ZEB, a vertebrate homolog of *Drosophila* Zfh-1, is a negative regulator of muscle differentiation

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A number of genes, spanning the evolutionary scale from yeast to mammals, that are involved in spatial and temporal patterning during development contain zinc finger and homeodomain motifs. One such zinc finger/homeodomain protein is *Drosophila* Zfh-1, a member of the *zfh* family of *Drosophila* genes, which is expressed in muscle precursors and is critical for the proper development of muscle. Here we demonstrate that a vertebrate homolog of Zfh-1 (ZEB) is a negative regulator of muscle differentiation. We show that ZEB binds to a subset of E boxes in muscle genes and functions by actively repressing transcription. One target of this repression is the members of the MEF-2 family, which synergize with proteins of the myogenic basic helix–loop–helix family (bHLH) (myoD, myf-5, myogenin and MRF-4) to induce myogenic differentiation. As muscle differentiation proceeds, myogenic bHLH proteins accumulate to levels sufficient to displace ZEB from the E boxes, releasing the repression and allowing bHLH proteins to further activate transcription. This mechanism of active transcriptional repression distinguishes ZEB from other negative regulators of myogenesis (Id, Twist and I-mfa) that inhibit muscle differentiation by simply binding and inactivating myogenic factors. The relative affinity of ZEB versus myogenic bHLH proteins varies for E boxes in different genes such that ZEB would be displaced from different genes at distinct times as myogenic bHLH proteins accumulate during myogenesis, thus providing a mechanism to regulate temporal order of gene expression.

Keywords: α 4 integrin/MEF-2/MRF proteins/transcriptional repression/ZEB

Introduction

Transcriptional repression plays a crucial role in determining patterns of tissue-specific gene expression during development, although the precise mechanisms are poorly understood in most cases (Johnson, 1995; Gray and Levine, 1996b). A number of transcriptional repressors contain zinc finger and homeodomain motifs (Gray and Levine, 1996b). For example, the *Drosophila* zinc finger protein Krüppel represses the *even-skipped* stripe 2 enhancer, which is important in segmentation (Stanojevic *et al.*, 1991), and *Drosophila* Snail represses *rhomboid* expression in the mesoderm, which seems to be important in

maintaining the mesoderm/neuroectoderm boundary (Ip *et al.*, 1992). Both proteins, Krüppel and Snail, appear to repress transcription by quenching or direct repression of the transcription complex depending on their location on a promoter (Gray and Levine, 1996a). In vertebrates, the zinc finger neuron-restrictive silencer factor (NRSF) has been shown to play an important role in regulation of mouse neuronal genes (Schoenherr and Anderson, 1995) and PRDI-BF1 has been shown to repress transcription of the β -interferon gene (Keller and Maniatis, 1992).

Another family of zinc finger/homeodomain proteins is Zfh, which is required for differentiation of the central nervous system and in regulation of cell fate determination and positioning of mesoderm derivatives, such as somatic musculature in *Drosophila* (Lai *et al.*, 1991, 1993; Lundell and Hirsh, 1992). The number and position of homeodomains and zinc finger motifs varies significantly among the different Zfh family members [i.e. Zfh-1 has a single central homeodomain with five N-terminal zinc fingers, four C-terminal zinc fingers and one zinc finger located just N-terminal of the homeodomain (Lai *et al.*, 1991); Zfh-2 has 16 zinc fingers, 14 of them at the C-terminal end, and three homeodomains (Lai *et al.*, 1991); Zfh-4 has only two internal zinc fingers and one homeodomain at the N- and C-terminal ends (Kostich and Sanes, 1995)]. Recently, a new zinc finger/homeodomain protein (ZEB) has been identified that appears to be a vertebrate member of the Zfh family (Fortini *et al.*, 1991; Genetta *et al.*, 1994). The sequence similarity in the zinc fingers and homeodomain motifs indicate that ZEB is a Zfh family member. In addition, the number and location of zinc fingers and homeodomains in ZEB is the same as Zfh-1, indicating that ZEB is a Zfh-1 homolog. *Drosophila* *zfh-1* mutants have important errors in the segregation of muscle precursors, which are mispositioned in the embryo and show a loss of precursors in some areas and extra precursors in others. This phenotype suggests that while Zfh-1 is not essential for muscle differentiation, it may have a regulatory role in the timing of muscle differentiation and positioning. Like Zfh-1, we present evidence here that ZEB has a regulatory role in vertebrate myogenesis.

ZEB was originally cloned as a protein that binds the consensus site [CAC (C/G) (T/G) (G/T)]. This sequence has sufficient wobble such that it includes a subset of E boxes (CANNTG). Indeed, ZEB has been shown to bind E boxes with the sequence CACCTG, but not other E boxes (Genetta *et al.*, 1994). Additionally, as the consensus sequence indicates, ZEB binds to other sites that are not E boxes (Genetta *et al.*, 1994). The N- and C-terminal zinc fingers of ZEB serve as DNA binding domains and appear to have similar affinity and binding specificity (Genetta *et al.*, 1994). Although it has been found that ZEB can compete with the basic helix–loop–helix (bHLH) protein E2.5 for binding to the μ E5 E box in the Ig

heavy chain gene enhancer (Genetta *et al.*, 1994), no transcriptional activity of ZEB was demonstrated (Genetta *et al.*, 1994). Previous studies have found that some E box-like sequences can negatively regulate genes (Simon and Burden, 1993; Weintraub *et al.*, 1994; Desbarats *et al.*, 1996; Duncan *et al.*, 1996), but it is not known whether these sites bind ZEB or the mechanism through which they regulate gene expression.

ZEB appears identical to the chicken δ EF1 protein, overexpression of which inhibits myoD-induced expression of some muscle genes (e.g. troponin T) in 10T1/2 cells (Sekido *et al.*, 1994). Indeed, we have found that a number of muscle-restricted genes contain ZEB consensus sites in their promoter regions (see below). Moreover, like Zfh-1, ZEB is expressed in muscle cell precursors: it is found in the epithelium of the undifferentiated somite and the dermomyotome preceding myogenic differentiation in embryonic mice (Funahashi *et al.*, 1993). Therefore, ZEB is present before active myogenic regulatory factors (MRFs) appear and is expressed in the proper spatial and temporal pattern to regulate muscle genes in undifferentiated cells. All these results together further suggest a role for ZEB during muscle differentiation.

Muscle differentiation is regulated by two families of positive factors: (i) the MRF family of bHLH proteins (myoD, myf-5, myogenin and MRF-4) that induce muscle differentiation by binding E box sequences in the regulatory regions of muscle genes activating their transcription; (ii) the MEF-2 proteins, which synergize with MRF proteins to regulate muscle differentiation and activate transcription either by binding to specific DNA sequences or by interaction with the MRF proteins (Molkentin *et al.*, 1995). Initially, it was assumed that myogenic differentiation was dependent only on the activity of MRF and MEF-2 proteins (and their synergistic activity), however, recent evidence indicates that muscle differentiation is also under negative regulation and that a proper temporal and spatial pattern of muscle gene expression is the result of a fine balance between positive and negative factors (Benezra *et al.*, 1990; Chen *et al.*, 1996; Spicer *et al.*, 1996). Negative regulators of myogenesis, such as Twist, members of the Id family and I-mfa, all act by binding MRF and/or MEF-2 proteins and blocking their activity (Benezra *et al.*, 1989; Chen *et al.*, 1996; Spicer *et al.*, 1996), however, we show here that ZEB inhibits muscle differentiation through a unique mechanism that involves interaction with the promoter of muscle genes and active repression of transcription. We mapped the region in ZEB needed for such repression and we show that ZEB is able to block transcriptional activation mediated by MEF-2 as well as the synergism between MRF and MEF-2, which is thought to be important for myogenic differentiation (Molkentin *et al.*, 1995, 1996; Lilly *et al.*, 1995; Olson *et al.*, 1995). Finally, we suggest a mechanism whereby ZEB can regulate the temporal pattern of muscle gene expression during development.

Results

ZEB is a negative regulator of muscle differentiation

Zfh-1 in *Drosophila* is expressed in muscle precursors and is required for proper muscle formation (Lai *et al.*,

1993). The vertebrate homolog of Zfh-1, ZEB, is also expressed in muscle precursors, in the undifferentiated somite and in the dermatomyotome (Funahashi *et al.*, 1993), suggesting a role for ZEB in vertebrate myogenesis. In support of this possibility, we found that a number of muscle-restricted genes contain ZEB consensus binding sequences [i.e. the bHLH MRF genes (Edmondson *et al.*, 1992; Tapscott *et al.*, 1992; Black *et al.*, 1995), muscle creatine kinase (MCK) (Amacher *et al.*, 1993) and the acetylcholine receptor δ subunit (Simon and Burden, 1993)]. Indeed, ZEB has been shown to interact with the MEF-1 site in the MCK gene (Genetta *et al.*, 1994; Sekido *et al.*, 1994). Moreover, ZEB appears identical to the chicken protein δ EF1, which has been shown to block myoD-induced expression of muscle genes such as troponin T in 10T1/2 cells (Sekido *et al.*, 1994), further suggesting a role for ZEB in vertebrate myogenesis.

To demonstrate that ZEB has a role in regulating muscle differentiation we used the differentiation of C2C12 myoblasts into myotubes as a myogenic assay (Rosen *et al.*, 1992). Stable expression of ZEB in C2C12 cells blocked myotube formation and expression of differentiation-specific markers such as myosin heavy chain, myogenin and α 4 integrin (Figure 1A–J and data not shown), providing further evidence for ZEB as a negative regulator of myogenesis.

Likewise, myotube formation and expression of differentiation markers such as myosin heavy chain by overexpression of myoD into 10T1/2 cells (Thayer and Weintraub, 1990) was inhibited by co-transfection of an expression vector for ZEB (Figure 2A and B).

Since recombinant ZEB can compete with recombinant E2.5 for binding to the μ E5 E box *in vitro* (Genetta *et al.*, 1994), we presumed that ZEB may inhibit myogenesis by displacing MRF proteins from E boxes as suggested (Genetta *et al.*, 1994; Sekido *et al.*, 1994). To test this hypothesis, we examined the activity of a ZEB construct expressing only the DNA binding domain of ZEB (DB-ZEB) in the myogenic assay (Figure 2A and B). We reasoned that if ZEB functions simply by displacing MRF proteins from E boxes, then the DNA binding domain should be sufficient for full activity. DB-ZEB bound to ZEB sites with greater affinity than the full-length protein and it displaced myoD from CACCTG sequences more efficiently than ZEB (data not shown). However, surprisingly, DB-ZEB had little effect on myogenic differentiation compared with full-length ZEB (Figure 2A and B). Many muscle genes contain multiple E boxes and the ZEB binding sequence CACCTG only constitutes a small subset of these E boxes. Our results with DB-ZEB indicate that displacing MRF proteins from these CACCTG sites is not sufficient to block myogenic differentiation (i.e. binding of MRF proteins to the other non-CACCTG E boxes appears to be sufficient to induce differentiation). Instead, we concluded that full-length ZEB is likely to be an active transcription factor when it is bound to CACCTG and to other non-E box sequences and that this activity of the full-length protein is required for inhibition of myogenic differentiation. We demonstrate below that ZEB is indeed an active transcriptional repressor and that its interaction with E boxes is not a mechanism for displacement of MRF proteins, but instead is an important regulatory

Fig. 1. Overexpression of ZEB blocks myogenic differentiation. Control C2C12 cells and cells stably overexpressing ZEB were switched to differentiation medium. (A–C) Control cells transfected with the empty ZEB expression vector; (D–F) clone 8 transfected with ZEB; (G–I) clone 17 of cells transfected with ZEB. (A), (D) and (G) are phase contrast micrographs of cells in differentiation medium. Immunofluorescent staining for $\alpha 4$ (B, E and H) and myosin heavy chain (C, F and I), which are expressed during myogenic differentiation, is shown at higher magnification. Transfected ZEB mRNA was detected by Northern blot (J) using an oligonucleotide encoding a HA-tag located at the 5'-end of ZEB cDNA; total ZEB mRNA was detected by reprobing the blot with an *NdeI-HindIII* fragment of ZEB cDNA.

feature that allows MRF proteins to displace ZEB and derepress transcription during myogenic differentiation.

ZEB blocks muscle differentiation by active transcriptional repression

As a first step in analyzing the transcriptional activity of ZEB, we examined the activity of ZEB sites present in the promoters of different genes expressed in muscle. One such gene that contains consensus ZEB sites is $\alpha 4$ integrin, whose expression is activated during muscle differentiation (Rosen *et al.*, 1992). Previously we found that interaction between $\alpha 4$ integrins and their ligand VCAM-1 is important in myogenic differentiation and that $\alpha 4$ expression is induced during differentiation of C2C12 cells (Rosen *et al.*, 1992). Expression of the $\alpha 4$ gene is dependent upon constitutively active Ets sites in the first 76 bp of the promoter (Rosen *et al.*, 1994). Transfection of $\alpha 4$ gene promoter constructs into the $\alpha 4(-)$ C2C12 myoblast cell line revealed that the gene is under negative regulation that inhibits the activity of these Ets sites in undifferentiated cells (Figure 3A). Silencer activity is dependent upon two consensus ZEB sites (CACCTG) at -361 and -399 . Consensus ZEB sites from other muscle genes, such as

MCK (the MEF-1 site; Amacher *et al.*, 1993) and the acetylcholine receptor δ subunit (the E1 box; Simon and Burden, 1993), showed similar silencer activity (Figure 3B). In addition, non-E box ZEB sites (Genetta *et al.*, 1994; Sekido *et al.*, 1994) were also effective repressors of -76 CAT (results not shown). Gel retardation assays with the -361 and -399 sites indicated that they do indeed bind ZEB (Figure 3C). The ubiquitous bHLH activating protein USF (Sawadogo and Roeder, 1985) also binds these sites, however, the ZEB sites only bind weakly to USF and overexpression of USF did not activate or repress transcription through the ZEB sites (results not shown). Expression of DB-ZEB blocked silencer activity of the ZEB sites in myoblasts by displacing endogenous ZEB (Figure 3B) and, accordingly, it restored $\alpha 4$ gene promoter activity in myoblasts (results not shown). Therefore, DB-ZEB appears to act efficiently as a dominant negative that blocks repression by ZEB. Together, the above results suggest that ZEB is an active transcriptional repressor and that this repressor activity is important for its negative role in myogenesis.

To demonstrate directly that ZEB is an active transcriptional repressor, it was fused to the DNA binding domain

Fig. 2. ZEB does not block myogenesis simply by displacing MRF proteins from E boxes. 10T1/2 fibroblasts were transfected with a myoD expression vector along with an expression vector for full-length ZEB, a truncated form of ZEB (DB-ZEB) expressing only the DNA binding domain or the empty vector as a control. After 4 days, cells were immunostained for myosin heavy chain. **(A)** Photomicrographs of cells expressing equal amounts of ZEB and DB-ZEB that have been immunostained for myosin heavy chain. **(B)** Quantification of the experiments in **(A)**. 100 corresponds to ~5% myosin-positive cells. The expression of ZEB and DB-ZEB proteins and their relative affinity for a ZEB DNA binding site were determined by Western blot and gel shift analysis respectively (results not shown).

of the yeast transcription factor Gal4 (Figure 4A). Gal4-ZEB efficiently inhibited transcriptional activation by the $\alpha 4$ gene Ets sites as well as a number of other transcription factors (including the SV40 enhancer) (Figure 4B and results not shown). Neither the N- nor C-terminal zinc finger DNA binding domains were required for repressor activity, as suggested above. In contrast, the central region containing the homeodomain showed full repressor activity. However, the homeodomain itself was not required for repressor activity, instead the repressor domain lies between the homeodomain and the N-terminal zinc fingers. A Western blot analysis revealed that all Gal4-ZEB proteins were of the predicted size (results not shown). The degree of repression by ZEB was similar to that of retinoblastoma protein (Figure 4B), which we have demonstrated previously is a potent transcriptional repressor (Weintraub *et al.*, 1992, 1995).

MyoD displaces ZEB from ZEB binding sites and activates transcription in a ZEB-resistant fashion

Transcriptional repression through ZEB sites is lost upon myogenic differentiation of C2C12 cells (Figures 3A and 5A). However, ZEB expression does not diminish upon

differentiation of C2C12 cells into myotubes (results not shown) nor does its level decrease following muscle differentiation in the mouse (Funahashi *et al.*, 1993). Therefore, it was unclear how repressor activity is lost during myogenesis. Since this loss of repressor activity occurs as active MRF proteins appear, we reasoned that the MRF proteins may block ZEB activity. Indeed, forced expression of either myoD or myf-5 in undifferentiated cells released repression (Figure 5A). These results suggest that MRF proteins may displace ZEB from CACCTG sites as they accumulate during differentiation. Consistent with such a model, we found that myoD binds to CACCTG sequences with greater affinity than ZEB (Figure 5B), implying that ZEB would be displaced from these sites as active MRF proteins appear during myogenic differentiation. However, the affinity of ZEB and MRFs for E boxes is dependent upon sequences surrounding the CACCTG core, such that their relative affinity varies significantly for sites in different muscle genes (results not shown). Thus, the type of site in a promoter determines the level of MRF protein required to displace ZEB and activate the muscle gene.

Interestingly, we found that ZEB is unable to block

A **B** **C**

Fig. 3. ZEB binding sites are transcriptional silencers in undifferentiated myoblasts. (A) $\alpha 4$ gene promoter activity is under negative regulation in myoblasts. Sixty millimeter dishes of C2C12 cells were transfected with 2 μ g of the indicated $\alpha 4$ gene CAT expression vectors and cells were either maintained as myoblasts or stimulated to differentiate into myotubes. Numbers in the construct name indicate position in the flanking sequences. X indicates a mutation. Results in this figure and Figures 4–6 are an average of duplicate assays and are all representative of at least five separate experiments with SD <15%. In all transfection experiments in Figures 3–6, empty expression vectors were co-transfected as controls and they had no effect on CAT activity. Similar results were obtained with 10T1/2 cells. (B) ZEB sites from different genes act as silencers. E boxes with ZEB consensus sequences (CACCTG) were cloned upstream of the Ets sites in -76CAT. -361 and -399 indicate E boxes in the $\alpha 4$ gene. MEF-1 indicates the MEF-1 site from the MCK gene and AchR the E1 box from the acetylcholine receptor δ subunit gene. For DB-ZEB overexpression, 0.2 μ g pci-FLAG-DB-ZEB were co-transfected with 3 μ g reporter plasmid. (C) Interaction of ZEB and MRF proteins with ZEB sites. Gel retardation assay with nuclear extract from 10T1/2 cells or 10T1/2 cells transfected with a myf-5 expression vector (identical results were obtained with a myoD expression vector). A probe containing the E361 and E399 sites was used in the assay. Competitor indicates addition to the binding assay reaction of a 50-fold excess of unlabeled E361 probe or 0.5 μ g antibodies against E2, myf-5, ZEB and c-myb. ZEB* indicates supershifted complexes when using the anti-ZEB antibody in the binding reaction. myf-5/E2 indicates the binding of complexes containing myf-5 and E proteins in cells transfected with myf-5. Antibodies against both E2 and myf-5 proteins blocked the binding of such complexes.

transcriptional activation by either myoD or myf-5 (Figure 6A), suggesting a second mechanism by which MRF proteins can overcome transcriptional repression by ZEB. This observation may be important because, as noted above, not all ZEB sites are E boxes (and non-E box ZEB sites are also effective repressor sites; results not shown) and MRF proteins would be unable to displace ZEB from such non-E box sites. Many muscle genes contain multiple E boxes (CANNTG), but most of these sites do not bind ZEB (CACCTG). Therefore, if a muscle gene contains an E box sequence that does not bind ZEB, then MRF proteins would be expected to activate transcription in a ZEB-resistant fashion through this site, even if the promoter also contains a ZEB site (of any type).

ZEB blocks transactivation by MEF-2 and its synergism with MRF proteins

While transcriptional activation through the MRF proteins appears resistant to ZEB, the activity of another muscle transcription factor, MEF-2, is efficiently inhibited (Figure

6B and C). MEF-2 is essential for myogenesis in *Drosophila* (Lilly *et al.*, 1995) and it has been shown during development to form an amplification loop with MRF proteins that increases the level of both MEF-2 and the MRFs (Olson *et al.*, 1995). MEF-2 and MRF proteins interact such that one protein can tether the other to the promoter (Molkentin *et al.*, 1995; Figure 6B). The resulting transcriptional synergy between MEF-2 and MRFs has been shown to augment myogenic differentiation (Molkentin *et al.*, 1995). We found that ZEB blocks the transcriptional synergy between myoD/E12 and MEF-2C, bringing activation down to the level of myoD/E12 alone (Figure 6B). When MEF-2 was brought directly to the promoter as a Gal4 fusion protein, its activity was also blocked by ZEB (Figure 6C), suggesting that ZEB blocks the synergy because it represses the transactivating domain of MEF-2. Our results then suggest that one target of negative regulation by ZEB during myogenesis may be MEF-2 activation and transcriptional synergy between MEF-2 and MRF proteins.

Fig. 4. ZEB is an active transcriptional repressor. (A) Diagram of Gal4-ZEB; numbers are bp and ZF and HD indicate zinc fingers and a homeodomain respectively. (B) Identification of the repressor domain. ZEB and fragments of ZEB were fused to the DNA binding domain of Gal4 and resulting expression vectors were co-transfected into C2C12 cells with -76CAT-G, which contains Gal4 sites upstream of the Ets sites, or pGXSV, which contains four Gal4 sites upstream of the SV40 enhancer and the E1B TATA box. Repression by Gal4-ZEB was similar with both reporters. One microgram of the reporter was transfected along with 0.5 μ g Gal4-ZEB constructs and CAT activity was determined.

Discussion

Here we have demonstrated that ZEB, a vertebrate homolog of the *Drosophila* Zfh-1 protein, is a negative regulator of myogenesis and presented evidence that it functions through a mechanism and at a level distinct from previously identified negative regulators, such as Id proteins, Twist and I-mfa. We also provide evidence that one target of ZEB is MEF-2 and the transcriptional synergy between MEF-2 and MRF proteins, which is thought to promote myogenic differentiation (Molkentin *et al.*, 1995).

Members of the Zfh family of zinc finger/homeodomain proteins are expressed in the central nervous system (CNS) and, in the case of Zfh-1 and -4, also in mesoderm derivatives, including muscle (Lai *et al.*, 1991; Kostich and Sanes, 1995). A human homolog of *Drosophila* Zfh-2 (ATBF-1) has been cloned and it has been shown to repress the α -fetoprotein promoter by binding to AT-rich areas, although the precise mechanism of repression is not understood (Fortini *et al.*, 1991; Lundell and Hirsh, 1992; Yasuda *et al.*, 1994). Like its *Drosophila* homolog Zfh-1 (Lai *et al.*, 1991), ZEB is expressed in muscle precursors and we present evidence that it regulates muscle differentiation by inhibiting muscle gene expression and myotube formation. Taken together, these results suggest that the Zfh family may be comprised of transcriptional repressors involved in differentiation of various tissues.

It is interesting to note that mutation of *zfh-1*, rather than blocking muscle differentiation, results in multiple abnormalities with mispositioning of muscles and too few

Fig. 5. MyoD blocks the silencer activity of ZEB sites in myoblasts and displaces ZEB from E boxes. (A) MRF proteins block silencer activity by ZEB in myoblasts. ZEB sites lose silencer activity upon myogenic differentiation. Similar blocking in the silencer activity of ZEB was obtained by forced overexpression of MyoD (similar results were obtained with myf-5). Two micrograms of reporter were co-transfected with 5 μ g pemsv-myod expression vector. (B) MyoD displaces ZEB sites in gel retardation assays. ZEB and myoD/E47 (Neuhold and Wold, 1993) were synthesized by coupled *in vitro* transcription/translation. The relative concentration of the proteins were estimated by SDS-PAGE (data not shown) and the relative amount of myoD/E47 is indicated above the lanes.

or too many precursors in some muscles (Lai *et al.*, 1993), suggesting a role for Zfh-1 in the timing of differentiation and/or the positioning of muscle precursors when they differentiate. Although there is no information about the mechanism through which Zfh-1 functions, the phenotype of *zfh-1* mutants is consistent with Zfh-1 as a negative regulator of muscle differentiation, as we propose here for its vertebrate homolog ZEB.

Zfh-1 is also expressed in the CNS and overexpression of Zfh-1 in *Drosophila* during the embryonic, larval and pupal periods results in high lethality and severe defects in the developing CNS, suggesting that Zfh-1 also has a regulatory role in CNS differentiation (Lai *et al.*, 1991). In addition to muscle, ZEB is also expressed in the developing CNS (Funahashi *et al.*, 1993). Thus, it is reasonable to assume that ZEB may also have a role in regulation of CNS differentiation.

Recently, several genes that negatively regulate myogenesis have been identified, such as members of the Id family, Twist and I-mfa (Benezra *et al.*, 1990; Chen *et al.*, 1996; Spicer *et al.*, 1996). These other proteins inhibit

Fig. 6. Activation by MRF is resistant to ZEB but activation by MEF-2 is not. **(A)** Aliquots of 0.8 μ g myoD and myf-5 expression vectors were co-transfected into 10T1/2 cells (similar results were obtained with C2C12 cells) with 1.5 μ g p72-4E-5G reporter and 1 μ g G-ZEB 918–2717 (which lacks the DNA binding domain but contains its repressor domain). Activation of this reporter was observed when Gal4 activators in Figure 4B were co-transfected, indicating that the Gal4 sites in the construct are functional. Also, note that the amount of Gal4–ZEB 918–2714 transfected was even greater than in Figure 4, where it efficiently blocked transcription. Gal4–ZEB fs (see Figure 4) was used as a negative control in these transfections (not shown). **(B)** G-ZEB 918–2717 blocks transcriptional synergy between MEF-2 and myoD/E12. A reduced amount of expression vectors (0.03 μ g myoD and E12 and 0.15 μ g MEF-2C) was co-transfected with 1.5 μ g p72-4E-5G reporter in order to observe synergy between myoD and MEF-2C. One microgram of G-ZEB 918–2717 was co-transfected. Note that expression was reduced to the level of myoD/E12 alone when G-ZEB 918–2717 was co-expressed; increasing the amount of G-ZEB 918–2717 transfected had no additional effect. Gal4–ZEB fs was used as a negative control and had no effect (results not shown). **(C)** ZEB inhibits the transactivation domain of MEF-2. Fusion proteins of myoD and MEF-2C with the DNA binding domain of Gal4 were co-transfected with LexA–ZEB (L-ZEB) into C2C12 cells (similar results were seen in 10T1/2 cells). Again, as a control, transactivation by Gal4 activators was not affected by LexA–ZEB in the absence of LexA sites in the reporter (results not shown). One microgram of the reporter plasmid was co-transfected with 0.5 μ g Gal4–myoD (G-myoD) or Gal4–MEF2C (G-MEF-2) and 1 μ g LexA–ZEB (L-ZEB).

myogenesis by binding directly to myogenic factors like myoD (and its E protein partners) or MEF-2 proteins and preventing their translocation to the nucleus or their binding to DNA. However, ZEB works as a bona fide transcription factor, binding specific DNA sequences in the promoters of muscle genes and actively repressing transcriptional activity mediated by other factors, including MEF-2 proteins.

During muscle differentiation there is a precise temporal pattern of gene expression, such that some genes appear early (e.g. myf-5 and myoD), others are intermediate (e.g. myogenin) and some appear late in myogenesis (e.g. acetylcholine receptor). The precise molecular mechanism that accounts for this temporal order is not understood and simple activation of transcription by MRF and MEF-2 proteins may not be sufficient to explain the entire pattern (Olson *et al.*, 1995; Molkentin and Olson, 1996). We found that E boxes from different genes vary significantly in their affinity for ZEB versus MRF proteins. Thus, as the level of active MRF proteins accumulates during myogenesis, ZEB would be displaced from some genes at low concentrations of MRF proteins (early myogenesis), whereas higher levels of MRF (late myogenesis) would be needed to displace ZEB from other genes. Therefore, the interplay between ZEB and MRF proteins on different genes may be a mechanism for imposing temporal order on expression on some muscle genes.

ZEB was first identified in B cells as a protein that binds *in vitro* to the IgH enhancer, however, no direct evidence for a repressor role for ZEB was reported

(Genetta *et al.*, 1994). We have examined ZEB activity in hematopoietic cells and found that it also functions as a repressor (Postigo *et al.*, 1997), suggesting a role for ZEB in hematopoietic cells. As in muscle, one target of such repression is the α 4 integrin gene. In addition to its role in myogenesis (Rosen *et al.*, 1992), α 4 integrin has a well-studied role in hematopoietic differentiation (Arroyo *et al.*, 1996) and function (e.g. leukocyte trafficking, the immune response and targeting of leukocytes to sites of inflammation; Lobb and Hemler, 1994). These results suggest a key role for ZEB in regulating gene expression in different tissues.

Materials and methods

Cell culture

C2C12 and CH3-10T1/2 cells were obtained from the American Tissue Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 13% fetal bovine serum (FBS), glutamine and antibiotics (Life Technologies, Gaithersburg, MD). For differentiation of C2C12 myoblasts to myotubes, cells were changed to DMEM with 2% horse serum (Life Technologies) as described (Rosen *et al.*, 1992) and maintained for 4 days, by which time >90% of cells had fused into myotubes.

Plasmid construction

DB-ZEB was created by PCR amplification of the region of ZEB cDNA encoding the DNA binding domain (2740–3136) (Funahashi *et al.*, 1993; Genetta *et al.*, 1994). Full-length and DB-ZEB were cloned into the *Mlu*I (5') and *Xba*I sites of the pCI neo expression vector (Promega Corp., Madison, WI). Annealed oligonucleotides containing an in-frame ATG and a Flag antibody recognition sequence were cloned upstream of the ZEB cDNA sequence into the *Mlu*I and *Xho*I sites. Additionally,

annealed oligonucleotides containing an SV40 nuclear localization signal and an in-frame stop codon were cloned immediately downstream of the cDNA sequence into *XbaI* and *NotI* sites.

Fragments of the $\alpha 4$ gene promoter were obtained by PCR with primers containing *KpnI* (5') and *Apal* (3') sites (Rosen *et al.*, 1994). The products were digested with *KpnI* and *Apal* and cloned into the corresponding sites upstream of the Ets sites in -76CAT (Rosen *et al.*, 1994). For E box constructs, annealed oligonucleotides containing the indicated E box(es) along with either 3 or 10 bp of flanking sequence (results were identical with both) and containing *KpnI* and *Apal* sites were cloned into the corresponding sites upstream of the Ets sites in -76CAT. In E361M the CACCTG core was mutated to TTGGCC or TTCCCC and in E399 to ACGCCT or ACCCCA, which blocked nuclear protein binding. To create -400CAT-E361M-E399M, the region between -400 and +15 was amplified by PCR, using a 5'-primer with the above mutations in E361 and E399 along with a previously described 3'-primer, and the resulting product was cloned into SKCAT as described (Rosen *et al.*, 1994). To construct full-length ZEB and ZEB fragment expression vectors, cDNA was amplified using PCR and the products were digested with *BamHI* and *XbaI* and cloned in-frame into the corresponding sites of the Gal4 expression vector pM1 [pM2 for the frame shift mutant (fs)] (Weintraub *et al.*, 1995).

PGXSV contains four Gal4 sites upstream of the SV40 enhancer and E1B TATA box (Chow *et al.*, 1996). The reporter p72-4E-5G was created by cloning the CAT gene into *EcoRV* and *BglII* sites of pSP72 (Promega), the E1B TATA box into the *XbaI* and *BamHI* sites, four MEF-1 E boxes from the MCK gene (Amacher *et al.*, 1993) into *XbaI* and *HindIII* sites and five Gal4 sites into the *XhoI* site (~120 bp upstream of the E boxes). To construct LexA-ZEB (L-ZEB), full-length ZEB was cloned in the *BamHI* and *XbaI* sites of PBXL-3 (Weintraub *et al.*, 1995).

E361 and E399 from the $\alpha 4$ promoter (Rosen *et al.*, 1994), three copies of the MEF-1 site from the MCK gene (Amacher *et al.*, 1993) or three copies of the E1 box from the acetylcholine receptor δ subunit gene (Simon and Burden, 1993) were cloned in the *KpnI* and *Apal* sites of the 76 $\alpha 4$ CAT construct (Rosen *et al.*, 1994).

The MEF-2C expression vector pcDNZ3-MEF-2C was constructed by cloning the MEF2C cDNA sequence (*HindIII-XbaI*) into the corresponding sites of pcDNA3.

Myogenic conversion assays and immunostaining

C2C12 were stably transfected with CMV-ZEB (Genetta *et al.*, 1994) and a plasmid encoding resistance to puromycin (puroBABE; Dr D.J.Templeton, Case Western Reserve University, Cleveland, OH). Puromycin-resistant clones overexpressing ZEB were isolated, switched to differentiation medium (DMEM, 2% horse serum) and stained with antibodies against mouse $\alpha 4$ integrin (PS/2, ATCC), myosin heavy chain (MF-20; Dr Kopan, Washington University, St Louis, MO) and myogenin (FD5; Pharmingen, San Diego, CA) as previously described (Rosen *et al.*, 1992). Northern blots for ZEB expression were performed as described in Rosen *et al.* (1992).

For 10T1/2 assays, 10T1/2 cells were transfected by the lipofectamine method in OptiMem medium (Life Technologies). After 12 h, medium was removed and replaced with 2% horse serum, DMEM. After 4–5 days, cells were fixed in methanol and stained with anti-myosin antibody MF-20. After washing to remove unbound antibody, cultures were incubated sequentially with horseradish peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) and with DAB substrate (Vector Labs, Burlingame, CA). Nuclei were counterstained with hematoxylin (Zymed, San Francisco, CA).

Electrophoretic mobility shift assays

For nuclear extracts from cells expressing myf-5, 10 cm plates were transfected with 10 μ g pEMSV-myf-5, cells were harvested after 48 h and nuclear extracts were prepared (Osborn *et al.*, 1989). The gel shift assay was performed as described (Rosen *et al.*, 1994). Antibodies were either purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) or prepared as described previously (Funahashi *et al.*, 1993).

Transient transfections and CAT assays

Cells were transfected by the calcium phosphate method. After 48 h, lysates were collected and CAT assays were performed as described (Rosen *et al.*, 1994). Transfection efficiency was corrected for by co-transfection of 0.25 μ g TKluc and CAT and luciferase activities were determined as described (Rosen *et al.*, 1994). DNA was brought to a total of 6 μ g for 60 mm (or 20 μ g for 100 mm) dishes. For transfection in C2C12 myotubes, myoblast cells were switched to medium containing

2% horse serum and cells were maintained for 4 days before harvesting for analysis of CAT activity (Rosen *et al.*, 1992).

In vitro transcription/translation

For transcription/translation assays, the *XhoI-NotI* fragment containing the pci-flag-ZEB and pci-flag DB-ZEB cDNAs were cloned into the corresponding sites in pBluescript-KS (Stratagene, La Jolla, CA). MyoD-E47 (Neuhold and Wold, 1993) and the ZEB proteins were synthesized *in vitro* using the Promega TNT kit and T3 RNA polymerase (Weintraub *et al.*, 1995).

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