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The δ-Crystallin Enhancer-Binding Protein δEF1 Is a Repressor of E2-Box-Mediated Gene Activation

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The repressor δEF1 was discovered by its action on the DC5 fragment of the lens-specific δ1-crystallin enhancer. C-proximal zinc fingers of δEF1 were found responsible for binding to the DC5 fragment and had specificity to CACCT as revealed by selection of high-affinity binding sequences from a random oligonucleotide pool. CACCT is present not only in DC5 but also in the E2 box (CACCTG) elements which are the binding sites of various basic helix-loop-helix activators and also the target of an unidentified repressor, raising the possibility that δEF1 accounts for the E2 box repressor activity. δEF1 competed with E47 for binding to an E2 box sequence in vitro. In lymphoid cells, endogenous δEF1 activity as a repressor was detectable, and exogenous δEF1 repressed immunoglobulin κ enhancer by binding to the κE2 site. Moreover, δEF1 repressed MyoD-dependent activation of the muscle creatine kinase enhancer and MyoD-induced myogenesis of 10T1/2 cells. Thus, δEF1 counteracts basic helix-loop-helix activators through binding site competition and fulfills the conditions of the E2 box repressor. In embryonic tissues, the most prominent site of δEF1 expression is the myotome. Myotomal expression as well as the above results argues for a significant contribution of δEF1 in regulation of embryonic myogenesis through the modulation of the actions of MyoD family proteins.

Cumulative evidence has indicated that repressors interacting with activators play crucial roles in developmental gene regulation. In the best-studied cases of transcriptional regulatory elements generating developmental specificities, it is found that a binding site of a repressor overlaps with a binding site of an activator so that the regulators of the opposite effects compete for occupancy of the same site (16, 28, 29, 32). In general, the repressor is more widely distributed in spatial and temporal terms than the activators. Thus, in the majority of cell types the repressor occupies the element and shuts off expression of the gene, and only under conditions in which binding of the activator to the element dominates over that of the repressor is expression of the gene turned on. This seems one of the basic mechanisms to elicit stage-specific or cell-type-specific gene expression.

An example is found in immunoglobulin enhancers in which E2 box activator binding sites with the core sequence of CACCTG have been identified. A group of basic helix-loop-helix (bHLH) activator proteins (25) which are encoded by E2A and E2-2 genes (3) and bind to the E2 site of the immunoglobulin κ enhancer (and the μE5 site of the immunoglobulin heavy-chain enhancer as well) are in competition with a repressor, and only in differentiated lymphoid cells is the action of the activator effectuated and immunoglobulin κ enhancer activated (29). The same scenario holds true for regulatory elements of myocyte-specific genes such as AChRδ in which activators containing myogenic MyoD family proteins as their component bind to an E2-box-activating element (32). In these cases, action of the repressor(s) has been indicated by mutational analysis of the regulatory elements, but the repressor molecules themselves have not been identified.

Analogous counteraction of an activator and a repressor through an overlapping binding site has also been demonstrated for the lens-specific δ1-crystallin enhancer which we have studied (16). Lens specificity of the δ1-crystallin enhancer (11, 12) is determined by a short DNA segment of the enhancer called DC5 in which two activator-binding sites have been mapped and the binding site of the repressor δEF1 overlaps with one of the activator sites (16). The repressor δEF1, which binds to the DC5 segment, was initially identified by gel mobility shift assay (9), and subsequently its cDNAs were molecularly cloned on the basis of binding of their encoding proteins to the DC5 sequence (10). In this particular case, the repressor has been well characterized.

It was revealed that δEF1 has multiple potential DNA-binding domains, two separated zinc finger clusters near N and C termini, and a homeodomain in between. There are several other nuclear factors reported to have both zinc fingers and homeodomains (7, 24). Among them, Zfh-1, a mesoderm-specific zinc finger-homeodomain protein in *Drosophila melanogaster* (7, 19), was found to be similar to δEF1 in the organization of domains and the amino acid sequences of the zinc fingers (10).

Histological analysis of early chicken embryos with specific antisera indicated that δEF1 is expressed in a temporally regulated manner in various tissues, e.g., the lens, the central nervous system, the neural crest derivatives, and various mesodermal tissues (10). Among these tissues, δEF1 expression is conspicuously high in the notochord and the myotome of early embryos.

This work was undertaken to investigate possible roles of the repressor δEF1 other than crystallin gene regulation in the

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lens, with anticipation that δEF1 may function as an activator-counteracting repressor in various genetic systems. An analysis of δEF1 binding specificity indicated that δEF1 selectively binds to CACCT included in the E2 box sequence CACCTG, which is the binding site of bHLH activators and at the same time the target of the unidentified repressors (29, 32). This raised the possibilities that δEF1 regulates activity of bHLH proteins through competition of the same binding sites and even that δEF1 is one of the repressors of the E2 box so far unidentified. The results reported below demonstrate that δEF1 does repress E2 box activators collectively called BCF (25) in myeloma cells and MyoD in myogenesis of 10T1/2 cells and lend strong support to the model that δEF1 is a widely employed repressor in developmental gene regulations.

MATERIALS AND METHODS

Nuclear extracts of COS-7 cells expressing exogenous δEF1. cDNA for δΔC-fin was constructed by removing from the full-length of δEF1 cDNA (10) the *Pst*I-*Sph*I fragment spanning the C-proximal zinc finger cluster region. δJF12 cDNA was made by fusion of the sequences coding for the N-terminal 18 amino acids and the JF12 portion (10) of δEF1. cDNAs for δEF1, δJF12, and δΔC-fin were inserted into the *Not*I site of pCDM8 (31) and transfected to COS-7 cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in 9-cm-diameter dishes and transfected with 10 μg of DNA by a DNA-calcium phosphate coprecipitation method (5). Transfected cells were cultured for 48 h, and nuclear extracts were prepared by the method of Schreiber et al. (30), using buffers containing a protease inhibitor cocktail (5 μM leupeptin, 5 μM pepstatin A, 50 μM bestatin, 5 μg of aprotinin per ml, 2.5 mM phenylmethylsulfonyl fluoride). In Western blotting (immunoblotting), 6 μg of protein of a COS-7 cell extract was electrophoresed in a sodium dodecyl sulfate (SDS)–7.5 or 15% polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed with anti-JF12 antibodies (10), using the ECL system (Amersham). Gel mobility shift assay was done by the method of Kamachi and Kondoh (16).

Selection of high-affinity binding sequences of δEF1. Oligonucleotides of 73 bases containing 15-base-long random sequences, 5′-GTAAAACGACGGCCAGTGGATCCAGAT CT-$(N)_{15}$-GAATTCCTGCAGGTCGTGACTGGGAAAAC-3′, were chemically synthesized and rendered double stranded with primer B (5′-GTTTTCCCAGTCACGAC-3′) and *Taq* DNA polymerase. The double-stranded 73-mers were amplified by PCR with primer A (5′-GTAAAACGACGGCCAGT-3′) and primer B. The amplified 73-mers of 1 μg were mixed with 0.2 mg of GST-JF12 fusion protein (10) attached to glutathione-Sepharose 4B beads (Pharmacia) in binding buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 0.1 mM $ZnSO_4$, 1 mM phenylmethylsulfonyl fluoride and 5 μg of poly(dA-dT). After incubation at room temperature for 30 min, the beads were precipitated and washed three times with the same buffer, and bound oligonucleotides were eluted from the beads in G buffer (33). After phenol extraction and ethanol precipitation, the selected oligonucleotides were amplified by PCR with primers A and B. The products were subjected to another two rounds of binding site selection and sequence amplification with primers C (5′-CCAGTGGATCCAGATCT-3′) and D (5′-AC GACCTGCAGGAATTC-3′). The 73-mers collected after the three rounds of selection were digested with *Bam*HI and *Pst*I, cloned into pBluescriptKS+, and sequenced.

Gel mobility shift assay. To obtain GST-E47, E47 cDNA was excised from pMNT/E47 (2) with *Eco*RI-*Bam*HI, blunt ended, and inserted into the *Not*I site of pGEX-3X(NotI) (10), and the plasmid was transformed in *Escherichia coli*. Fusion proteins GST-JF12 and GST-E47 were synthesized in *E. coli* and affinity purified as described by Funahashi et al. (10). The fusion proteins as well as nuclear extracts from COS-7 cells (as described above) or from myelomas prepared by the method of Dignam et al. (6) were analyzed by gel mobility shift assay. Two different probes were used: the DC5 probe, carrying the δ1-crystallin enhancer sequence described by Kamachi and Kondoh (16), and the κE2 probe, which was prepared by inserting the synthetic oligonucleotide

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gatctGGCAGGTGGCg
  aCCGTCCACCGcctag
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at the *Bam*HI and *Bgl*II sites of pUC19BEX (16) and then by excising the *Hin*dIII-*Bam*HI fragment. The probes were end labeled by filling in with [α-^{32}P]dCTP. Sequence-specific competitors were oligonucleotides of the immunoglobulin κ enhancer sequence centered by the κE2 sequence (W) and its mutant forms (m1, m2, and m4):

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W,  gatcTCCTCCCAGGCAGGTGGCCCAGATTACg
        AGGAGGGTCCGTCCACCGGGTCTAATGcctag

m1, gatcTCCTCCCAGGTAGGTGGCCCAGATTACg
        AGGAGGGTCCATCCACCGGGTCTAATGcctag

m2, gatcTCCTCCCAGGCATTTTGCCCAGATTACg
        AGGAGGGTCCGTAAAACGGGTCTAATGcctag

m4, gatcTCCTCCCAGGCAGATGGCCCAGATTACg
        AGGAGGGTCCGTCTACCGGGTCTAATGcctag
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Immunoglobulin κ enhancer fragments A to C (see Fig. 4A) and B sequence mutant forms (m1, m2, and m4) were also prepared by PCR with specific primers. A total of 0.5 ng of each probe and various amounts of competitors was incubated with 2 μg of nuclear extract or 5 ng of glutathione *S*-transferase (GST) fusion proteins, unless otherwise indicated, at room temperature for 30 min in 10 μl of reaction mixture. Three binding conditions were chosen: A (optimized for δEF1 binding), 20 mM HEPES (pH 7.9), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 1 mM DTT, 15% glycerol, 0.1 μg of bovine serum albumin (BSA) per μl, and 0.1 μg of poly(dA-dT) per μl; B (optimized for BCF binding), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol (25), 0.1 μg of BSA per μl, 0.1 μg of poly(dI-dC) per μl; and C (composite), 10 mM Tris (pH 7.5), 50 mM NaCl, 0.1 mM $ZnSO_4$, 1 mM DTT, 15% glycerol, 0.1 μg of BSA per μl, and 5 ng of poly(dA-dT) per μl. The complexes were resolved by electrophoresis through 4 or 6% polyacrylamide gels containing 22 mM Tris-borate (pH 8.0)–0.5 mM EDTA.

Construction of luciferase reporter genes. Various regulatory elements were inserted at the *Sal*I-*Bam*HI site upstream of the promoter of the basic luciferase reporter gene δ51LucII (16). The 475-bp *Alu*I-*Alu*I fragment containing the immunoglobulin κ enhancer (26) was cloned at the *Eco*RV site of pUC19BEX (16) and employed as the template for PCR to introduce point mutations into the enhancer by using a series of primers containing the *Sph*I site: common primer, GCAT GCCCAGATTACAGTTGAC; mutagenic m1 primer, GCAT GCCACCTACCTGGGAGGAACT; m2 primer, GCATGCA AAATGCCTGGGAGGAACTG; and m4 primer, GCATGC CATCTGCCTGGGAGGAACT. PCR was performed between the common primer and the M13 forward primer which

produced the fragment downstream of the κE2 site, and between each of the mutagenic primers and the M13 reverse primer which produced the mutated fragment supstream of the κE2 site. These fragments were digested with *Sph*I and blunt ended with Klenow fragment. The mutated enhancers were reconstructed on pUC19BEX by ligating the common downstream fragment with each of the upstream fragments. Duplicated E2 box elements of the mouse muscle creatine kinase (MCK) enhancer (2R sites [37])

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gatctGGCAGCAGGTGTTGGGAGGCAGCAGGTGTTGGGAG
    aCCGTCGTCCACAACCCTCCGTCGTCCACAACCCTCctag
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were chemically synthesized and inserted into the *Bam*HI site of δ51LucII.

Transfection of luciferase and β-galactosidase genes. Myeloma cells P3U1 (P3-X63.Ag8.U1) and J558L were grown in RPMI medium containing 10% FCS and suspended at 10^7/ml in serum-free RPMI medium containing 10 mM HEPES. Aliquots of 0.25 ml were mixed with DNA containing 5 μg of luciferase reporter plasmids, various amounts of activator/repressor plasmids, and stuffer pCMVX/pUC19 plasmids so that the total amount of DNA was 10 μg. The cells were transfected by electroporation with a Gene Pulser (Bio-Rad) at 200 V/0.4 cm with 960 μF capacitance. The cells were cultured for a further 24 h for luciferase expression. 10T1/2 cells were grown in DMEM containing 10% FCS. Cells (5×10^4) were plated on 3.5-cm-diameter dishes 1 day before transfection. Cells were transfected with 2 μg of DNA containing 0.4 μg of the luciferase gene carrying the MCK-2R element, 1 μg of pβactMyoD [MyoD expression vector; pβA.D(+) of Fujisawa-Sehara et al. (8)] and various amounts of pCMVX-δEF1 (δEF1 expression vector) and pCMVX/pUC19 by a DNA-calcium phosphate coprecipitation method (5). In a few experiments, 0.1 μg of pβactβgal was included in the transfection mixture to examine the effects of δEF1 on the β-actin promoter. pβactβgal was constructed by inserting the *Sph*I fragment of pMoZtk (36) into the *Sal*I site of pβA (8) after making the ends blunt with T4 DNA polymerase. The medium was changed after 10 h, and cells were harvested after an additional 48 h. Cell extracts were prepared from the same number of transfected cells, luciferase activity was measured by the method of Kamachi and Kondoh (16), and β-galactosidase activity was measured by the method of Fiering et al. (6a), using 4-methylumbelliferyl-β-D-galactoside as a substrate.

Myogenesis of 10T1/2 cells. 10T1/2 cells were cultured in 3.5-cm-diameter dishes and transfected in quadruplicate with 0.75 μg of DNA containing 0.075 μg of pβactβgal, 0.1125 μg of pβactMyoD, various amounts of pCMVX-δEF1, and insert-free pCMVX. After 2 days, two of the dishes were fixed and stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (34), and the rest were fed with DMEM containing 10% horse serum for a further 3 days. The cultures were fixed with 3.5% paraformaldehyde in HEPES-buffered saline and treated with anti-troponin T (TN-T) monoclonal antibody NT302 (1), biotinylated anti-mouse immunoglobulin antibodies (Amersham), and avidin-biotinylated peroxidase complexes (Vector). Peroxidase color reaction was carried out as described by Kondoh et al. (18).

RESULTS

Specific binding sequence of δEF1. The protein δEF1 has multiple domains potentially involved in DNA binding, two zinc finger clusters separated to near N and C termini and a homeodomain in the middle (10). Among them, the C-proximal three zinc fingers alone bind to the DC5 fragment of the δ1-crystallin enhancer (10).

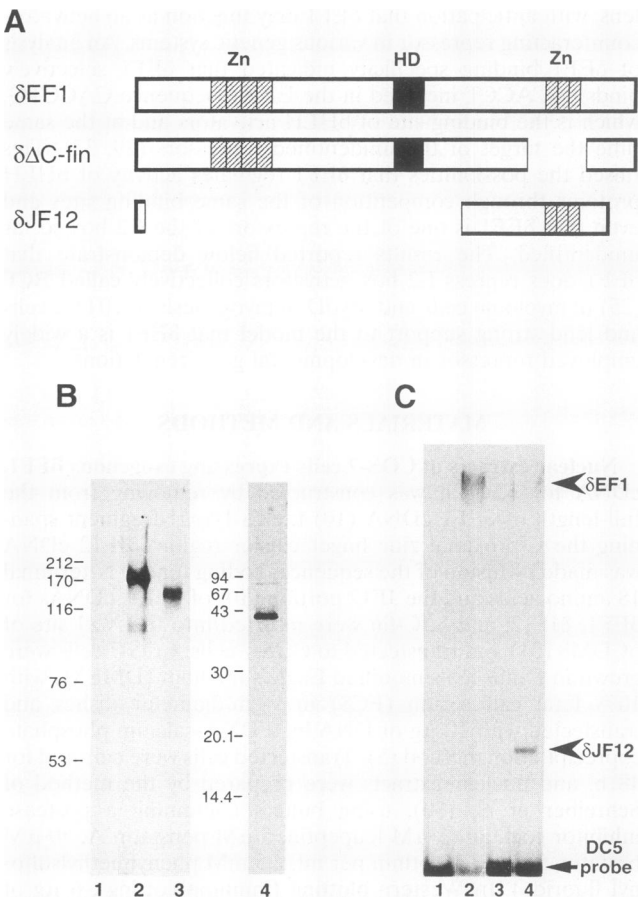

FIG. 1. Binding of δEF1 and its derivatives to the DC5 sequence. (A) Scheme of protein organization of δEF1 and its derivatives, δΔC-fin and δJF12. N terminus is to the left. Zinc finger clusters (Zn) are indicated by hatched boxes, and the homeodomain (HD) is shown by the solid box. (B) Western blotting of δEF1 and its derivatives expressed in COS-7 cells. cDNAs coding for the proteins shown in panel A were placed in expression vector pCDM8 and transfected to COS-7 cells. Nuclear extracts from COS-7 cells transfected with pCDM8 carrying no cDNA (lane 1), δEF1 cDNA (lane 2), δΔC-fin cDNA (lane 3), and δJF12 cDNA (lane 4) were processed for immunoblot analysis. Lanes 1 to 3, 7.5% polyacrylamide gel; lane 4, 15% gel. The molecular masses (in kilodaltons) of the reference proteins are shown to the left. δEF1 is 124 kDa in size but runs at the position of 170 kDa (10). (C) The same nuclear extracts as in panel B were analyzed by gel mobility shift assay (condition A) for binding to the probe of the DC5 sequence of the δ1-crystallin enhancer.

We examined whether the C-terminal zinc fingers are essential for binding to the DC5 sequence. δEF1, δΔC-fin (a mutant form of δEF1 lacking the C-proximal zinc fingers), and δJF12 proteins carrying only the C-proximal zinc fingers as a DNA-binding domain (Fig. 1A) were expressed in COS-7 cells by transfection of cDNAs under regulation of the cytomegalovirus promoter in vector pCDM8 (31). Nuclear extracts of the transfected COS-7 cells contained a comparable amount of the protein coded by the cDNAs, as shown by Western blot (Fig. 1B). In a gel mobility shift assay with the same nuclear extracts, it was shown that δEF1 and δJF12 bound the DC5 probe but δΔC-fin bound to the probe was not detectable (Fig. 1C). This binding was specifically inhibited by the DC5 sequence (10). In

analogous experiments, isolated homeodomain or N-proximal zinc fingers did not bind with the same high affinity to the probe (data not shown). Thus, the C-proximal zinc fingers were shown to be primarily responsible for binding to the DC5 sequence.

To determine binding specificity of the C-proximal zinc fingers, the JF12 portion of δEF1 fused to GST was used to select high-affinity binding sequences from a pool of random synthetic oligonucleotides. The fusion protein was expressed in *E. coli*, affinity purified, and bound to glutathione-agarose beads. Oligonucleotides which had randomized 15-bp-long sequences flanked by the sequences for *Pst*I-*Bam*HI restriction and for primer binding sites were synthesized. They were mixed with GST-JF12 beads, and the base sequences of those bound to the beads were amplified by PCR. The amplified sequences were again selected for binding to the fusion protein. The cycles of selection and amplification of bound sequences were repeated three times, the final products were cloned into a Bluescript plasmid vector, and 41 arbitrarily chosen clones were sequenced for *Bam*HI-*Pst*I inserts. The sequences were then aligned for a possible consensus sequence. As shown in Fig. 2A, most of the sequences contained CACCT and the rest had pentanucleotide sequences which differ from CACCT by only a single base. The CACCT consensus (Fig. 2B) was, in fact, found in the δEF1 binding site of the δ1-crystallin enhancer (Fig. 2C).

C-proximal zinc fingers of δEF1 bind to the E2 box sequence and interfere with E2 box binding of bHLH protein E47. The consensus binding sequence of δEF1, CACCT, is included in the E2 box sequence CACCTG, to which a variety of bHLH protein dimers bind as activators (Fig. 2C). There is concrete evidence that E2 box sequences are targets of a repressor(s), though the repressor(s) has not been biochemically identified (29, 32). Thus, a model in which δEF1 is one of the unidentified repressors which bind to E2 box sequences and interfere with the binding and/or action of the bHLH activators was considered. To test this model, we examined whether δEF1 through its C-proximal zinc fingers binds to the E2 element of the immunoglobulin κ enhancer (κE2 site), which is the best-studied E2-box-activating element, and also whether the binding of δEF1 and a bHLH protein is mutually exclusive.

Various mutant forms of the κE2 sequence were synthesized and used as competitors in a gel mobility shift assay (Fig. 3A). GST-JF12 fusion protein carrying the C-proximal zinc fingers, in fact, bound to the κE2 element, as shown in a gel mobility shift assay (condition A) (Fig. 3B). The wild-type κE2 sequence competed with the probe in binding to GST-JF12, and a mutant form of κE2 with a sequence closer to the one found in the δ1-crystallin enhancer (m1) competed more efficiently than the wild-type sequence, whereas mutant sequences that had base alterations in CACCT (m2 and m4) failed to act as competitors. On the other hand, the GST fusion protein with the bHLH region of E47, which bound to the E2 box sequence as a homodimer (35), responded differently to the mutations (condition B) (Fig. 3C). The mutation in the central part of the E2 box which conserved the generalized E box sequence CANNTG (m4) still retained, though at somewhat lower levels, the binding capacity, whereas the mutations disrupting the E box (m1 and m2) failed to compete.

To see whether C-proximal zinc fingers of δEF1 interfere with binding of the E47 bHLH domain to an E2 box, increasing amounts of GST-JF12 protein were added to GST-E47 protein, and complexes with the probes of κE2 sequence were examined (Fig. 3D). The condition of DNA binding adopted in this analysis (condition C) was a compromise between those chosen for δEF1 and E47, because optimizing binding of one

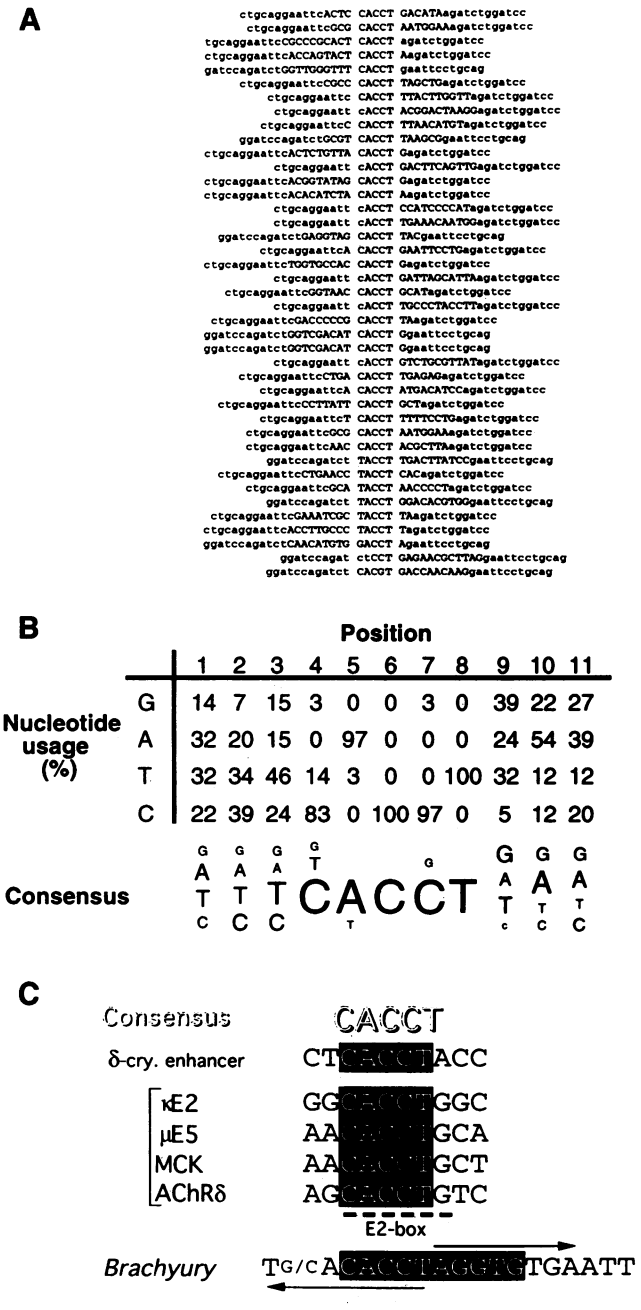

FIG. 2. Determination of the binding specificity of the C-proximal zinc fingers. (A) Base sequences of 41 oligonucleotides, which were randomly chosen from those collected after three rounds of binding site selection with GST-JF12, are aligned at the consensus core sequence, 5′-CACCT-3′. Linker sequences are shown in lowercase letters. (B) Analysis of nucleotide usage at each position relative to the consensus 5′-CACCT-3′ (positions 4 to 8). At each position, the percentage of occurrences of the four bases is shown. (C) Occurrence of CACCT in various known DNA-binding sites: δ-crystallin enhancer DC5 fragment (δ-cry. enhancer), E2 box sequences (κE2; μE5; MCK, MCK enhancer; and AChRδ, acetylcholine receptor δ-subunit gene regulatory sequence), and the Brachyury binding sequence (17).

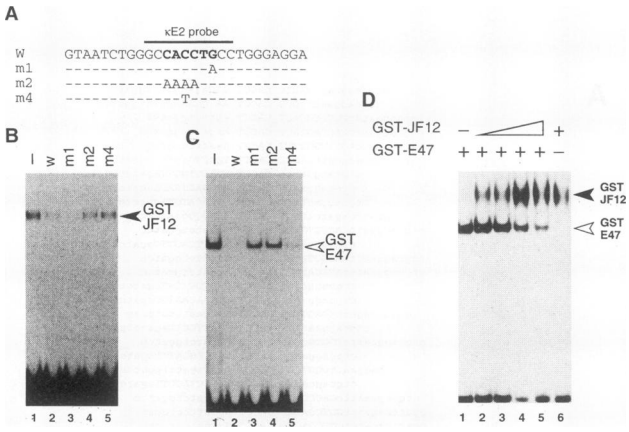

FIG. 3. Interaction of DNA-binding domains of δEF1 and E47 with the κE2 sequence. (A) The κE2 site and flanking sequences (22) used as the probe and competitors. The 10-bp κE2 sequence in the probe is indicated. W, wild-type sequence; m1, m2, and m4, mutant sequences in which altered nucleotides are indicated. (B) GST-JF12, the C-proximal zinc fingers of δEF1 fused to GST, was examined for binding to κE2 probe in the presence of various competitor sequences. The probe was the 10-bp κE2 sequence flanked by polylinker sequences, and the competitors carried the 27-bp immunoglobulin κ sequences centered by the κE2 site shown in panel A. Gel mobility shift assay was done under condition A. Lane 1, no specific competitor; lanes 2 to 5, 10 ng each of wild-type (w), m1, m2, and m4 sequences, respectively. (C) GST-E47, the bHLH domain of E47 fused to GST, was examined for binding to the κE2 sequence under condition B. Lanes are as in panel B. (D) Competition between GST-JF12 and GST-E47 for binding to the κE2 sequence. A constant amount (10 ng) of GST-E47 and increasing amounts of GST-JF12 were mixed with κE2 probe, and the complexes made were analyzed by gel mobility shift assay under condition C. Lanes 1 to 5, 10 ng of GST-E47 and 0 ng (lane 1), 5 ng (lane 2), 10 ng (lane 3), 25 ng (lane 4), and 50 ng (lane 5) of GST-JF12; lane 6, 10 ng of GST-JF12 alone.

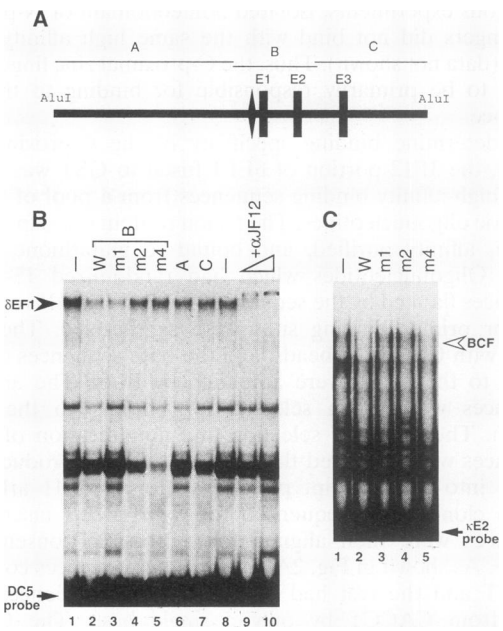

FIG. 4. δEF1 and BCF binding activities in myeloma cells. (A) Organization of the immunoglobulin κ enhancer of the mouse (*Alu*I-*Alu*I fragment, 475 bp), and its subfragments A to C used as competitors in the gel mobility shift assay in panel B. Solid boxes E1 to E3 indicate the defined activating sites, and the diamond indicates the NF-κB binding site (22). The B subfragment (110-bp) sequence was altered at the κE2 site to the mutant sequences m1, m2, and m4 (Fig. 3A), and these were used as competitors in the analysis. (B) Detection of δEF1 by gel mobility shift assay. Nuclear extracts from P3U1 myeloma cells were incubated with the DC5 probe in the presence of specific competitors (10 ng) or antibodies and complexes with the probe were analyzed by gel mobility shift assay under condition A. Lanes 1 and 8, no competitor; lane 2, 110-bp wild-type (W) B fragment; lanes 3 to 5, B fragment containing m1, m2, and m4 mutations, respectively; lane 6, 230-bp A fragment; lane 7, 141-bp C fragment; lanes 9 and 10, 0.15 and 1.5 μg, respectively, of anti-JF12 antibodies. (C) Detection of BCF (25). The same nuclear extract as in panel B was analyzed for BCF binding to the κE2 probe under condition B. δEF1 is not detectable in this condition (9). Lane 1, no specific competitor; lanes 2 to 5, wild-type (W) and mutant κE2 sequences as in Fig. 3A. The protein-probe complex representing BCF is indicated.

of the proteins significantly reduced the affinity of the other. GST-JF12 at high concentrations inhibited complexing of GST-E47 with the probe, supporting the model of competitive binding to the same site by δEF1 and bHLH proteins.

δEF1 is present in lymphoid cells, and overexpression of δEF1 inhibits the immunoglobulin κ enhancer by binding to the κE2 site. Given the observation that the C-proximal zinc fingers of δEF1 bind to the κE2 site and can displace a bHLH protein at the same site, it became conceivable that δEF1 could act as a repressor of immunoglobulin κ enhancer by binding to the κE2 site. In addition, considering the wide distribution of δEF1 activity among tissues, it appeared possible that lymphoid cells express endogenous δEF1 as well. To test the latter possibility, we examined myeloma nuclear extracts for δEF1 activity, using immunoglobulin κ enhancer fragments as competitors (Fig. 4A). Nuclear extracts of mouse myeloma P3U1 yielded a complex with DC5 probe which showed a very low mobility characteristic of δEF1 (Fig. 4B). This complex was inhibited by the immunoglobulin κ B fragment carrying the wild-type κE2 sequence or the m1 mutant sequence with a higher affinity to δEF1 C-proximal zinc fingers, but not by mutant sequences m2 and m4 in which CACCT was altered (Fig. 3) or by immunoglobulin κ enhancer fragments A and C. Thus, in the entire span of the immunoglobulin κ enhancer, κE2 is the only site for binding of the putative δEF1. Moreover, this complex was supershifted by anti-chicken δEF1 antibodies (10). The same results were obtained with extracts of another myeloma cell line, J558L (data not shown). Together, these demonstrated that myeloma nuclei contained a significant level of endogenous δEF1, which binds to the κE2 site with the same specificity as the C-proximal zinc fingers.

The same nuclear extract contained a group of proteins making complexes with κE2 probe and collectively called BCF (25) (Fig. 4C). BCF had been shown to represent bHLH proteins in lymphoid cells (3, 25). Complexes made between BCF and κE2 were specifically inhibited by the competitors of the κE2 sequence and its mutant forms in the same way as GST-E47 (compare Fig. 3C and 4C).

Does δEF1 then repress activity of the immunoglobulin κ enhancer (22, 26), which is dependent on BCF (25)? To the myeloma cells, a luciferase gene carrying the immunoglobulin κ enhancer was introduced together with various amounts of δEF1 expression vector. In addition to wild-type immunoglobulin κ enhancer sequence, mutant enhancers which carried the same base alterations as in Fig. 3A were used. In the absence of added δEF1 expression vector, the immunoglobulin κ enhancer supported a high luciferase expression, whereas this

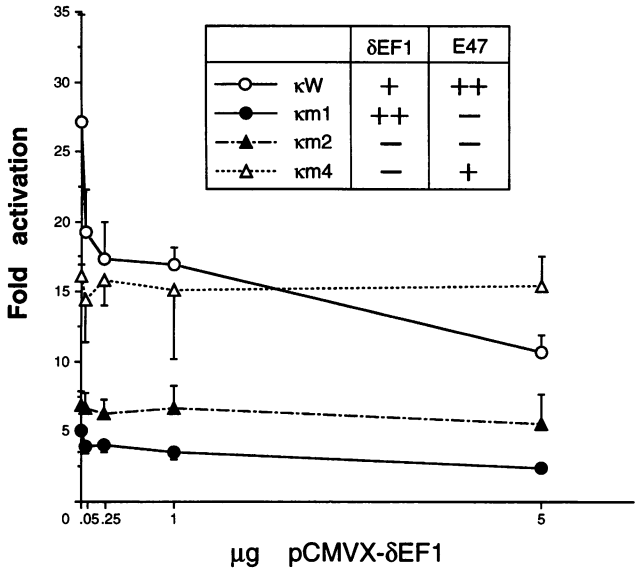

FIG. 5. Effects of mutations in the κE2 sequence on immunoglobulin κ enhancer activity and on susceptibility to repression by δEF1. P3U1 myeloma cells (2.5×10^6) were transfected with a constant amount (5 μg) of luciferase reporter gene carrying an immunoglobulin κ enhancer and with various amounts of pCMVX-δEF1 expression vector. The total amount of the DNA was adjusted to 10 μg with the insert-free vector plasmid. Relative luciferase activity, averaged for three or four independent transfections, was plotted against the amount of cotransfected pCMVX-δEF1. The bars represent standard deviations. In the inset, the kind of κE2 mutation and binding of the factors to the sequences are indicated (− to ++) as determined in the experiments shown in Fig. 3 and 4. The values at 0 μg indicate the levels of luciferase expression in the myeloma cells activated by the immunoglobulin κ enhancer. Similar results were obtained in three different experiments.

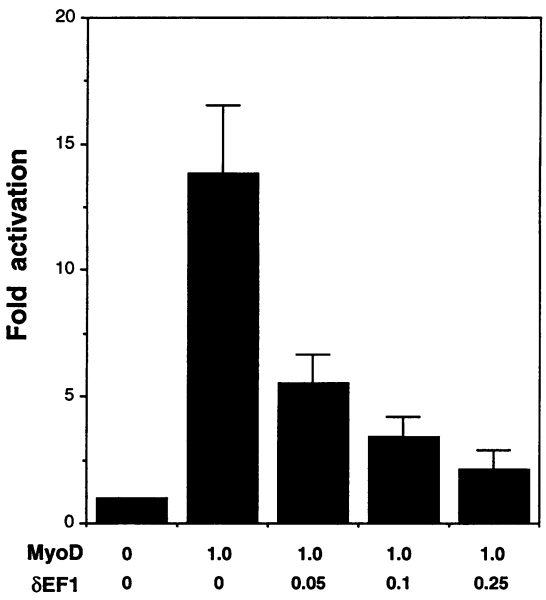

FIG. 6. Repression of MyoD-activated MCK enhancer element by exogenous δEF1. A luciferase reporter gene (0.5 μg) carrying two copies of the E2 box element of the mouse MCK enhancer [2R sites (37)] was transfected to 10T1/2 fibroblasts (5×10^4 cells), and the effect of cotransfected expression vectors for MyoD (constant, 1 μg of pβactMyoD) and for δEF1 (variable, the numbers indicating micrograms of pCMVX-δEF1) was measured. Relative luciferase activities averaged for four independent transfections are shown with standard deviations. Exogenous MyoD activated the luciferase expression, and δEF1 repressed the activation in a dose-dependent manner. In an experiment in which 0.1 μg of pβactβgal was included in the transfection, the maximum amount (0.25 μg) of pCMVX-δEF1 allowed β-galactosidase expression 87% as high as without pCMVX-δEF1, indicating that δEF1 did not repress the β-actin promoter.

was lowered to one-fifth that level when the enhancer carried the mutation m2, which abolished binding of both δEF1 and bHLH proteins (Fig. 5). The activation level was lowered further with the mutant sequence m1, which lost E47 binding and gained a stronger δEF1 binding. The mutant enhancer κm4, which had no δEF1 binding but retained a significant binding affinity for bHLH proteins, displayed an enhancer activity 60% as high as that of the wild-type immunoglobulin κ enhancer.

By increasing the amount of δEF1 cDNA expression vector, luciferase expression was repressed by 60% if the reporter gene carried the wild-type immunoglobulin κ enhancer (Fig. 5). This repression, however, was not observed with use of cDNA-free expression vector or when the exogenous δEF1 lacked its C-proximal zinc fingers (data not shown). When the immunoglobulin κ enhancer had a mutated κE2 sequence lacking binding to δEF1 (κm2 or κm4), luciferase expression was not repressed by exogenous δEF1. When the mutation of the κE2 sequence caused loss of E47 binding and an increase of δEF1 affinity (κm1), immunoglobulin κ enhancer activity was further lowered by exogenous δEF1. These results indicated that δEF1 repressed the immunoglobulin κ enhancer through binding to the κE2 element.

Thus, exogenous δEF1 repressed the immunoglobulin κ enhancer in a binding-dependent manner, and the data without exogenous δEF1 are consistent with the same activity of the endogenous δEF1. Residual enhancer activities were noted under repression by δEF1 or in the absence of active κE2 element. They are accounted for by activating elements other than κE2 which had been identified in the immunoglobulin κ enhancer (22).

δEF1 represses MyoD-induced activation of the MCK enhancer element and myogenesis of 10T1/2 fibroblasts. The above results have shown that δEF1 represses E2-box-mediated gene activation. Given the facts that MyoD family proteins most often utilize E2 box sequences as their targets (25, 32, 37) and that δEF1 protein is conspicuously abundant in embryonic myotomes (10) where MyoD family myogenic genes are highly active (27), it is possible that δEF1 modulates the activity of the MyoD family proteins.

To test this possibility, we examined the effect of δEF1 on transactivation of the MCK enhancer by MyoD. To this end, duplicated MyoD binding sites of the MCK enhancer (37) were placed upstream of the same luciferase gene used for analysis of the immunoglobulin κ enhancer and transfected to 10T1/2 fibroblasts. Expression of the luciferase gene was activated nearly by 14-fold by cotransfection of MyoD cDNA under the regulation of β-actin promoter (MyoD expression vector; pβactMyoD) (Fig. 6). However, transactivation by MyoD was efficiently repressed by increase of cotransfected δEF1 expression vector (pCMVX-δEF1), down to one-sixth the level of the maximum activation (Fig. 6). Thus, δEF1 efficiently blocked transactivation by MyoD. In untransfected 10T1/2 cells, endogenous δEF1 was detectable, though the level was lower than in the myeloma cells (data not shown).

Exogenous MyoD expression not only activates MCK and

other muscle-specific enhancers but induces myogenesis of 10T1/2 fibroblasts [Fig. 7B(a)]. To test the effect of δEF1 expression on MyoD-induced myogenesis, various amounts of the δEF1 expression vector were mixed with a constant amount of the MyoD expression vector and transfected into 10T1/2 cells (Fig. 7A). To control variation of transfection efficiency, pβactβgal was also included in the transfection mixture. MyoD-induced myogenesis was significantly repressed by exogenous δEF1. The number of cells stained with anti-TN-T antibody (not present without MyoD transfection) was reduced to one-fourth that by exogenous δEF1 (Fig. 7A). This population of TN-T-positive cells remained constant in increasing amounts of δEF1 expression vector. Repression of the myogenesis was dependent on the DNA-binding capacity of the exogenous δEF1, since no effect was observed with δΔC-fin lacking the C-proximal zinc fingers of δEF1 (Fig. 7A).

In addition to this decrease in the number of TN-T-positive cells, there was a significant change in the morphology of these stained cells (Fig. 7B). In the absence of exogenous δEF1, a large fraction of the TN-T-positive cells had thick and elongated cytoplasm and were often multinucleated, characteristic of differentiated myocytes/myotubes [Fig. 7B(a)]. However, when δEF1 cDNA was expressed by cotransfection, there were very few cells with myotubular morphology, and TN-T-expressing cells generally had very small cytoplasms [Fig. 7B(b)]. This also indicated that exogenous δEF1 severely interfered with myogenesis. Some myogenic activity remained, as indicated by TN-T-expressing small cells. Considering the fact that E2 box sequences are the major portion, but not all, of the binding sites of MyoD family proteins, it seems likely that δEF1-insensitive pathways somehow lead to production of such cells.

DISCUSSION

δEF1 was discovered as a nuclear factor which binds to the DC5 fragment of the chicken δ1-crystallin enhancer carrying regulatory elements essential for the lens-specific enhancer action (9, 16). Mutational analysis of the δEF1 binding site and examination of the effect of overexpression of δEF1 from an exogenous gene both indicated that δEF1 is the repressor of the δ1-crystallin enhancer which counteracts an activator and modulates the enhancer activity (10, 16).

Analysis of δEF1 expression in developing embryos (10) provided us with a very interesting finding. Expression of δEF1 was not confined to the lens but was widespread among the central nervous system, the neural crest derivatives, and the mesoderms, and the most prominent sites of its expression were the notochord and the myotome. This prompted us to explore possible functions of δEF1 in various embryonic tissues, and the initial attempt was to determine consensus high-affinity binding sequences from which we may infer the possible regulatory target genes of δEF1.

δEF1 has specificity to E2 box sequences and acts as a repressor of E2-box-mediated gene activation. δEF1 protein has multiple domains potentially involved in DNA binding, two separated zinc finger clusters, and a homeodomain in between. The primary DNA-binding domain specific to the δ-crystallin enhancer sequence DC5 was shown to be the cluster of three zinc fingers proximal to the C terminus which are classified as Krüppel type (10). A mutant protein lacking the C-proximal zinc fingers (ΔC-fin) failed to bind DC5, while a fragment of δEF1 carrying the C-proximal fingers (JF12) bound to DC5 (Fig. 1C) with the same sequence specificity as the native δEF1 (Fig. 3B and 4B).

Selection of oligonucleotides which bound with high affinity to the JF12 portion of δEF1 identified the consensus binding

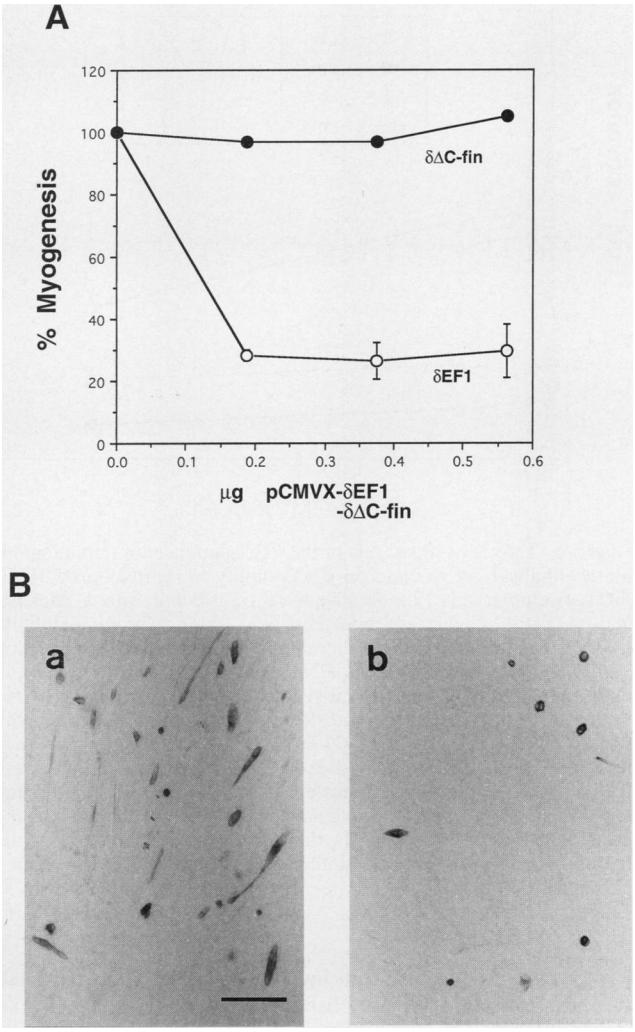

FIG. 7. Repression of MyoD-induced myogenesis of 10T1/2 fibroblasts by exogenous δEF1. 10T1/2 cells (5×10^4) were transfected with constant amounts of pβactMyoD (MyoD expression vector driven by a β-actin promoter) (0.1125 μg) and pβactβgal (0.075 μg) and with variable amounts of δEF1 expression vector pCMVX-δEF1. The total amount of DNA was made 0.75 μg with insert-free pCMVX plasmid. After 2 days, parts of the transfected cultures were fixed to count β-galactosidase-expressing cells as a score of transfection efficiency, and the rest were cultured in the myogenic medium for a further 3 days to examine myogenesis by staining with an anti-TN-T antibody. (A) Relative number of TN-T-positive cells (counted as nuclei) in a culture as an index of myogenesis. Open circles represent data for wild-type δEF1, while solid circles represent those with a mutant form, δΔC-fin, lacking the C-proximal zinc fingers (Fig. 1). The data are normalized to the number of β-galactosidase-positive cells in sibling cultures receiving the same DNA-calcium phosphate coprecipitates and are averaged for triplicate transfections. Similar results were obtained in three independent experiments. The 100% value in the absence of exogenous δEF1 roughly corresponded to 1×10^4 TN-T-positive cells and 4×10^4 β-galactosidase-positive cells in a culture. (B) Immunohistological detection of TN-T-expressing cells in MyoD-transfected 10T1/2 cultures. (a) Culture without exogenous δEF1 expression; (b) culture with 0.2 μg of δEF1 expression vector. Cultures receiving larger amounts of the δEF1 expression vector were indistinguishable. Bar, 200 μm.

sequence CACCT (Fig. 2A and B). This consensus sequence was found in the DC5 fragment of the δ1-crystallin enhancer and in the E2 box sequences (Fig. 2C). An analogous attempt to define the consensus sequence of the entire δEF1 molecule resulted in the same CACCT (24a). Specific DNA-binding capacities possibly associated with N-proximal fingers and the homeodomain are currently under investigation, and we anticipate auxiliary functions to these domains in binding of the entire δEF1 protein to the DNA.

Specificity of δEF1 toward CACCT not only is consistent with its action on the δ1-crystallin enhancer but indicates its possible involvement in regulation of a wide variety of enhancers/regulatory regions which are dependent on the E2 box (CACCTG) elements themselves serving as binding sites of bHLH activators while at the same time being the target of unidentified repressors (29, 32). We showed first that the CACCT-binding zinc fingers of δEF1 interfere with the binding of a bHLH protein to an E2 box sequence. Second, we demonstrated that exogenous δEF1 repressed E2-box-dependent immunoglobulin κ enhancer activity, transactivation of a MCK enhancer element by MyoD, and MyoD-induced myogenesis, which is thought to involve a number of E2 box elements. Endogenous mouse δEF1 protein has been shown to be present in these cells. Thus, δEF1 is likely to be one of the repressors of the E2 boxes which have been predicted (29).

Possible function of δEF1 in the myotome. In chicken embryos, δEF1 expression commences after somites are formed, and the expression steeply increases in the myotome when somites are compartmentalized (10). It is intriguing that this is the stage when MyoD family myogenic genes are activated (27). Proteins encoded by this gene family have bHLH DNA-binding motifs, in most instances to heterodimerize with E2A/E2-2 gene products and activate muscle-specific genes through binding to E box (largely, E2 box) elements (3, 21, 25). In the process of somitegenesis, expression of the MyoD family of genes appears to commit the cells to the muscle lineage, but muscular differentiation seems temporally regulated and occurs much later. This implies that activity of the MyoD family proteins is negatively regulated, and growth factor-dependent phosphorylation of MyoD family proteins (23) and formation of DNA-binding-incompetent complexes with Id (14) have been considered as candidate mechanisms for the negative regulation. In addition to these mechanisms, we speculate that counteraction by δEF1 has a significant contribution to differential regulation of MyoD family proteins. The action of δEF1 must be specific to the E2 box (CACCTG) among the E box elements (CANNTG) and therefore allows E box elements other than those of the E2 type to be fully active under its action. In fact, exogenous δEF1 in MyoD-transfected 10T1/2 cells repressed full differentiation of the myocytes but still allowed expression of some muscular traits. This differential action of δEF1 on E box elements may be significant in the temporal regulation of muscle differentiation starting from myotome segregation.

δEF1 in the notochord. Development of the notochord, and possibly the notochordal activity to induce various axial/paraaxial structures as well, is under regulation of the Brachyury (T) gene product (13). Brachyury expression is confined to premesodermal cells in the primitive streak and in the axial mesoderm to form notochord as well as the notochord itself (38). It has recently been demonstrated that Brachyury codes for a DNA-binding nuclear protein which is likely a transcriptional factor. Most intriguingly, the Brachyury binding consensus turned out to be TTTCA CACCT AGGTG TGAAA (17), in which the δEF1 consensus CACCT occurs twice as an inverted repeat. It is thus reasonable to assume that δEF1 also binds to the Brachyury binding site. In the notochord of the chicken, δEF1 expression is turned on at stage 12, which is much later than Brachyury. δEF1 may modulate binding of Brachyury protein to the target site and as a consequence be involved in temporal change of the activity of the notochord, such as induction of floor plate and motor neurons (39).

***D. melanogaster* counterpart of δEF1.** In *D. melanogaster*, two proteins are known to have multiple zinc fingers and homeodomains (7). One of them, Zfh-1, is very similar to δEF1 (10) not only in the organization of DNA-binding domains but in amino acid sequences of 3′ C-proximal zinc fingers, especially in those involved in nucleotide recognition (25a). This raises the possibility that Zfh-1 also binds to the E2 box sequence. In addition, a high expression level of δEF1 in the mesodermal tissues, notochord, and myotome is reminiscent of mesodermal expression of Zfh-1 in *D. melanogaster* (19). These suggest that Zfh-1 has a regulatory function in *D. melanogaster* analogous to that of δEF1. Null *zfh-1* mutants have been isolated from *D. melanogaster*, and consistent with the site of expression of Zfh-1, the homozygous mutants have been reported to be lethal with defects of muscle development (20).

δEF1 as a repressor in developmental gene regulation. δEF1 described here satisfies the characteristics of the repressors predicted for tissue-specific enhancers. First, the DNA-binding sites of the repressors overlap with the binding sites of activators, and as a result the repressors compete with the activators for DNA binding. The binding specificity of δEF1 predicts that it competes with bHLH proteins and also with Brachyury protein for DNA binding. It was demonstrated that δEF1 and a bHLH protein, E47, compete for a binding site in vitro, and δEF1 repressed gene activation by bHLH proteins in transfected cells. Second, the repressors are also expected to suppress the effect of such activators that bind to neighboring sites (28, 29). δEF1, in fact, has the capacity to repress neighboring activation elements of the δ1-crystallin enhancer (16).

In addition to these characteristics, expression in embryonic tissues and the experimental results reported here argue that δEF1 is of general use in developmental gene regulation. The major target sites of δEF1 action seem to be the bHLH protein binding E2 boxes to which the C-proximal zinc fingers also bind. In analogy to the MyoD family proteins in myotome, we speculate that in developing neural tissues δEF1 possibly counteracts bHLH proteins such as Mash-1 (15).

The mechanism of the action of δEF1 to regulate DNA binding of bHLH proteins is fundamentally different from those previously known. Phosphorylation (23) or heterodimerization with Id (4) or with HES (2) totally abolishes DNA-binding activity of the proteins, while δEF1 competitively inhibits a subset of bHLH protein-binding sites, which allows δEF1 to modulate bHLH activators in a gene-specific manner. These negative regulations may be relevant to the long-held premise that cell differentiation proceeds in two steps, cell commitment and terminal differentiation. It is conceivable that cell commitment is marked by expression of the activator proteins, although their action is largely suppressed by the negative regulations, including the one mediated by δEF1. When these activators are relieved from negative regulation to execute their action, the terminal differentiation will occur.

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