) (34), 1 to 2 μg of a nonspecific competitor [poly(dI-dC) or poly(dA-dT)], and 0.5 to 3 μl of nuclear extract or purified proteins in amounts determined by prior titrations. Probes were used at 0.5 to 1 ng, and 1 to 30 ng of oligonucleotide competitors was used. Experiments using antisera were performed as follows. The nuclear extract and antiserum were incubated at room temperature for 10 to 15 min without DNAs. Subsequently, 1

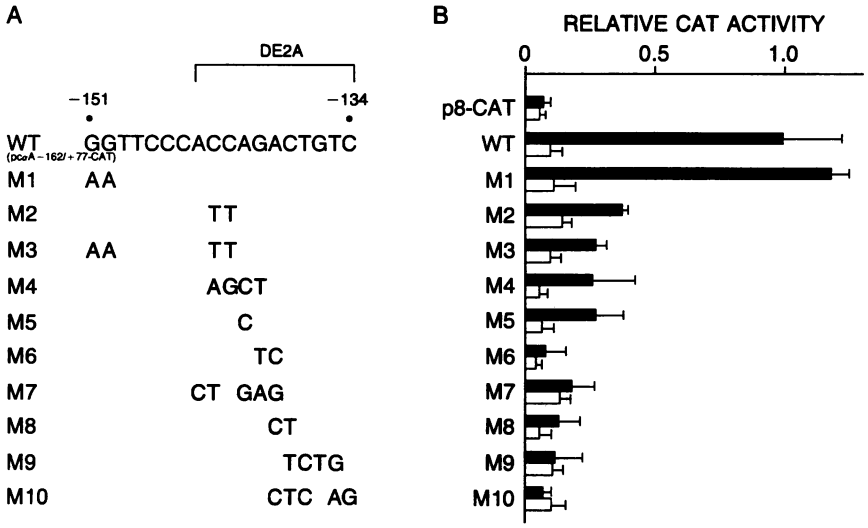

FIG. 2. Transfection of PLEs and CEFs, using promoter-CAT constructs mutated within DE2A. (A) Point mutants (M1 to M10) of the −151/−134 region made within the wild-type (WT) chicken αA-crystallin −162/+77 promoter fragment fused to the CAT gene (pcαA−162/+77-CAT). (B) Average CAT activity and standard deviation for each plasmid transfected into either PLEs (solid bars) or CEFs (open bars). CAT activities are relative to that of pcαA−162/+77-CAT (wild type [WT]) in PLEs. The promoterless p8-CAT vector is shown as a negative control.

μg of poly(dI-dC) or poly(dA-dT) or 250 ng of NF-κB oligonucleotide was added as a nonspecific competitor, and then the probe was added; samples were incubated for an additional 10 min and electrophoresed as described previously (34). In vitro DNase I footprinting using purified USF and an end-labeled −242/+77 chicken αA-crystallin gene fragment was performed in the presence of 1 μg of poly(dA-dT) as described elsewhere (34).

Immunoprecipitation and Western blot (immunoblot) analysis of proteins. Protein A-Sepharose 6B (Pharmacia) was equilibrated in buffer A50 (20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol), and 3 to 7 μl of antiserum supplemented with A50 was bound to 25 μl of the gel in a total volume of 50 μl at 28°C for 30 min. The gel was washed with an excess of A50 and incubated with either 200 μg of lens nuclear extract or 42 μg of CEF nuclear extract in a total volume of 50 μl of buffer A50 on ice overnight with gentle agitation. Four microliters of the resulting supernatant fraction was used for EMSA experiments. Western immunoblotting was done as described elsewhere (2).

RESULTS

DE2A is functionally important in lens and nonlens cells and consists of two adjacent *cis* elements. To understand the structure and function of the DE2A site of the chicken αA-crystallin gene (Fig. 1), the −151/−134 region of the −162/+77 promoter fragment fused to the CAT gene was mutated (Fig. 2A) and transfected into PLEs and CEFs (Fig. 2B). All but one mutation (M1, located outside DE2A) significantly decreased CAT activity in PLEs, in agreement with previously published data (34). Mutations M4, M5, M6, and M8 reduced promoter activity in transfected CEFs, suggesting the presence of at least one *cis* element which functions in fibroblasts. Overall promoter strength in CEFs was very low, as expected (35).

We next examined the possibility that DE2A, previously defined by two linker-scanning mutations (−144/−139 and −139/−134) (34), comprises two adjacent *cis* elements. EMSAs were performed with radiolabeled oligonucleotides spanning either the −150/−138 (oligonucleotide A) or −144/−127 (oligonucleotide B) region of the chicken αA-crystallin gene (Fig. 1C). The probes were incubated with lens or CEF nuclear extracts in the presence of specific oligonucleotide competitors corresponding to the functional mutations examined above (Fig. 2A). Nuclear extracts from intact lenses and CEFs of 14-day-old chicken embryos incubated with oligonucleotide A formed specific complex A1 (Fig. 3A, lanes 2 and 9). A1 was eliminated by self-competition with oligonucleotide A (lanes 3 and 10), while competition with an oligonucleotide containing mutation M4 (lanes 4 and 11), M6 (lanes 5 and 12), or M7 (lanes 6 and 13) had little if any effect, in agreement with the transfection data shown in Fig. 2B. In contrast, competition with an oligonucleotide including mutation M10 strongly reduced the formation of complex A1 (lanes 7 and 14), even

TABLE 1. DE1B mutagenesis expression experiments

Plasmid	Insert[a]	Sequence from −106 to −86 (5′−3′)	Relative CAT activity	
			PLEs	CEFs
pcαA(i55)	None		1.00	1.00
pcαA(i55D)	D	CTGACCACGTTGCCTTCGTCG	0.35 ± 0.12	0.57 ± 0.03
pcαA(i55DM1)	D-M1	CTGACTCGAGTGCCTTCGTCG	0.67 ± 0.07	0.90 ± 0.11
pcαA(i55DM2)	D-M2	CTGACGTGGCTATCTTCGTCG	1.27 ± 0.13	1.28 ± 0.07

[a] Nucleotides used for subcloning are not shown.

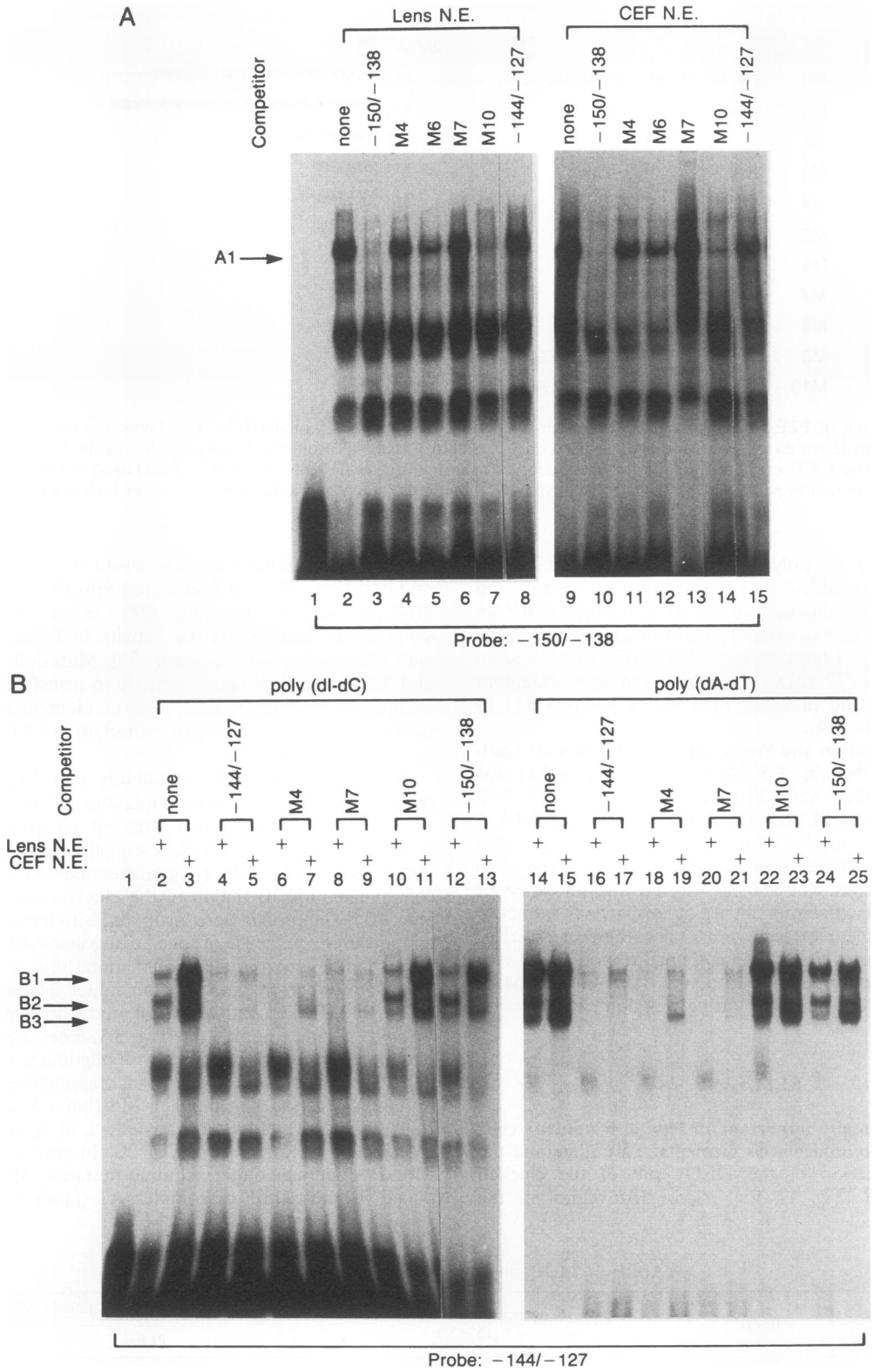

FIG. 3. EMSA analysis of lens and CEF nuclear proteins binding to DE2A. See Fig. 1 and 2 for sequences of the competing oligonucleotides. (A) A1 is the major specific complex formed on oligonucleotide A (−150 to −138). Poly(dA-dT) was used as the nonspecific competitor. (B) B1, B2, and B3 are the major specific complexes formed on oligonucleotide B (−144 to −127). N.E., nuclear extract.

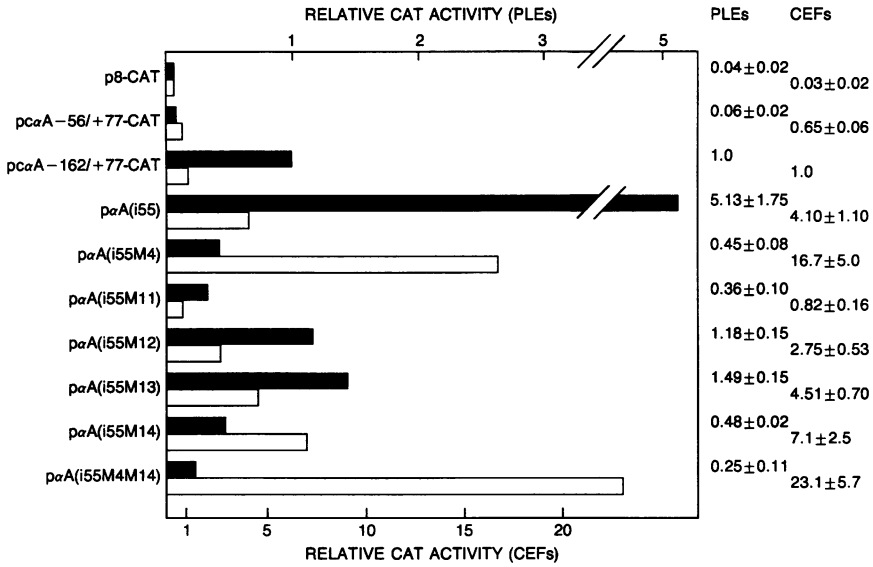

FIG. 4. Transfection of PLEs and CEFs with pαA(i55) and its derivatives. pαA(i55) and its derivatives were constructed by inserting wild-type and mutant −148/−96 oligonucleotides (see Fig. 1B) upstream of the chicken αA-crystallin −56/+77 basal promoter in pcαA−56/+77-CAT. The average CAT activity and standard deviation generated for each plasmid transfected into either PLEs (solid bars) or CEFs (open bars) is given. The promoterless p8-CAT vector is shown as a negative control.

though this mutation virtually eliminated promoter activity. The fact that M4, M6, and M7 reduced promoter activity and were poor competitors for binding to the wild-type DE2A site, while M10 reduced promoter activity and competed for binding to DE2A, suggested that there are two adjacent protein-binding sites. This idea was supported by the fact that oligonucleotide B, which spans positions −144 to −127, had no effect on the formation of complex A1 (lanes 8 and 15), suggesting that the putative downstream protein-binding site requires nucleotides downstream from position −138.

Evidence for the binding of another protein to DE2A was obtained in EMSA experiments using oligonucleotide B (Fig. 1C). Incubation of radiolabeled oligonucleotide B with lens or CEF nuclear extracts resulted in the formation of complexes B1, B2, and B3 (Fig. 3B). B1 was a relatively weak complex in lens nuclear extracts in the presence of poly(dI-dC) (lane 2), while it was the major complex in the presence of poly(dA-dT) (lane 14). B1 was the major complex in CEF nuclear extracts in the presence of either copolymer (lanes 3 and 15). B1 was reduced in lens and CEF nuclear extracts in the presence of oligonucleotides containing mutation M4 (lanes 6, 7, 18, and 19) or M7 (lanes 8, 9, 20, and 21). In contrast, oligonucleotides containing either mutation M10 (lanes 10, 11, 22, and 23) or oligonucleotide A (−150/−138 sequence) (lanes 12, 13, 24, and 25) did not compete for the formation of complex B1. These data are consistent with the interpretation that the putative downstream binding site is not fully covered by oligonucleotide A (−150 to −138). Taken together, the EMSA competition and transfection data support the idea that DE2A (34) consists of two adjacent protein-binding sites. Inspection of our protein-binding data suggest that mutations M4, M6, and M7 define one *cis* element (−144 to −139) and M10 defines a different *cis* element (−139 to −134).

Since previous experiments have suggested that DE1B (−102 to −91) contains a repressor element (see below) (34), new constructs were made in order to more carefully study the activating effects of DE2A in the absence of DE1B (Fig. 1A). Two constructs [pαA(i55M4) and pαA(i55M11)] contained a mutation in each of the two proposed DE2A protein-binding sites and were made within pαA(i55) (Fig. 1B). pαA(i55) consisted of the chicken αA-crystallin −56/+77 basal promoter fragment fused to the CAT gene, to which was added the αA-crystallin −148/−96 oligonucleotide upstream of the basal promoter (Fig. 1B). pαA(i55) lacks the 3′ half of the DE1B sequence. The basal pcαA−56/+77-CAT construct was essentially inactive in transfected PLEs and CEFs (35), while pcαA(i55) was fivefold more active in the PLEs and CEFs than pcαA−162/+77-CAT, supporting the idea that DE1B is a repressor (Fig. 4).

In the constructs lacking the DE1B repressor, mutations in DE2A [pαA(i55M4) and pαA(i55M11)] generated significantly less CAT activity relative to the control pαA(i55) in transfected PLEs (Fig. 4). In contrast, pαA(i55M4) gave higher CAT activity than did pαA(i55) in transfected CEFs, suggesting a negative role for this site in CEFs, while pαA(i55M11) gave less CAT activity than pαA(i55) in CEFs. A double mutation [pαA(i55M4M14); Fig. 1B] confirmed the functional properties of the individual M4 and M14 mutants in both PLEs and CEFs (Fig. 4). These transfection data provide additional evidence that DE2A consists of two protein-binding sites and raise the possibility that DE2A behaves as a composite element (for a review, see reference 46).

USF, CREB and/or CREM, and AP-1 proteins interact with the DE2A site. The idea that DE2A is a composite element was explored further by examining the proteins interacting with the two putative *cis* elements comprising this site. Inspection of the DE2A sequence revealed similarity to a CANNTG (E-box) motif (underlined) at −148 to −138 (5′ TCCCACCAGAC 3′) and a GTCA motif (−136 to −133) resembling half consensus sites for CREs (TGACGTC/AA/G) and AP-1 (TGAC/GTA/CA). Numerous transcription factors have been shown to interact with E-box (helix-loop-helix family including USF and c-Myc [31]), CRE (ATF/CREB family [23, 28, 45, 47]), and AP-1 (Fos/Jun family [57, 66]) consensus sequences. To assess whether any of these known transcription factors interact with the two adjacent protein-binding sites in DE2A, a series of

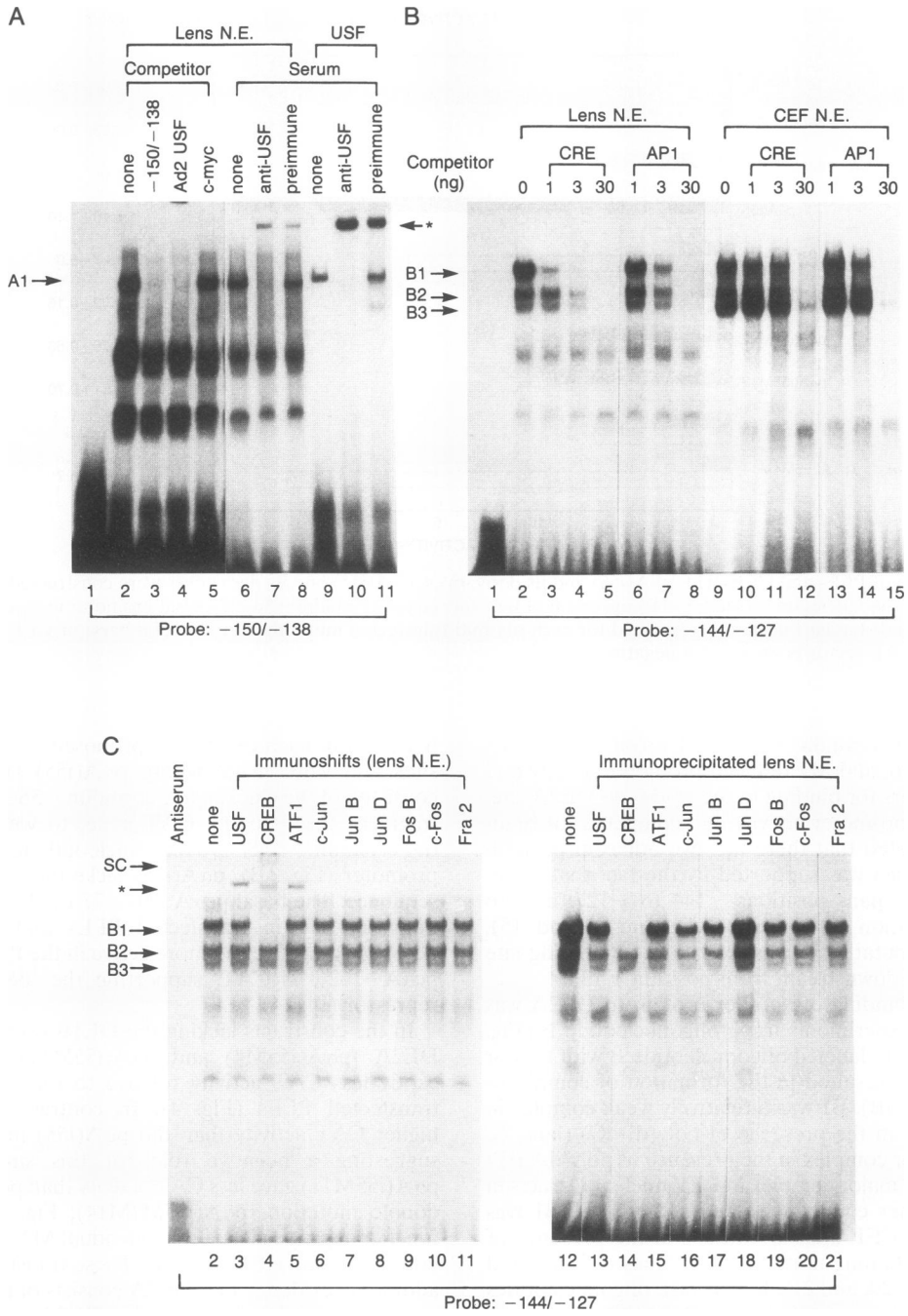

FIG. 5. Identification of proteins binding to the DE2A region in EMSAs. (A) A1 is the major specific complex formed on oligonucleotide A (−150 to −138). The asterisk indicates the band detected with both anti-USF and preimmune serum. N.E., nuclear extract; Ad2, adenovirus type 2. (B) B1, B2, and B3 are major specific complexes formed on oligonucleotide B (−144 to −127). Competitor oligonucleotides were the rat somatostatin CRE and rat elastase AP-1 sites. (C) The effects of specific antisera on B1, B2, and B3 were studied by EMSA-immunoshift experiments in a normal (left) and immunoprecipitated (right) lens nuclear extract. An NF-κB oligonucleotide (250 ng) was used in the immunoshift experiments and poly(dA-dT) was used in the immunoprecipitation experiments as nonspecific DNA competitors. Supershifted complexes (SC) as well as bands detected with the preimmune sera or antisera in the absence of nuclear extracts (*) are denoted. (D) Experiment similar to that in panel C, using CEF nuclear extract. Poly(dI-dC) was used as a nonspecific competitor in the immunoprecipitation experiments. (E) Effects of anti-ΔCREB, anti-αCREB, and anti-CREMS4 antisera on the formation of lens and fibroblast complexes and binding of purified ΔCREB, CREMτ, and c-Jun with oligonucleotide B (−144 to −127).

EMSAs was performed with either oligonucleotide A or oligonucleotide B (Fig. 1C).

First, the identity of the proteins forming complex A1 in the lens nuclear extract complexes was examined (Fig. 5A). A1 was almost eliminated in the presence of an oligonucleotide competitor containing the adenovirus type 2 major late promoter USF-binding site (60) (lane 4). In contrast, an oligonucleotide containing a c-Myc-binding site (underlined) from the duck

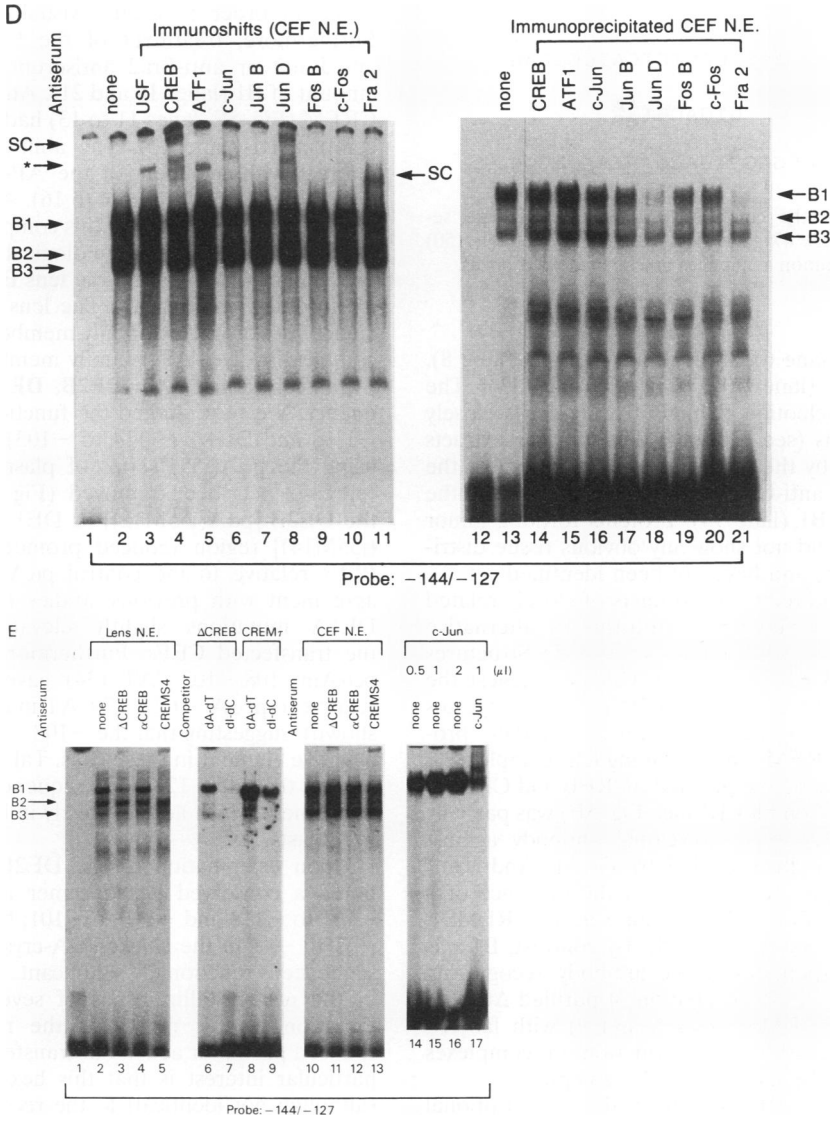

FIG. 5—*Continued.*

τ-crystallin/α-enolase gene (5′ CCACCACGTGCTGCT 3′) (lane 5) (33) did not prevent the formation of A1. A1 formed in the lens nuclear extract comigrated with a complex formed with purified human USF (lane 9). Moreover, an anti-USF antiserum reduced the formation of A1 (lane 7), while a preimmune serum had no apparent effect (lane 8). The anti-USF antiserum also eliminated the A1 complex formed with purified human USF (lane 10). Parallel experiments also associated USF with A1 formed in nuclear extracts of CEFs (data not shown). Finally, similar amounts of the expected 43-kDa immunoreactive USF protein were detected by Western immunoblotting in the lens and CEF nuclear extracts (data not shown). We conclude that USF (5, 60) binds to the −150/−138 sequence of the αA-crystallin gene.

We next addressed the identity of the proteins interacting with oligonucleotide B. In view of the similarity of the −138/−130 sequence with CRE and AP-1 consensus sequences, we performed competition experiments with three concentrations of oligonucleotides containing the rat somatostatin gene CRE site (5′ TGACGTCA 3′) or the rat collagenase gene AP-1 site (5′ TGACTCA 3′) (Fig. 5B), using 0.5 to 1 ng of the labeled oligonucleotide. The wild-type CRE oligonucleotide was the most efficient competitor of B1 formed in a lens nuclear extract (lanes 3 to 5 versus 6 to 8), with only 3 ng virtually eliminating B1 formation (lane 4). In contrast, 30 ng of the AP-1 oligonucleotide was required to eliminate complex B1 in the lens extract (lane 8). The fibroblast B1 complex was affected nearly equally by the CRE (lanes 10 to 12) and AP-1 (lanes 13 to 15) oligonucleotides, with 30 ng being required for strong reduction in both cases. Although the difference was slight, B2 and B3 appeared to be competed for less well than B1 with both CRE and AP-1 oligonucleotides.

Antisera against different members of the ATF/CREB and AP-1 families of transcription factors were used to identify proteins involved in forming complex B1 (Fig. 5C). An anti-CREB antiserum raised against a synthetic peptide corresponding to the carboxy-terminal 10 amino acids of CREB (27) reduced lens complex B1 and produced the expected supershifted complex (lane 4). Formation of B1, B2, and B3 was not affected by the presence of antisera specific for USF (lane 3),

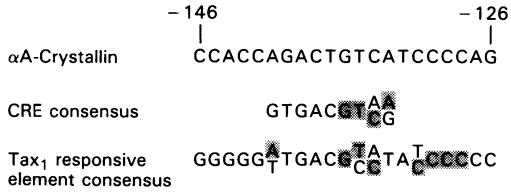

FIG. 6. Comparison of the chicken αA-crystallin CRE-like sequence with the CRE (23, 47) and Tax_1-responsive element (50) consensus sequences. Common sequences are in shadowed boxes.

ATF1 (lane 5), c-Jun (lane 6), JunB (lane 7), JunD (lane 8), FosB (lane 9), c-Fos (lane 10), or Fra2 (lane 11). The possibility that oligonucleotide B binds CREB or its closely related CREM proteins (see below) in lens nuclear extracts was further supported by the fact that prior treatment of the lens extract with the anti-CREB antiserum prevented the formation of complex B1 (lane 14). Proteins forming minor complexes B2 and B3 did not show any obvious tissue distribution (data not shown) and have not been identified yet.

CREB and CREM proteins are products of closely related genes which generate a number of isoforms by alternative RNA splicing (for reviews, see references 8 and 45). Structures of the C termini of CREB and several CREMs suggest the possibility that the antiserum against CREB (27) recognizes CREMs as well (17). To further identify candidate proteins from the CREB/CREM family forming lens complex B1, we used additional antisera and purified ΔCREB and CREMτ in EMSA experiments. Complex B1 (see Fig. 5E) was partially reduced in the presence of a polyclonal antibody against ΔCREB (CREB327, kindly provided by O. M. Andrisani) (lane 3) and was completely abolished in the presence of a polyclonal antibody against CREM (antiserum CREMS4, kindly provided by J. Habener) (lane 5). By contrast, B1 was not affected by the α-peptide-specific antibody recognizing αCREB (CREB341) (lane 4). Incubation of purified ΔCREB (lanes 6 and 7) and CREMτ (lanes 8 and 9) with labeled oligonucleotide B resulted in the formation of complexes which comigrated with the lens-specific B1 complex.

The involvement of a CRE-like site in the transcriptional regulation of the chicken αA-crystallin gene in PLEs was supported by a cotransfection experiment using the human T-cell leukemia virus type I Tax_1 expression plasmid $ptax_1$ (49). Tax_1 activates transcription from numerous CREs (see reference 19 and references therein). Tax_1 is a non-DNA-binding protein which interacts specifically at least with CREB homodimers and ATF1-CREB heterodimers (1, 50, 67). This interaction is controlled by amino acid residues 282 to 284 of CREB (1). In addition, specific sequences flanking the core CRE are required to mediate a positive response to Tax_1 (50). The Tax_1-responsive element consensus is present between positions −141 and −128 of the αA-crystallin gene (Fig. 6), consistent with the possibility that this region can be activated by Tax_1. Indeed, cotransfection of pcαA−162/+77-CAT with increasing amounts (0.1 to 3.0 μg) of $ptax_1$ into PLEs resulted in a corresponding enhancement of αA-crystallin promoter activity, with a maximum stimulation of fourfold (data not shown).

In contrast to the binding of CREB and/or CREM from the lens nuclear extract, the B1 complex formed in the CEF nuclear extract was partially supershifted in the presence of anti-CREB, anti-JunD, and anti-Fra2 antisera (Fig. 5D, lanes 4, 8, and 11). The B1, B2, and B3 complexes are not easily discerned in Fig. 5D because the autoradiogram was overexposed in order to clearly visualize the supershifted band. Furthermore, treatment of the CEF nuclear extracts with anti-JunD or anti-Fra2 antiserum significantly reduced the amount of B1 (lanes 18 and 21). Antisera against ΔCREB and CREM (Fig. 5E, lanes 11 to 13) had no effect on the fibroblast complex B1 (lane 10). The ability of oligonucleotide B to interact with members of the AP-1 family is demonstrated directly for c-Jun (lanes 14 to 16). An antiserum against c-Jun inhibited the formation of this complex (lane 17). Collectively, EMSA competition, immunological data, and Tax_1 activation experiments indicate that the lens B1 complex is distinct from its fibroblast counterpart. The lens complex B1 is formed by CREB and/or CREM family members, while the fibroblast B1 complex involves AP-1 family members.

Pax-6 interacts with DE2B, DE1A, and the −60/−29 sequence. We next studied the function of the DE2B (−128 to −119) and DE1A (−114 to −103) regions on expression by using the pαA(i55) series of plasmids, in which the DE1B repressor has been removed (Fig. 1B). Mutation of either the DE2B [pαA(i55M12)] or DE1A [pαA(i55M13) and pαA (i55M14)] region reduced promoter activity in transfected PLEs relative to the control pαA(i55) plasmid (Fig. 4), in agreement with previous studies (34). In contrast, the two DE1A mutations slightly elevated promoter activity in the transfected CEFs. Furthermore, linker-scanning mutant pcαAm−108/−103-CAT (34) gave threefold-higher activity relative to pcαA−162/+77-CAT in transfected CEFs (data not shown), suggesting that the −108/−103 sequence behaves as a negative element in these calls. Taken together, these findings suggest that the −128/−101 sequence may contain at least one *cis* element which acts positively in lens cells and negatively in fibroblasts.

Upon examination of the DE2B and DE1A regions, we noted a conserved hexadecamer motif located at positions −131 to −116 and −116 to −101; this motif is also present at −51 to −41 in the chicken αA-crystallin gene. The −57/−41 sequence is functionally significant, since it is highly conserved in the αA-crystallin genes of several species (30, 62) and mutation of this region in the mouse αA-crystallin gene reduced promoter activity in transfected lens cells (13, 48). Of particular interest is that this hexadecamer motif is similar (although not identical) to the recently described Pax-6 consensus binding site derived by the PCR-based selection method (16). It also shares sequence similarity with the binding site for the paired-box protein BSAP/Pax-5. Certain BSAP/Pax-5-binding sites can interact in vitro with Pax-6 (7).

We investigated whether the −133/−98 (oligonucleotide C; Fig. 1C) and −60/−29 (oligonucleotide E) sequences can interact with Pax-6 in chicken lens nuclear extracts (Fig. 7). The −60/−29 oligonucleotide was incubated with a chicken lens nuclear extract and with a whole-cell extract from COP-8 cells transfected with a mouse Pax-6 cDNA expression vector (pKW10-Pax-6 [7]). Two major complexes, E1 and E2, and several minor complexes were detected (Fig. 7A, lanes 2 and 9). E1 was virtually abolished by competition with oligonucleotides comprising three different BSAP/Pax-5-binding sites (H2B2.2 [lane 4], e5 [lane 5], and H2A2.1 [lane 7]), and both E1 and E2 were eliminated by competition with an oligonucleotide containing a fourth BSAP/Pax-5-binding site (CD19-1; lane 6). All four competitor oligonucleotides have been shown to interact with Pax-6, albeit with a lower affinity than with BSAP/Pax-5 (7). E1 was not detected in the presence of anti-Pax-6 antiserum 11 (lane 8), which recognizes a Pax-6 paired domain (4). Similar data were obtained for whole-cell extract of COP-8 cells transfected with pKW10-Pax-6 (lanes 9 and 10). In contrast to results for lens and Pax-6-containing

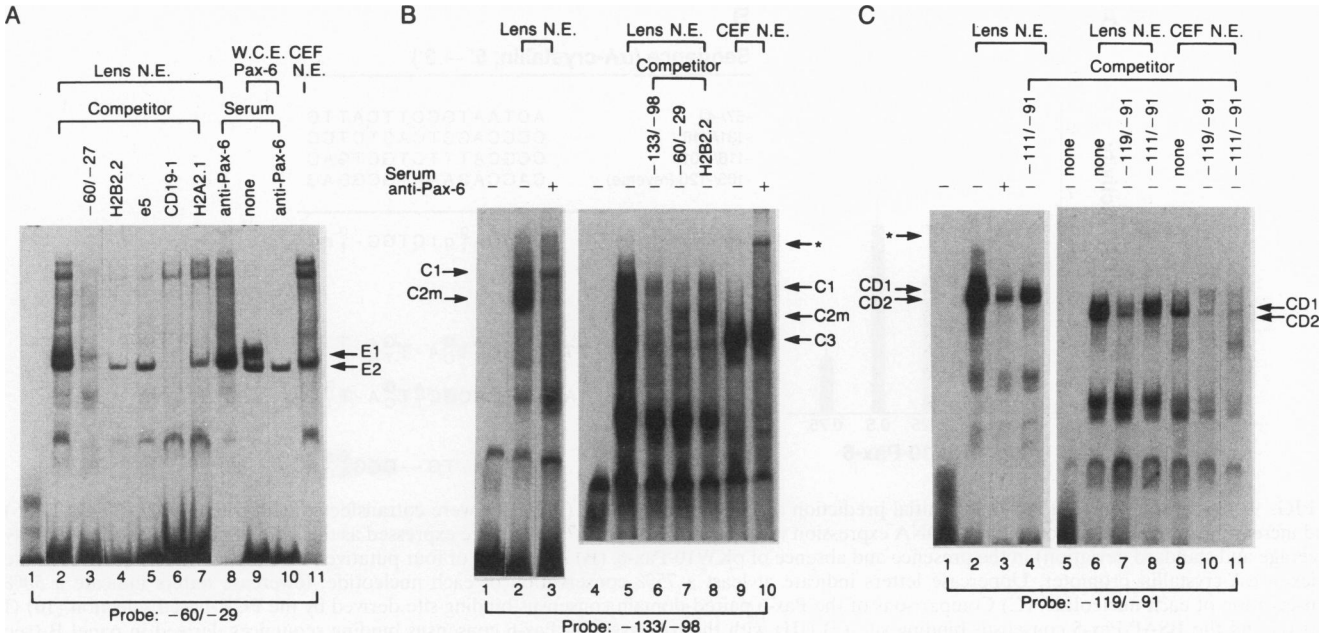

FIG. 7. EMSA analysis of Pax-6 protein binding to the chicken αA-crystallin promoter. (A) E1 and E2 are major specific complexes formed with oligonucleotide E (−60 to −29) in the presence of poly(dA-dT) as a nonspecific competitor. W.C.E. Pax-6 designates a whole-cell extract from COP-8 transfected with pKW10-Pax-6. (B) C1, C2m, and C3 are major specific complexes formed with oligonucleotide C (−133 to −98) in the presence of poly(dI-dC) as a nonspecific competitor. The autoradiogram showing lanes 4 to 10 was overexposed to show both the C2m and C3 bands. (C) CD1 and CD2 are major specific complexes formed with oligonucleotide CD (−119 to −91) in the presence of poly(dI-dC).

extracts, incubation of the −60/−29 oligonucleotide with CEF nuclear extracts did not result in the formation of E1, although E2 was observed (lane 11). These data support the idea that Pax-6 binds to the −60/−29 sequence of the chicken αA-crystallin promoter in lens cells but not in CEFs.

The binding of proteins to the DE2B/DE1A sequence of the chicken αA-crystallin gene was studied next by incubating oligonucleotide C (−133 to −98) and oligonucleotide CD (−119 to −91) (Fig. 1C) with the lens and CEF nuclear extracts. The complexes generated with oligonucleotide C were generally less discrete than those formed with oligonucleotide CD. Nevertheless, oligonucleotide C, which contains three hexadecamer motifs, generated several retarded complexes (C1, C2m, and C3 in Fig. 7B). The major, broad lens-specific band appeared to contain multiple complexes labeled C2m (lanes 2 and 5); C2m was not formed in the CEF nuclear extract (lane 9). C2m was reduced in the presence of anti-Pax-6 antiserum (lane 3) and by competition with oligonucleotides known to bind Pax-6 (lanes 7 and 8). CEF nuclear extracts formed another complex called C3 with oligonucleotide C (lane 9), which was insensitive to the anti-Pax-6 antiserum (lane 10).

We next incubated oligonucleotide CD, which contains two hexadecamer motifs, with lens or CEF nuclear extracts (Fig. 7C). The lens extract formed two specific complexes, CD1 and CD2 (lanes 2 and 6); these were reduced significantly by competition with itself (lane 7) but only weakly by competition with oligo D (lanes 4 and 8), which lacks the hexadecamer motif. Furthermore, the anti-Pax-6 antiserum reduced the amount of CD1 (lane 3). These observations are consistent with the presence of at least one putative Pax-6 recognition site in the −119/−91 sequence. In contrast, the CEF nuclear extract formed one major complex, CD2 (lane 9), which was virtually eliminated by competition with both oligonucleotide CD (lane 10) and oligonucleotide D (lane 11), consistent with the absence of Pax-6 binding in CEFs.

The presence of Pax-6 in the lens nuclear extracts was demonstrated by Western immunoblotting using the antiserum recognizing the Pax-6 paired domain (Fig. 8). The major immunoreactive products detected in the nuclear extracts from the lens and brain and in whole-cell extract of COP-8 cells transiently transfected with pKW10-Pax-6 had an expected mobility of a 46- to 48-kDa protein. Minor bands of 32 to 33 kDa probably represent different Pax-6 isoforms, by analogy with the analysis of Pax-6 in the quail neuroretina (4). The 30-kDa band detected in the CEF nuclear extract may be artifactual since it did not appear with a different anti-Pax-6 antiserum (kindly provided by I. Mikkola and T. Johansen)

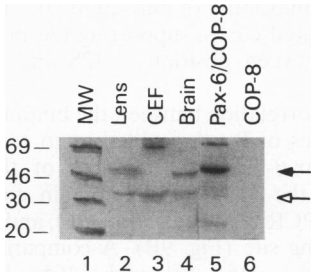

FIG. 8. Western immunoblotting of various extracts (see Materials and Methods) and anti-Pax-6 antiserum. The solid arrow indicates the major 46- to 48-kDa immunoreactive band known to be Pax-6 (4); the open arrow indicates bands near 32 to 33 kDa which are probably alternatively spliced products of the Pax-6 gene (4). A sodium dodecyl sulfate–4 to 20% polyacrylamide gradient gel (Novex, San Diego, Calif.) was used. Positions of molecular weight (MW) standards are shown in thousands.

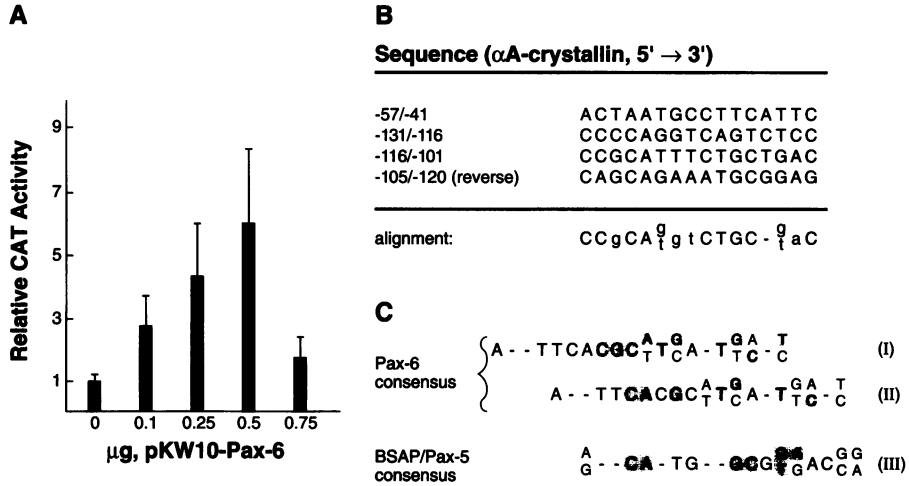

FIG. 9. Cotransfection with Pax-6 and initial prediction of Pax-6-binding sites. (A) CEFs were cotransfected with pcαA−162/+77-CAT (35) and increasing amounts of a mouse Pax-6 cDNA expression vector (pKW10-Pax-6 [7]). Data are expressed as the ratio of normalized CAT activity (average and standard deviation) in the presence and absence of pKW10-Pax-6. (B) Alignment of four putative Pax-6 recognition sequences in the chicken αA-crystallin promoter. Uppercase letters indicate at least a 75% conservation of each nucleotide; lowercase letters indicate a 50% conservation of each nucleotide. (C) Comparisons of the Pax-6 paired-domain consensus binding site derived by the PCR-based selection (16) (I and II) and the BSAP/Pax-5 consensus binding site (7) (III) with the αA-crystallin Pax-6 consensus binding sequences derived in panel B (see alignment). Two possible alignments (I and II) between the αA-crystallin-derived Pax-6 consensus binding sequence and the PCR-derived Pax-6 paired-domain consensus binding sequence (16) are shown. Common nucleotides in the αA-crystallin alignment and the Pax-6 and BSAP/Pax-5 consensus sites are shaded.

recognizing the highly conserved C terminus of zebrafish Pax-6 (6a).

Pax-6 activates the chicken αA-crystallin promoter in cotransfected CEFs. The possibility that Pax-6 activates the chicken αA-crystallin promoter was examined by cotransfecting CEFs with pcαA−162/+77-CAT (35) and the mouse Pax-6 cDNA expression vector (pKW10-Pax-6 [7]). Increasing amounts of pKW10-Pax-6 stimulated pcαA−162/+77-CAT activity in cotransfected CEFs (Fig. 9A); the parental vector (pKW10) lacking Pax-6 coding sequences had no effect on pcαA−162/+77-CAT activity (data not shown). A maximum stimulation of sixfold was found with 500 ng of pKW10-Pax-6. Therefore, Pax-6 is capable of activating the chicken αA-crystallin gene promoter in cells which do not normally express the αA-crystallin gene. In addition, we tested two plasmids (pcαAm−128/−123-CAT and pcαAm−108/−103-CAT [34]) with mutations within the indicated sequences (−128 to −123 or −108 to −103) in a similar cotransfection experiment using CEFs and 0, 250, and 500 ng of pKW10-Pax-6. CAT activity was increased a maximum of only 20 to 40% by pKW10-Pax-6 in the cotransfected CEFs, supporting the presence of Pax-6-binding sites between positions −128 and −103 (data not shown).

The positive correlation between the binding and promoter-activating abilities of Pax-6 enabled us to compare and align the putative Pax-6-binding sequences of the chicken αA-crystallin gene, the Pax-6 paired-domain consensus binding sites derived by PCR-based selection (16), and the BSAP/Pax-5 consensus binding site (Fig. 9B). A comparison of the −57/−41, −131/−116, −116/−101, and −105/−120 (reverse) regions yielded a common pattern related to both the Pax-6 paired-domain (16) and BSAP/Pax-5 (7) consensus binding sites. The alignment of chicken αA-crystallin Pax-6-binding sites also agrees with a general model of the Pax bipartite recognition site derived from binding sequences known for Pax-1, Pax-5, and Pax-8 (7).

DE1B (−102 to −91) is a repressor site. In our previous investigation, mutations pcαAm−102/−96-CAT and pcαAm −96/−91-CAT within the DE1B site stimulated activity of the αA-crystallin promoter in transfected PLEs (34). The same mutants stimulated promoter activity 12- and 3-fold, respectively, in transfected CEFs (34a). These findings suggested that DE1B acts as a repressor in lens and nonlens cells. To investigate further the possible repressing function of DE1B, oligonucleotides containing the wild-type (D) or mutated (D-M1 and D-M2) −106/−86 region were subcloned into pαA (i55) between the −148/−96 insert and the basal −56/+77 promoter (Table 1). The resulting plasmids [pαA(i55D), pαA (i55D-M1), and pαA(i55D-M2)] maintained the linear order of individual sites present in the normal chicken αA-crystallin gene. Insertion of the wild-type −106/−86 sequence into pcαA (i55D) resulted in a nearly threefold reduction of CAT activity relative to pcαA(i55) levels in transfected PLEs (Table 1). In contrast, mutation D-M1 produced a slight reduction and mutation D-M2 produced a marginal elevation of promoter activity in transfected PLEs. Similar results were obtained for transfected CEFs.

We next investigated the binding of nuclear factors to oligonucleotide D (−111 to −91) (Fig. 10). At least two specific complexes, D1 and D2, were detected after incubating oligonucleotide D with the lens (lane 2) or CEF (lane 6) nuclear extracts.

Inspection of the −104/−94 sequence (5′ GACCACGTT GC 3′) revealed a similarity with the E-box motif (underlined), as exists in DE2A which binds the helix-loop-helix protein, USF (see above). Consequently, we investigated the possibility that USF also binds to DE1B by immunoshift experiments (Fig. 10). An anti-USF antiserum prevented the formation of D1 and D2 in lens (lane 4) and CEF (lane 8) nuclear extracts, while the preimmune serum did not (lanes 5 and 9, respectively). In addition, when the lens nuclear extract was immunoprecipitated with an anti-USF antiserum before incubation with oligonucleotide D, complexes D1 and D2 were not detected (lane 10). Incubation of oligonucleotide D with purified USF

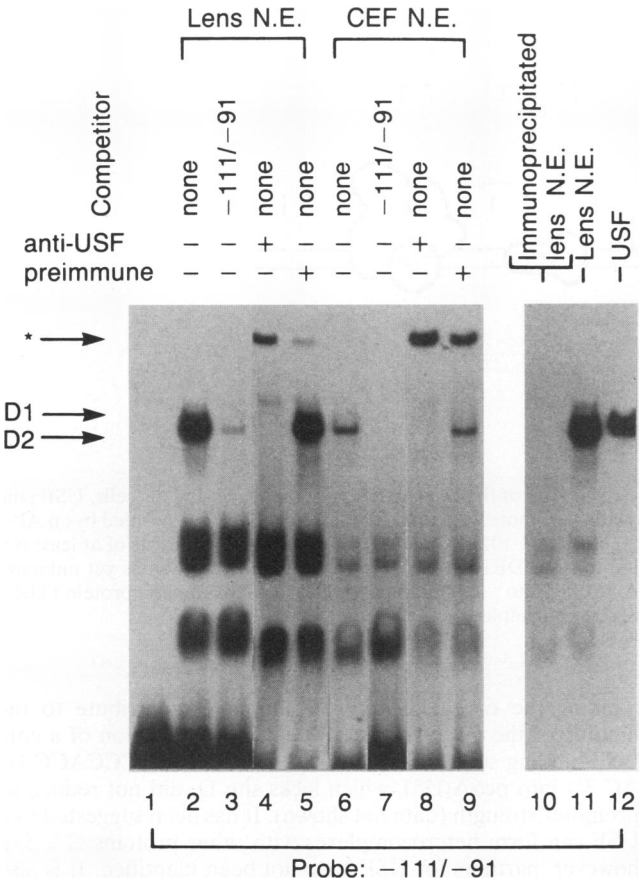

FIG. 10. EMSA analysis of proteins interacting with the DE1B sequence. D1 and D2 are major specific complexes formed on oligonucleotide D (−111 to −91) in the presence of poly(dI-dC) as a nonspecific competitor. In one case (lane 10), the anti-USF antiserum was used to immunodeplete the lens nuclear extract (N.E.) prior to incubation with the probe; the antiserum was used in direct binding experiments in all other cases (lanes 4 and 8).

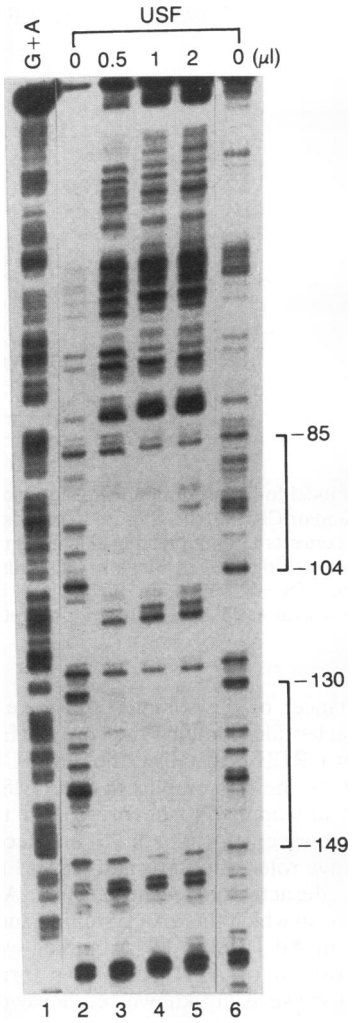

FIG. 11. In vitro DNase I footprinting of the chicken αA-crystallin promoter. The chicken αA-crystallin −242/+77 promoter fragment was footprinted in vitro, using 0 (lanes 2 and 6), 0.5, 1, or 2 μl of purified USF protein (300 ng/μl, 90% purity). Protected regions (lanes 3 to 5) are bracketed. G+A (lane 1) is a sequencing ladder.

resulted in the formation of a specific complex that migrated closely with D1 and D2 (lane 12). We presume that the residual CD1 and CD2 complexes remaining with oligonucleotide D in lens nuclear extracts treated with anti-Pax-6 antiserum (Fig. 7C, lane 3) is due to binding of USF to this probe (Fig. 10).

Finally, DNase I footprinting of the −242/+77 promoter fragment incubated with purified USF was used to test directly the possibility that USF binds to DE2A and DE1B. The results showed clearly that positions −149 to −130 (DE2A) and −104 to −85 (DE1B) were protected from DNase I digestion after incubation with purified USF protein (Fig. 11).

DISCUSSION

Our previous mutagenesis and protein-binding experiments have identified DE2A (−144 to −134), DE2B (−128 to −119), DE1A (−114 to −104), and DE1B (−102 to −92) as regulatory regions of the chicken αA-crystallin gene (34). These are similar but not identical to the αCE1 (−162 to −134), αCE2 (−119 to −99), and αCE3 (−135 to −121) regulatory regions identified by Matsuo and Yasuda (42). Our present investigation has resulted in an initial molecular model for the transcriptional regulation of the chicken αA-crystallin gene. We now call the control sites A (including the 5′ half of DE2A), B (including the 3′ half of DE2A), C (including DE2B and DE1A), D (including DE1B), and E (−57 to −42). These are diagrammed in Fig. 12. Our EMSA, immunological, mutagenesis, and transfection data indicate that these five sites bind at least USF, CREB, and/or CREM (or AP-1 proteins) and Pax-6 to create a complex array of positive and negative regulatory elements that confers high expression in the lens and repression in fibroblasts.

Site A binds the ubiquitous factor USF in nuclear extracts of both lens and fibroblasts and forms half of a composite element. A hallmark of composite elements is that the activating or repressing potential of a bound factor is determined by the protein(s) occupying an adjacent site (12, 59). Site B is adjacent to the USF-binding site A. Site B behaves as a CRE in the lens, where the gene is active, and binds CREB and/or CREM. The chicken αA-crystallin recognition sequence (5′ GACTGTCA 3′) diverges substantially from the consensus CRE, TGACGTC/AA/G (23, 47). A similar divergent CREB-binding site (5′ GTGCGTAA 3′) was recently found in the

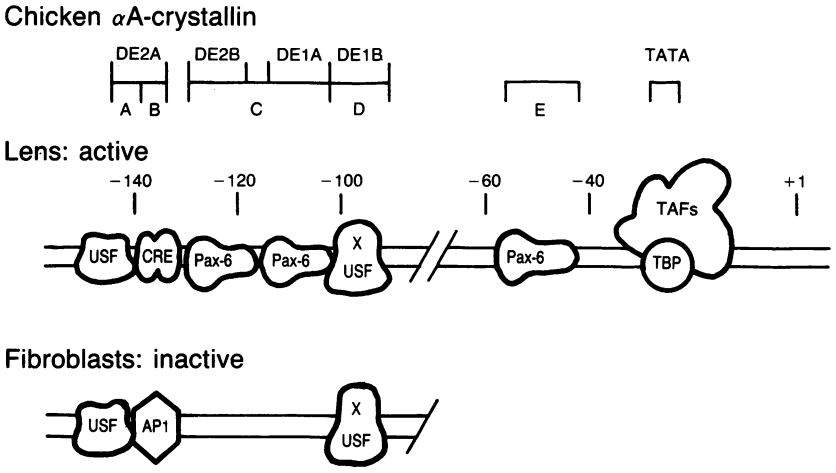

FIG. 12. A model for transcription factor interactions contributing to the regulation of the chicken αA-crystallin gene. In lens cells, USF (site A) and CREB and/or CREM (site B) form a composite element (DE2A) activating promoter function. In fibroblasts, site B is occupied by an AP-1 complex which converts USF interacting at site A into a repressor. Region C (−130 to −101, including DE2B and DE1A) consists of at least two Pax-6-binding sites recognized by Pax-6 in lens cells. Repressor site D (−102 to −91, DE1B) binds USF in a complex with an as yet unknown protein (X). The −56/−42 region also binds Pax-6, and the canonical TATA box (−30 to −23) interacts with TATA-box binding protein (TBP), its associated coactivators (TAFs), and other components of an active transcription complex.

upstream enhancer of the somatostatin gene (65). It is also known that nucleotides flanking the core CRE are involved in the binding of CREB (10). Since the anti-αCREB antiserum did not eliminate the B1 complex in our EMSA experiments, αCREB does not appear to be present in the lens-specific complex B1. However, further studies are necessary to distinguish the relative roles of CREB and CREM family members in the lens-specific activation of the chicken αA-crystallin gene.

In fibroblasts, in which the αA-crystallin gene is inactive, site B behaves as an AP-1 site in DNA-binding experiments. The precise composition of AP-1 complexes formed in nuclear extracts of fibroblasts is not known yet; however, the immunoprecipitation results suggest the involvement of Fra2 and JunD, with a possible role for ATF/CREB family members. Thus, our results demonstrate that USF, in addition to the previously characterized AP-1, CREB, and steroid hormone receptor-binding factors (46), can be a component of composite element complexes. The specificity of the adjacent binding sites was illustrated in the EMSA tests showing the absence of cross-competition between oligonucleotides A (−150 to −138) and B (−144 to −127). This is explained by oligonucleotide A lacking a complete CRE-like site and oligonucleotide B lacking complete USF site. The binding of factors to these adjacent regulatory elements is consistent with our previous methylation interference experiments (35).

The present data extend our previous mutagenesis experiments (34) and indicate that site D is a negative element in both lens and fibroblasts. Both site A and site D bind USF. At least two models can be invoked to explain the dual role for USF in the regulation of the chicken αA-crystallin gene. In one model, USF is complexed either with itself, forming a homodimer to activate promoter activity (site A), or with a different, unidentified protein, forming a heterodimer to repress promoter activity (site D). The similar intensities of the complexes formed with site A in nuclear extracts derived from the lens and fibroblasts (Fig. 3A) contrast with the different intensities of the complexes formed with site D in the extracts derived from these two sources (Fig. 10). This observation supports the idea that the A and D complexes are not composed of identical proteins. It is likely that sequences flanking the core USF-binding site in D contribute to the binding of the putative heterodimer, since insertion of a core USF-binding sequence (−150 to −139, 5′ GTTCCCACCAG AC 3′) into pcαA(i55), which lacks site D, did not reduce its promoter strength (data not shown). It has been suggested that USF can form heterocomplexes with other proteins (14, 55); however, partners for USF have not been identified. It is also possible that the activating effect of USF at site A and its repressing effect at site D may be due to the different relative positions of these control elements with respect to the other regulatory sites or factors of the αA-crystallin gene. These possibilities are not mutually exclusive.

Finally, the present investigation indicates that Pax-6 acts in concert with USF and CREB/CREM to activate chicken αA-crystallin gene expression in the lens. That Pax-6 does not bind to the chicken αA-crystallin gene regulatory elements in nuclear extracts from fibroblasts is consistent with its importance as a positive control factor specifically for lens expression (Fig. 12). Indeed, Pax-6 has been reported in numerous tissues of the developing eye, including the presumptive lens ectoderm and the embryonic lens (37, 39, 68). The necessity for Pax-6 binding to sites C and E for αA-crystallin promoter activity is supported by the fact that the −154/−90 sequence can activate the −56/+77 promoter fragment but not the −35/+77 promoter fragment in transfection experiments (data not shown). However, despite the beneficial contribution of site E for optimal promoter activity of the chicken αA-crystallin gene, linker-scanning mutations throughout this region did not reduce promoter strength in transfected PLEs (34). Perhaps this reflects some functional redundancy when the complete −162/+77 sequence was used, as was done in the previous experiments (34). The additive effect of multiple control elements for efficient enhancer activity was demonstrated in transfection experiments using a heterologous promoter driven by different combinations of *cis* elements of the chicken αA-crystallin gene (42).

Apart from the autoregulated expression of Pax-6 in the quail neuroretina (54), our data provide the first reported target gene for Pax-6. Thus, we have performed preliminary sequence alignments as a basis for eventually deriving a Pax-6

consensus binding site, using naturally occurring sequences (Fig. 9B). Inspection of the database for this putative consensus binding site has identified potential Pax-6-binding sites among the regulatory sequences that have been shown previously to be required for lens expression of the mouse αA-crystallin (6, 6a, 31) and αB-crystallin (15), chicken βB1-crystallin (56), βA3/A1-crystallin (44) and δ1-crystallin (24), and guinea pig ζ-crystallin (38) genes. Indeed, recent experiments have indicated that Pax-6 binds to and activates the lens-specific enhancer of the chicken δ1-crystallin gene (6b) and that Pax-6 binds to the lens-specific regulatory sequence (ZPE) of the guinea pig ζ-crystallin gene (55a). The number of Pax-6-binding sites and multiple forms of Pax-6 (4) may both contribute to the selective regulation of Pax-6 target genes.

In summary, the present experiments indicate that Pax-6, USF, CREB and/or CREM, and AP-1 proteins are involved in a complex regulatory network including positive and negative elements controlling the high expression of the αA-crystallin gene in the lens and its repressed state in nonlens tissues of the chicken. Additional levels of regulation of αA-crystallin gene activity may, of course, be determined by chromatin structure as well as by other *cis* elements and *trans* factors. For example, the −242/−162 sequence lowers promoter activity in transfected PLEs and CEFs (35), suggesting another negative element in this region. Moreover, another protein binding site, DE3, was found upstream of the site A (34), and binding of a heat shock transcription factor to this region was recently obtained (18). Thus, our present data provide an initial model upon which to base future experiments on the regulated expression of this highly expressed lens gene.

ACKNOWLEDGMENTS

We are grateful to O. M. Andrisani, M. Busslinger, T. Czerny, J. Habener, H. C. Hurst, K. A. W. Lee, and S. Saule for clones and antisera. We thank J. N. Brady and F. Kashanchi for $ptax_1$ and help with Western immunoblottings. J. F. Klement prepared the mutant M1-M3 plasmids of pcαA−162/+77-CAT. We thank B. Norman and K. Cveklova for help with preparation of oligonucleotides and plasmids. We thank Z. Kozmik and J. B. McDermott for helpful suggestions. Nucleotide sequence analyses were performed on the Advanced Scientific Computing Laboratory supercomputer facilities located at NCI/FCRDC (Frederick, Md.).

REFERENCES

1. **Adya, N., L.-J. Zhao, W. Huang, I. Boros, and C.-Z. Giam.** 1994. Expansion of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282-284 near the conserved DNA-binding domain of CREB. Proc. Natl. Acad. Sci. USA **91**:5642–5646.
2. **Bresnick, E. H., and G. Felsenfeld.** 1993. Evidence that the transcription factor USF is a component of the human β-globin locus control region heteromeric protein complex. J. Biol. Chem. **268**:18824–18834.
3. **Bresnick, E. H., and G. Felsenfeld.** 1994. The leucine-zipper is necessary for stabilizing a dimer of the helix-loop-helix transcription factor USF but not for maintenance of an elongated conformation. J. Biol. Chem. **269**:2110–2116.
4. **Carriere, C., S. Plaza, P. Martin, B. Quatannens, M. Bailly, D. Stehelin, and S. Saule.** 1993. Characterization of quail Pax-6 (Pax-QNR) proteins expressed in the neuroretina. Mol. Cell. Biol. **13**:7257–7266.
5. **Carthew, R. W., L. A. Chodosh, and P. A. Sharp.** 1987. The major late transcription factor binds to and activates the mouse metallothionein I promoter. Genes Dev. **1**:973–980.
6. **Chepelinsky, A. B., B. Sommer, and J. Piatigorsky.** 1987. Interaction between two different regulatory elements activates the murine αA-crystallin gene promoter in explanted lens epithelia. Mol. Cell. Biol. **7**:1807–1814.

6a. **Cvekl, A., F. Kashanchi, C. M. Sax, J. N. Brady, and J. Piatigorsky.** Unpublished data.

6b. **Cvekl, A., C. M. Sax, X. Li, and J. Piatigorsky.** Unpublished data.

7. **Czerny, T., G. Schaffner, and M. Busslinger.** 1993. DNA sequence recognition structure of the paired domain and its binding site. Genes Dev. **7**:2048–2061.
8. **deGroot, R. P., and P. Sassone-Corsi.** 1993. Hormonal control of gene expression: multiplicity and versatility of cyclic adenosine 3′,5′-monophosphate-responsive nuclear regulators. Mol. Endocrinol. **7**:145–153.
9. **de Jong, W. W., J. A. M. Leunissen, and C. E. M. Voorter.** 1993. Evolution of the α-crystallin/small heat shock protein family. Mol. Biol. Evol. **10**:103–126.
10. **Deutsch, P. J., J. P. Hoeffler, J. L. Jameson, J. C. Liu, and J. F. Habener.** 1988. Structural determinants for transcription for transcriptional activation by cAMP-responsive DNA elements. J. Biol. Chem. **263**:18466–18472.
11. **Deutsch, U., and P. Gruss.** 1991. Murine paired domain proteins as regulatory factors of embryonic development. Semin. Dev. Biol. **2**:413–424.
12. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto.** 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science **249**:1266–1272.
13. **Donovan, D. M., C. M. Sax, J. F. Klement, X. Li, A. B. Chepelinsky, and J. Piatigorsky.** 1992. Conservation of mouse αA-crystallin promoter activity in chicken lens epithelial cells. J. Mol. Evol. **35**:337–345.
14. **Du, H., A. L. Roy, and R. G. Roeder.** 1993. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. EMBO J. **12**:501–511.
15. **Dubin, R. A., E. F. Wawrousek, and J. Piatigorsky.** 1989. Expression of the murine αB-crystallin gene is not restricted to the lens. Mol. Cell. Biol. **9**:1083–1091.
16. **Epstein, J., J. Cai, T. Glaser, L. Jepeal, and R. Maas.** 1994. Identification of a *Pax* paired domain recognition sequence and evidence for DNA-dependent conformational change. J. Biol. Chem. **269**:8355–8361.
17. **Foulkes, N. S., B. Mellstrom, E. Benusiglio, and P. Sassone-Corsi.** 1992. Developmental switch of CREM function during spermatogenesis: from antagonist to activator. Nature (London) **355**:80–84.
18. **Frederikse, P., and J. Piatigorsky.** 1994. Normal and heat inducible binding of HSF proteins to α-crystallin regulatory sequences. Invest. Ophthalmol. Visual Sci. **35**(Suppl.):2073.
19. **Fujii, M., H. Tsuchiya, T. Chuhjo, T. Akizawa, and M. Seiki.** 1992. Interaction of HTLV-I Tax_1 with $p67^{SRF}$ causes the aberrant induction of cellular immediate early genes through CArG boxes. Genes Dev. **6**:2066–2076.
20. **Glaser, T., D. S. Walton, and R. L. Maas.** 1992. Genomic structure, evolutionary conservation and aniridia mutations in the human Pax6 gene. Nature Genet. **2**:232–239.
21. **Grainger, R. M.** 1992. Embryonic lens induction: shedding light on vertebrate tissue determination. Trends Genet. **8**:349–355.
22. **Gruss, P., and C. Walther.** 1992. Pax in development. Cell **69**: 719–722.
23. **Habener, J. F.** 1990. Cyclic AMP response element binding proteins: a cornucopia of transcription factors. Mol. Endocrinol. **4**: 1087–1094.
24. **Hayashi, S., K. Goto, T. S. Okada, and H. Kondoh.** 1987. Lens-specific enhancer in the third intron regulates expression of the chicken δ1-crystallin gene. Genes Dev. **1**:818–828.
25. **Hill, R. E., J. Favor, B. L. M. Hogan, C. T. T. Ton, G. F. Saunders, I. M. Hanson, J. Prosser, T. Jordan, N. D. Hastie, and V. van Heyningen.** 1991. Mouse *Small eye* results from mutations in a paired-like homeobox-containing gene. Nature (London) **354**: 522–525.
26. **Horwitz, J.** 1992. α-Crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. USA **89**:10449–10453.
27. **Hurst, H. C., N. Mason, N. C. Jones, and K. A. W. Lee.** 1990. The cellular transcription factor CREB corresponds to activating transcription factor 47 (ATF-47) and forms complexes with a group of polypeptides related to ATF-43. Mol. Cell. Biol. **10**:6192–6203.
28. **Hurst, H. C., N. F. Totty, and N. C. Jones.** 1991. Identification and

functional characterization of the cellular activating transcription factor 43 (ATF-43) protein. Nucleic Acids Res. **19:**4601–4609.
29. **Ingolia, T. C., and E. A. Craig.** 1982. Four small Drosophila heat shock proteins are related to each other and to mammalian α-crystallins. Proc. Natl. Acad. Sci. USA **79:**2360–2364.
30. **Jaworski, C. J., A. B. Chepelinsky, and J. Piatigorsky.** 1991. The αA-crystallin gene: conserved features of the 5′ flanking regions in human, mouse, and chicken. J. Mol. Evol. **33:**495–505.
31. **Kadesch, T.** 1993. Consequences of heteromeric interactions among helix-loop-helix proteins. Cell Growth Differ. **4:**49–55.
32. **Kantorow, M., A. Cvekl, C. M. Sax, and J. Piatigorsky.** 1993. Protein-DNA interactions of the mouse αA-crystallin control regions. J. Mol. Biol. **230:**425–435.
33. **Kim, R. Y., T. Lietman, J. Piatigorsky, and G. J. Wistow.** 1991. Structure and expression of the duck α-enolase/τ-crystallin-encoding gene. Gene **103:**193–200.
34. **Klement, J. F., A. Cvekl, and J. Piatigorsky.** 1993. Functional elements DE2A, DE2B and DE1A and the TATA box are required for activity of the chicken αA-crystallin gene in transfected lens epithelial cells. J. Biol. Chem. **268:**6777–6784.
34a. **Klement, J. F., and J. Piatigorsky.** Unpublished data.
35. **Klement, J. F., E. F. Wawrousek, and J. Piatigorsky.** 1989. Tissue-specific expression of the chicken αA-crystallin gene in cultured lens epithelia and transgenic mice. J. Biol. Chem. **264:**19837–19844.
36. **Krauss, S., T. Johansen, V. Korzh, and A. Fjose.** 1991. Expression pattern of zebrafish genes suggests a role in early brain regionalization. Nature (London) **353:**267–270.
37. **Krauss, S., T. Johansen, V. Korzh, U. Moens, J. U. Ericson, and A. Fjose.** 1991. Zebrafish pax[zf-a]: a paired box-containing gene expressed in the neural tube. EMBO J. **10:**3609–3619.
38. **Lee, D. C., P. Gonzales, and G. Wistow.** 1994. ζ-Crystallin: a lens-specific promoter and the gene recruitment of an enzyme as a crystallin. J. Mol. Biol. **236:**669–678.
39. **Li, H.-S., J.-M. Yang, R. Jacobson, D. Pasko, and O. Sundin.** 1994. Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. Dev. Biol. **162:**181–194.
40. **Martha, A., R. E. Ferrell, H. Mintz-Hittner, L. A. Lyons, and G. F. Saunders.** 1994. Paired box mutations in familial and sporadic aniridia predicts truncated aniridia proteins. Am. J. Hum. Genet. **54:**801–811.
41. **Matsuo, I., M. Kitamura, K. Okazaki, and K. Yasuda.** 1991. Binding of a factor to an enhancer element responsible for the tissue-specific expression of the chicken αA-crystallin gene. Development **113:**539–550.
42. **Matsuo, I., and K. Yasuda.** 1992. The cooperative interaction between two motifs of an enhancer element of the chicken αA-crystallin gene, αCE1 and αCE2, confers lens-specific expression. Nucleic Acids Res. **20:**3701–3712.
43. **McAvoy, J. W.** 1980. Induction of the eye lens. Differentiation **17:**137–149.
44. **McDermott, J. B., C. A. Peterson, and J. Piatigorsky.** 1992. Structure and lens expression of the gene encoding chicken βA3/A1-crystallin. Gene **117:**193–200.
45. **Meyer, T. E., and J. F. Habener.** 1993. Cyclic adenosine 3′,5′-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. Endocrine Rev. **14:**269–290.
46. **Miner, J. N., M. I. Diamond, and K. R. Yamamoto.** 1991. Joints in the regulatory lattice: composite regulation by steroid receptor-AP1 complexes. Cell Growth Differ. **2:**525–530.
47. **Montminy, M. R., G. A. Gonzales, and K. K. Yamamoto.** 1990. Regulation of cAMP-inducible genes by CREB. Trends Neurochem. Sci. **13:**184–188.
48. **Nakamura, T., D. M. Donovan, K. Hamada, C. M. Sax, B. Norman, J. R. Flanagan, K. Ozato, H. Westphal, and J. Piatigorsky.** 1990. Regulation of the mouse αA-crystallin gene: isolation of a cDNA encoding a protein that binds to a *cis* sequence motif shared with the major histocompatibility complex class I gene and other genes. Mol. Cell. Biol. **10:**3700–3708.
49. **Nerenberg, M. I., S. H. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay.** 1987. The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. Science **237:**1324–1328.
50. **Paca-Uccaralertkun, S., L.-J. Zhao, N. Adya, J. V. Cross, B. R. Cullen, I. M. Boros, and C.-Z. Giam.** 1994. In vitro selection of DNA elements highly responsive to the human T-cell lymphotropic virus type I transcriptional activator, Tax. Mol. Cell. Biol. **14:**456–462.
51. **Piatigorsky, J.** 1981. Lens differentiation in vertebrates. A review of cellular and molecular features. Differentiation **19:**134–158.
52. **Piatigorsky, J.** 1992. Lens crystallins. Innovation associated with changes in gene regulation. J. Biol. Chem. **267:**4277–4280.
53. **Piatigorsky, J., and P. S. Zelenka.** 1992. Transcriptional regulation of crystallin genes: *cis* elements, trans-factors, and signal transduction systems in the lens. Adv. Dev. Biochem. **1:**211–256.
54. **Plaza, S., C. Dozier, and S. Saule.** 1993. Quail PAX-6 (PAX-QNR) encodes a transcription factor able to bind and transactivate its own promoter. Cell Growth Differ. **4:**1–10.
55. **Pognonec, P., and R. G. Roeder.** 1991. Recombinant 43-kDa USF binds to DNA and activates transcription in a manner indistinguishable from that of natural 43/44-kDa USF. Mol. Cell. Biol. **11:**5125–5136.
55a. **Richardson, J., A. Cvekl, and G. J. Wistow.** Submitted for publication.
56. **Roth, H. J., G. C. Das, and J. Piatigorsky.** 1991. Chicken βB1-crystallin gene expression: presence of conserved functional polyomavirus enhancer-like and octamer binding-like promoter elements found in non-lens genes. Mol. Cell. Biol. **11:**1488–1499.
57. **Ryseck, R.-P., and R. Bravo.** 1991. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene **6:**533–542.
58. **Saha, M. S., M. Servetnick, and R. M. Grainger.** 1992. Vertebrate eye development. Curr. Opin. Genet. Dev. **2:**582–588.
59. **Sakai, D. D., S. Helms, J. Carlstedt-Duke, J.-A. Gustafsson, F. M. Rottman, and K. R. Yamamoto.** 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2:**1144–1154.
60. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43:**165–175.
61. **Sax, C. M., F. X. Farrell, Z. E. Zehner, and J. Piatigorsky.** 1990. Regulation of vimentin gene expression in the ocular lens. Dev. Biol. **139:**56–64.
62. **Sax, C. M., and J. Piatigorsky.** 1994. Expression of the α-crystallin/small heat shock protein/molecular chaperone genes in the lens and other tissues. Adv. Enzymol. Relat. Areas Mol. Biol. **69:**155–201.
63. **Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller.** 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA **7:**47–55.
64. **Ton, C. C., H. Hirvonen, H. Miwa, M. Weil, P. Monaghan, T. Jordan, V. van Heyningen, N. Hastie, H. Meijers-Heijboer, M. Drechsler, B. Z. Royer-Pokora, F. Collins, A. Swaroop, L. C. Strong, and G. F. Saunders.** 1991. Positional cloning and characterization of a paired-box- and homeobox-containing gene from the aniridia region. Cell **67:**1059–1074.
65. **Vallejo, M., L. Penchuk, and J. F. Habener.** 1992. Somatostatin gene upstream enhancer element activated by a protein complex consisting of CREB, Isl-1-like, and α-CBF-like transcription factors. J. Biol. Chem. **267:**12876–12884.
66. **Vogt, P. K., and T. J. Bos.** 1990. *jun*: oncogene and transcription factor. Adv. Cancer Res. **55:**1–35.
67. **Wagner, S., and M. R. Green.** 1993. HTLV-I Tax protein stimulation of DNA binding of bZIP proteins by enhancing dimerization. Science **262:**395–399.
68. **Walther, C., and P. Gruss.** 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. Development **113:**1435–1449.
69. **Williams, J. S., J. E. Dixon, and O. M. Andrisani.** 1993. Binding constant determination studies utilizing recombinant ΔCREB protein. DNA Cell Biol. **12:**183–190.
70. **Wistow, G. J., and J. Piatigorsky.** 1988. Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. Annu. Rev. Biochem. **57:**479–504.