A Complex Enhancer of the Chicken βA3/Al-Crystallin Gene Depends on an AP-1–CRE Element for Activity

Joan B. McDermott, Aleš Cvekl, and Joram Piatigorsky

Purpose. To define transcriptional regulatory elements of the chicken βA3/Al-crystallin gene.

Methods. Reporter genes were made with fragments of the chicken βA3/Al-crystallin gene fused to the bacterial gene encoding chloramphenicol acetyltransferase (CAT). The reporter plasmids were transfected into primary cultures of chicken-patched lens epithelium or fibroblast cells, and the CAT activity of cellular extracts was measured. The binding of lens nuclear proteins to βA3/Al sequences was tested in electrophoretic mobility shift assays.

Results. Sequences from –287 to –254 bp relative to the transcriptional start site function as an enhancer in transfected lens and nonlens cells. The length of a T-rich sequence downstream of the enhancer influences its activity. Minimal enhancer activity depends on sequences between –270 and –254 bp, and full activity requires additional upstream sequences. The minimal enhancer includes a consensus sequence (TGAGTCA) for basic region-leucine zipper (bZIP) proteins of the AP-1–CREB superfamily. Lens nuclear proteins bind the enhancer sequences to form several specific complexes, some of which are related antigenically to members of the AP-1 and CREB families of proteins.


The spatial and temporal expression of the crystallin genes contributes to the refractive properties of the eye lens. In the chicken, the α-, β-, and δ-crystallins are the major refractive proteins. δ-crystallin is expressed first, followed by the β-crystallins, and finally by the α-crystallins. There are at least six β-crystallins in the vertebrate lens. The βA3- and βA1-crystallins are derived from alternative start codons in a single transcript and are a major component of vertebrate lenses. Transcription of the βA3/Al-crystallin gene is regulated spatially and temporally during development. In situ hybridization of the rat lens showed that βA3–Al mRNA is detectable in the elongating cells of the epithelium and in the fiber layer.

Lenticular expression of the crystallin genes generally depends on promoter elements, whereas additional elements help determine the quantity and spatial pattern of crystallin expression within the lens. Expression of transgenes in mice has shown that fragments –88/+34 of the murine αA-, –164/+44 of the murine αB-, –67/+45 of murine γF-, –242/+77 of the chicken αA-, –143/+22 of chicken βA3/Al-, and –101/+50 of the chicken βB1-crystallin genes are sufficient to direct lens-specific expression. Tissue-restricted transcription factors such as Pax-6, Sox 1, and Sox 2 have been implicated in the lens-specific activity of crystallin promoters. Additional enhancer elements elevate the activity of crystallin promoter–reporter transgenes in the lens and in transfected lens cells. Enhancer elements, generally upstream, have been identified in the murine αB-, chicken αA-, murine γF-, and chicken δ1-crystallin genes by transfection and transgenic mouse experiments. Unlike the other crystallin enhancers, the δ1 enhancer is situated within intron 3 and is required for lens-specific expression of this crystallin gene. The enhancer elements of the murine γF-crystallin gene broaden expression of the reporter β-galactosidase gene in the lens fiber cells of transgenic mice. The hamster αA enhancer
also expands the expression from the murine γF promoter in recombinant transgenes in lens fiber cells in transgenic mice. The enhancer-binding proteins often are proteins expressed ubiquitously. For example, the chicken αA-162/-88 enhancer includes a cyclic adenosine monophosphate-responsive element (CRE), which binds CREB or CREM basic region-leucine zipper proteins (bZIP) proteins in the lens, and a related group of bZIP proteins, within the AP-1 family, in fibroblasts. USF binds an adjacent site. Despite the fact that these enhancer-binding proteins lack tissue specificity, transcription of the chicken αA-crystallin promoter is stimulated in lens cells and repressed in fibroblasts by these ubiquitous regulatory proteins.

In our initial studies of regulation of chicken βA3/A1, we identified transcriptionally active promoter sequences by transfection of primary lens epithelial (PLE) cells with chloramphenicol acetyltransferase (CAT) reporter genes. PLE cells from chicken lenses express the endogenous βA3/A1 gene (5 and data not shown). A fragment from −382 to +22 bp functions as a promoter in PLE cells and contains at least one positive regulatory element between −290 and −253 bp. In transgenic mice, fragment −143 to +22 is shown. A fragment from −382 to +22 bp functions positive regulatory element between −382 and −143 bp. In transgenic mice, fragment −143 to +22 is sufficient for lens-specific expression of the βA3/A1-crystallin reporter gene. In vitro DNase I footprinting showed binding of lens nuclear proteins to regions both within the −143/+22 minimal promoter and between −290 and −253 bp. In this study, we have examined whether this upstream footprinted sequence, which contains a consensus-binding site for the AP-1 family, in fibroblasts. USF binds an adjacent site. Despite the fact that these enhancer-binding proteins lack tissue specificity, transcription of the chicken αA-crystallin promoter is stimulated in lens cells and repressed in fibroblasts by these ubiquitous regulatory proteins.

Below is a list of the name, position, and sequence for each upstream strand oligonucleotide. The numbers correspond to the inserted nucleotides (capital letters); mutated bases are underlined; vector sequences are in lower case.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
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<tr>
<td>E34 -287/-254</td>
<td>5’-gatccAAATGCCAGAAGCTC-AACCTGTTGATGTTCGTCATCG</td>
</tr>
<tr>
<td>E22u -287/-266</td>
<td>5’-gatccAAATGCCAGAAGCTC-AACCTGTTGATGTTCGTCATCG</td>
</tr>
<tr>
<td>E27 -280/-254</td>
<td>5’-gatccGAAGGCTCAAATCTGT-TGACGTACGTTCG</td>
</tr>
<tr>
<td>E22d -275/-254</td>
<td>5’-gatccAAATGCCAGAAGCTC-AACCTGTTGATGTTCGTCATCG</td>
</tr>
<tr>
<td>E22d -275/-254</td>
<td>5’-gatccGAAGGCTCAAATCTGT-TGACGTACGTTCG</td>
</tr>
<tr>
<td>E17 -270/-254</td>
<td>5’-gatccAAATGCCAGAAGCTC-AACCTGTTGATGTTCGTCATCG</td>
</tr>
<tr>
<td>E12 -265/-254</td>
<td>5’-gatccAAATGCCAGAAGCTC-AACCTGTTGATGTTCGTCATCG</td>
</tr>
<tr>
<td>E34m1 -287 -254</td>
<td>5’-gatccGAAGGCTCAAATCTGT-TGACGTACGTTCG</td>
</tr>
<tr>
<td>E34m2 -287 -254</td>
<td>5’-gatccAAATGCCAGAAGCTC-AACCTGTTGATGTTCGTCATCG</td>
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Transfections and Enzyme Essays

Primary cultures of PLE cells and breast muscle fibroblasts were made from 14-day-old chicken embryos as described previously. Animals used in this study were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Six 14-day lenses or cells from one breast muscle were plated per 60-mm dish in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Gaithersburg, MD) with 10% fetal bovine serum (Gibco-BRL) in 5% CO2. Fibroblasts were prepared by trypsin digestion of breast muscle in Hank’s F-10 medium (Gibco-BRL) for 30 minutes, disrupted with a pipette, pelleted with 0.1 volume fetal bovine serum, and resuspended in complete DMEM.
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Cells were transfected two days after plating with 10 μg of a test plasmid and 1 μg of pTBI encoding β-galactosidase under the control of RSV promoter. Transfection was done by the calcium phosphate precipitation method without glycerol shock. The cells were collected 2 days after transfection and lysed in 250 mM TRIS-HCl, pH 7.5, by repeated freezing and thawing. The β-galactosidase activity and CAT activity of cellular extracts were determined by the methods of Nielsen et al using 5 μl of extract and Neumann et al using 50 μl of extract, respectively. The rate of acetylation of chloramphenicol was normalized to the activity of β-galactosidase for each dish. Each plasmid was tested in triplicate dishes in at least two experiments except for pl43E34m2 (n = 3). The normalized CAT activity in cells transfected with each plasmid was calculated relative to that of cells transfected with the parent vector pBLCAT3 set to 1.0, except in Figure 1C (see legend).

Electrophoretic Mobility Shift Assay

Nuclear extracts were made from lenses collected from 14- and 15-day-old chicken embryos as described by Digmann et al with several modifications, or according to Shapiro et al. The following oligonucleotide probes were used: 5’-GGAAAATGCCAGAG-CCGTCAA-3’, -290 to -270; 5’-AGAAGCGTGCAA-CTGTTTGAAT, -280 to -260; 5’-AAACTGTTGAGT-CATTCCGC, -271 to -251; AP-1 oligonucleotides were end-labeled with 32P and polynucleotide kinase. Samples containing 1 - 2 × 10⁶ cpm of the probe were incubated at room temperature for 15 minutes in 12.5 μl with 1X buffer, 1 μg poly d(A-T), 0.25 μg tRNA, 2.5 μg bovine serum albumin, and 4% Ficoll 400. 10X buffer is 85 mM Hepes, pH 7.9, 300 mM potassium chloride, 15 mM magnesium chloride, 4 mM dithiothreitol, and 3 mM phenylmethyl sulfonyl fluoride. Nuclear extracts were depleted of specific proteins by precipitation with antibodies to: CREB; ATF-1; c-Jun; JunB; JunD; c-Fos; FosB or Fra-2 (Santa Cruz Biotechnology, Santa Cruz, CA) as described elsewhere. Recombinant human c-Jun was obtained from Promega (Madison, WI), and recombinant human CREM (CREM-1) and the bZIP domain of CREB (amino acids 254 to 327) were obtained from Santa Cruz Biotechnology.

RESULTS

Identification of a Nonspecific Enhancer

Previous studies indicated that sequences between -382 and -143 bp relative to the βA3/A1 transcriptional start site contain a positive regulatory element, and in vitro footprinting of the region with partially purified lens nuclear proteins showed consistent protein protection from -287 to -254 bp. We noted that this region contains a consensus AP-1 binding site (TGAG(G/C)(C/A)).

FIGURE 1. Relative transcriptional activity of plasmids containing truncated fragments of βA3/A1 5’-flanking sequences. Reporter plasmids carrying fragments of the βA3/A1 promoter from -382, -287, -270, -238, and -143 to +22 bp were transfected into primary lens epithelial cells. The graph shows the average and standard deviation of the chloramphenicol acetyltransferase (CAT) activity of cells transfected with each plasmid, relative to the average normalized activity of the parent plasmid, pBLCAT3.
Figure 2. Transcriptional activation of the βA3/Δ1 promoter by βA3/Δ1 sequences between −287 and −254 bp. (A) The diagram indicates the name and sequence of each oligonucleotide inserted into p143CAT. The underlined sequence is the AP-1 consensus binding site. (B) Fragment −287/−254 functions as an enhancer. The graph shows the chloramphenicol acetyltransferase (CAT) activity of primary lens epithelial (PLE) cells transfected with each plasmid, relative to the average normalized CAT activity of pBLCAT3 for each experiment. (C) Enhancer activity requires sequences from −270 to −254 bp. The average CAT activity of extracts from PLE cells (black bars) or fibroblasts (gray bars) transfected with each plasmid is expressed as the percentage of the CAT activity in p143E34-transfected cells.

The transfection results showed that fragment −287/−254 is an orientation-independent activator (enhancer) of the homologous βA3/Δ1 promoter in lenticular cells (Fig. 2B). PLE cells transfected with p143E34 and p143E34r contained more than 10-fold higher levels of CAT activity than cells transfected with the enhancerless plasmid, p143CAT. The CAT activities recovered from PLE cells transfected with p143E22u and p143E12 did not differ significantly from that of p143CAT-transfected cells, indicating that neither of these segments alone has enhancer activity. The p143E34m1 mutation, but not the p143E34m2 mutation, decreased the activity of the full-length enhancer, consistent with the expected effects on an AP-1 site. The reduced promoter strength of p143E34m1 indicates that enhancer activity depends on the G at position −264; however, the putative AP-1 site is not sufficient for enhancer activity because E12 (−265/−254) was not able to increase promoter strength (Fig. 2B). Therefore, additional 5’ flanking sequences not contained in oligonucleotide E12 may be required for binding of bZIP proteins or enhancer activity may depend on synergy with proteins bound upstream from the putative AP-1 site.

A series of oligonucleotides containing the putative AP-1 site and progressively truncated at the 5’ end were tested for enhancer activity. Oligonucleotides E27, E22d, and E17 included sequences from −280 to −254, from −275 to −254, and from −270 to −254, respectively (Fig. 2A). Each oligonucleotide containing sequences from −270 to −254 bp functioned as an enhancer in PLE cells, albeit to different extents (black bars, Fig. 2C). Significantly more activity was recovered from cells transfected with plasmids containing the full-length enhancer than with any of the truncated enhancers. From these data, we conclude that full-enhancer activity depends on the AP-1 consensus sequence as well as additional sequences between −287 and −280 bp. Unexpectedly, similar results were obtained on transfection of chicken embryonic fibroblasts (gray bars, Fig. 2C). In contrast to the results in PLE cells, however, fragment −265/−254 (E12) was sufficient to enhance activity in fibroblasts. Enhancer constructs also were tested using the heter-
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Relative CAT Activity

FIGURE 3. Promoter activation by the enhancer from positions -238, -200, -181, -163, and -143 bp. Reporter plasmids carrying βA3/Al promoter fragment -287/+22 or fragments from -238, -200, -181, -163, and -143 bp to +22 bp with (gray bars) and without (black bars) oligonucleotide E34 were transfected into primary lens epithelial (PLE) cells. The diagram shows the 5' endpoint of the /βA3 to Al sequences in each plasmid and the sequence of the T-rich region. Asterisks mark positions -200, -181, and -163 bp. The graph shows the average and standard deviation of the chloramphenicol acetyltransferase (CAT) activity of PLE cells transfected with each plasmid, relative to the average normalized activity of pBLCAT3.

ologous thymidine kinase (tk) promoter. Both orientations of the wild-type sequence E34 (pTKE34 and pTKE34r) elevated the basal pTK activity fourfold to fivefold (data not shown). We conclude that the -287/-254 sequence is a nonspecific enhancer of the chicken βA3/Al-crystallin gene.

T-Rich Sequence

Because the CAT activity of cells transfected with p143E34 was much higher than that of cells transfected with p287CAT, we explored the possibility that the stimulation of the βA3/Al promoter by the -287/-254 enhancer is modulated by downstream sequences, which contain a thymidine-rich (T-rich) region. We made three promoter fragments that delete some or all of the intervening T-rich sequences to generate enhancerless plasmids p200CAT, p181CAT, and p163CAT. The E34 enhancer then was ligated upstream of the promoter fragments to generate plasmids p200E34, p181E34, and p163E34. We compared the activities generated by these plasmids with CAT activity from p287CAT, in which the enhancer is in its native position. The transcriptional activity in PLE cells of all the plasmids containing E34 was greater than that of the enhancerless plasmids (gray bars, Fig. 3). In general, stimulation of promoter strength by the enhancer increased with deletion of the T-rich region. A linear relation between the activity of the enhancer and its distance from the basal promoter may have been obscured by variability between experiments.

Electrophoretic Mobility Shift Assays

We examined binding of the enhancer sequences by chicken lentil nuclear proteins by electrophoretic mobility shift assay. We used three partially overlapping oligonucleotides as probes because probes spanning the enhancer generated a large number of complexes. Six specific complexes numbered in order of increasing mobility were detected. Complexes 1, 3, and 6 bound upstream probe -290/-270, and complexes 2, 4, and 5 bound downstream probe -271/-251 (Fig 4). Complex 4 also formed with the middle probe, -280/-260. All the probes competed effectively with themselves for complex formation. Except for oligo D on the middle probe -280/-260, the non-identical oligos competed to a lesser extent with
FIGURE 4. Electrophoretic mobility shift assay of -290/270 (upstream, U), -281/-261 (middle, M), and -271/-251 (downstream, D) probes with lens nuclear extracts. For each probe, the lanes contain no extract (free), 2 µl of lens extract with no competitor (--), lens extract with 100-fold molar excess of competitor oligonucleotides -290/-270 (U), -280/-260 (M), or -271/-251 (D). The complexes formed are numbered according to mobility. Complexes 1, 3, and 6 form with U; complex 4 forms with M; complexes 2, 4, and 5 form with D.

Each of the labeled probes. AP-1 and CRE consensus oligonucleotides competed with the -271/-251 probe for the formation of complexes 2 and 4 equally well (data not shown).

Attention was focused on the -271/-251 sequence because it contains an essential regulatory element for enhancer activity. The -271/-251 probe is centered on the heptameric sequence of the putative AP-1 site. As an initial step toward identifying the nuclear proteins that bind to the -271/-251 oligonucleotide, proteins of the AP-1 and ATF-CREB families were depleted from crude lens nuclear extract by immunoprecipitation. Complex 2 was eliminated by depletion of JunD and reduced by depletion of c-Jun (Fig. 5A). Depletion of CREB or CREM proteins or both by an antisera against the C-terminus of CREB virtually eliminated complex 4. In addition, direct binding of c-Jun, CREM (CREM-1), and a truncated bZIP fragment of CREB established that these proteins can interact with the -271/-251 sequence (Fig. 5B). Recombinant human c-Jun formed a complex of approximately the same mobility as complex 1, and recombinant human CREM formed a complex with the mobility of complex 2 (Fig. 5B). We conclude that proteins of both the AP-1 and CREB families are present in lens nuclei and are able to bind the enhancer sequences. In addition, similar experiments using immunoprecipitated fibroblast nuclear extracts showed that the major complex formed with this probe contains AP-1 family members JunD and Fra2 (data not shown).

DISCUSSION

We have identified an enhancer element at -287 to -254 in the chicken βA3/Al-crystallin gene. The results of deletion experiments suggest that the activating sequences detected previously between -382 and -143 bp are located between -287 and -238 bp, although additional regulatory elements may lie between -382 and -287 bp. Insertion of sequence -287/-254 bp (E34) upstream of the βA3/Al or tk promoters enhances the activity of reporter plasmids in transfected PLE cells. Surprisingly, the enhancer also activates the βA3/Al --143/+22 promoter in transfected fibroblasts. This contrasts with our previous demonstration that p382CAT, which contains the enhancer, is not active transcriptionally in transfected fibroblasts. A possible explanation for this discrepancy is that a repressor of nonlens expression lies between positions -143 and -382 bp or that placing...
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the enhancer next to the −143/+22 promoter fragment may have affected its lens specificity; these possibilities are not mutually exclusive. In any case, these assays identify and characterize a powerful enhancer, which we postulate couples with a lens-specific region downstream of −143 bp11 to contribute to the high expression of the endogenous gene in the chicken lens.

The position of the enhancer also affects its transcriptional stimulation of the βA3/A1 promoter in PLE cells, because promoter strength increased as the enhancer was brought closer to the structural gene. However, the current experiments did not separate the effects of altering the enhancer’s position and the influence of the T-rich sequence on enhancer activity. The possible function of T-rich sequences in the upper strand of the 5′-flanking sequences of crystallin genes is not known. There are T-rich sequences in the 5′-flanking regions of the chicken βA3/A1-, squid SL11,38 and guinea pig γ-crystallin genes39 covering approximately 60 bp and a 180 bp-long T-rich sequence in the 5′-flanking region of the duck τ gene.40 The role of the T-rich sequences, which should bend readily,41 may be to facilitate interactions between proteins bound to enhancer elements and the promoter.

The current transfection and electrophoretic mobility shift assay experiments indicate that the βA3/A1-crystallin enhancer is a complex element whose activity depends on a bZIP binding site between −270 and −254 bp. Fragment −270/−254 (E17) was the smallest fragment tested that enhanced plasmid activity. Additional sequences between −287 and −270 bp are necessary but not sufficient for full-enhancer activity. As expected from the size of the footprinted region, multiple lens nuclear proteins bind the enhancer, forming complexes with probes to both ends. βA3/A1 enhancer activity apparently depends on bZIP protein binding because mutation of the full-length enhancer at a critical site for bZIP binding (bp −264 in E34ml) eliminated its activity, and because bZIP proteins of the CREB–CREM and AP-1 families bind the −271/−251 probe. We expected to see binding of AP-1 proteins to the −264/−258 sequence, which matches the AP-1 consensus binding site. Surprisingly, complex 2, the dominant complex formed by lens nuclear proteins with the probe, contains proteins of the CREB family. The lens nuclear extract was made from whole lenses of 14-day embryonic chickens, and the relative amount of the complexes presumably reflects the relative abundance of proteins in the whole lens. Nevertheless, AP-1 and CRE consensus oligonucleotides competed equally well for binding of the −271/−251 probe.

Binding of the βA3/A1 enhancer by bZIP proteins suggests mechanisms for spatial and temporal regulation of βA3/A1 expression in the lens. The regulatory elements of the chicken20 and murine42 (unpublished data, 1994) α-crystallin genes are bound by members of the CREB family in lens cells and by members of the AP-1 family in fibroblasts to activate and repress transcription, respectively. We found that cotransfection of p143E34 with ptax, (an expression vector of HTLV-1 Tax, protein known to interact with specific CREB-like proteins to stimulate transcription)20,42 raised CAT levels threefold in PLE cells (data not shown). In contrast, the CAT activity of PLE cells transfected with p143E34 was lowered on cotransfection of a c-Jun expression vector (Rinaudo JAS, Zelenka PS, unpublished data, 1993). In addition, Rinaudo et al15 found that infection of PLE cells with a retroviral c-Jun expression vector lowered the level of endogenous βA3/A1 mRNA. Thus, βA3/A1 may be activated by CREB family proteins and inactivated by AP-1 proteins. Rinaudo et al15 have suggested that AP-1–CRE elements function as enhancer or repressor elements of the crystallin genes as the AP-1- and CREB-like protein ratios change during lens development.

Finally, the chicken βA3/A1 enhancer may contribute to the species-specific expression pattern of the crystallin. Sequences within the chicken −143/+22 minimal promoter are well conserved in the mammalian homologues, but the available sequences of the murine and human βA3/A1-crystallin promoters do not contain a consensus bZIP binding site. The reports of Hejtmancik et al17 and Yancey et al43 indicate that βA3/A1 is transcribed in the central epithelium of chickens but not in rats. Further analyses are required to establish the normal pattern of expression in each species and to elucidate the role of the enhancer in transcriptional expression of this gene.

Key Words
AP-1, CREB, β-crystallins, enhancer, gene regulation, lens

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References


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