c-Myb and Ets proteins synergize to overcome transcriptional repression by ZEB

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The Zfh family of zinc finger/homeodomain proteins was first identified in *Drosophila* where it is required for differentiation of tissues such as the central nervous system and muscle. ZEB, a vertebrate homolog of Zfh-1, binds a subset of E boxes and blocks myogenesis through transcriptional repression of muscle genes. We present evidence here that ZEB also has an important role in controlling hematopoietic gene transcription. Two families of transcription factors that are required for normal hematopoiesis are c-Myb and Ets. These factors act synergistically to activate transcription, and this synergy is required for transcription of at least several important hematopoietic genes. ZEB blocks the activity of c-Myb and Ets individually, but together the factors synergize to resist this repression. Such repression imposes a requirement for both c-Myb and Ets for transcriptional activity, providing one explanation for why synergy between these factors is important. The balance between repression by ZEB and transcriptional activation by c-Myb/Ets provides a flexible regulatory mechanism for controlling gene expression in hematopoietic cells. We demonstrate that one target of this positive/negative regulation *in vivo* is the $\alpha 4$ integrin, which play a key role in normal hematopoiesis and function of mature leukocytes.

Keywords: c-Myb/Ets/hematopoiesis/ $\alpha 4$ integrin/repression/ZEB

Introduction

The Zfh family is comprised of zinc finger/homeodomain proteins that were first identified in *Drosophila* where they are required for proper differentiation of tissues such as the central nervous system (CNS) and muscle (Fortini *et al.*, 1991; Lai *et al.*, 1993). However, the mechanism of action of the Zfh protein in *Drosophila* is unknown. A vertebrate homolog of *Drosophila* Zfh-2, ATBF-1, has been identified and shown to repress the expression of the α -fetoprotein gene (Yasuda *et al.*, 1994). We have found recently that the vertebrate homolog of *Drosophila* Zfh-1, ZEB, represses muscle genes and regulates myogenic differentiation (Postigo and Dean, 1997). Thus, the Zfh family appears to regulate tissue differentiation by repressing gene transcription.

ZEB binds to a subset of E boxes (CANNTG) with the

sequence CACCTG (Genetta *et al.*, 1994). Once bound to these sites in the promoter region of muscle genes, ZEB represses transcription and blocks muscle differentiation. ZEB is displaced from these E boxes as myogenic basic helix–loop–helix proteins (bHLH) (myoD, myogenin, myf-5, MRF-4), that also bind to these sites, accumulate during muscle differentiation (Postigo and Dean, 1997). The interplay between ZEB and bHLH proteins at different genes may provide a mechanism for imposing temporal order on the expression of muscle genes.

Zfh-1 is expressed in the CNS, and its overexpression results in an early embryonic lethal phenotype with severe CNS defects in *Drosophila* (Fortini *et al.*, 1991). ZEB is also expressed in the CNS, suggesting that it may have a role in differentiation of other tissues in addition to muscle. In fact, ZEB was described originally in B cells, although no transcriptional activity was reported (Genetta *et al.*, 1994). Therefore, we investigated a role for ZEB in hematopoietic cells. One target of ZEB in myogenesis is the $\alpha 4$ integrin gene (Postigo and Dean, 1997). $\alpha 4$ is one of a number of integrins that associate with a common $\beta 1$ subunit (Lobb and Hemler, 1994; Springer, 1994). $\alpha 4$ integrin is expressed early in muscle development where it interacts with its ligand vascular cell adhesion molecule 1 (VCAM-1) to mediate cell–cell interactions important for myogenesis (Rosen *et al.*, 1992; Sheppard *et al.*, 1994). Interestingly, $\alpha 4$ integrin is also expressed in hematopoietic cells, where it has a key role in the differentiation of all hematopoietic lineages through its interaction with fibronectin and VCAM-1 in the stromal matrix and stromal cells in the bone marrow and fetal liver (Miyake *et al.*, 1991; Roldan *et al.*, 1992; Teixido *et al.*, 1992). $\alpha 4$ is expressed before most lineage-specific markers on all hematopoietic cell lineages during the early stages of hematopoiesis (Sanchez *et al.*, 1993; Hamamura *et al.*, 1996). However, as hematopoietic differentiation proceeds, its expression becomes restricted to only a few lineages such as lymphocytes and some subsets of myeloid cells, where it continues to function by targeting these leukocytes to sites of inflammation (Lobb and Hemler, 1994). These observations, together with recent reports showing that $\alpha 4$ is required *in vivo* for normal hematopoiesis in mice (Hamamura *et al.*, 1996; Arroyo *et al.*, 1996), indicate an important role for $\alpha 4$ in hematopoiesis and leukocyte function. $\alpha 4$ integrin expression continues to be regulated in mature leukocytes as $\alpha 4$ expression is up-regulated during mitogenic activation (Sanchez-Madrid *et al.*, 1986) and differentially regulated among lymphoid subsets (Shimizu *et al.*, 1990; Picker *et al.*, 1991).

We demonstrate that ZEB activity, and hence $\alpha 4$ integrin expression, is regulated in hematopoietic cells through a mechanism distinct from that which we have characterized in muscle (Postigo and Dean, 1997). Instead of being displaced from the promoter by bHLH proteins, the

hematopoietic transcription factor c-Myb synergizes with Ets proteins to resist repression by ZEB in hematopoietic cells. In addition to regulating the $\alpha 4$ gene, c-Myb and Ets also play a crucial role in the regulation of other hematopoietic genes and thus in hematopoiesis itself (Graf, 1992; Lipsick, 1996). Disruption of *ets* and *c-myb* genes has been shown to severely affect the proper development of multiple hematopoietic lineages (Mucenski *et al.*, 1991; Scott *et al.*, 1994; Muthusamy *et al.*, 1995; Shivdasani and Orkin, 1996). Interestingly, the E26 virus, which carries the *v-myb* oncogene as a fusion with *v-ets*, efficiently induces erythroleukemia, whereas avian myeloblastosis virus (AMV; which carries *v-myb* but lacks *v-ets*) does not (Radke *et al.*, 1982; Metz and Graf, 1991a,b), providing evidence of the potent effect of the synergism between c-Myb and Ets in the regulation of hematopoietic genes and specific steps in hematopoiesis.

Only a few hematopoietic genes have been identified as targets of c-Myb, and it is interesting to note that several of these genes which have been examined in detail are dependent upon synergism between c-Myb and Ets. Such genes include the early myeloid markers *mim-1* (Ness *et al.*, 1989; Dudek *et al.*, 1992) and CD13 (Shapiro, 1995), CD4 (Siu *et al.*, 1992), *p56^{lck}* (McCracken *et al.*, 1994) and the early hematopoietic marker CD34 (Melotti and Calabretta, 1994; Melotti *et al.*, 1994). It has been demonstrated with some of these genes that expression is not induced by c-Myb or Ets separately, but is dependent on the combination of both factors (McCracken *et al.*, 1994), suggesting that, as with the $\alpha 4$ gene, this synergy may overcome a silencer that blocks activity of the factors individually. Indeed, such silencer elements have been reported in some of the above genes (Allen *et al.*, 1992; Sawada *et al.*, 1994; Perrotti *et al.*, 1995; Duncan *et al.*, 1996). Although the repressor protein has not been characterized for CD4 and *p56^{lck}*, ZEB sites are evident in both genes, suggesting that ZEB may be responsible for the silencer activity.

Here, we demonstrate that ZEB is important in regulating gene expression in hematopoietic cells. Regulation of ZEB activity in hematopoietic cells is distinct from that in muscle, and our results suggest that a fine balance between repression by ZEB and synergistic activation by c-Myb and Ets regulates gene expression in hematopoietic cells.

Results

ZEB is expressed and binds ZEB sites in hematopoietic cells, but it does not repress gene expression

ZEB blocks muscle differentiation by repressing muscle genes. One such gene is $\alpha 4$ integrin (Postigo and Dean, 1997). In addition to its expression in muscle, $\alpha 4$ integrin has a key role in hematopoietic differentiation, leukocyte trafficking and recruitment of leukocytes to sites of inflammation (Miyake *et al.*, 1991; Yednock *et al.*, 1992; Lobb and Hemler, 1994). The interaction between $\alpha 4$ integrin on hematopoietic cells and VCAM-1 and fibronectin on stromal cells and stromal matrix in the fetal liver and bone marrow is important for normal hematopoiesis (Miyake *et al.*, 1991; Roldan *et al.*, 1992). Previous results demonstrated that Ets sites in the first

76 bp of the $\alpha 4$ promoter gene are active in most cell types (Rosen *et al.*, 1994), making it unlikely that these sites alone could account for the restricted pattern of $\alpha 4$ expression. In order to understand how the $\alpha 4$ gene is regulated in hematopoietic cells, we transfected $\alpha 4$ gene promoter constructs into $\alpha 4$ (+) and $\alpha 4$ (–) cells.

In $\alpha 4$ (–) cells, the activity of the promoter was blocked in constructs containing sequences upstream of –300 bp (Figure 1A–D). This pattern of promoter activity was the same as the pattern we found in $\alpha 4$ (–) myoblasts (Figure 1D, and Postigo and Dean, 1997). Two ZEB sites (at positions –361 and –399 bp) are responsible for this silencer activity in myoblasts (Postigo and Dean, 1997). When the same constructs were transfected into the $\alpha 4$ (+) T-cell line Jurkat, the erythroleukemia cell line HEL or the B cell line Raji, we found that the activity is maintained in promoter constructs extending to –2.0 kb (Figure 1E and F) as $\alpha 4$ (–) myoblasts differentiate into $\alpha 4$ (+) myotubes (Figure 1H). The lack of repressor activity suggested that ZEB may not be expressed in hematopoietic cells. However, we found that Jurkat cells express high levels of ZEB (Figure 2A), and ZEB has also been shown to be expressed in B cells (Genetta *et al.*, 1994).

In muscle, ZEB is displaced from E361 and E399 by bHLH proteins as $\alpha 4$ (–) myoblasts differentiate into $\alpha 4$ (+) myotubes (Figure 1D and H, and Postigo and Dean, 1997). Even though ZEB is expressed in hematopoietic cells, it was possible that the ratio of ZEB to bHLH proteins (E proteins) is low and ZEB is prevented from binding ZEB sites. However, increasing this ratio by overexpression of ZEB in hematopoietic cells did not result in repressor activity (Figure 2B), contrary to myotubes where overexpression of ZEB displaced myoD/E proteins complexes from E boxes and blocked $\alpha 4$ promoter activity (Figure 2C, and Postigo and Dean, 1997).

Next, we examined the capacity of ZEB to bind ZEB sites from the $\alpha 4$ promoter. Gel retardation assays with E361 and E399 gave the same pattern of nuclear protein binding with nuclear extracts from $\alpha 4$ (+) hematopoietic cells and with $\alpha 4$ (–) cells; we found binding of ZEB along with the ubiquitous bHLH protein USF (upstream stimulatory factor) (Sawadogo and Roeder, 1985) (Figure 3A and B). However, the ZEB sites bind only weakly to USF, and overexpression of USF did not activate or repress transcription through the ZEB sites (results not shown). Therefore, the lack of repression of the $\alpha 4$ promoter observed in hematopoietic cells was not due to the lack of binding of ZEB to its sites in the $\alpha 4$ promoter.

ZEB sites are silencers in hematopoietic cells

One explanation for the lack of ZEB repressor activity in hematopoietic cells was that a hematopoietic-specific transcription factor may block ZEB function in the context of the $\alpha 4$ gene promoter. To investigate this possibility, we first examined the activity of ZEB sites out of the context of the $\alpha 4$ promoter. ZEB sites efficiently blocked the activity of the Ets sites from the $\alpha 4$ promoter in $\alpha 4$ (+) hematopoietic cells (Figure 4A) as well as in $\alpha 4$ (–) cell types (Figure 4B). These results are in contrast to $\alpha 4$ (+) myotubes where the loss of ZEB activity (E361 and E399 did not have any repressor effect on Ets activity) was the result of the displacement of ZEB by myoD/E12

Fig. 1. $\alpha 4$ gene promoter activity is under negative regulation in $\alpha 4(-)$ cells but not in $\alpha 4(+)$ cells. Numbers in the construct name indicate the position in the flanking sequences. Cells were transfected as described in Materials and methods. Results are the average of duplicate assays and are representative of at least five separate experiments. For transfection in C2C12 myotubes, C2C12 myoblast cells were switched to media containing 2% horse serum and maintained for 4 days before harvesting for analysis of CAT activity as described (Rosen *et al.*, 1992).

during myogenic differentiation (Figure 4C, and Postigo and Dean, 1997). Overexpression of the bHLH E proteins E12 and E47 did not have any effect on repression by ZEB in Jurkat cells (Figure 4A), further suggesting that bHLH proteins do not displace ZEB in hematopoietic cells as they do in muscle cells (Postigo and Dean, 1997).

The hematopoietic transcription factor c-Myb is necessary but not sufficient to overcome repression by ZEB

The results described above suggest that a hematopoietic-specific transcription factor may overcome ZEB repression

in the context of the $\alpha 4$ gene promoter. c-Myb is one of the first transcription factors detected during hematopoiesis, where it has been shown to play a key role in the differentiation process (reviewed in Graf, 1992). Additionally, c-Myb has been shown to synergize with Ets factors in the regulation of hematopoietic genes. The early appearance of $\alpha 4$ during hematopoiesis suggests that $\alpha 4$ expression is likely to depend upon a transcription factor such as c-Myb. Indeed, a number of potential c-Myb sites are evident upstream and downstream of the ZEB sites (from -130 bp to -2.0 kb) in the $\alpha 4$ promoter (Figure 5A and data not shown). Gel-shift experiments demonstrated that

Fig. 2. Lymphoid cells express high levels of ZEB mRNA and overexpression of ZEB does not silence $\alpha 4$ promoter activity. (A) mRNA levels for ZEB in $\alpha 4$ (-) C2C12 myoblasts, $\alpha 4$ (-) HT1080 fibrosarcoma cells and $\alpha 4$ (+) T-lymphoid Jurkat cells were determined by Northern blot analysis using a *NdeI*-*HindIII* fragment of ZEB cDNA. (B) Overexpression of ZEB in $\alpha 4$ (+) Jurkat lymphoid cells does not silence $\alpha 4$ promoter activity. Twenty-five μ g of $\alpha 4$ CAT reporter constructs were co-transfected with 60 μ g of an expression vector for ZEB. Similar results were obtained with the erythroleukemia cell line HEL (data not shown). (C) Overexpression of ZEB in $\alpha 4$ (+) C2C12 myotubes represses $\alpha 4$ gene promoter activity and restores silencer activity. C2C12 myoblasts were transfected with 8 μ g of $\alpha 4$ CAT reporters and 12 μ g of ZEB and stimulated to fuse into myotubes as in Figure 1.

some of these sites bind efficiently to recombinant c-Myb (data not shown). In addition, the -130 to -400 region as well as individual sites in the $\alpha 4$ promoter were tested

Fig. 3. ZEB binds ZEB sites in both $\alpha 4$ (-) and $\alpha 4$ (+) cells. Gel retardation assays using a probe containing ZEB sites E361 and E399 from the $\alpha 4$ promoter gave a similar pattern of binding with nuclear extracts from $\alpha 4$ (+) Jurkat cells (A) and $\alpha 4$ (-) HT1080 cells (B). Antibodies (0.5 μ g) against ZEB, USF, E12, HEB (this figure) and MyoD and c-Myb (not shown) were added in the binding assays. Only ZEB and USF show binding, as demonstrated by supershift of the corresponding bands (indicated as *). 'NS' indicates non-specific complexes. ZEB and USF binding was competed with a 50-fold excess of unlabeled probe, but not with the corresponding mutant probe (not shown).

for c-Myb binding with nuclear extracts from Jurkat cells (Figure 5A and B and data not shown). Binding was evident with extracts from Jurkat cells but not with extracts from the control $\alpha 4$ (-)/c-myb (-) fibrosarcoma cell line HT1080 (Figure 5A). This binding was blocked by unlabeled c-Myb sites from the $\alpha 4$ gene promoter and other genes regulated by c-Myb [such as the T-cell receptor (TCR) δ enhancer (Hernandez-Munain and Krangel, 1994)], but not by their corresponding mutated sites (Figure 5B). Additionally, binding was inhibited by anti-c-Myb antibodies (Figure 5B).

To determine whether c-Myb was sufficient to activate the $\alpha 4$ gene promoter in $\alpha 4$ (-) cells, an expression vector for c-Myb was co-transfected with $\alpha 4$ gene promoter constructs into the $\alpha 4$ (-)/c-myb (-) HT1080 fibrosarcoma cells. c-Myb overcame the repressor activity of ZEB and activated the $\alpha 4$ gene promoter (Figure 6A).

Reporter constructs containing different combinations of Ets sites, ZEB sites and c-Myb sites were then transfected into Jurkat cells [as well as $\alpha 4$ (-) HT1080 cells] (Figure 6A and B). The activity of the ZEB sites in Jurkat cells was blocked by the combination of Ets and c-Myb sites; however, mutation of either the Ets or c-Myb sites restored repressor activity (Figure 6B). These results demonstrate the requirement for both Ets and c-Myb sites to overcome repression by ZEB. It is interesting to note that addition of the c-Myb sites to the Ets sites did not change the level of transcription significantly above that of the Ets sites alone. Therefore, c-Myb does not overcome ZEB repression by increasing transcription when combined with Ets factors and simply overwhelming repression by ZEB. Instead, the combination of c-Myb and Ets activates transcription through a mechanism distinct from either factor alone and in a fashion resistant to ZEB.

ZEB fused to the DNA-binding domain (DBD) of the yeast protein Gal4 blocked the activity of the Ets sites (Figure 6C), demonstrating directly that ZEB blocks Ets transactivation. Additionally, ZEB fused to the DBD of the bacterial protein LexA blocked transactivation by

Fig. 4. ZEB sites are silencers in $\alpha 4$ (+) Jurkat cells when taken out of the context of the $\alpha 4$ gene promoter. E361 and E399 ZEB binding sites were cloned upstream of the $\alpha 4$ Ets sites in the -76 $\alpha 4$ CAT, and the resulting constructs were transfected in $\alpha 4$ (+) Jurkat cells. Repressor activity was found in $\alpha 4$ (+) Jurkat cells (A) (similar results were obtained with the erythroleukemia HEL cell line) as well as in $\alpha 4$ (-) HT1080 cells (B) but not in $\alpha 4$ (+) C2C12 myotubes (C). The control E box at -585 bp which is not a ZEB site did not repress Ets activity. Overexpression of the bHLH E proteins E12 and E47 in Jurkat cells did not block repression by ZEB (A). Twenty five μ g of $\alpha 4$ CAT reporter constructs were co-transfected in Jurkat with or without 60 μ g of E12 or E47 expression vectors. In HT1080 cells and C2C12 myotubes, 3 μ g of the reporter constructs were transfected.

c-Myb fused to the DBD of Gal4 (Figure 6C), indicating that ZEB blocks the transactivation domain of c-Myb. Therefore, ZEB blocks the transactivation activity of Ets and c-Myb individually, but the combination of these sites synergistically overcomes the repression.

These results suggest that expression of c-Myb is an important determining factor in the control $\alpha 4$ expression in hematopoietic cells. In support of this conclusion, forced expression of c-Myb in $\alpha 4$ (-)/c-Myb (-) cells led to expression of endogenous $\alpha 4$ on the cell surface (Figure 7).

As another way of demonstrating an important role for c-Myb in the regulation of $\alpha 4$ gene expression, we used a dominant-negative form of c-Myb (MT) that contains the DBD of c-Myb but lacks the transactivation domain (Badiani *et al.*, 1994). This dominant-negative c-Myb has been shown previously to block c-Myb function *in vivo* and *in vitro* (Badiani *et al.*, 1994; Taylor *et al.*, 1996). Co-transfection of the MT expression vector with the -2.0 $\alpha 4$ CAT reporter in Jurkat cells repressed the activity of -2.0 $\alpha 4$ CAT to a level similar to that found in $\alpha 4$ (-) HT1080 cells (Figure 8A). These results provide additional evidence that c-Myb is required for $\alpha 4$ gene promoter activity in hematopoietic cells.

Loss of $\alpha 4$ expression on hematopoietic progenitors in c-Myb (-/-) mice

c-Myb (-/-) mice die *in utero* due to severe anemia, showing a reduced number of hematopoietic precursors in the liver (Mucenski *et al.*, 1991). We examined the expression of $\alpha 4$ integrin on hematopoietic cells in embryonic day 14.5 c-Myb (-/-) mice. If c-Myb is indeed important for expression of $\alpha 4$, then $\alpha 4$ should be absent from hematopoietic cells in these mice. We found that expression of $\alpha 4$ integrin in the hematopoietic cell pro-

genitors present in the fetal liver was abrogated in the c-Myb (-/-) mice (Figure 8B), but was high in c-Myb (+/+) mice (Figure 8C), indicating that *in vivo* expression of $\alpha 4$ requires c-Myb. As controls, expression of other markers seemed to be unaffected in c-Myb (-/-) mice (results not shown). Expression of $\alpha 4$ in other tissues such as skeletal and smooth muscle and neural crest derivatives was similar in both the (-/-) and (+/+) genotypes (data not shown), indicating a selective role for c-Myb in the expression of $\alpha 4$ in the hematopoietic system.

These results demonstrate a key role for c-Myb in the regulation of the $\alpha 4$ gene, and place $\alpha 4$ as one of the first genes demonstrated to be regulated by c-Myb *in vivo*.

Discussion

ZEB is a vertebrate homolog of the *Drosophila* Zfh-1 protein. Zfh-1 has been shown to be important in differentiation of tissues such as the CNS and muscle in *Drosophila* (Fortini *et al.*, 1991; Lai *et al.*, 1993). We have found previously that ZEB is an active transcriptional repressor that, like Zfh-1, regulates muscle differentiation (Postigo and Dean, 1997). ZEB is also expressed in other tissues, and we present evidence here that it may also have a regulatory role in hematopoiesis. Both c-Myb and Ets are required for normal hematopoiesis (Shivdasani and Orkin, 1996). These factors can act in synergy to activate transcription, and this synergy is essential for activation of important hematopoietic genes (reviewed in Graf, 1992). ZEB blocks the activity of Ets and Myb individually, but repressor activity is overcome by the c-Myb/Ets synergy. ZEB sites in the promoter of the $\alpha 4$ integrin gene render expression dependent upon the synergy between c-Myb and Ets and may provide an explanation for why this synergy

Fig. 5. The hematopoietic transcription factor c-Myb binds to the $\alpha 4$ promoter. **(A)** A number of c-Myb sites are evident in the $\alpha 4$ promoter between -130 bp and -2.0 kb (this figure and data not shown) (arrows on the top indicate c-Myb sites, whereas arrows on the bottom indicate the ZEB sites). Gel retardation assay using as a probe from -130 to -400 of the $\alpha 4$ gene promoter shows the binding of c-Myb in nuclear extracts from $\alpha 4$ (+) lymphoid cell line Jurkat but not from $\alpha 4$ (-) HT1080 fibrosarcoma cells, as evidenced by competition with a 50-fold excess of unlabeled probe containing a consensus c-Myb sequence. 'NS' indicates non-specific complexes. **(B)** Gel retardation assay using nuclear extracts from $\alpha 4$ (+)/c-*myb* (+) Jurkat cells and the c-Myb site at position -134 bp of the $\alpha 4$ gene promoter as a probe. 'Competitor' indicates addition to the binding reaction of a 50-fold excess of unlabeled wild-type and mutant (mut) c-Myb sites from the $\alpha 4$ gene (-134) or the TCR δ enhancer [$\delta E3(3')$], Hernandez-Munain and Krangel, 1994]. 'NS' indicates non-specific complexes.

is required. During differentiation and cell activation, the level of c-Myb varies and, when it is expressed, it synergizes with Ets factors to overcome ZEB repression and activate hematopoietic genes. We presented *in vitro* and *in vivo* evidence that in the absence of c-Myb, ZEB

blocks $\alpha 4$ expression throughout all hematopoietic cell lineages. Conversely, we found that without ZEB, the $\alpha 4$ gene would be expressed constitutively as a result of the Ets sites in the promoter. This mechanism of regulating ZEB activity and $\alpha 4$ expression in hematopoietic cells is different from what we found previously in muscle cells, where ZEB is displaced from ZEB sites by myogenic bHLH factors that appear during myogenic differentiation (Postigo and Dean, 1997).

During development, initial hematopoiesis takes place in the yolk sac (or analogs in mammals) and paraortic compartments (reviewed in Dorshkind, 1994; Orkin, 1995). Hematopoietic stem cells migrate from those sites to the fetal liver of the embryo where most of the embryonic hematopoiesis occurs. Perinatally, stem cells migrate to the thymus and bone marrow. Recently, chimeric animal experiments have shown that $\beta 1$ integrins (a larger subclass of integrins including $\alpha 4\beta 1$) are involved in this migration process (Hirscht *et al.*, 1996). Hematopoiesis in the fetal liver, thymus and bone marrow requires the interaction of adhesion molecules on the stem cells with ligands in stromal cells. One of the interactions that has been shown *in vivo* and *in vitro* to be crucial for normal hematopoietic development is the binding of the $\alpha 4$ integrin on the surface of stem cells to its ligands VCAM-1 and fibronectin in the stroma of these organs (Miyake *et al.*, 1991; Roldan *et al.*, 1992). $\alpha 4$ appears on the surface of precursor cells very early during hematopoiesis, before most lineage-specific markers (Sanchez *et al.*, 1993). Antibodies against $\alpha 4$ and VCAM-1 have been shown to block differentiation of cultured precursors (Miyake *et al.*, 1991; Roldan *et al.*, 1992; Teixido *et al.*, 1992). Likewise, injection of anti- $\alpha 4$ antibodies into pregnant mice blocked erythropoiesis and inhibited other lineages in the embryos (Hamamura *et al.*, 1996). Additionally, it has been shown in mice chimeric for $\alpha 4$ that this integrin is required for lymphoid differentiation in the bone marrow (Arroyo *et al.*, 1996).

Disruption of the *c-myb* gene causes mice to die *in utero* due to a failure in liver hematopoiesis, resulting in severe anemia (Mucenski *et al.*, 1991). Interestingly, the precursors cells present in *c-myb* ($-/-$) animals are able to differentiate in culture to their corresponding mature phenotypes, indicating that the defect is in the failure of the precursor population in the liver to expand, instead of in the differentiation process *per se* (Mucenski *et al.*, 1991). c-Myb has been shown to be required for hematopoietic cells to enter S phase, suggesting that c-Myb is important for efficient cell division (Stern and Smith, 1986; Gewirtz *et al.*, 1989). Moreover, recent evidence has demonstrated that inactivation of c-Myb in hematopoietic cells triggers apoptosis and that c-Myb regulates expression of *bcl-2*, which protects against apoptosis (Frampton *et al.*, 1996; Taylor *et al.*, 1996).

We found that one result of the disruption of the *c-myb* gene is the loss of $\alpha 4$ integrin expression. As outlined above, the failure of hematopoiesis in the *c-myb* ($-/-$) mice appears to result from a failure in proliferation and/or triggering of apoptosis (Mucenski *et al.*, 1991; Frampton *et al.*, 1996; Taylor *et al.*, 1996). Interestingly, $\alpha 4$ integrin can transmit signals that trigger cell proliferation (Nojima *et al.*, 1990; Damle and Aruffo, 1991), and adhesion of integrins to ligand has been shown to prevent apoptosis

(Koopman *et al.*, 1994). Therefore, the loss of $\alpha 4$ integrin in *c-myb* ($-/-$) mice may contribute to the failure of the hematopoietic population to expand, a phenotype observed in these animals (Mucenski *et al.*, 1991).

Early during hematopoiesis, $\alpha 4$ integrin is expressed in all cell lineages: lymphoid and myeloid precursors, erythroblasts and megakaryocytes (Miyake *et al.*, 1991; Rosemblatt *et al.*, 1991; Teixido *et al.*, 1992; Avraham *et al.*, 1993; Sadahira *et al.*, 1995; Hamamura *et al.*, 1996). As these precursors mature, $\alpha 4$ expression becomes restricted to lymphocytes and some myeloid cells (Lobb and Hemler *et al.*, 1994). Thus, control of $\alpha 4$ expression is very tightly regulated during hematopoiesis. Regulation of $\alpha 4$ integrin expression continues to be critical beyond hematopoiesis, where it is expressed differentially on the different lymphocyte subsets (Shimizu *et al.*, 1990; Picker *et al.*, 1991), and its expression is up-regulated upon mitogenic stimulation (Sanchez-Madrid *et al.*, 1986). Deregulation of $\alpha 4$ expression has been associated with inflammatory diseases and hematologic malignancies (Freedman *et al.*, 1992; Moller *et al.*, 1992; Juneja *et al.*, 1993; Kuriyama *et al.*, 1994). We propose that control of $\alpha 4$ integrin expression in hematopoietic cells is the result of a fine balance between repression by ZEB and activation by *c-Myb*/Ets. ZEB imposes a requirement for both *c-Myb* and Ets to activate the $\alpha 4$ gene (and perhaps other

Fig. 6. *c-Myb* regulates $\alpha 4$ gene promoter activity.

(A) Overexpression of *c-Myb* in the $\alpha 4 (-)$ HT1080 cell line overcomes repression by ZEB. Three μ g of the -76 α 4CAT, -2.0 α 4CAT, pETS-ZEB-CAT and pETS-ZEB-MYB-CAT reporter constructs were co-transfected with 0.9 μ g of a PSV-*c-Myb* expression vector. pETS-ZEB-CAT indicates that the ZEB site E361 was cloned upstream of the Ets sites in the -76 α 4CAT reporter. pETS-ZEB-MYB-CAT indicates that four copies of the *c-Myb* site at -134 bp were cloned upstream of the ZEB sites of the pETS-ZEB-CAT construct. (B) The activity of the $\alpha 4$ gene promoter in Jurkat cells depends on synergistic activity of *c-Myb* and Ets transcription factors. Mutation of the Ets sites (indicated by pETSmut) abolishes the capacity of *c-Myb* to overcome ZEB repression. Thirty μ g of the different α 4CAT reporters were transfected in Jurkat cells (similar results were obtained using the erythroleukemia HEL cell line). (C) ZEB represses transcriptional activation by Ets and *c-Myb* factors individually. Three μ g of -76-G4-CAT (containing five copies of the Gal4 DNA-binding site upstream of the -76 α 4CAT) was co-transfected in HT1080 cells with 2 μ g of G-ZEB which encodes a fusion protein of ZEB with the DBD of Gal4. One μ g of pL-G (containing six LexA-binding sites and two Gal4 sites upstream of the E1B TATA box) was co-transfected in HT1080 with 0.5 μ g of a Gal4-*c-Myb* expression vector with or without 2 μ g of L-ZEB which encodes a fusion protein between the DBD of LexA and ZEB.

Fig. 7. *c-Myb* stimulates expression of the endogenous $\alpha 4$ gene in $\alpha 4 (-)$ cells. Flow cytometric analysis showing that $\alpha 4$ appears on the surface of the $\alpha 4 (-)$ /*c-myb* ($-$) T98G glioblastoma cell line (dashed line) after stable expression of *c-Myb* [SV-*myb*-M-T98G cell line (Melotti *et al.*, 1994)] (solid line). As a control, $\beta 1$ integrin showed no significant change in expression. Likewise, no change was seen in the level of expression of the $\alpha 5$ integrin (not shown). The fluorescence intensity of an isotype-matched negative control antibody overlaid with the expression of $\alpha 4$ integrin in T98G cells (not shown).

hematopoietic genes). We present evidence that regulation of $\alpha 4$ expression can result from variations of *c-Myb* expression: *c-Myb* levels (in parallel to $\alpha 4$ expression) decline during hematopoiesis as precursors mature (Graf, 1992) and increases as a result of mitogenic activation (Torrelli *et al.*, 1985; Pauza, 1987). Erythroblasts and megakaryocytes lose $\alpha 4$ expression in parallel with the

Fig. 8. Inactivation or disruption of the *c-myc* gene abolishes expression of $\alpha 4$ integrin in hematopoietic cells. (A) Overexpression of a dominant-negative form of c-Myb (DN-myc; MT) blocked the activity of the $\alpha 4$ gene promoter in $\alpha 4$ (+) Jurkat cells. Twenty-five μ g of -76 $\alpha 4$ CAT and -2.0 $\alpha 4$ CAT reporter constructs were co-transfected in $\alpha 4$ (+) Jurkat cells with 25, 50 and 75 μ g of DN-myc. Note that DN-myc blocked the activity of -2.0 $\alpha 4$ CAT (contains c-Myb sites) but not of -76 $\alpha 4$ CAT (lacks c-Myb sites). (B and C) $\alpha 4$ is expressed on all hematopoietic cells in E14.5 liver of *c-myc* (+/+) mice (B) but was not evident on hematopoietic cells in the E14.5 liver of *c-myc* (-/-) mice (C). Frozen sections of E14.5 mice were immunostained with anti- $\alpha 4$ antibody as described (Rosen *et al.*, 1992). Precursors in the fetal liver of *c-myc* (-/-) embryos expressed other hematopoietic markers (results not shown).

disappearance of c-Myb as they mature to erythrocytes and platelets (Rosemblatt *et al.*, 1991; Avraham *et al.*, 1993; Sadahira *et al.*, 1995; Hamumura *et al.*, 1996). Lymphoid and some subsets of myeloid cells also down-regulate $\alpha 4$ expression as c-Myb decreases during their differentiation, although they still express $\alpha 4$ when they reach the bloodstream (Lobb and Hemler, 1994). Expression is again up-regulated upon cellular activation (Sanchez-Madrid *et al.*, 1986).

The Ets family comprises an increasing number of members (Leiden, 1993; Wasyllick *et al.*, 1993; Crepieux *et al.*, 1994). The set of Ets factors that bind the $\alpha 4$ promoter seems to vary from one cell type to another and, at least, includes Ets-1 and GABP α and β (Rosen *et al.*, 1994 and unpublished results). The expression and activity of Ets proteins are regulated during T lymphocyte differentiation and activation (Pognonec *et al.*, 1988; Bhat *et al.*, 1989, 1990; Muthusamy *et al.*, 1995). Although we have found that the Ets sites in the $\alpha 4$ gene promoter are active

in most cells that we have tested, it is possible that, like those of c-Myb, their activity varies during hematopoietic differentiation and in different hematopoietic lineages, providing an additional level of regulation. Likewise, it is also possible that the level of ZEB in hematopoietic cells changes during hematopoiesis and/or cell activation. Therefore, in addition to c-Myb, regulation of Ets and/or ZEB activities could add further flexibility to the regulation of the $\alpha 4$ gene (and perhaps other hematopoietic genes regulated by c-Myb/Ets). Although the studies presented here have relied on the comparison of promoter activities in hematopoietic cells versus non-hematopoietic cells, they provide strong evidence that c-Myb and Ets factors synergize to overcome repression by ZEB. While these experiments do not provide conclusive evidence that the c-Myb/Ets-ZEB regulatory pathway plays a critical role in hematopoietic cell differentiation and function, taken together with patterns of c-Myb and Ets expression and the experiments in the *c-myc* (-/-) mice, these experiments suggest that this balance between repression by ZEB and activation by c-Myb/Ets is involved in the regulation of the $\alpha 4$ gene and possibly several other hematopoietic genes.

Interestingly, the combination of c-Myb and Ets did not increase transcription significantly above the level with Ets alone. Therefore, the c-Myb/Ets combination does not overcome repression by ZEB simply by increasing transcription but, instead, the c-Myb/Ets synergy activates transcription through a mechanism distinct from that of either factor alone. It will be of interest to determine the molecular basis for the resistance to ZEB repression. However, further information about how c-Myb/Ets synergize to activate transcription is needed. ZEB may provide a tool to analyze such mechanisms of transcriptional synergy. A target of future studies will be the components of the basal transcription complex that interact with c-Myb/Ets. It is likely that the combination of c-Myb and Ets proteins interact with basal transcription complex components in a fashion distinct from either factor alone and in a manner that is resistant to disruption by ZEB.

Materials and methods

Cell culture

The HT1080 fibrosarcoma [American Type Culture Collection (ATCC), Rockville, MD] and C33A cervical carcinoma cell lines (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) containing 5% fetal calf serum (FCS) and 5% calf serum (Life Technologies). The T-cell line Jurkat (ATCC), B-cell line Raji (ATCC) and the erythroleukemia HEL cell line (ATCC) were maintained in RPMI 1640 media containing 10% FCS. C2C12 myoblasts (ATCC) and C3H10T1/2 fibroblasts (ATCC) were grown in DMEM containing 13% FCS. For differentiation of C2C12 myoblasts to myotubes, cells were changed to DMEM-2% horse serum (Life Technologies) as described (Rosen *et al.*, 1992) and maintained for 4 days; by this time >90% of cells had fused into myotubes. The glioblastoma cell line T98G and its *c-myc* overexpression derivative SV-*myb*-T98G were obtained from B. Calabretta (Jefferson University, Philadelphia, PA) (Melotti *et al.*, 1994) and maintained in the same media as the HT1080 cell line.

Plasmid construction

$\alpha 4$ promoter deletion constructs have been described previously (Rosen *et al.*, 1994). Numbers in the construct name indicate the amount of 5'-flanking sequence present. PSV-CAT has been described previously (Rosen *et al.*, 1994; Weintraub *et al.*, 1995). Fragments of the $\alpha 4$ gene promoter were obtained by PCR with primers containing *Kpn*I (5') and *Apa*I (3') sites (Rosen *et al.*, 1994). The products were digested with

KpnI-*ApaI* 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 *ApaI* 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 ACGCGT or ACCCCA, which blocked nuclear protein binding (data not shown).

Prokaryotic expression vector for human *c-myb* Flag (Flag-*c-myb*) and eukaryotic expression vectors pSV and pSV-*c-myb* were obtained from B.Calabretta. Expression vectors for E12 and E47 bHLH E proteins were obtained from F.Peverali (EMBL, Heidelberg, Germany). The expression vector for ZEB was constructed as described previously (Postigo and Dean, 1997). The dominant-negative c-Myb MT construct was obtained from K.Weston (Institute for Cancer Research, London, UK). Gal4-*c-Myb* CD was obtained from J.S.Lipsick (State University of New York, Stony Brook, NY). pG-L was described previously (Weintraub *et al.*, 1995). Full-length ZEB cDNA was fused in-frame with the DBDs of Gal4 and LexA proteins by cloning in *Bam*HI-*Xba*I sites of PM1 and pBXL3 (Weintraub *et al.*, 1995) to make Gal4-ZEB (G-ZEB) and LexA-ZEB (L-ZEB) fusion proteins, respectively.

The pETS-ZEB-MYB-CAT reporter construct containing Ets sites, ZEB sites and c-Myb sites was constructed in the following way: two copies of the E361 site in $\alpha 4$ integrin (including 6 bp upstream and downstream of the E box consensus site) were cloned in the *KpnI*-*ApaI* sites of 76 α 4CAT (pETS-CAT) to make pETS-ZEB-CAT. Then, four copies of the c-Myb site at -134 bp in the $\alpha 4$ gene (other c-Myb sites from the $\alpha 4$ promoter were also used with identical results) were cloned in *Clal*-*StuI* sites of a polylinker blunt ended and cloned at the *KpnI* site to make pETS-ZEB-MYB-CAT. pETS-MYB-CAT was constructed in a similar way but lacking the two copies of the E361 site.

Transient transfections and CAT assays

Jurkat, HEL and Raji cells were transfected by electroporation, and other cells were transfected by the calcium phosphate method (Rosen *et al.*, 1994). After 48 h, lysates were collected, the transfection efficiency was corrected and CAT assays were performed 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 media containing 2% horse serum and cells were maintained for 4 days before harvesting for analysis of CAT activity (Rosen *et al.*, 1992).

Gel retardation assays

Nuclear extracts were prepared as described (Osborn *et al.*, 1989). Oligonucleotide probes were end labeled with [γ - 32 P]ATP using T4 polynucleotide kinase. The *Eco*RI ends of the -130/400 $\alpha 4$ fragment were labeled with Klenow fragment and [α - 32 P]dATP. Two to three μ g of nuclear protein extracts or 0.1 μ l of bacterial extracts from GST-ZEB-transformed bacteria were incubated with 1 μ g of bovine serum albumin (BSA) and 0.5 μ g of poly(dIdC) in 25 μ l of reaction mix containing 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 1 mM EDTA, 10% glycerol for 10 min on ice in the presence or absence of a 50-fold excess of unlabeled probe or 0.5 μ g of antibodies per reaction. After 10 min, 6 fmol of labeled probe was added for 10 min and incubated on ice, followed by another 10 min at room temperature. Then, samples were subjected to electrophoresis as described (Rosen *et al.*, 1994). Antibodies against USF, myoD, HEB, E2 proteins and c-Myb were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibody against ZEB was obtained from H.Kondoh (Osaka University, Osaka, Japan) (Funahashi *et al.*, 1993).

FACS analysis

Approximately 1×10^5 cells were incubated at 4°C with 50 μ l of hybridoma culture supernatant of TS2/16 ($\beta 1$ integrin) or HP2/1 ($\alpha 4$ integrin) mAbs (F.Sanchez-Madrid, Hospital de la Princesa, Spain) (Pulido *et al.*, 1991) or 50 μ l of 10 μ g/ml solutions of PID6 mAb ($\alpha 5$ integrin, Becton Dickinson, Palo Alto, CA) or control mouse IgG1 or IgG2a (Sigma Chem. Co, St. Louis, MO) for 30 min. After two washes with phosphate-buffered saline (PBS), cells were incubated with a goat anti-mouse Ig F(ab')₂ fragment-fluorescein isothiocyanate (FITC) (1:100 dilution) (Dako, Glostrup, Denmark) for another 30 min at 4°C, and green fluorescence was examined by flow cytometry using a FACScan.

Tissue staining

Tissue sections from *c-myb* (-/-), (-/+) and (+/+) mice (Mucenski, 1991) were immunostained with anti- $\alpha 4$ integrin (PS/2, Pharmingen,

San Diego, CA), TER 119 antigen (TER119, Pharmingen) and CD45 (I-3/2.3, M.Thomas, Washington University, MO) and examined by fluorescence microscopy. Cell staining was performed as described (Mucenski *et al.*, 1991).

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