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IDENTIFICATION OF THE CONTACT SITES OF A FACTOR THAT INTERACTS WITH MOTIF I (αCE1) OF THE CHICKEN αA-CRYSTALLIN LENS-SPECIFIC ENHANCER

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SUMMARY: A lens-specific enhancer, an 84bp element between base pairs -162 and -79, of the chicken αA-crystallin gene is composed of two motifs, αCE1 (-162 and -134) and αCE2 (-119 and -99). Previous studies showed that a nuclear factor which binds to αCE1,termed αCEF1, is present at high levels in lens cells. Methylation interference analysis identified an inverted repeat of 5bp separated by 4bp, 5'-CTGGTTCCCACCAG-3', between positions -153 and -140 as an αCEF1-binding site. Gel mobility shift assays using synthetic oligonucleotides with site-directed mutations revealed that the αCEF1-binding consensus sequence is 5'-C(T/A)GGN$_6$CC(A/T)G-3'. Comparison of this binding motif with regulatory sequences of diverse crystallin genes from diverse species suggests that αCE1 may be a ubiquitous crystallin gene enhancer.

Recent studies on the regulation of eukaryotic gene expression have focused on interactions between regulatory DNA sequences and sequence-specific DNA binding proteins (1, 2). These elements are identified by transient transfection and DNA-binding experiments. The expression of lens crystallin genes is regulated temporally and spatially during lens development (3,4). We have shown that an 84bp element located between -162 and -79 of the chicken αA-crystallin gene is essential and sufficient for lens-specific expression (5, 6). Our recent studies revealed that the 84bp element appears to be composed of two motifs, αCE1 (-162 to -134) and αCE2 (-119 to -99). αCE1 exhibits strong lens-specific enhancer activity when combined with αCE2, but neither alone show any enhancer activity. We have also indicated that a 61kD nuclear protein, termed αCEF1, present predominantly in chicken lens cells, interacts with αCE1 (6). Therefore, it is likely that αCEF1 plays an important role in activating lens-specific expression of the chicken αA-crystallin gene. To determine the contact points and sequence-specificity of αCEF1, we performed methylation interference assays and electrophoretic mobility shift experiments using synthetic oligonucleotides with site-directed mutations. Our findings indicates that a dyad symmetry of 5bp separated by 4bp (5'-CTGGTTCCCACCAG-3') binds αCEF1 and that the Guanosines within this dyad are critical for αCEF1-binding.

MATERIALS AND METHODS

Preparation of nuclear extracts: Lens nuclear extracts were prepared by the method of Dignam et al. (7).

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Methylation interference analysis: For this experiment we cloned a 84bp DNA fragment corresponding to a sequence -162 to -79 of the αA-crystallin gene into the BamHI site of pUC18. The resulting plasmid was cleaved with both EcoRI and Hind III endonucleases to obtain a DNA fragment containing the 84bp sequence. After labelling both ends of this fragment by a filling in reaction, using Klenow fragment and [α-^{32}P]dATP, end-labeled DNA fragments were obtained via cleavage it with Pst I or Kpn I endonucleases. The probe was partially methylated at guanine residues as described by Maxam and Gilbert (8). A five-fold scaled-up binding reaction mixture with the methylated DNA was electrophoresed on a native 4% polyacrylamide gel. The wet gel was monitered with a BioImage Analyzer (Fuji Films Co., Inc.). Free and complexed probes were cut out from the gel, transferred separately to DEAE papers, and then eluted by an incubation in 1M NaCl solution for 30 min at 20°C. Each DNA was cleaved with piperizine and then equal amounts of radioactive materials were run separately on a sequencing gel containing 10% polyacrylamide and 7.5M urea.

Probes and gel mobility shift assay: The oligonucleotides, whose nucleotide sequences are presented in Figure 2, were synthesized using a DNA synthesizer 370A (Applied Biosystems Inc.). Complimentary oligonucleotides were annealed and then end-filled with Klenow fragment and [α-^{32}P]dCTP. The binding reactions and electrophoresis were carried out as described previously (6).

RESULTS

To determine the contact points of αCEF1 within αCE1 of the chicken αA-crystallin promoter, we performed DNA methylation interference assay with a partially methylated probe (Fig. 1A). Methylation of guanine (G) residues at positions -140, -150 and -151 of the

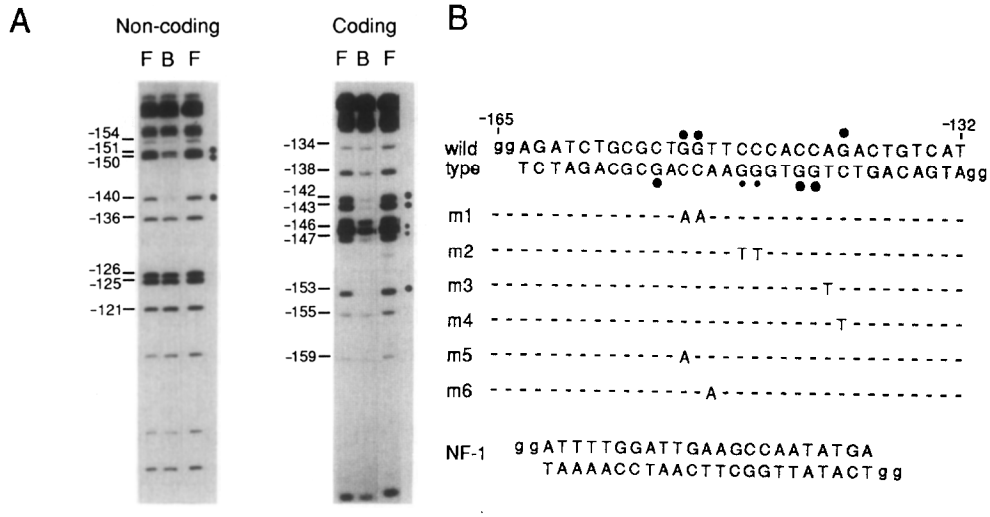

Fig. 1. (A) Methylation interference analysis of the αCEF1 binding site. A DNA fragment containing a sequence -162 to -79 of the chicken αA-crystallin promoter was used as a probe. Non-coding and coding refer to the sense and anti-sense strands, respectively. F and B represent free and αCE1-bound probes, respectively. The nucleotide positions of the G residues in the αA-crystallin promoter are numbered at the left of each panel. (B) Sequences of oligonucleotides used for electrophoretic mobility shift assays. The sequence of the wild type oligonucleotide corresponding to residues -165 to -132 of the chicken αA-crystallin promoter is shown in capitals. G residues whose methylation reduced binding strongly and weakly are indicated at the right of each panel by large and small dots, respectively. Only the mutated nucleotide(s) in each mutant are indicated, and a dash within the sequence indicates the wild type nucleotide at that position. NF1 binding site represents a sequence of the human adenovirus 2 replication origin.

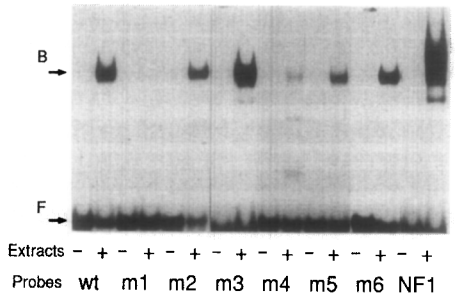

Fig. 2. Gel mobility shift assays using wild-type and mutant αCE1 sites, and NF1-binding site. Probes (wt, m1, m2, m3, m4, m5, m6 and NF1) below the lanes correspond to those in Fig. 2, except that wt corresponds to the wild type in Fig. 1B. Each probe (10,000cpm/0.2 ng) was incubated in the absence (-) or presence (+) of 10 mg of lens nuclear extract for 30 min at 20°C. Bound (B with arrow) and free probes (F with arrow) were visualized by autoradiography.

coding sequence and methylation of Gs at positions -142, -143 and -153 on the non-coding sequence interfered with αCEF1-binding. On the non-coding sequence, methylation of Gs at positions -146 and -147 showed partial interference. Interestingly, the region between -153 and -140 contains a dyad of symmetry of 5bp separated by 4bp, 5'-CTGGTTCCCACCAG-3'. Thus the interfered G residues, within the dyad of symmetry, show radial symmetry and are essential for αCEF1-binding.

To determine whether the guanine residues whose methylation interfered with the complex formation are essential for αCEF1-binding, we performed electrophoretic mobility shift assays using lens nuclear extract, synthetic oligonucleotides with site-directed mutations between positions -153 and -140 and one of an unrelated NF1-binding site used as a control (Fig. 1B) (9). To allow direct comparison of factor binding to each mutant, we used an equal amount of lens nuclear extract in each gel mobility shift assay.

Incubation of wt oligonucleotide containing the sequence corresponding to those between -165 and -132 of the chicken αA-crystallin promoter with lens nuclear extract yielded a major slowly migrating DNA-protein complex, termed B (Fig. 2, lane wt). This confirms our previous results that a sequence between -162 and -127 of the chicken αA-crystallin promoter produced a single slowly migrating complex (6).

Mutation of two G residues at positions -150 and -151, whose methylation efficiently interfered with binding, into two As resulted in a complete loss of B complex formation (lane m1). However, when one of these two G residues, at position -151, was changed into an A residue, the resulting oligonucleotide m5 retained the ability to form the B complex, however the band intensity was about a half of that of the bound wt probe (lane m5). Changing another G residue at position -140 whose methylation had strongly interfered with binding into a T residue remarkably reduced binding activity (lane m4). These results demonstrate that the interfered G residues within the inverted repeat are critical for αCEF1-binding.

Oligonucleotide m3 with a point mutation of A to T at position -141, which was not found to interfere with binding, always produced a broader band than the wt probe. Under the same binding conditions the radioactivity of bound m3 probe was about 1.5 times higher than that of

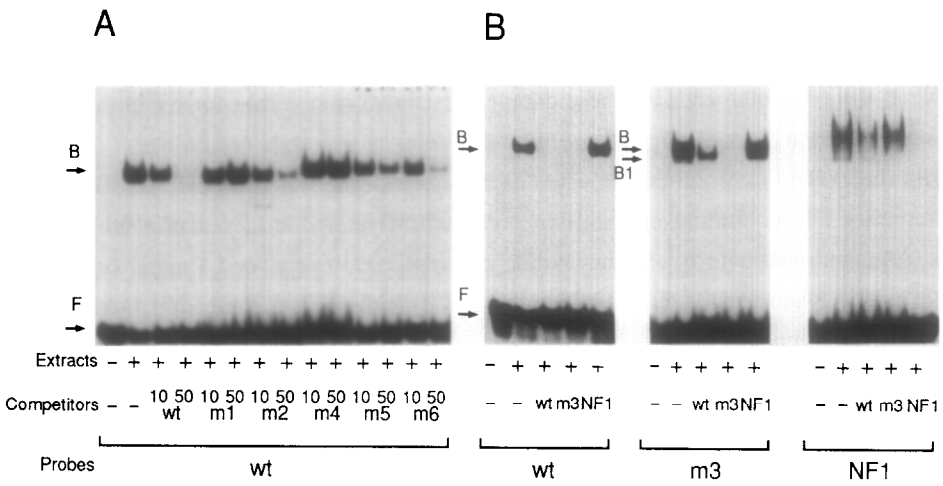

Fig. 3. (A) Binding competition experiments using wild-type probe and unlabeled competitor oligonucleotides (wt, m1, m2, m4, m5, and m6). Nuclear extract was incubated with each unlabeled oligonucleotide at a 10- (10) or 50- (50) fold molar excess for 10 min at 0°C, subsequently wild type probe was added to each mixture, followed by incubation for further 30 min 20°C. (B) Binding competition experiments using the wild type, m3 and NF1 oligonucleotides. Nuclear extract was incubated without (-) or with (+) a 200-fold molar excess unlabeled competitor oligonucleotide (wt, m3 or NF1) for 10 min, and then probe (wt, m3 or NF1) was added to each mixture, followed by a further 30 min incubation.

bound wt probe. When G residues at positions -146 and -147 on the lower strand, which showed partial interference, were mutated into A residues, the resulting oligonucleotide m2 was able to form the B complex but was a poorer binding site for αCEF1 than the wild type sequence (lane m2). Transversion of a T residue at position -149 to an A residue did not affect B complex formation (lane m6), suggesting that a complete inverted repeat may not be necessary for binding and whilst the G residues within the inverted repeat are critical for αCEF1-binding. Moreover, a specific nucleotide sequence between this repeat is not be required for αCEF1-binding, as mutations of these sequence did not affect this binding.

As a control sequence with well-defined binding sites and characteristics, we selected a binding site for NF1 of the human adenovirus 2 replication origin (9) and tested its binding activity in lens nuclear extracts. NF1/CTF is a ubiquitous nuclear factor binding to the pentanucleotide sequence CCAAT, commonly found 50 to 100 base pairs upstream from the transcription start site of eukaryotic genes that encode mRNA (1). Incubation of the NF1 probe with lens nuclear extract yielded slowly migrating multimeric bands (lane NF1), which are probably due to complex formation with several CCAAT-binding proteins, including C/EBP and NF1/CTF (1, 9).

The sequence specificity of αCEF1-binding was further demonstrated by competitive binding assays using the wild type and mutated oligonucleotides. After incubation of an appropriate amount of unlabelled competitors with lens nuclear extract at 0°C for 10 min, radiolabelled crystallin wild type probe was added and incubated 20°C for further 30 min (Fig. 3A and B). Addition of at least a 50-fold molar excess of unlabeled wild type oligonucleotide was enough to completely inhibit the complex formation (Fig. 3A). Unlabeled oligonucleotides m2, m5 and m6 affected binding of αCEF1 to the wild type probe but a small amount of the B

band still remained at a 50-fold molar excess, indicating that they were relatively poor competitors compared to the wt oligonucleotide. Since oligonucleotides m1 and m4 had no or only weak ability to bind αCEF1, these competitors failed to compete for complex formation even when added at 50-fold (Fig. 3A) or 500-fold molar excess (data not shown).

With the exception of m3 probe, when each mutant probe was competed for binding with wt competitor DNA, binding competition occurred efficiently at less than a 50-fold molar excess. As mentioned before, oligonucleotide m3 appeared to produced a major broader band than wt probe when incubated with lens nuclear extract (Fig. 3, lane m3), and this band appeared to be composed of two very closely apposed bands, suggesting that a point mutation of a T residue to an A residue at position -141 might generate a site for a new factor. To verify this issue, we performed competitive binding assays using m3 probe, and unlabeled wt, m3 and NF1 oligonucleotides. As expected, NF1 which has no relation with the αCEF1-binding sequence did not interfere with any complex formation except its own (Fig. 3B). The B complex formed with the wt probe was competed for efficiently by unlabeled m3 oligonucleotide. In contrast, wt competitor failed to fully compete for m3-protein complex formation even at a 200-fold excess, the slightly faster migrating complex, termed B1 complex, remained. This B1 complex was not competed out even when wt competitor DNA was added at more than 500-fold excess, indicating that a point mutation of A to T at position -141 produced a new binding site for a DNA-binding factor in lens nuclear extract.

DISCUSSION

In this report we have identified the contact sites of the factor αCEF1 that interacts with a distal motif, αCE1, of an 84bp lens-specific enhancer element of the chicken αA-crystallin gene. A factor with binding properties similar to αCEF1 has also been elsewhere (10). This other study identified a factor in lens nuclear extract binding to the sequences from -170 to -111 of the chicken αA-crystallin gene. Moreover, they also reported very similar methylation interference patterns. G residues within the dyad of symmetry between -153 and -140 showed an identical interference pattern, but there was difference in that slight interferences were observed outside this region. G residues at positions -156 and -154 on the non-coding sequence and -138, -134 and -131 on the coding sequence appeared to be weak methylation-sensitive sites in this previous study. In our hands G residues at positions -154 and -134 are probably methylation sensitive (Fig. 1). These differences may be due to several experimental variables including preparations and sources of lens nuclear extract, binding assay conditions and so on. As they and we also did not perform gel retardation assays using probes mutated in these regions, it is not clear whether these Gs might play a pivotal role in αCEF1-binding.

Based on electrophoretic mobility shift assays using mutant probes and their competition efficiency, the order of binding affinity to αCEF1 is wt (the native sequence), m3 > m6, m2 > m5 > m4 > m1. Briefly, a lone G at position -140 within a dyad of symmetry located between -153 and -140 of the chicken αA-crystallin promoter is indispensable for binding, since point mutation of this residue resulted in a complete loss of binding (Fig. 2, lane m4). Either of two adjacent G residues at positions -150 and -151 in this inverted repeat is required for binding, as

Gene	Location	Sequence	Citation
Chick αA	-153/-140	CTGGTTCCCACCAG	I. Matsuo unpublished
Chick αA	-178/-165	-A-CA----T----	M. Thompson *et al.* (15)
Mouse αA	-99/-86	-A-CC--T-C--C-	T. Nakamura *et al.* (14)
Mouse γF	-39/-26	-A-CAGA--T--T-	Q. Liu *et al.* (13)
Mouse γA	-39/-26	-A-CAG--AT--T-	S. Lok *et al.* (16)
Mouse γD	-39/-26	-A-CAGA--T--T-	S. Lok *et al.* (16)
Human γD	-39/-26	-A-CAG---T--T-	S. Lok *et al.* (16)
Rat γA	-49/-36	-A-CAG--AT--T-	R. Peek *et al.* (17)
Rat γD	-49/-36	-A-CAGA--T--T-	R. Peek *et al.* (17)
5'-flanking consensus		GCAGCA(T/G)CCCTCC(T/A)G	M. Thompson *et al.* (15)
αCEF1 binding consensus		C(A/T)GGNNNNNNCC(T/A)G	

Fig. 4. Comparison of regulatory sequences of several crystallin genes. Sequences are numbered from the transcription initiation site of each gene. They are taken from the published papers shown in the column of citation. A dash within the sequence indicates the nucleotide of the chicken αA-crystallin promoter at that position. Sequences of which mutation resulted in a loss of transcription in transfected lens cells are underlined. G residues in the upper and lower strands whose methylation reduced binding are indicated by closed and open circles, respectively.

mutation of either of these Gs does not abolish αCEF1-binding (lane m5), but mutation of both does (lane m1). Either T of this inverted repeat was not critical for binding, since mutating them had no effect on binding. Moreover, when nucleotides of the 6bp spacer between the inverted repeat were changed, binding activity was unaffected. Thus, we could conclude that a possible αCEF1-binding motif is 5'-C(T/A)GGN$_6$CC(A/T)G-3'.

Most crystallin genes are expressed in lens cells and are conserved among species. As these genes are a suitable system to analyze molecular events of tissue-specific gene expression, the regulatory mechanisms of some of crystallin gene expression have been studied by transfection experiments in chick lens cells (6, 10-14). For example, a sequence -111 to -60 upstream of the mouse αA-crystallin promoter is required for lens-specific expression (12), and that between -67 and -25 of the mouse γF-crystallin promoter is sufficient to activate transcription when duplicated immediately 5' to a basal promoter (13). Studies using site-directed mutagenesis defined the regulatory sequences required for lens-specific expression within these promoter regions (Fig. 4, 12-14). Moreover, one consensus sequence, GCAGCA(T/G)CCCTCC(T/A)G, was found in the 5' flanking sequences of several crystallin genes (15). We compared these sequences with the αCEF1 binding motif described here, since there could be similar sequences that could bind a common factor to exert the proper tissue fidelity of expression in the chicken cells. The result is summarized in Fig.4. It is apparent that a sequence -39 to -26 of the mouse γA-, γF- and γD- genes, and the rat A- and γD-crystallin promoters are well conserved. The 5bp incomplete inverted repeat found in these genes is expected to bind αCEF1, since the second G residue in αCEF1 binding consensus sequence (Fig. 4) can be replaced by a C residue

(Fig. 2, m5). Introduction of mutation into one of the repeat sequence of the mouse γF-crystallin (13), the mouse αA-crystallin (14) and the chicken αA-crystallin promoters resulted in a loss of transcription activity in transfected chicken lens cells. It was shown that a complete or incomplete inverted repeat sequence binds a homo- or heterodimer of transcription factors, leading to activate transcription of genes containing the repeat sequence (18, 19). αCEF1 appeared to bind as a heterodimer to the target sequence, since western blot analysis using poly- and monoclonal antibodies raised against purifed αCEF1 revealed that αCEF1 is composed of two immunologically distinct peptides (unpublished results). Therefore, we propose that αCEF1 may be one of a common transcription factor for these crystallin genes. Future functional studies should reveal whether αCEF1 plays a key role in regulation of these various crystallin genes.

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