

## B-Cell- and Myocyte-Specific E2-Box-Binding Factors Contain E12/E47-Like Subunits

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**Recent studies have identified a family of DNA-binding proteins that share a common DNA-binding and dimerization domain with the potential to form a helix-loop-helix (HLH) structure. Various HLH proteins can form heterodimers that bind to a common DNA sequence, termed the E2-box. We demonstrate here that E2-box-binding B-cell- and myocyte-specific nuclear factors contain subunits which are identical or closely related to ubiquitously expressed (E12/E47) HLH proteins. These biochemical data suggest a pleiotropic function for E12/E47-like molecules in mammalian differentiation, similar to the genetically defined function of daughterless in *Drosophila* development.**

One class of DNA-binding proteins contains a region of homology that has the potential to form a helix-loop-helix (HLH) structure preceded by a highly basic region (22); this region has been shown to contain a DNA-binding and dimerization domain (12, 22, 29). Certain HLH homo- and heterodimers bind to a common DNA sequence, termed the E2-box. The E2-box is present in the immunoglobulin heavy- and light-chain gene enhancer, the insulin gene enhancer, and the muscle creatine kinase gene enhancer (4, 7, 19, 20). In each of these enhancers it plays a crucial role in the regulation of tissue-specific gene expression. Representative members of the HLH family that bind the E2-box are E12 (pan-1), E47 (E2-5; pan-2), and E2-2 (16, 22, 24) immunoglobulin enhancer-binding proteins; MyoD (12), myogenin (3, 31), and myf-5 (2), three proteins that induce myogenesis; and daughterless (*da*) (6, 8-11) and achaete-scute (28), *Drosophila* genes that are involved in cell determination and differentiation. In vitro evidence implies that classes of HLH proteins use combinatorial interactions to control tissue-specific gene expression in various developmental pathways (21). Class A includes ubiquitously expressed proteins *da*, E12, E2-2, and E47. E12 and E47 are differentially spliced products of the same gene (26b) and share most of their amino acid sequence. All four proteins have very closely related HLH motifs. Class B includes tissue-specific proteins, MyoD, myogenin, myf-5, and members of the achaete-scute gene family. Class B proteins bind in vitro with high affinity to the E2-box as heterodimers with class A proteins. In addition, there is a class of HLH proteins which lack a basic region and thus are negative regulators of DNA binding (*Id*, extramacrochaete) (1, 14, 15). To examine whether HLH heterodimers are also formed in vitro, E2-box-binding proteins from a variety of cell lines were characterized. We show here that B-cell- and muscle-specific E2-box-binding factors contain components that are identical or antigenically related to the E12/E47 proteins.

### MATERIALS AND METHODS

**Cell lines.** HeLa and LA-N-5 (26) cells were grown in Dulbecco modified Eagle medium supplemented with 5%

(vol/vol) fetal calf serum. PD31 and WEHI 231 cells were grown in RPMI medium containing 10% fetal calf serum. Ba/F and LyD9 were grown in RPMI medium containing 20% fetal calf serum and interleukin-3 (27).

**Plasmids.** A 2-kb cDNA fragment containing the entire coding region of E47 was inserted into the *Xba*I site of the pBluescript II SK<sup>-</sup> vector (Stratagene) (28a) for in vitro transcription with T3 RNA polymerase. A CDM8 vector containing the 2-kb E47 cDNA insert at its *Bst*XI site was used for expression in eucaryotic cells (25).

**Extract preparation and gel shift assays.** Nuclear extracts were prepared exactly as described by Dignam et al. (13). Oligonucleotides containing the wild-type monomeric  $\mu$ E2 and muscle creatine kinase E2-box sequence were <sup>32</sup>P labeled (50,000 cpm/ng), and 0.5 ng of DNA probe was incubated with 3 to 10  $\mu$ g of nuclear extract. The  $\kappa$ E2 probe was derived from a restriction fragment as described previously (22). The binding reaction mixtures were incubated at room temperature for 15 min and contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 0.5 or 1  $\mu$ g of poly(dIC-dIC) as the nonspecific competitor. The complexes were resolved by electrophoresis through 4% polyacrylamide gels containing 50 mM Tris, 0.38 M glycine, and 2 mM EDTA. For competition experiments the conditions were exactly as described above, except that specific and nonspecific competitor DNAs were included in the mixture (in the amounts detailed in the figure legends) prior to the addition of protein. For supershift assays, nuclear extracts were incubated for 15 min with antibody prior to incubation with labeled DNA.

**In vitro transcription and translation.** RNA was synthesized in a 100- $\mu$ l reaction for 2 h at 40°C with a substrate of 2  $\mu$ g of linearized DNA and with T3 or T7 RNA polymerase (Stratagene) in the presence of RNasin (Boehringer Mannheim). The RNA was phenol and chloroform extracted once and ethanol precipitated. The ethanol precipitate was dried and resuspended in 20  $\mu$ l of water. In vitro translation of the RNAs was done by using a rabbit reticulocyte lysate (Promega) in a 50- $\mu$ l reaction with 10% of the synthesized RNA as a substrate. Proteins were synthesized at 30°C for 1 h. Reaction conditions were as recommended by the manufacturer (Promega).

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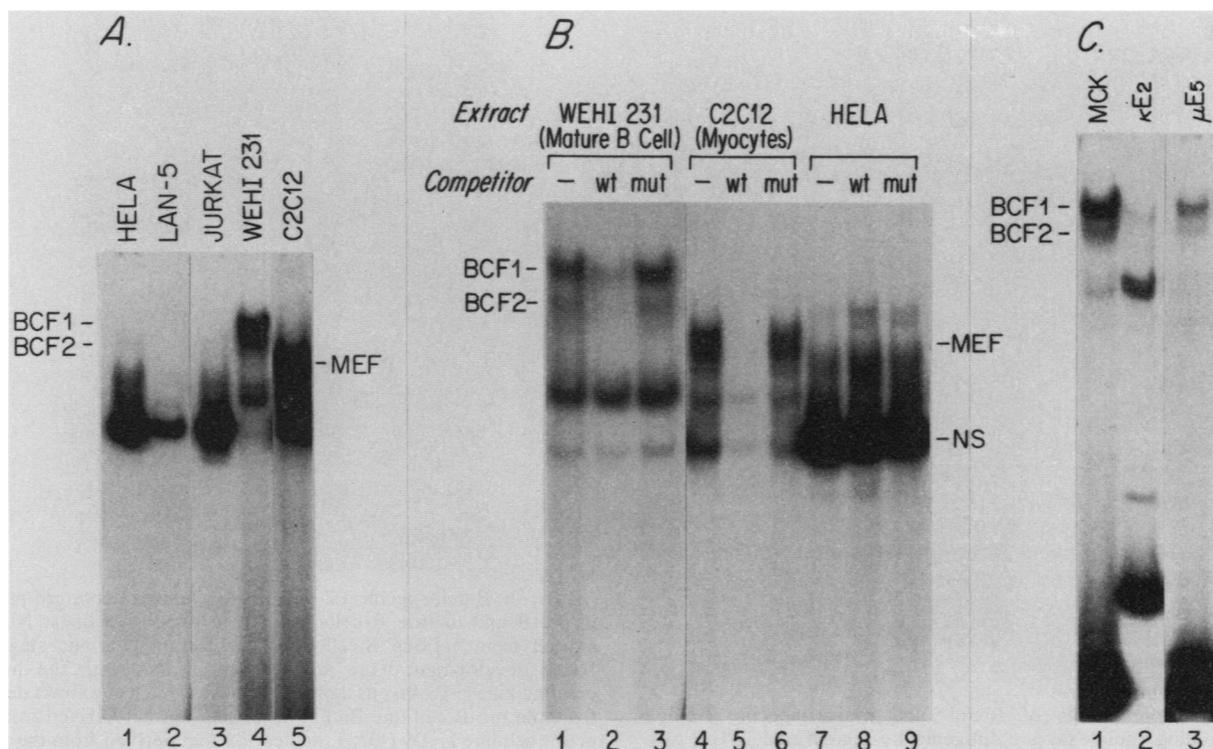


FIG. 1. Binding of proteins to the E2-box motif. (A) B-cell and myocyte nuclear extracts contain tissue-specific muscle creatine kinase enhancer E2-box DNA-binding proteins. Lanes: 1, HeLa extract; 2, LA-N-5 extract (neuroblastoma); 3, Jurkat extract (T cell); 4, WEHI 231 extract (mature B cell); 5, C2C12 extract (muscle cell). (B) B-cell- and myocyte-specific proteins bind specifically to the muscle creatine kinase enhancer E2-box. Lanes: 1, WEHI 231 extract with no competitor added (-); 2, WEHI 231 extract in the presence of 10 ng of unlabeled wild-type (wt) E2-box competitor DNA; 3, WEHI 231 extract in the presence of 10 ng of unlabeled mutant (mut) E2-box competitor DNA; 4, C2C12 extract with no competitor DNA added; 5, C2C12 extract in the presence of 10 ng of unlabeled E2-box competitor DNA; 6, C2C12 extract with 10 ng of unlabeled mutant E2-box competitor DNA; 7, HeLa extract with no competitor DNA added; 8, HeLa extract with 10 ng of unlabeled E2-box competitor DNA added; 9, HeLa extract with 10 ng of unlabeled mutant competitor DNA added. (C) B-cell-specific DNA-binding proteins bind to the E2-box present in the muscle creatine kinase enhancer and to  $\mu$ E5 and  $\kappa$ E2 sequences present in the immunoglobulin enhancers. Lanes: 1, WEHI 231 extract analyzed by using the E2-box present in the muscle creatine kinase enhancer as a probe; 2, WEHI 231 extract analyzed by using the E2-box present in the  $\kappa$ E2 site of the kappa light-chain enhancer as a probe; 3, WEHI 231 extract analyzed by using the E2-box present in the  $\mu$ E5 site of the immunoglobulin heavy-chain enhancer as a probe. MEF, Muscle-specific complexes; NS, nonspecific DNA-binding proteins.

## RESULTS

**B-cell-specific E2-box DNA-binding proteins.** B-cell-specific proteins binding to the E-box sites in the immunoglobulin heavy-chain enhancer were first detected by *in vivo* methylation protection experiments (7). Previous studies with low-ionic-strength gel systems failed to detect such B-cell-specific E2-box-binding proteins *in vitro* (17, 19). Recently, tissue-specific E2-box-binding proteins were found in muscle-derived nuclear extracts (3, 4, 18). To look for other tissue-specific E2-box-binding proteins, we screened a wide variety of nuclear extracts by using an electrophoretic mobility shift assay (EMSA) at high ionic strength. As demonstrated before (3, 4, 18), muscle-specific proteins, designated as MEF-1, are present in myocyte nuclear extracts and bind the E2-box sequence present in the muscle creatine kinase enhancer (Fig. 1A, lane 5). Nuclear extracts derived from HeLa cells (lane 1), the T-cell line Jurkat (23) (lane 3), and the neuroblastoma cell line LA-N-5 (26) (lane 2) did not contain complexes that migrated in a cell-type-specific manner. However, nuclear extracts derived from a mature B-cell line, WEHI 231, contained two B-cell-specific complexes, termed BCF1 and BCF2, which

were not found in extracts of the other tested cell types (lane 4). Thus, in addition to myocytes, distinct but tissue-specific E2-box-binding proteins are present in nuclear extracts derived from B cells.

To determine whether the B-cell- and myocyte-specific complexes interact with the E2-box in a specific manner, we performed a competition analysis. DNA containing the wild-type E2-box sequence, derived from the muscle creatine kinase enhancer (GCAGGTGT), did compete for BCF1 and BCF2 binding (Fig. 1B, lane 2), whereas DNA containing a mutant E2-box sequence (TACCGTGT) did not compete (lane 3). Similarly, DNA containing the wild-type E2 sequence competed for binding to muscle-specific complexes (18) (Fig. 1B, lane 5), whereas DNA containing the mutant E21 sequence did not compete (18) (lane 6).

To test whether BCF1 and BCF2 can interact with the E2-box sequences present in the heavy-chain and kappa light-chain enhancer, nuclear extracts from WEHI 231 were incubated with probes containing the  $\kappa$ E2 (GGCAGGTGG) (22) and  $\mu$ E5 (TGCAGGTGT) sites. Both  $\kappa$ E2 and  $\mu$ E5 are crucial for immunoglobulin heavy- and light-chain gene expression (19). BCF complexes bind to  $\mu$ E5 (Fig. 1C, lane

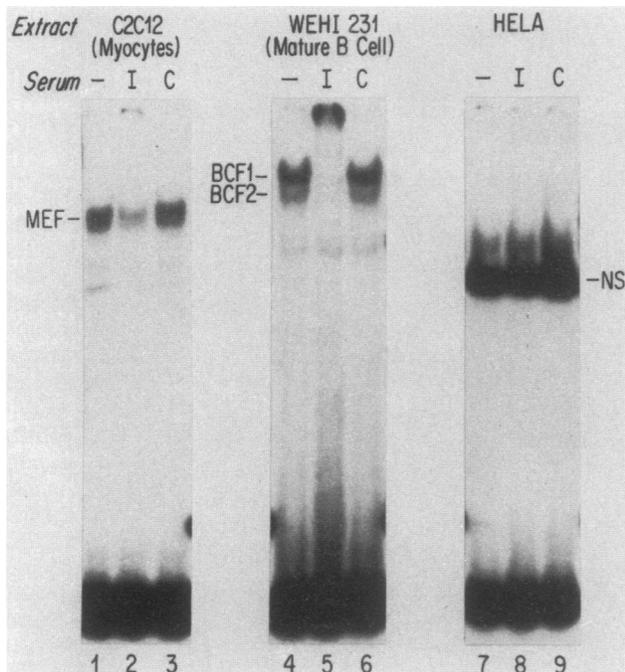


FIG. 2. Components of B-cell- and muscle-specific E2-box DNA-binding complexes are antigenically related to E12/E47 proteins. Lanes: 1, muscle (C2C12) nuclear extracts incubated in the absence of antiserum; 2, muscle extract incubated in the presence of anti-E12/E47 antiserum; 3, muscle extract incubated in the presence of preimmune antiserum; 4, B-cell extract incubated in the absence of antiserum; 5, B-cell extract incubated in the presence of anti-E12/E47 antiserum; 6, B-cell extract incubated in the presence of preimmune serum; 7, HeLa extract incubated in the absence of antiserum; 8, HeLa extract incubated in the presence of anti-E12/E47 serum; 9, HeLa extract incubated in the presence of preimmune serum. MEF, Muscle-specific complexes; NS, nonspecific DNA-binding proteins.

3) with high affinity, but binding to the  $\kappa$ E2 site was weaker and qualitatively different (lane 2). The faster-migrating complex binding to the  $\kappa$ E2 site was nonspecific. Thus, BCF complexes bind to sequences that are important for immunoglobulin heavy- and light-chain gene expression (17, 19).

**B-cell-specific E2-box DNA-binding complexes contain E12/E47-like subunits.** E47 protein can transactivate a reporter gene containing multiple  $\mu$ E5 and  $\kappa$ E2 sequences positioned 5' of its promoter (16, 29a), suggesting that E47 is part of a transcriptional complex binding to the  $\mu$ E5 and  $\kappa$ E2 motifs. To test whether the BCF complexes contain E12/E47 molecules, B-cell nuclear extracts derived from WEHI 231 were incubated with anti-E12/E47 antiserum and assayed by EMSA. The antiserum does not recognize determinants present in the conserved HLH motif or determinants present in other related HLH proteins, for example MyoD, Id, and myogenin among the tested molecules (1, 21, 31). Thus, it is specific for E12 and E47 polypeptides, although we cannot exclude the possibility that other closely related polypeptides share determinants with E12/E47. The anti-E12/E47 antibody, however, does not distinguish between E12 and E47 polypeptides (21). Both the BCF1 and BCF2 complexes supershifted (migrated more slowly) in the presence of the anti-E12/E47 antibody (Fig. 2, lane 5), whereas preimmune serum did not affect complex mobility (lane 6). Thus, BCF1 and BCF2 contain E47- and/or E12-like molecules.

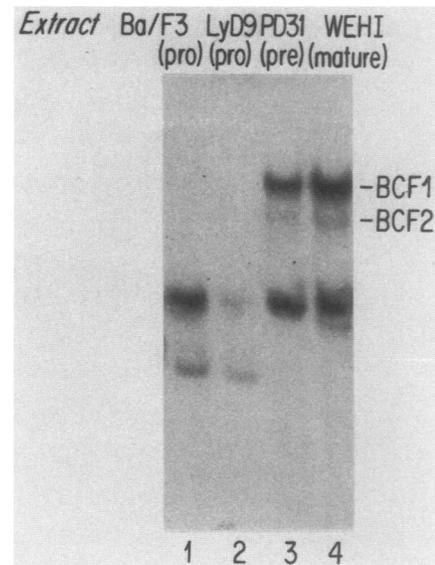


FIG. 3. B-cell-specific E2-box DNA-binding factors are present in pre-B and mature B cells but not in pro-B cell lines. Nuclear extract from various B-cell lines representing various stages of B-cell development were analyzed by EMSA with the muscle creatine kinase E2-box as a probe. Lanes: 1, nuclear extract derived from the pro-B cell line Ba/F3; 2, nuclear extract derived from the pro-B cell line LyD9 (13); 3, nuclear extract derived from the pre-B cell line PD31; 4, nuclear extract derived from the mature B-cell line WEHI 231. Only the region of the gel containing the shifted probe is shown.

**BCF1 and BCF2 are present in pre-B and mature B cells but not in pro-B cells.** To determine at what stages of B-cell development BCF1 and BCF2 are present, nuclear extracts derived from different B-cell lines were examined by EMSA with the E2-box, derived from the muscle creatine kinase enhancer, as a probe. Both BCF1 and BCF2 were detected in pre-B-cell and mature B-cell lines (Fig. 3, lanes 3 and 4), but not in pro-B-cell lines (a kind gift of R. Palacios) (lanes 1 and 2) (27). We have examined four additional pre-B-cell and mature B-cell lines; they all contain the B-cell-specific BCF1 and BCF2 complexes (data not shown). The faster-migrating band present in pro-B cells is nonspecific (20a). Mature B and pre-B-cell lines, but not the pro-B-cell lines examined here, express immunoglobulin heavy chain. The presence of BCF1 and BCF2 therefore correlates with immunoglobulin heavy-chain gene expression.

**Myocyte-specific E2-box DNA-binding proteins contain E12/E47-like subunits.** Previously we have shown that E12 and E47 proteins can interact with MyoD *in vitro* to form heterodimers that bind to the E2-box motif (21). Myocyte nuclear extracts contain muscle-specific complexes that are identical or antigenically related to MyoD and myogenin (18). To determine whether E12/E47-like proteins form heterodimers with MyoD and myogenin *in vivo*, myocyte nuclear extracts were incubated with an anti-E12/E47 antibody and analyzed by EMSA. The muscle-specific complexes reacted with the antibody (Fig. 2, lane 2) but not with preimmune serum (lane 3). Thus, myocyte-specific complexes contain E12/E47 molecules or ones closely related to E12/E47.

**BCF complexes comigrate with homo-oligomers of E47 in an EMSA.** Although the anti-E12/E47 antibody did react with

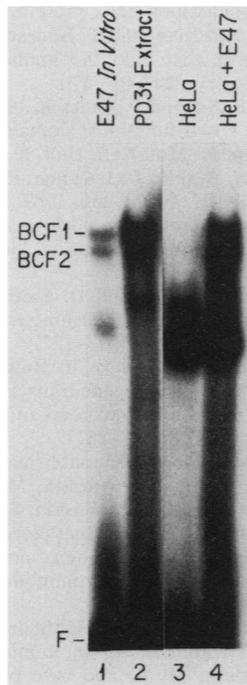


FIG. 4. BCF1 and BCF2 proteins comigrate in an EMSA with homo-oligomers of E47. BCF1 and BCF2 were analyzed by EMSA and compared in vitro and in vivo by using a radiolabeled muscle creatine kinase E2-box as a probe. Lanes: 1, in vitro-translated E47; 2, nuclear extract derived from a pre-B cell line, PD31; 3, nuclear extract derived from HeLa cells; 4, nuclear extract derived from HeLa cells transiently transfected with an E47 cDNA placed under the control of the cytomegalovirus promoter. F, Probe not bound to protein.

the muscle-specific complexes, the relative ability of the antiserum to supershift the complexes was 100-fold lower for the muscle-specific complexes than for the BCF proteins (20a). A trivial explanation for this difference in affinity could be that BCF1 and BCF2 are homo-oligomers of E12/E47. To address this issue, we have examined the relative affinity of the anti-E12/E47 antibody for in vitro-synthesized E47 homo-oligomers and E47-MyoD heterodimers. E47 homo-oligomers have a 100-fold-higher affinity for the antibody than do E47-MyoD heterodimers (20a). In addition, in vitro-translated full-length E47 generated two complexes that comigrated with the BCF1 and BCF2 complexes present in nuclear extracts from a pre-B-cell line, PD31 (Fig. 4, lanes 1 and 2). Why the in vitro E47-synthesized molecules generate two complexes with different mobilities in an EMSA is not clear. They either could represent variable complexes of E47, for example a dimer (BCF2) and a tetramer (BCF1), or could result from posttranslational modification. To determine whether the product of E47 cDNA migrates with similar mobility when expressed in vivo, a full-length E47 cDNA placed under the control of the cytomegalovirus promoter was introduced transiently into HeLa cells. Nuclear extracts derived from HeLa cells transfected with E47 cDNA were then examined by EMSA. Two complexes were apparent in the extracts prepared from the transfected cells but not from the control cells. These complexes comigrated exactly with BCF1 and BCF2 (Fig. 4, lanes 3 and 4) and were supershifted by the anti-E12/E47 antibody (data not shown). These data suggest that the BCF proteins might consist of

oligomers of E47 or E12 or a closely related protein. In addition, in vitro-translated E47 polypeptide and BCF proteins have very similar affinities to anti-E12/E47 antibody (20a), which further supports the idea that the BCF proteins are indeed oligomers of E12 or E47. It is unlikely that BCF1 and BCF2 represent homo-oligomers of E12, because E12 homo-oligomers have a relatively weak affinity for the E2 sequence under the conditions described here (22). Thus, BCF1 and BCF2 most probably represent homo-oligomers of E47 or hetero-oligomers containing E12 and E47. We note, however, that further analysis of these complexes is required to exclude the possibility that a B-cell-specific polypeptide associates with E47 or E12 in BCF1 and BCF2.

## DISCUSSION

We have presented data showing that both myocyte and B-cell E2-box DNA-binding proteins contain E12/E47-like subunits. In B-cell nuclear extracts, complexes containing E47 subunits—very probably consisting of a homo-oligomer of E47—are present that cannot be detected in non-B cells. E12 and E47 mRNA has been detected in all cell types examined (22). We have immunoprecipitated E12/E47 polypeptides from Jurkat and HeLa cells (20a). Why are apparent E47 homo-oligomers detected in nuclear extracts derived from B cells but not in those of other cell types? One possibility is that in many cells E47 polypeptides are complexed with other HLH polypeptides, such as Id, that would inhibit their DNA binding (1). Id was shown to form heterodimers with E47 in vitro, inactivating its ability to bind to DNA. Id levels are low in differentiated cell types, whereas they are high in undifferentiated cells (1). High levels of Id might inhibit the DNA-binding activity or HLH proteins such as E47. In pro-B and other cell types, levels of inhibitory molecules identical or related to Id might be high, preventing E47 from forming homo-oligomers (24a). During B-cell differentiation, similarly to myogenesis, levels of such inhibitory molecules might drop, allowing E47 polypeptides to form oligomers that can now bind to regulatory sequences present in the immunoglobulin heavy- and light-chain gene enhancers, inducing their transcription and subsequently B-cell differentiation. In other cells, the presence of class B HLH proteins such as MyoD would lead to preferential heterodimer formation (26b) and suppress the homo-oligomers.

These data suggest that the role of E12/E47-like proteins in mammalian development might be similar to that of *da* in *Drosophila*. *da* is a gene whose various mutants show pleiotropic effects. *da* controls both sex determination and neurogenesis (9). A role for *da* in other developmental pathways is likely, but evidence is lacking because of the lethality of *da* mutations. The physical similarity between *da* and E12/E47 proteins is striking. The E12/E47 sequences are as related to *da* in the region surrounding the HLH region as they are to each other (22). In addition, each of the proteins is ubiquitously expressed. In this paper we present data suggesting a role for E12/E47-like proteins in myogenesis and B-cell development. A role for E12/E47 proteins in pancreatic  $\beta$ -cell differentiation is likely as well. The E2-box sequence is present in the insulin enhancer, a site that has been shown to be crucial for its tissue-specific expression (20, 24). Indeed, Stein and collaborators recently found that a  $\beta$ -cell-specific complex that binds the E2-box is supershifted with an anti-E12/E47 antibody (26a, 30). They also found that the activity of the E2 regulatory sequence present in the insulin enhancer is responsive to Id. Thus, E12/E47

and related molecules may well function in controlling development by associating either with tissue-specific components, such as MyoD in MEF-1, or as homo-oligomers in the BCF proteins, inducing the differentiated-muscle-, B-cell-, and  $\beta$ -cell-specific phenotypes.

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