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Overlapping Positive and Negative Regulatory Elements Determine Lens-Specific Activity of the δ1-Crystallin Enhancer

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Lens-specific expression of the δ1-crystallin gene is governed by an enhancer in the third intron, and the 30-bp-long DC5 fragment was found to be responsible for eliciting the lens-specific activity. Mutational analysis of the DC5 fragment identified two contiguous, interdependent positive elements and a negative element which overlaps the 3′-located positive element. Previously identified ubiquitous factor δEF1 bound to the negative element and repressed the enhancer activity in nonlens cells. Mutation and cotransfection analyses indicated the existence of an activator which counteracts the action of δEF1 in lens cells, probably through binding site competition. We also found a group of nuclear factors, collectively called δEF2, which bound to the 5′-located positive element. δEF2a and -b were the major species in lens cells, whereas δEF2c and -d predominated in nonlens cells. These δEF2 proteins probably cooperate with factors bound to the 3′-located element in activation in lens cells and repression in nonlens cells. δEF2 proteins also bound to a promoter sequence of the γF-crystallin gene, suggesting that δEF2 proteins are involved in lens-specific regulation of various crystallin classes.

It is believed that cell-type-specific transcription of genes is regulated by the activities of sequence-specific transcription factors which bind to modular *cis*-acting regulatory elements in promoters and/or enhancers. These regulatory elements are classified as either positive or negative, depending on which kind of nuclear factors they bind, activators or repressors (for reviews, see references 14, 17, and 19). Many cell-type-specific promoters and enhancers contain positive regulatory elements for both ubiquitous and tissue-specific factors. The transcription factors which bind to these elements are thought to interact directly or indirectly to elicit temporally and spatially specific gene expression. However, the mechanism by which overall specificity is generated remains to be elucidated. We have studied this problem by using crystallin genes, which are expressed in lens-specific or lens-preferred manners.

Lens cells are one of the few cell types which undergo terminal differentiation early in the embryogenic period. Lens cell differentiation is associated with concomitant and abundant expression of crystallins. In the amniote vertebrates, crystallins are divided into four classes, α, β, γ (found in mammals), and δ (found in birds and reptiles). Lens differentiation proceeds in a series of steps, and different crystallin classes respond to these steps (for a review, see reference 22). Initially, lens placode differentiates from ectoderm, and this differentiation is accompanied by the onset of δ-crystallin expression in the chicken. This process is followed by α-crystallin expression, which commences when the vesicular lens is formed. Then the cells on the internal side elongate and become terminally differentiated lens fiber cells. The terminal differentiation is paralleled by the expression of β-crystallins (and also γ-crystallins in mammals) and a steep increase in δ-crystallin.

Most molecular analyses of crystallin gene expression, focusing on determination of lens-specific regulation, have been carried out via transfection of cultured cells and/or by assays in transgenic mice. The tissue-specific regulatory elements thus identified appear quite diverse among the crystallin genes in their genomic localization and in base sequences (for a review, see reference 23), probably reflecting the complexity of crystallin gene regulation. However, the mechanisms governing overall lens specificity appear highly conserved among vertebrates. Mouse cells lacking the δ-crystallin gene regulate the chicken δ-crystallin gene correctly in terms of lens specificity (11, 13, 31); conversely, chicken lens cells, which lack the γ-crystallin gene, can support specific expression of mouse γ-crystallin genes (15, 16).

We have focused on the molecular basis of lens-specific transcriptional activation of the chicken δ1-crystallin gene because of its earliest expression in lens differentiation (6–8). It was found that the lens-specific expression of this gene is determined by an enhancer in the third intron (8). Through dissection of the entire enhancer region, we identified a core fragment which is essential for lens-specific activity of the enhancer (7). It was found that the core and its 55-base-long subfragment, HN, were sufficient for eliciting lens-specific enhancer action in their multimeric forms (6, 7). The effect of blockwise base substitutions on the enhancer activity of the HN fragment was investigated, and the region essential for the lens-specific enhancer activity was defined (6). A nuclear factor, δEF1, which bound to this region was identified, but this factor was not lens specific, suggesting that additional nuclear factors or modification events are also involved in generating and regulating the enhancer activity. In addition, there were various other regions of the HN fragment assignable as functional elements whose sequence alterations significantly decreased the enhancer activity of the fragment

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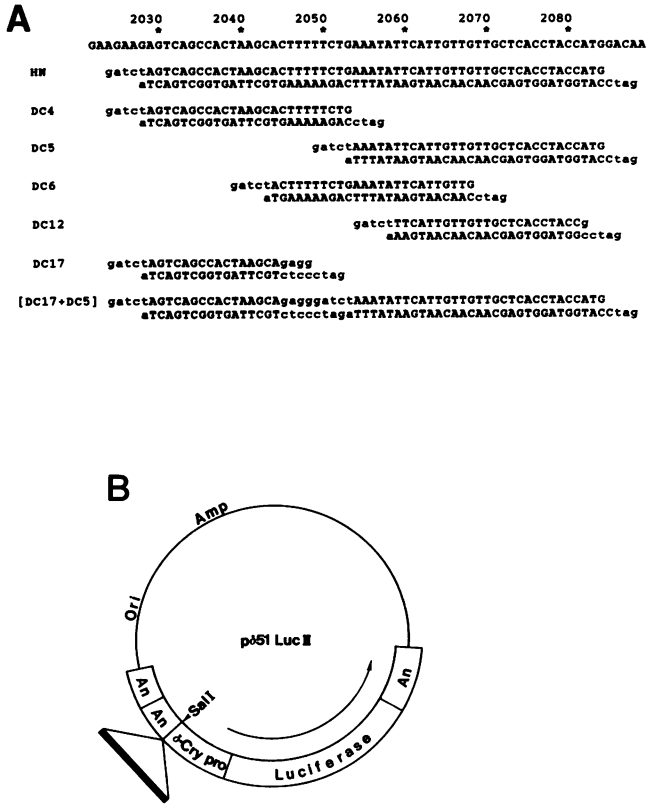

FIG. 1. Fragments of the δ1-crystallin enhancer core region tested and the reporter luciferase plasmid. (A) Nucleotide sequences of HN and DC fragments, compared with the corresponding genomic sequence shown at the top. Positions of nucleotides from the transcriptional initiation site (8) are also indicated. Lowercase letters indicate bases introduced for generating *Bgl*II-*Bam*HI sites and not present in the original genomic sequence. (B) Structure of plasmid pδ51LucII. The arrow indicates the direction of transcription. An, polyadenylation signal; δ-Cry pro, δ-crystallin promoter. Various enhancers were inserted at the *Sal*I site. The diagram is schematic and not drawn to scale.

(6). Thus, this investigation was undertaken to delineate the elements on the HN fragments, to define them in functional terms, and to characterize the nuclear factors other than δEF1 which interact with these elements.

MATERIALS AND METHODS

DC fragments and their octamerization. Various HN subfragments were prepared and designated DC fragments. DC fragments with *Bgl*II and *Bam*HI sites at their termini (Fig. 1A) were synthesized, annealed, and cloned as monomers into pUC19BEX digested with *Bgl*II and *Bam*HI. pUC19 BEX is a derivative of pUC19 made by replacing the *Sal*I-*Sma*I portion of the polylinker sequence with the following sequence to introduce restriction sites for *Bgl*II, *Eco*RV, and *Xho*I:

TCGACAGATCTGATATCGGATCCTCGAGCCC
GTCTAGACTATAGCCTAGGAGCTCGGG
SalI BglII EcoRV BamHI XhoI SmaI

Unidirectional octamers of DC fragments were made in the following way. pUC19BEX containing a monomer was digested with *Sca*I, which cleaves in the middle of the ampicillin resistance gene, and then with *Bam*HI or *Bgl*II. The *Bgl*II-*Sca*I and *Sca*I-*Bam*HI fragments, each carrying a monomeric DC fragment, were ligated so that a direct repeat of the DC sequence was made, joining *Bgl*II and *Bam*HI termini. By repeating this procedure three times, the octameric DC sequence was generated.

Construction of luciferase plasmids. pδ51LucII (Fig. 1B), used to test various octamerized sequences for enhancer activity, was constructed by linking the following sequences in a plasmid: duplicated poly(A) addition signals to prevent read-through from the vector sequence (2), polylinker sequence of pUC19 to insert DC fragments, minimal δ1-crystallin promoter sequence excised from pδ(−51)Ztk (12), which is active in driving luciferase expression, luciferase-coding sequence and a poly(A) addition signal from pSV232A/L-AΔ5′ (2), and replication origin of pUC19. pSV232A/L-AΔ5′II, derived from pSV232A/L-AΔ5′ by replacing the downstream *Bam*HI site with *Xho*I, was digested with *Sca*I and *Tth*111I, and the replication origin-containing half was replaced by the corresponding *Sca*I-*Pvu*II fragment of pUC19. The resulting plasmid, pSVLucII, was digested with *Bam*HI and *Hin*dIII, and the fragment containing the simian virus 40 promoter was replaced by the *Bam*HI-*Hin*dIII sequence of the pUC19 polylinker, yielding pPL-LucII. The δ1-crystallin promoter sequence (−51 to +57) was excised from pδ(−51)Ztk (12) by digestion with *Pst*I and *Pvu*II, the *Pvu*II terminus was converted to a *Hin*dIII site by linker addition, and the fragment was inserted into the *Pst*I-*Hin*dIII sites of pPLLucII. The resulting plasmid was pδ51LucII.

Octamerized DC sequences on the pUC19BEX vector were excised at *Sal*I and *Xho*I sites and inserted into the *Sal*I site of pδ51LucII. Octamerized HN sequence (6) was excised from pUC19 with *Sal*I and *Sac*I, blunt ended, and then inserted into the blunt-ended *Sal*I site of pδ51LucII.

Construction of a δEF1 expression plasmid. Expression vector pCMV was derived from pCDM8 (28). The segment containing cytomegalovirus promoter, stuffer, and splice and polyadenylation signals was excised from pCDM8 with *Pvu*II and *Bam*HI, blunt ended, and then inserted into pUC19 from which the *Pvu*II (276)-*Pvu*II (628) segment had been removed. In the resulting plasmid, pCMV, the insert was oriented so that the cytomegalovirus promoter was closer to the bacterial replication origin. pCMVX was made by removing the stuffer by *Xho*I digestion. Full-length δEF1 cDNA (6a) was cloned at the *Not*I site of pCMVX, which resulted in pCMVX-δEF1.

Cell culture and transfection. Primary cultures of chicken tissues were prepared from 15-day-old chicken embryos as described by Hayashi et al. (8). Plasmid transfections were carried out by the DNA-calcium phosphate coprecipitation method as modified by Chen and Okayama (1). In a typical assay, ca. 2×10^5 cells were seeded in a 3.5-cm-diameter dish and cultured for 24 h before transfection. The culture was transfected with 1 μg of luciferase plasmid, washed after 6 h, and harvested after 24 h for luciferase assay. For cotransfection assay, 1 μg of total DNA containing 0.5 μg of luciferase plasmid and variable amounts of pCMVX-δEF1, pCMVX, and pUC19 were transfected so that the molarity of pCMVX-δEF1 plus pCMVX remained constant.

Luciferase assay. Transfected cells were washed with phosphate-buffered saline three times and lysed in 300 μl of extraction buffer (0.1 M potassium phosphate [pH 7.8], 1% Nonidet P-40, 1 mM dithiothreitol). A 5- to 50-μl sample of the lysate was added to 350 μl of assay buffer (25 mM glycylglycine [pH 7.8], 2 mM ATP, 15 mM $MgSO_4$, 1 mM

dithiothreitol). The reaction was started by the injection of 30 μl of 1 mM luciferin, and light output was measured from 2 to 22 s at 25°C with a model 1251 luminometer (Wallac).

Preparation of nuclear extracts. Nuclear extracts were prepared from isolated tissues of 15-day-old chicken embryos by the method of Dignam et al. (3). For lung, liver, and heart nuclear extracts, the protease inhibitors leupeptin (1 μM), pepstatin (1 μM), bestatin (10 μM), and aprotinin (1 μg/ml), in addition to phenylmethylsulfonyl fluoride (0.5 mM), were included in all buffers.

Gel mobility shift assay. For the DC5 probe, a *Hin*dIII-*Bam*HI fragment was excised from pUC19BEX containing monomeric DC5 sequence and labeled by filling in the termini with [α-^{32}P]dCTP. Similarly, the DC12 probe was excised as a *Hin*dIII-*Xho*I fragment. Each probe (0.01 pmol) was incubated with nuclear extract (about 10 μg) in a total volume of 10 μl containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 15% glycerol, 50 to 100 mM KCl, 1 mM dithiothreitol, 2 μg of poly(dA-dT), or 2.5 μg of poly(dG-dC) for 30 min at room temperature. $MgCl_2$, $ZnSO_4$, or EDTA was also included when indicated. The mixtures were electrophoresed in a native 6% polyacrylamide (60:1, acrylamide/bisacrylamide) gel containing 22 mM Tris-borate and 0.5 mM EDTA, and the gels were autoradiographed after drying.

In the competition assay, appropriate amounts of synthetic DC fragments were added to the reaction mixture (see figure legends). Oligonucleotides of regulatory regions of non-δ-crystallin genes (15, 18, 26) were chicken αA
(−120 to −98; gatcTCTCCGCATTTCTGCTGACCACG
AGAGGCGTAAAGACGACTGGTGCctag);
chicken βB1
(−95 to −76; gatctACAGACACTGATGAGCTGGCg
aTGTCTGTGACTACTCGACCGcctag);
mouse γF
(−51 to −32; gatctTTCCTGCCAACACAGCAGACg
aAAGGACGGTTGTGTCGTCTGcctag);
and mouse γF
(−63 to −44; gatctCCTTTTGTGCTGTTCCTGCCg
aGGAAAACACGACAAGGACGGcctag).

RESULTS

HN subfragment DC5 carries lens-specific elements. Previous studies have shown that the element(s) that is essential for eliciting lens specificity of the δ1-crystallin enhancer lies within the 55-bp-long HN fragment (6–8). To delineate the element(s) further, we divided the HN fragment into three subfragments, DC4, DC5, and DC6 (Fig. 1A), and assessed their activities. Each nucleotide sequence was synthesized, unidirectionally octamerized, and placed upstream of the δ1-crystallin basal promoter (−51 to +57) linked to the luciferase gene (Fig. 1B). This promoter has been shown to have no cell type specificity (8, 12). We examined two orientations of the inserted fragments, normal and reverse, as designated in relation to the direction of transcription. These plasmids were transfected into lens epithelial cells and dermal fibroblasts in primary culture prepared from 15-day-old chicken embryos, and luciferase activities were measured after 24 h. The results are presented as relative luciferase activities compared with that of an enhancerless promoter.

As shown in Fig. 2A to C, octamerized DC4, DC5, and DC6 fragments showed nonspecific, lens-specific, and no enhancer activity, respectively. The DC5 fragment not only activated luciferase expression in lens cells but appeared to have a down-regulating activity in fibroblasts: the plasmids containing octamerized DC5 showed luciferase activity which was about half the level of the enhancerfree plasmid. This result indicated that the regulatory element(s) responsible for lens specificity lies within the DC5 fragment.

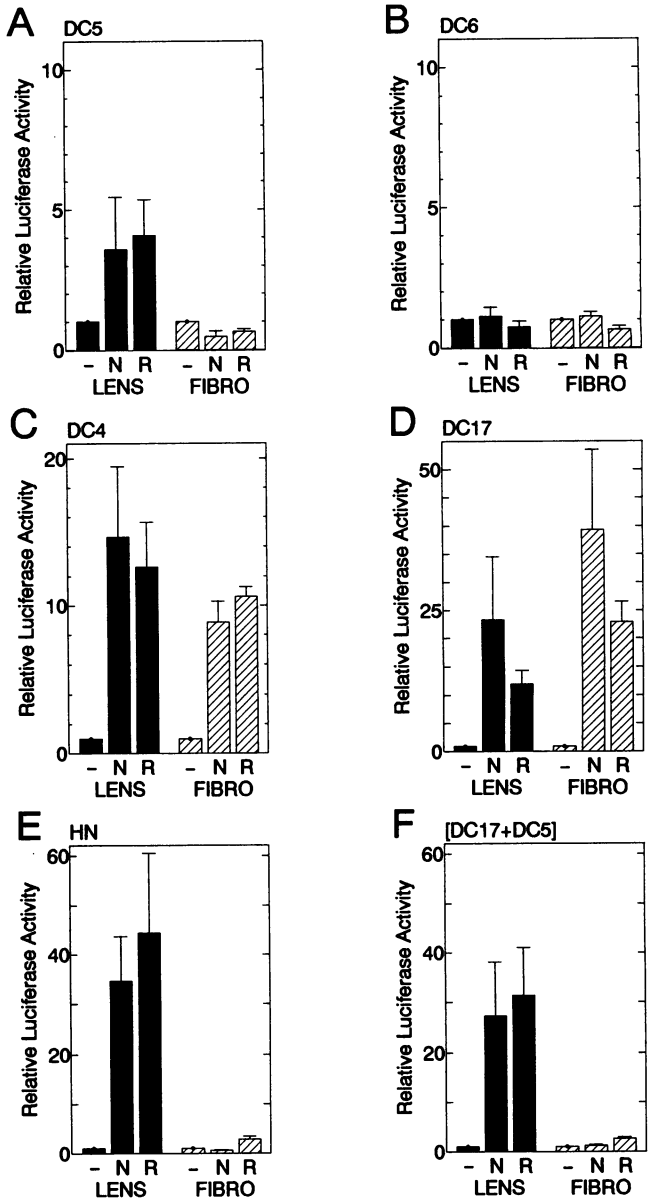

FIG. 2. Enhancer activities of HN subfragments. The octamers of DC5 (A), DC6 (B), DC4 (C), DC17 (D), HN (E), and [DC17+DC5] (F) fragments were cloned at the *Sal*I site of pδ51LucII in both normal (N) and reverse (R) orientations with respect to the direction of transcription. In [DC17+DC5], the DC17 fragment was linked to the DC5 fragment and then the combined fragment was octamerized. Lens epithelial cells (LENS) and dermal fibroblasts (FIBRO) were transfected with each luciferase plasmid. Relative luciferase activity was normalized to the activity of enhancerless pδ51LucII. Each bar represents the average of at least three independent transfections; thin lines represent standard deviations.

The results described above suggest that the DC5 fragment harbors elements responsible not only for activation in

the lens but also for repression in nonlens cells. This possibility was corroborated by the enhancer activity of recombined DC17 and DC5 fragments. DC17 lacks nine 3′ base pairs of DC4 which had been shown to be dispensable in the HN fragment (6), and DC17 octamers displayed nonspecific enhancer activity as expected (Fig. 2D), indicating that the element responsible for the nonspecific activity of DC4 lies within DC17. The composite enhancer unit [DC17+DC5] was of the same length as the HN fragment, and the octamers were comparable in specificity and magnitude of activation to the HN fragment (Fig. 2E and F). In other words, enhancer activity of DC5 in the lens was boosted by combination with DC17, and nonlens activity of DC17 was totally repressed by DC5.

Characterization of positive and negative elements in DC5. To characterize the putative elements for activation in the lens and for repression in nonlens cells, mutations were introduced in the DC5 fragment in the form of three consecutive base alterations which spanned the entire DC5 length: M1, M11, and M3 to M10 (from 5′ to 3′) (Fig. 3A). These mutant DC5 fragments were octamerized and assessed for enhancer activity (Fig. 3B and D).

M1 and M11, which mutated the 5′ proximal base sequences, did not significantly alter the enhancer activity in lens cells. However, mutations M3 to M10, except for M9, all abolished activation in the lens, indicating that the region which spans 24 bp is essential for the enhancer activity. Interestingly, mutation M9 resulted in a low but significant enhancer activity in both lens cells and fibroblasts. Mutation M13, in which the same three-base block as in M9 was changed to a different base sequence (Fig. 3A), also produced nonspecific enhancer activity (Fig. 3C and E). It is therefore likely that mutation of the block represented by M9/M13 resulted in derepression rather than that these mutations created a binding site for a nonspecific activator.

To characterize the repressing mechanism dependent on M9/M13 as well as the activating mechanism derepressed by the mutations, single-point mutations were introduced within and in the vicinity of the block represented by M9/M13 (Fig. 3A). Mutations were also designed so as to take into account the binding sequence of δEF1 described below. In lens cells, these point mutations had strikingly high derepressing effects. Three-base mutations produced at most 5-fold augmentation of luciferase expression, but M14, a point mutation in the neighboring block defined by M8, elicited 20-fold augmentation, and mutation M15, which was in the block of M9/M13, produced as much as 60-fold augmentation (Fig. 3C). In fibroblasts, however, these point mutations exhibited lower magnitudes of derepression, and the spectrum of the derepressing effect among the mutations was somewhat different from that in the lens cells: M13 and M14 produced larger derepression than did M9 and M15 (Fig. 3E).

The observation that point mutations resulted in larger augmentation (derepression) in lens cells is explained if one assumes that the repressing element, which is inactivated by mutations M9 and M13, is located inside an activating region. Mutations M9 and M13 abolish the repressing mechanism but still allow some residual activation. Mutation M8 destroys activating and repressing mechanisms simultaneously. The point mutation M14, in the same block as M8, abolishes the repressing mechanism but retains a level of activation. The point mutation M15 in the block of M9/M13, where the activating mechanism tolerates alteration of all three bases, allows high activation but no repression. The same explanation may apply to the derepression in fibroblasts, but the different spectrum and the extent of mutational effects suggest that the details of the mechanism of activation dependent on the M8-M9/M13 region are different between lens cells and fibroblasts.

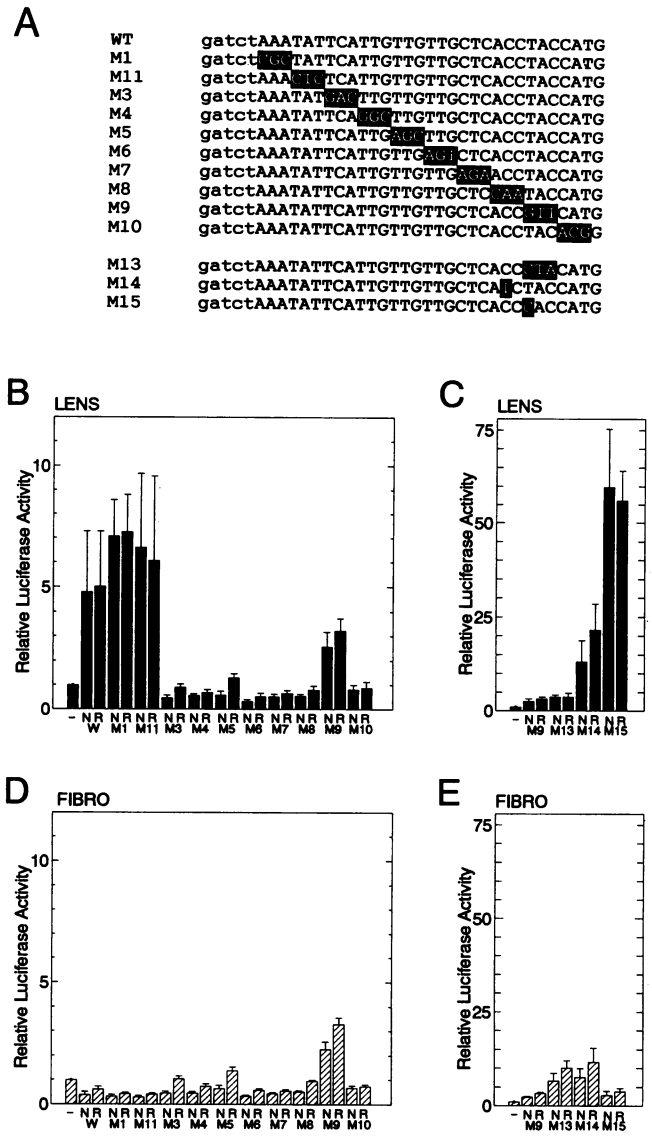

FIG. 3. Mutational analysis of the enhancer activity of the DC5 fragment. (A) Original (wild-type [WT]) and mutated (M1 to M15) DC5 fragments are shown by the top-strand sequence. Altered bases are highlighted. (B to E) Enhancer activities of the octamerized DC5 fragments, measured as in Fig. 2, in lens cells (B and C) and fibroblasts (D and E). For abbreviations, see the legend to Fig. 2.

Effect of DC5 activity on a neighboring element. As described above, DC17 activity, which by itself is nonspecific, came under the control of DC5 activity when the two fragments were combined (Fig. 2). We examined the effects of mutated DC5 fragment on the enhancer activity of the composite fragment [DC17+DC5]. The result shown in Fig. 4, compared with that shown in Fig. 3, indicated that the activity of the composite fragment paralleled the activity of DC5. When mutated DC5 was defective as an enhancer, the composite fragment [DC17+DC5] did not possess enhancer activity. On the other hand, none of the mutations which

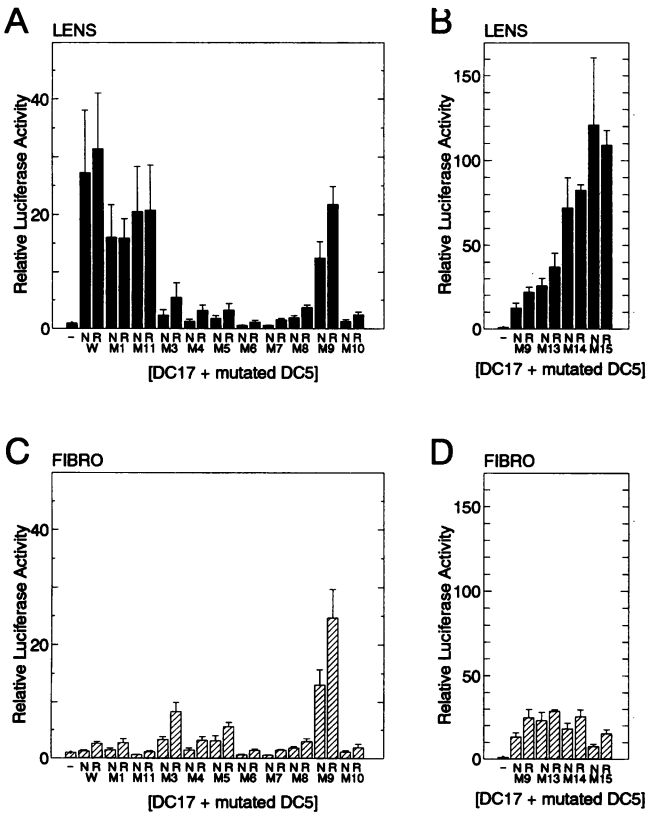

FIG. 4. Effects of DC5 mutations on enhancer activity when assessed in combination with the DC17 fragment. Enhancer activities of octamerized [DC17 + mutated DC5] fragments were measured as in Fig. 3. For abbreviations, see the legend to Fig. 2.

caused derepression of DC5 activity (M9, M13, M14, and M15) repressed the activity of DC17 in fibroblasts (Fig. 4D). Thus, the repressed state of DC5 was extended to DC17 in the composite fragment.

In the DC5 mutant fragments M3, M4, and M5, repression of DC17 activity in the composite fragment appeared incomplete (Fig. 4C). This point is discussed below in conjunction with δEF2 binding.

Repression correlates with binding of δEF1. We previously identified a nuclear factor, δEF1, that binds to the sequence of the HN fragment centered by the block represented by M8 (6). This binding region covered the area where activating and repressing elements overlapped. We therefore sought to determine whether the binding of δEF1 is correlated with activation or repression. For this purpose, DC5 mutant sequences were examined as competitors of δEF1 binding to the DC5 fragment in a gel mobility shift assay.

δEF1 activity with the same sequence specificity of binding was always detectable in nuclear extracts prepared from various organs of 15-day-old chicken embryos (6) (Fig. 5A). Representative data for binding specificity with use of brain δEF1 are shown in Fig. 5B. The mutant sequences that caused derepression in enhancer activity showed impaired binding of δEF1, in a way parallel to the extent of derepression in lens cells. Mutation M9, which showed the lowest derepression level (Fig. 3C and 4B), retained significant δEF1 binding capacity (Fig. 5B, lane 6), while mutation M15, which showed the highest derepression level, had no binding activity (Fig. 5B, lane 10). The effects of mutations M7 and

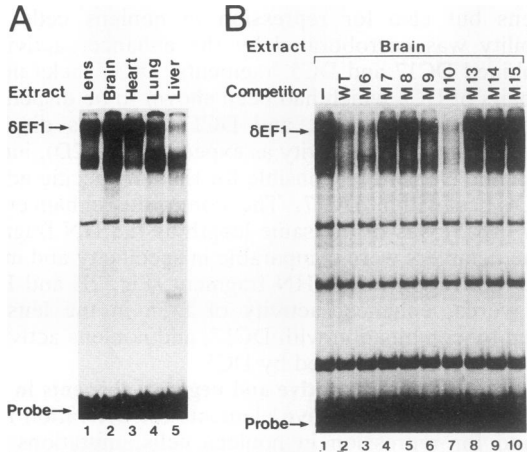

FIG. 5. Gel mobility shift analysis of δEF1 binding. (A) The DC5 probe was incubated with nuclear extracts of lens (lane 1), brain (lane 2), heart (lane 3), lung (lane 4), and liver (lane 5). The binding reaction was carried out in the presence of 2 μg of poly(dA-dT) and 0.1 mM $ZnSO_4$ in addition to the standard components. (B) The DC5 probe was incubated with brain nuclear extract in the absence (−) or presence of 100-fold molar excesses of unlabeled synthetic oligonucleotides (wild type [WT] to M15). The reaction mixture included 2 μg of poly(dA-dT), 0.1 mM $ZnSO_4$, and 2 mM $MgCl_2$ in addition to the standard components.

M8, which abolished the capacity to bind δEF1, were consistent with our previous data for HN mutant sequences (6); nevertheless, these mutations failed to derepress the enhancer activity, probably because these three-block mutations simultaneously impaired the activation mechanism(s) as discussed above. Thus, we concluded that binding of δEF1 is correlated with repressing activity.

Cotransfection of a δEF1 expression plasmid in lens cells. We have recently isolated a full-length cDNA that encodes δEF1 (6a) (EMBL-GenBank-DDBJ nucleotide sequence accession number D14313). This cDNA was placed in expression vector pCMVX. Various amounts of the δEF1 expression plasmid were cotransfected with a constant amount of reporter plasmids carrying octamerized DC5 or [DC17+ DC5]. As shown in Fig. 6A and C, enhancer activity was progressively repressed as the amount of the δEF1 expression plasmid increased. This effect was caused by binding of δEF1, because no repression was observed when the DC5 fragment on the reporter plasmids contained mutation M15, which lacked δEF1 binding activity (Fig. 6B and D). This result demonstrated that δEF1 acts as the transcriptional repressor on DC5.

Identification of δEF2 proteins which bind to the activating region. The findings presented above indicated that one or more activators in lens cells must interact with the DC5 fragment. To identify such factors in lens nuclear extract, we carried out a gel mobility shift assay using a DC5 probe under various binding conditions which differed in nonspecific competitors and in the composition of divalent cations.

When poly(dG-dC) was used as a nonspecific competitor, we detected two groups of specific DNA-protein complex, A and B, that were abolished by inclusion of an excess of unlabeled DC5 fragment (Fig. 7, lanes 1 and 2).

The group A complex of lens nuclear extract was composed of three subspecies, a, b, and d (in order of mobility). These complexes were detectable only when poly(dG-dC) was used as a nonspecific competitor and showed the same

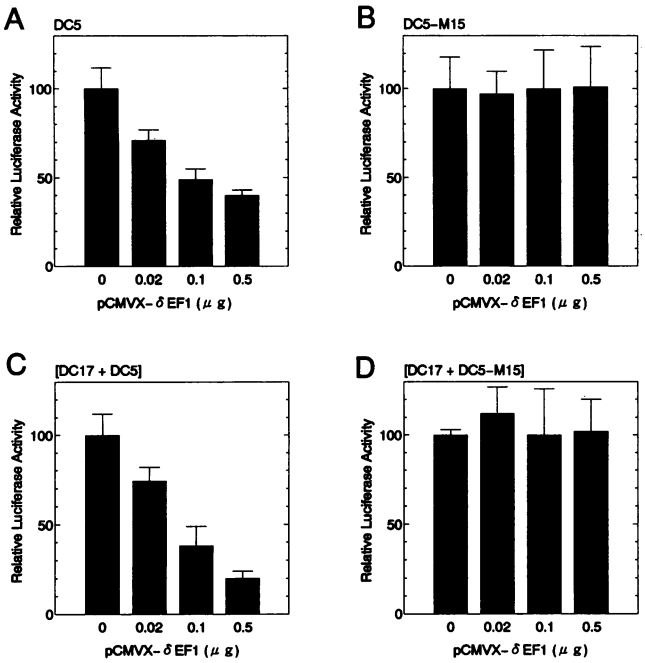

FIG. 6. Cotransfection of luciferase gene with δEF1 expression plasmid in lens cells. Lens cells were cotransfected with pCMVX-δEF1 (0.02, 0.1, or 0.5 μg) and 0.5 μg of luciferase plasmids containing the octamerized DC5 (A), DC5-M15 (B), [DC17+DC5] (C), and [DC17+DC5−M15] (D) fragments. Luciferase activities were normalized against those obtained without pCMVX-δEF1 cotransfection, which were assigned a value of 100. Averages of five independent transfections and standard deviations are shown.

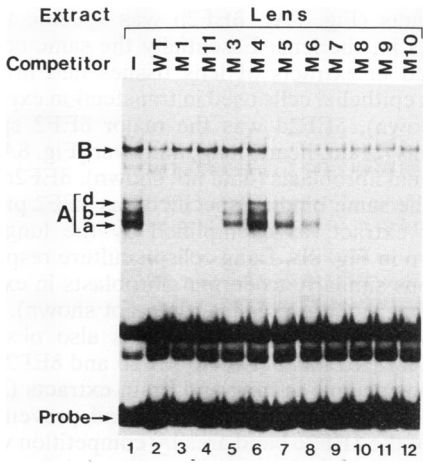

FIG. 7. Detection of nuclear factors interacting with the DC5 fragment. Labeled DC5 probe was incubated with lens nuclear extract in the absence (−) or presence of 50-fold molar excesses of the unlabeled synthetic oligonucleotides (wild type [WT] to M10). The binding mixture included 2.5 μg of poly(dG-dC) and 1 mM EDTA in addition to standard components. A and B indicate sequence-specific protein-DNA complexes. Three subspecies of A complexes, a, b, and d, are also indicated.

nucleotide sequence dependence. The proteins in complex A were collectively called δEF2; those in individual complexes were called δEF2a, -b, and -d. Competition using various mutated DC5 fragments in a gel mobility shift assay indicated that mutation M4 totally abolished δEF2 binding (Fig. 7, lane 6), while neighboring M3, M5, and M6 mutations reduced binding (Fig. 7, lanes 5, 7, and 8). Therefore, the recognition sequence of the δEF2 proteins must lie in the region covered by M3 to M6, in the base sequence TCAT TGTTGTTG.

Comparison of this result with enhancer activity of various mutant DC5 sequences (Fig. 3) indicated that the δEF2 binding sequence corresponded to the 5′ half of the region required for activation in the lens cells and suggested that some or all of the δEF2 proteins are involved in the lens-specific activation mechanism.

Complex B was formed by binding of a protein to the region covered by mutations M1 to M4. The protein in complex B was assigned to Oct-1 (30) for the following reasons (data not shown). First, the nucleotide sequence involved in complex B formation included ATATTCAT, which resembles the octamer consensus sequence ATTTG CAT; second, formation of complex B was more efficiently prevented by the authentic octamer binding consensus sequence than by DC5 itself; and third, a complex with the same mobility as complex B was observed when a probe with the octamer consensus sequence (6) was used for Oct-1 binding. The enhancer activity of DC5 octamers was decreased slightly by mutations M1 and M11 only when combined with DC17 (Fig. 4). Therefore, the contribution of Oct-1 binding to the activity of the DC5 fragment seems marginal.

δEF2 binding activity was investigated in nuclear extracts prepared from various organs of 15-day-old chicken embryos (Fig. 8A). The DC12 probe, which contained δEF2 binding sequence but lacked the sequence for complex B formation (Fig. 1A), was used. Assignment of gel mobility shift bands to δEF2 was done by determining the effect of DC5 mutants as competitors. δEF2a, which was the major species of lens nuclear extract (Fig. 7), was also present in brain nuclear extract, but at a much lower level, and was absent in other

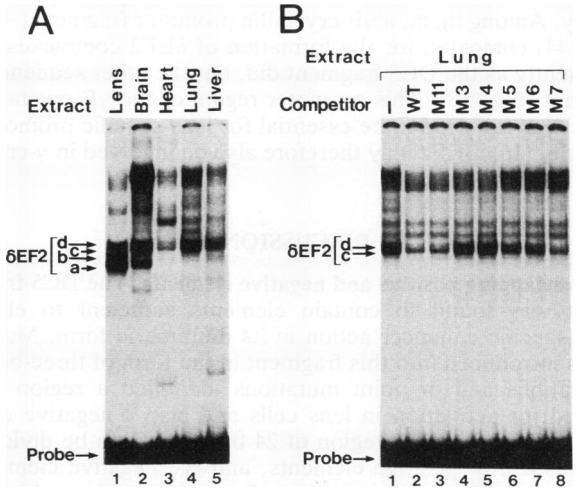

FIG. 8. Tissue distribution of δEF2 binding activity. (A) The DC12 probe was incubated with nuclear extracts of lens (lane 1), brain (lane 2), heart (lane 3), lung (lane 4), and liver (lane 5) under the same binding conditions as in Fig. 7. Four subspecies of δEF2 (a to d) are indicated. (B) The DC12 probe was incubated with lung nuclear extract in the absence (−) or presence of 50-fold molar excesses of unlabeled synthetic oligonucleotides (wild type [WT] to M7).

nuclear extracts (Fig. 8A). δEF2b was specific to, but a minor species in, the lens. Essentially the same complexes were detected in extracts of lens tissues and in those of cultured lens epithelial cells used in transfection experiments (data not shown). δEF2d was the major δEF2 species in nonlens organs (brain, heart, lung, and liver [Fig. 8A]) and in cultured dermal fibroblasts (data not shown). δEF2d in these organs had the same binding specificity as δEF2 proteins in lens nuclear extract, as exemplified by the lung nuclear extract shown in Fig. 8B. Lung cells in culture responded to DC5 mutations similarly to dermal fibroblasts in expression of DC5-bearing luciferase genes (data not shown). Another δEF2 complex, designated δEF2c, was also observed in nonlens nuclear extracts between δEF2b and δEF2d, which was most conspicuous in lung and brain extracts (Fig. 8A), although an overlapping nonspecific band prevented total elimination of the δEF2c band area by competition with DC5 (Fig. 8B).

Thus, the perfect match of the δEF2 binding site to the 5′ half of the activating region, as well as the presence of cell-type-dependent forms, strongly argues for involvement of this group of binding proteins in transcriptional activation by the DC5 fragment and possibly in the lens specificity of activation. δEF2 proteins in nonlens cells seem to tighten repression by δEF1, since mutations of the δEF2 binding site (M3 to M5) resulted in incomplete repression by δEF1 binding (Fig. 3C and 4C).

Detection of proteins binding to the 3′ half of the activating region has not been successful.

Binding of δEF2 to the γF-crystallin promoter. We examined the possibility that δEF2 proteins are involved in transcriptional regulation of other crystallin genes. Comparison was made of the δEF2 binding sequence and known regulatory regions of various crystallin genes. In chicken αA-crystallin (18) and βB1-crystallin (26) and mouse γF-crystallin (15, 16) genes, we found sequences which have some similarity to the δEF2 binding sequence and have been demonstrated to be required for proper promoter activities (Fig. 9A). We synthesized oligonucleotides of these sequences and tested them for δEF2 binding by competition assay. Among them, a γF-crystallin promoter fragment (−63 to −44) competed for the formation of δEF2 complexes as efficiently as the DC5 fragment did, but the other sequences did not (Fig. 9B). This promoter region of the γF-crystallin gene was reported to be essential for lens-specific promoter activity (16). δEF2 may therefore also be involved in γ-crystallin regulation.

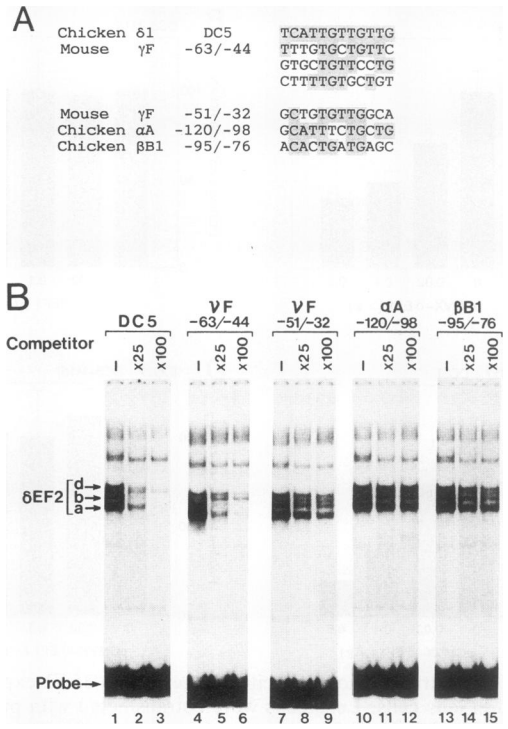

FIG. 9. Binding of δEF2 proteins to regulatory sequences of other crystallin classes. (A) Identified regulatory regions of various crystallin genes were searched for sequences with similarity to the δEF2 binding site, and high-scored sequences were tabulated. Twelve-nucleotide segments of the sequences were aligned for maximum matching with the δEF2 site in the DC5 fragment. In the γF (−63 to −44) sequence, two additional possible alignments are shown. Nucleotides matched to the DC5 sequence are stippled. (B) Oligonucleotides of the crystallin regulatory sequences which included those shown in panel A were synthesized (see Materials and Methods) as competitors of δEF2 binding. The DC12 probe was incubated with lens nuclear extract in the absence (−) or presence of 25- or 100-fold molar excesses of unlabeled competitor oligonucleotides under the same binding conditions as in Fig. 7.

DISCUSSION

Overlapping positive and negative elements. The DC5 fragment was found to contain elements sufficient to elicit lens-specific enhancer action in its multimeric form. Mutations introduced into this fragment in the form of three-base alterations and of point mutations identified a region required for activation in lens cells and also a negative element. The activating region of 24 bp appears to be divided into 5′ and 3′ positive elements, and the negative element overlaps the 3′ positive element. Repression observed in all cell types is attributable to binding of δEF1 to the negative element, and binding of δEF2a and -b to the 5′ positive element seems to be required for the enhancer activity in lens cells (Fig. 10). Although we have not been able to provide in vitro evidence for binding of an activator to the 3′ positive element which overlaps the δEF1 binding site, our in vivo functional data strongly argue for the existence of such an activator protein. The mechanism of repression by δEF1 is not fully understood, but the simplest model to account for the observed mutational effects is the competition of a binding site. Along with binding of this unidentified activator, concomitant binding of δEF2a and -b appears to be essential for generating the activating function, because all single mutations in the activating region of the DC5 fragment except M9 totally abolished the enhancer activity.

In nonlens cells, there may be some activators which bind to the 3′ positive element, as suggested by the derepressing effect of the mutations of δEF1 binding site. However, these activators seem to be completely negated by δEF1, and δEF2c and -d in the neighboring binding site probably contribute to the stringency of the repressed state (Fig. 10B). In lens cells, the 5′ positive element is occupied by δEF2a and -b, and binding of δEF1 and an unidentified activator is in competition for the 3′ site (Fig. 10A). Maturation of lens cells will shift the state from repressor dominant (Fig. 10A, right) to activator dominant (Fig. 10A, left). In the cultured lens epithelial cells used in our transfection assay, repression by δEF1 still had a substantial effect, as indicated by the high derepressing effect of the mutations which abolished δEF1 binding (Fig. 3 and 4). When δEF1 was overexpressed in the

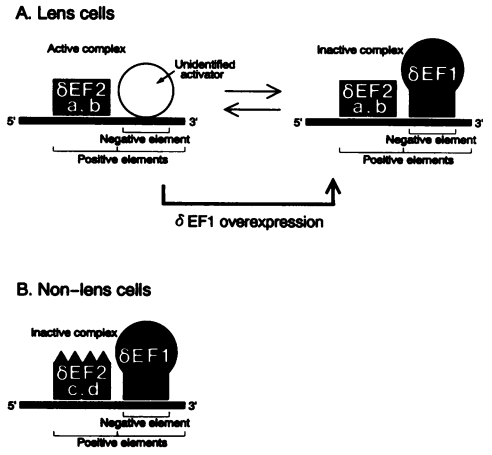

FIG. 10. Model for nuclear factor interactions on the DC5 fragment. (A) In lens cells, an unidentified activator and repressor δEF1 are in competition for the binding site. When the activator occupies the site, it forms an active complex with lens-type δEF2 (δEF2a and -b) (left) and activates transcription. However, when δEF1 occupies the site, the condition preferred in δEF1-overexpressed cells, δEF1 and δEF2 form an inactive complex. (B) In nonlens cells, δEF1 binds to the negative element and represses transcription. The repression is tightened by nonlens forms of δEF2 (δEF2c and -d).

cultured lens epithelial cells, the repressor molecules overwhelmed the activators (Fig. 6). In embryonic lens fiber cells, in which the δ1-crystallin gene is transcribed at the highest level (32), there are sufficient activator molecules to totally abolish the effect of the repressor δEF1.

Overlap of positive and negative elements analogous to the elements on the DC5 fragment has been reported for a few other systems determining cell type specificity. In the immunoglobulin heavy-chain enhancer, the μE5-μE3 region exhibits lymphoid cell-specific enhancer activity through interaction of two regulatory elements, μE3 and μE5. In nonlymphoid cells, binding of the repressor to the μE5 element inhibits activation by TFE3 on μE3, while displacement of the repressor by excess ITF-1, which occurs in lymphoid cells, results in synergistic activation by ITF-1 and TFE3 (27). It is interesting to note that the repressor binding site sequence CACCTGC (μE5 motif) resembles the δEF1 binding site sequence (TCACCT). In the glycophorin B promoter, erythroid cell-specific transcription requires binding sites for the nonspecific activator SP1, the erythroid cell-specific activator GATA-1, and a nonspecific repressor (24). In this case, erythroid cell-specific activity is achieved through displacement of the repressor by GATA-1.

Repressor and activator proteins. Nuclear factor δEF1, which was discovered in our previous investigation (6), has now been identified as the repressor which binds to the negative element. In support of this finding, forced expression of δEF1 repressed the enhancer activity in lens epithelial cells (Fig. 6).

Molecular cloning of cDNA encoding δEF1 has revealed that δEF1 is a 124-kDa protein containing two widely separated sets of C_2-H_2-type zinc fingers and one homeodomain between them (6a); these DNA-binding domains have significant similarity to *Drosophila* ZFH-1 (zinc finger homeodomain protein 1 [4]). Immunohistological staining of chicken embryonic tissues with anti-δEF1 revealed wide distribution of δEF1 protein in most mesoderm-derived tissues and in some ectodermal tissues, e.g., the lens and the central nervous system (6a). The tissue distribution of δEF1 and its repressor action in transfected lens epithelial cells suggested that δEF1 shuts down the δ1-crystallin enhancer in nonlens cells and that its effect is counteracted by an activator in lens cells.

Forms of protein factor δEF2 which bind to the 5′ half of the activating region of DC5 fragment were found. δEF2 consists of four subspecies, a, b, c, and d, which have the same binding specificity. Since the proportion of the subspecies varied considerably among the cell types, it is conceivable that differential occupancy by δEF2 subspecies of the same binding site provides a determinative cue of cell-type-dependent enhancer activity. It is possible that δEF2a and -b, which are present predominantly in lens cells, participate in the lens-specific enhancer activity of the DC5 fragment. On the other hand, δEF2c and -d, which are abundant in nonlens cells, presumably function as auxiliary negative regulators in nonlens cells. This latter model is supported by the observation that mutations M3, M4, and M5 resulted in incomplete repression of enhancer activity in fibroblasts (Fig. 3D and 4C).

The same binding specificity and similar extents of mobility shift of the probe among δEF2 subspecies indicated that these subspecies have similar protein structures. One possible mechanism for generating such multiple forms of DNA-binding proteins is that a single protein is posttranslationally modified without alteration of its DNA-binding specificity, e.g., by phosphorylation (29) and glycosylation (9). Another possibility is that variant proteins are produced by different forms of mRNA transcribed from a single gene (e.g., references 5, 10, and 25). There are cases (e.g., CREM and *erbA* genes) in which isoforms generated through developmentally regulated alternative splicing even have opposite functions in transcriptional regulation (5, 10). It is also possible that different proteins with the same DNA binding specificity are encoded by distinct genes, as has been demonstrated for the octamer-binding proteins (20, 21, 30).

Molecular cloning of the δEF2 gene(s) will clarify the origin of the multiple forms of δEF2 and will provide experimental means to test whether δEF2 subspecies have the activities proposed in the model (Fig. 10).

Long-range effect of repression. The HN fragment, previously demonstrated to have strict lens-specific enhancer activity, was divided into subfragments capable of lens-specific activation (DC5) and nonspecific activation (DC4 and DC17). Since individual activation levels of the subfragments were lower than that of the HN fragment, the regulatory elements on the subfragments cooperate to augment the activation level while maintaining the stringency of overall lens specificity (Fig. 2). This result extends our previous observation on larger fragments of the δ1-crystallin enhancer that lens-specific and nonspecific fragments functionally cooperate to give rise to higher yet still lens-specific enhancer action (7).

With respect to the activity of DC17, which by itself is nonspecific, the activity in nonlens cells was repressed when DC17 was combined with the DC5 fragment. In addition, in cases in which the DC5 mutation impaired the positive element but retained intact the negative element, DC17 combined with these DC5 mutants was also repressed in lens cells (Fig. 4A). Thus, when the DC5 fragment is in the repressed state, activity of the adjoining element is silenced. This silencing effect is probably exerted throughout the enhancer region and provides the basis of the stringent lens-specific enhancer action. The entire δ1-crystallin enhancer is composed of multiple nonspecific activating re-

gions and a region to trigger lens-specific activation (7) which is now recognized to be in the DC5 fragment. In most nonlens cells, the action of repressor δEF1 is in effect and nonspecific enhancer elements appear totally repressed. In lens cells, derepression as well as cooperative activation by various positive elements elicit a very strong enhancer activity.

Lens-specific regulation. There are a number of crystallin genes, each expressed in a unique spatiotemporal order. An interesting question is the extent to which the mechanism and/or the factors that we identified on the DC5 fragment of the δ1-crystallin enhancer are relevant to lens specificity determination of other crystallin genes. It is worth noting that the mechanism of lens-specific regulation is conserved across species. Although the mouse does not have the δ1-crystallin gene, this gene is expressed in a lens-specific manner in cultured mouse cells (13) and transgenic mice (11, 31). The lens-specific enhancer activity of the HN fragment has also been demonstrated in these mouse systems (8a). Conversely, the γF-crystallin promoter of the mouse is regulated with correct lens specificity in chicken cells lacking γ-crystallin genes (15, 16). Therefore, some of the regulatory factors and/or mechanisms must be conserved between different vertebrate species. In fact, δEF1-like binding activity was also observed in nuclear extracts of the mammalian cell lines HeLa (human), COS7 (monkey), and OTF9 (mouse) (our unpublished observation).

Investigation of different crystallin genes has identified various regulatory elements involved in lens-specific transcription of these genes, but these elements and the nuclear factors which bind to them have been found to be diversified (for a review, see reference 23). However, different assay systems have been used for different crystallin genes, and all of the important elements may not have been identified. We tested DNA sequences derived from regulatory regions of various crystallin genes sharing some similarity to the δEF2 binding site (Fig. 10A) for binding of δEF2. Among the sequences tested, a distal part of the promoter sequence (−63 to −44) of the γF-crystallin gene bound δEF2 proteins. Liu et al. (15) have reported that the sequence −67 to −25 is able to function as a strong enhancer when duplicated and placed upstream of the TATA box and that the lens-specific nuclear factor γF-1 binds to the sequence centered at −46 to −36 (15). As the sequence −67 to −47 is also important for promoter activity of the γF-crystallin gene (16), it is likely that δEF2a and -b along with γF-1 activate transcription in lens cells. Thus, the group of δEF2 proteins are among the candidate factors responsible for a possibly conserved lens-specific regulatory mechanism.

Generality of the mechanism. In conclusion, we have shown that two mechanisms with opposite effects interact via overlapping regulatory elements, and this interaction determines the lens-specific enhancer effect of the DC5 fragment: repression in nonlens cells by the binding of δEF1 to the negative element, and activation in lens cells by the action of factors which bind side-by-side in the activating region and by the counteraction of δEF1, probably through competitive displacement. One of the activating factors is ascribable to δEF2a and -b. The present investigation underscores the importance of counteracting positive and negative actions in determining stringent cell type specificity. In this respect, the broad distribution of δEF1 among cell types suggests that repressor δEF1 plays a central role in a variety of cell-type-specific regulations. Moreover, considering recently reported analogous cases (24, 27), overlapping positive and negative elements may be a widely employed strategy in cell-type-specific gene regulation.

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REFERENCES

1. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
2. **De Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–737.
3. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
4. **Fortini, M. E., L. Zhichun, and G. M. Rubin.** 1991. The Drosophila *zfh-1* and *zfh-2* genes encode novel proteins containing both zinc finger and homeodomain motifs. Mech. Dev. **34**:113–122.
5. **Foulkes, N. S., B. Mellström, E. Benusiglio, and P. Sassone-Corsi.** 1992. Developmental switch of CREM function during spermatogenesis: from antagonist to activator. Nature (London) **355**:80–84.
6. **Funahashi, J., Y. Kamachi, K. Goto, and H. Kondoh.** 1991. Identification of nuclear factor δEF1 and its binding site essential for lens-specific activity of the δ1-crystallin enhancer. Nucleic Acids Res. **19**:3543–3547.

6a. **Funahashi, J., R. Sekido, K. Murai, Y. Kamachi, and H. Kondoh.** δ-Crystallin enhancer binding protein δEF1 is a zinc-finger homeodomain protein implicated in postgastrulation embryogenesis. Development, in press.

7. **Goto, K., T. S. Okada, and H. Kondoh.** 1990. Functional cooperation of lens-specific and nonspecific elements in the δ1-crystallin enhancer. Mol. Cell. Biol. **10**:958–964.
8. **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.

8a. **Higashi, Y., Y. Kamachi, and H. Kondoh.** Unpublished data.

9. **Jackson, S. P., and R. Tjian.** 1988. O-glycosylation of eukaryotic transcription factors: implication for mechanisms of transcriptional regulation. Cell **55**:125–133.
10. **Koenig, R. J., M. A. Lazar, R. H. Hodin, G. A. Brent, P. R. Larsen, W. W. Chin, and D. D. Moore.** 1989. Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing. Nature (London) **337**:659–661.
11. **Kondoh, H., K. Katoh, Y. Takahashi, H. Fujisawa, M. Yokoyama, S. Kimura, M. Katsuki, M. Saito, T. Nomura, Y. Hiramoto, and T. S. Okada.** 1987. Specific expression of the chicken δ-crystallin gene in the lens and the pyramidal neurons of the piriform cortex in transgenic mice. Dev. Biol. **120**:177–185.
12. **Kondoh, H., Y. Ueda, I. Araki, S. Hayashi, and K. Goto.** 1988. Regulatory elements of the chicken δ1-crystallin gene. UCLA Symp. New Ser. **88**:189–196.
13. **Kondoh, H., K. Yasuda, and T. S. Okada.** 1983. Tissue-specific expression of a cloned chick δ-crystallin gene in mouse cells. Nature (London) **301**:440–442.
14. **Levin, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59**:405–408.
15. **Liu, Q., M. Tini, L.-C. Tsui, and M. L. Breitman.** 1991. Interaction of a lens cell transcription factor with the proximal

domain of the γF-crystallin promoter. Mol. Cell. Biol. **11**:1531–1537.
16. **Lok, S., M. L. Breitman, and L.-C. Tsui.** 1989. Multiple regulatory elements of the murine γ-2-crystallin promoter. Nucleic Acids Res. **17**:3563–3582.
17. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
18. **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.
19. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
20. **Müller, M. M., S. Ruppert, W. Schaffner, and P. Matthias.** 1988. A cloned octamer transcription factor stimulates transcription from lymphoid-specific promoters in non-B cells. Nature (London) **336**:544–551.
21. **Okamoto, K., H. Okazawa, A. Okuda, M. Sakai, M. Muramatsu, and H. Hamada.** 1990. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell **60**:461–472.
22. **Piatigorsky, J.** 1981. Lens differentiation in vertebrates. A review of cellular and molecular features. Differentiation **19**: 134–153.
23. **Piatigorsky, J., and P. S. Zelenka.** 1992. Transcriptional regulation of crystallin genes. Adv. Dev. Biochem. **1**:211–256.
24. **Rahuel, C., M.-A. Vint, V. Lemarchandel, J.-P. Cartron, and P.-H. Roméo.** 1992. Erythroid-specific activity of the glycophorin B promoter requires GATA-1 mediated displacement of a repressor. EMBO J. **11**:4095–4102.
25. **Roman, C., L. Cohn, and K. Calame.** 1991. A dominant negative form of transcription activator mTFE3 created by differential splicing. Science **254**:94–97.
26. **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.
27. **Ruezinsky, D., H. Beckmann, and T. Kadesch.** 1991. Modulation of the IgH enhancer's cell type specificity through genetic switch. Genes Dev. **5**:29–37.
28. **Seed, B.** 1987. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature (London) **329**:840–842.
29. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54**:855–864.
30. **Sturm, R. A., G. Das, and W. Herr.** 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Genes Dev. **2**:1582–1599.
31. **Takahashi, Y., K. Hanaoka, M. Hayasaka, K. Katoh, Y. Kato, T. S. Okada, and H. Kondoh.** 1988. Embryonic stem cell-mediated transfer and correct regulation of the chicken δ-crystallin gene in developing mouse embryos. Development **102**: 259–269.
32. **Thomas, G., P. S. Zelenka, R. A. Cuthbertson, B. L. Norman, and J. Piatigorsky.** 1990. Differential expression of the two δ-crystallin/argininosuccinate lyase genes in lens, heart, and brain of chicken embryos. New Biol. **2**:903–914.