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Histone H3 tail positioning and acetylation by the c-Myb but not the v-Myb DNA-binding SANT domain

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The c-Myb transcription factor coordinates proliferation and differentiation of hematopoietic precursor cells. Myb has three consecutive N-terminal SANT-type repeat domains (R1, R2, R3), two of which (R2, R3) form the DNA-binding domain (DBD). Three amino acid substitutions in R2 alter the way Myb regulates genes and determine the leukemogenicity of the retrovirally transduced v-Myb oncogene. The molecular mechanism of how these mutations unleash the leukemogenic potential of Myb is unknown. Here we demonstrate that the c-Myb–DBD binds to the N-terminal histone tails of H3 and H3.3. C-Myb binding facilitates histone tail acetylation, which is mandatory during activation of prevalent differentiation genes in conjunction with CCAAT enhancer-binding proteins (C/EBP). Leukemogenic mutations in v-Myb eliminate the interaction with H3 and acetylation of H3 tails and abolish activation of endogenous differentiation genes. In primary v-myb-transformed myeloblasts, pharmacologic enhancement of H3 acetylation restored activation of differentiation genes and induced cell differentiation. Our data link a novel chromatin function of c-Myb with lineage-specific expression of differentiation genes and relate the loss of this function with the leukemic conversion of Myb.

[*Keywords*: Myb; chromatin; hematopoiesis; leukemia; transcription]

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Classical retroviral oncogenes induce transformation and tumorigenic conversion of particular cell types at distinct differentiation stages and in distinct tissues. This suggests that retroviral oncoproteins interfere with epigenetic processes that direct cell maturation and multiplication.

The retroviral oncoprotein v-myb of avian myeloblastosis virus (AMV) is an example of an abducted and mutated version of a cellular transcription factor that plays a decisive role in blood cell development, proliferation, and differentiation (Hall et al. 1941; Mucenski et al. 1991). The cellular predecessor of *v-myb,* the proto-oncogene *c-myb,* encodes a metabolically labile gene regulatory protein that is essential for adult hematopoiesis. *Myb* has been repeatedly captured by avian leukemia viruses. Its genomic locus is also a frequent target of retroviral integration in mice. In addition, the *c-MYB* gene is overexpressed in a variety of human leukemias, and its down-regulation is a prerequisite for proliferation arrest (Wolff 1996). Transgenic mice that overexpress c-Myb protein, however, do not develop tumors (Furuta et al. 1993), suggesting that the proto-oncoprotein c-Myb requires further activation to unleash its tumorigenic potential (Graf 1992; Lipsick 1996). In addition to proliferation control, several lines of evidence also suggest that c-Myb is involved in cell differentiation (Ness et al. 1993; Kasper et al. 2002; Emambokus et al. 2003), raising the possibility that the cellular role of the Myb protein is to coordinate cell multiplication and cell specification.

The AMV strain transduces an acutely transforming N- and C-terminally truncated viral Myb (v-Myb) oncoprotein that also harbors 11 amino acid substitutions. AMV induces myeloid leukemia in chicken and transforms myeloid precursors (myeloblasts) in vitro (Graf and Stehelin 1982; Ness 1996; Lipsick and Wang 1999). Whereas truncation of Myb activates the protein, several of the amino acid substitutions sustain the transformation strength of v-myb (Dini et al. 1995). Three mutations in the DNA-binding domain (DBD) of *v-Myb,* however, determine both the cell lineage specificity and the leukemogenic potential in the animal (Introna et al. 1990). Interestingly, these amino acid substitutions do not affect recognition of *cis*-regulatory Myb-binding sites on DNA (Brendeford et al. 1997). Structural analysis showed that these three critical amino acid substitutions replace solvent-exposed hydrophobic amino acid side-chains (I91, L106, and V117 are replaced by N, H, and D, respectively) that are located on a side of Myb that faces away from the DNA (Tahirov et al. 2002). Accordingly,

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these data may indicate that v-Myb is devoid of protein interactions that normally prevent a leukemogenic activity of c-Myb.

The C-terminal leucine zipper of CCAAT enhancer-binding protein β (C/EBPβ) interacts with the surface of R2 in c-Myb, and the AMV-specific amino acid substitutions in R2 abrogate accommodation of C/EBPβ (Brendeford et al. 1997; Tahirov et al. 2002). Concomitantly, C/EBP and v-Myb fail to cooperate in the activation of the prototypal differentiation genes mim-1 and lysozyme (Introna et al. 1990; Kowenz-Leutz et al. 1997). Although the structural data provided a compelling explanation for failure of *v-myb* to participate in cell maturation and to block differentiation, individual back mutations, domain swap mutants of Myb (this study; A. Leutz and E. Kowenz-Leutz, unpubl.), and leucine zipper exchange mutants in C/EBP (swap of C/EBP leucine zipper to that of the cAMP response-binding protein, CREB; data not shown) indicated that the physical interaction between Myb and C/EBP and activation of endogenous genes could be separated. Accordingly, the R2 mutations in v-myb appear to extinguish another important function of Myb.

Each of the three Myb repeats—including R2 and R3, which together establish a compound DBD—carry the architectural signature of the chromatin-interacting SANT domain, previously identified in several chromatin regulatory proteins (e.g., the Swi3, Ada2, TFIIIB, NcoR, and ISWI proteins) (Aasland et al. 1996). However, a direct connection between Myb domains that bind to DNA and chromatin remodeling has not yet been shown. SANT domains appear to be devoid of enzymatic activity but are functionally involved in histone acetylation, deacetylation, and ATP-dependent remodeling (Boyer et al. 2002; Sterner et al. 2002). Several reports on SANT domain functions indicate that this domain serves as a protein interaction domain that binds to histone modifying enzymes while it simultaneously facilitates substrate recognition and enhances enzyme activity (see Discussion).

Here we show that the wild-type Myb–DBD, but not its leukemogenic v-Myb cousin, interacts with the N-terminal tail of histone H3 and positions it for acetylation. Both histone tail binding and acetylation are prerequisites for the activation of differentiation genes. These data thus demonstrate for the first time that Myb displays a function in chromatin organization by directing histone modifications through its SANT–DBD, and that these functions are lost in its leukemogenic counterpart.

Results

The Myb SANT–DBD binds to the N-terminal tail of histone H3

Protein interaction screening using the SANT-Myb domain of c-myb or v-myb to disclose binding partners has been largely deferred by the toxicity of the Myb–DBD in yeast. We argued that this could be due to the fact that the domain binds to yeast DNA and interferes with gene expression. We sought to overcome this limitation by introducing a mutation in the Myb–DBD (MybK128M) that is deficient for binding to *cis*-regulatory Myb sites but that retains the overall SANT domain structure and that could therefore be used as a bait for screening (Oda et al. 1998).

The mutant Myb–DBD-bait (MybK128M) was used in a yeast two-hybrid screen (Ansieau and Leutz 2002) of an adult human bone marrow cDNA library. A full-length clone of the human histone H3 variant H3.3 was isolated. The specificity of the interaction between c-Myb and histones was examined by subjecting purified human histones to a GST–c-Myb DBD construct. As shown in Figure 1A, only histone H3 interacts with GST–c-Myb–DBD, confirming the screening result.

Nucleosomal histone proteins consist of regulatory N-terminal flexible tail and nucleosomal C-terminal core domains. The H3.3 tail domain (amino acids 1–42) and the core domain (amino acids 42–136) were expressed separately as GST constructs and examined for interaction with the Myb–DBD. As shown in Figure 1B, only the N terminus, but not the core, of H3.3 interacts strongly with the Myb–DBD. Since H3 and H3.3 have highly conserved N-terminal domains (Fretzin et al. 1991; Akhmanova et al. 1995), we compared the inter-

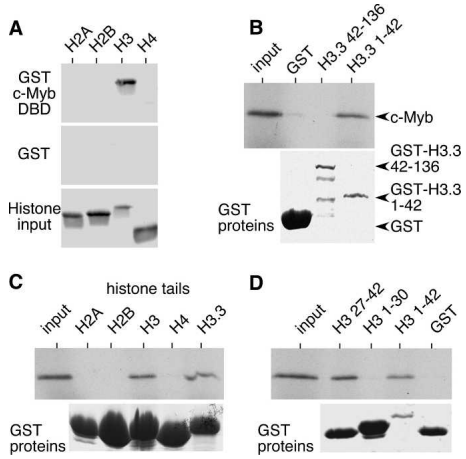

Figure 1. C-Myb–DBD Interacts with the histone H3 tail. (*A*) Matrix-bound GST–c-Myb–DBD was subjected to purified, recombinant human histones as indicated on the *top*. Beads were extensively washed, and bound protein was subjected to SDS-PAGE and revealed by silver staining. (*B*) GST–histone H3.3 fragments (amino acids 1–42 and 42–136), as indicated, or GST control was incubated with in vitro ^{35}S-methionine-labeled c-Myb–DBD. Specifically bound proteins were subjected to SDS-PAGE and revealed by fluorography. (*C*) Affinity binding as in *B* using GST–histone tail (human) constructs of H2A, (amino acids 1–35), H2B (amino acids 1–35), H3 (amino acids 1–46), H4 (amino acids 1–34), or H3.3 (amino acids 1–42). (*D*) Comparison of c-Myb binding to various GST–histone H3 tail constructs. *Bottom* panels (GST protein) in *B–D* show Coomassie stain of GST constructs used in the binding experiments as shown above. Input represents 15% of the material used for pull-down assays.

action between c-Myb and both H3 and H3.3 N-terminal histone tails to the N-terminal tails of other histones. As shown in Figure 1C, both H3 and H3.3 N termini bind to c-Myb–DBD, whereas the N termini of H2A, H2B, and H4 failed to do so. Further mapping of the H3 N-terminal tail, as shown in Figure 1D, suggests that the major binding site for c-Myb–DBD resides between amino acid residues 27 and 42 of H3.

Interaction between the N terminus of H3 and c-Myb or v-Myb

The retrovirally transduced v-Myb leukemia protein of AMV carries four amino acid substitutions in its v-Myb–DBD (three in R2—I91, L106, V117—and one, I181, in R3, are replaced by amino acids N, H, D, and V, respectively) (positions are indicated by dots in Fig. 2A). Whereas the R3 mutation represents a conservative amino acid exchange that has not been found to affect the Myb biology, single R2 back mutations of v-Myb to the wild-type configuration (N91I, H106L, or D117V) dramatically alter the phenotype and differentiation stage, restore the ability to activate differentiation genes

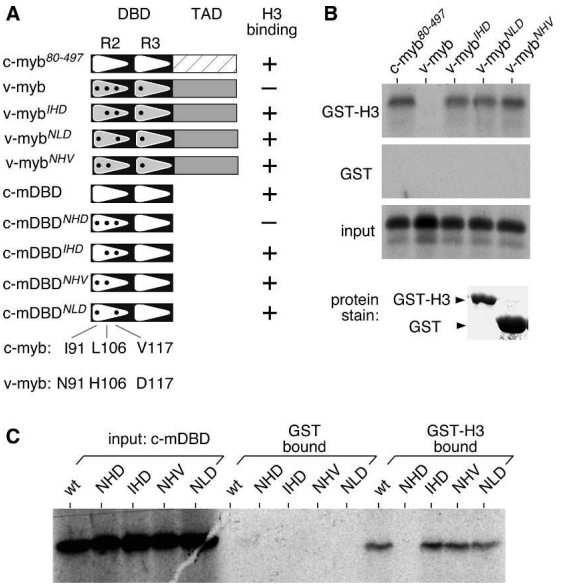

Figure 2. Configuration of the Myb–DBD and H3 tail binding. (*A*) Overview of Myb constructs and summary of H3 binding data as shown in *B* and *C*. (*B*) In vitro-translated, ^{35}S-methionine-labeled c-Myb (N- and C-terminally truncated, amino acids 80–497) or v-Myb was incubated with matrix-bound GST–histone tails or GST as a control. (*Upper* panels) Specifically bound proteins were revealed by SDS-PAGE and fluorography. GST panel shows unspecific binding to GST control, and input panel represents 15% of the labeled material used for pull-down assays. *Bottom* panel shows protein stains of GST–H3 and GST as indicated. (*C*) Pull-down of various ^{35}S-methionine-labeled Myb–DBD proteins by GST–H3. Matrix-bound GST fusion proteins were incubated with in vitro-translated, labeled Myb–DBD mutants. Input represents 15% of the labeled material used for pull-down assays.

in concert with C/EBP, and diminish the leukemogenic potential (Introna et al. 1990). Thus, the v-myb R2 mutations (I91N, L106H, and V117D) are essential for the myeloid differentiation block, maintenance of self-renewal during transformation and tumorigenicity.

Binding assays with v-Myb and c-Myb were performed to determine whether wild-type and leukemogenic Myb proteins interact differentially with the H3 tail. As shown in Figure 2B, truncated c-Myb binds to the H3 tail, whereas v-Myb did not. Individual back mutations in v-Myb to c-Myb–DBD that decrease its leukemogenicity and alter the transformed phenotype restored the interaction with the H3 tail (Fig. 2B).

Amino acid substitutions, such as those that occur in v-Myb, were introduced into R2 of c-Myb to further discriminate between effects of the DBD and mutations elsewhere in the oncoprotein. As shown in Figure 2C, the three AMV-specific amino acid substitutions of R2 (I91N, L106H, V117D) that are critical for its leukemogenic potential also abrogated binding to H3. Individual amino acid substitutions (data not shown) or double substitutions (Fig. 2C) in the c-Myb R2-SANT domain were not sufficient to abrogate interactions with the H3 tail. These results show that the c-Myb–DBD interacts with the H3 tail and that the triad of v-Myb–DBD amino acid substitutions, previously shown to determine both the leukemogenicity and the transformed phenotype, is simultaneously required to abolish the interaction with the H3 tail.

Although c-Myb and v-Myb display similar DNA sequence binding specificities, it has been suggested that the DNA–protein complexes exhibit different stabilities (Brendeford et al. 1997). To exclude the possibility that decreased DNA binding accounts for a loss of H3 interaction on endogenous Myb-target genes, we first compared binding of v-Myb and c-Myb to target genes by chromatin immunopreciptation assay (ChIP, schematically shown in Fig. 3A). As shown in Figure 3B, wild-type and all mutated Myb proteins interact with their natural, chromatin-embedded target genes in cells, although some quantitative differences of promoter-bound Myb variants were observed (Fig. 3B,C) that might be due to differences in local promoter architectures or antibody accessibility during ChIP. Nevertheless, these results show that the mutations in the v-Myb protein do not prevent its association with target promoters. Accordingly, the interaction with H3 tails is not required for Myb to find its binding sites in responsive genes. Of particular attention is the v-Myb N91I back mutation (Fig. 3C, v-myb^{IHD}) that was reported to display somewhat reduced DNA-complex stability similar to v-Myb (Brendeford et al. 1997). Similar ChIP quantities of both v-Myb and v-Myb N91I were recovered, but only the v-Myb N91I mutant regained H3 tail binding (Fig. 2) concomitantly with the activation of differentiation genes and the loss of leukemic differentiation block (Introna et al. 1990). The results shown in Figure 3 therefore demonstrate that the leukemogenic potential and the myelomonocytic differentiation block of v-Myb converge

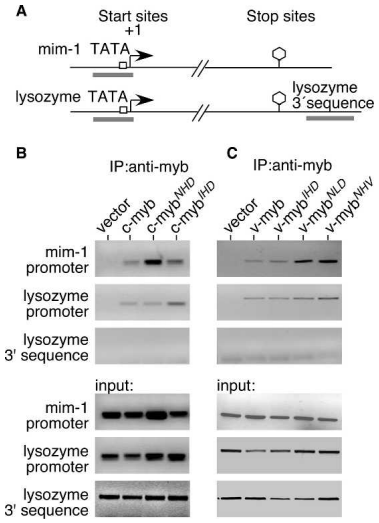

Figure 3. Target gene binding of Myb is independent of H3 tail binding. (*A*) Schematic representations of mim-1 and lysozyme genes indicating the location of amplification products (gray bars), TATA boxes, transcription start sites (+1), and transcription stop sites. (*B*) Truncated c-Myb proteins (amino acids 80–497), amino acid substitution mutants of c-Myb, or vector controls were transfected into QT6 fibroblasts. Cell lysates were analyzed by ChIP with anti-Myb antibody, and coimmunoprecipitated DNA was PCR amplified, separated by electrophoresis, and shown as a negative image of an ethidium bromide-stained gel. Input lanes (*lower* panel) represent 0.1% of material used for IP. Myb constructs as shown in Figure 2A. (*C*) Same as in *B* using v-Myb and mutants of v-Myb.

through the three amino acid substitutions in the abrogation of H3 interaction.

H3–Myb interaction and histone acetylation

The c-Myb and v-Myb proteins interact through their transactivation domain with the KIX domain of the histone acetyl transferase (HAT) p300 (Mink et al. 1997; Kasper et al. 2002). The interaction between Myb and p300 is essential for target gene activation and Myb-dependent megakaryocyte differentiation (Kasper et al. 2002). Although the p300–Myb interaction does not appear to involve the Myb–DBD, we assessed whether v-Myb–DBD mutations might nonetheless affect the interaction with p300 on cellular genes. As shown in Figure 4A, ChIP analysis revealed that enhanced association of p300 with Myb target genes depends on the presence of Myb but is not affected by v-Myb–DBD mutations. In addition, immunoprecipitation (Fig. 4B) showed that Myb associates with p300 irrespective of the amino acid substitutions in the DBD and thus independently of the interaction between the Myb–DBD and the histone H3 tail (Fig. 4B).

P300 and/or potentially p300-associated acetyl transferases may catalyze the acetylation of K18 and K23 of the H3 N terminus during gene activation in vivo (Daujat et al. 2002). We therefore determined whether various Myb derivatives would catalyze the acetylation of H3 tails at endogenous Myb target genes in the absence and presence of cotransfected p300. As shown in Figure 5A, acetylation of H3 K18 and K23 at both Myb target genes, mim-1 and lysozyme, was enhanced by c-Myb or Myb derivatives that contain one of the three critical R2 amino acids in wild-type configuration. Moreover, cotransfected p300 enhanced H3 acetylation under these conditions. No such H3 acetylation-dependent ChIP fragments were found in the vector controls, or with v-Myb or with c-myb that harbor the three critical v-Myb amino acid substitutions in R2. Furthermore, individual back mutations in v-Myb toward the c-Myb–DBD configuration, which abate leukemogenicity and change phenotype, also restored the ability to promote acetylation of H3 tails. In addition, methylation of H3 R17 was detectable in p300-transfected cells in combination with c-Myb but not with v-Myb, whereas no changes were seen on H4 K5 or K8 acetylation (data not shown). These data suggest that several histone modifications might occur when Myb interacts with p300 and H3 tails, potentially by p300-associated CARM1 (Xu et al. 2001; Daujat et al. 2002). Next, we examined the H3 acetylation stage in primary chick bone marrow cells infected

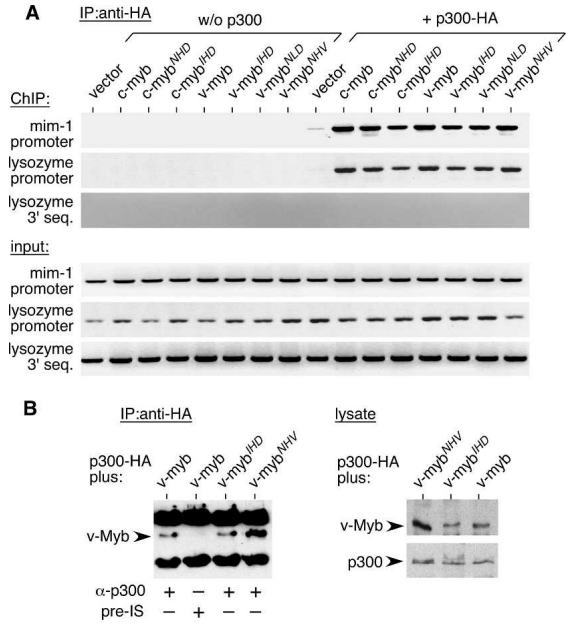

Figure 4. c-Myb and v-Myb proteins recruit p300 to chromatin-embedded promoters independently of H3 tail binding. (*A*) C-Myb and v-Myb proteins or mutants of c-Myb or v-Myb were transfected together with HA-tagged p300 into QT6 fibroblasts. Chromatin was immunoprecipitated with anti-HA antibody. Coprecipitated DNA was analyzed by PCR amplification. A negative image of an ethidium bromide-stained gel is shown. (*Lower* panel) Input lanes represent 0.1% of material used for IP. (*B*) Myb and p300 expression and IP controls. v-Myb proteins were expressed together with p300 in QT6 fibroblasts. (*Left*) IP with HA or preimmune serum samples were separated by SDS-PAGE, blotted, and revealed by anti-Myb immunostaining. Cell lysate controls of SDS-PAGE separated and blotted proteins show expression of transfected Myb and p300 proteins.

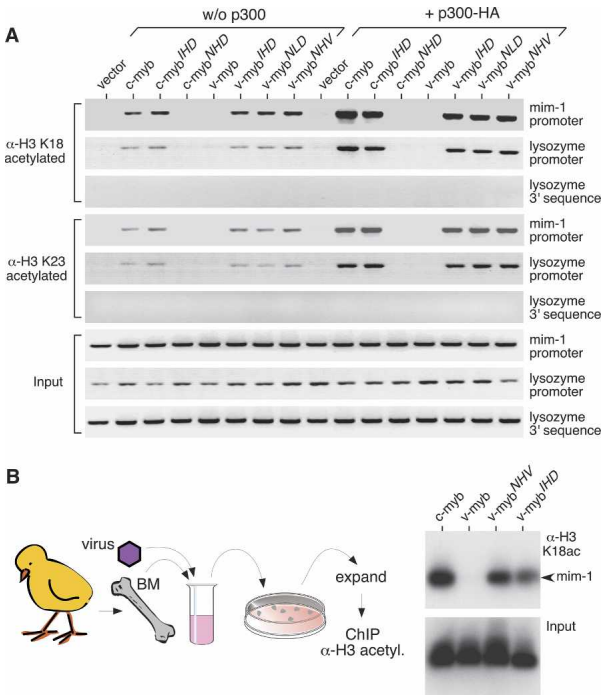

Figure 5. Acetylation of H3 K18 and K23 at Myb target genes depend on H3 tail binding and p300 recruitment by Myb. (*A*) Vector control or c-Myb proteins and mutants thereof were transfected into QT6 cells without (*left*) or together with (*right*) p300, as indicated on the *top*. Chromatin was immunoprecipitated with antibodies directed toward acetylated H3 K18 or K23 as shown on the *left*. Coprecipitated DNA was analyzed by PCR amplification. A negative image of an ethidium bromide-stained gel is shown. Input lanes represent 0.1% of material used for IP. (*B*) Bone marrow cells were isolated from a 1-d-old chicken and infected with recombinant viruses encoding truncated c-myb or myb–DBD mutants as shown on the *right* (retroviral constructs indicate lanes of ChIP analysis) and in Introna et al. (1990). Transformed myeloblasts were expanded under appropriate conditions, and ChIP analysis was performed from as indicated on the *right*.

with various retroviral Myb constructs (Fig. 5B). The results show that also in primary myeloblasts, under physiological conditions, acetylation of H3 at the endogenous mim-1 promoter reflects the configuration of the R2 SANT domain, confirming the data presented in Figure 5A. Thus, although all Myb derivatives that contain the transactivation domain can recruit p300 to their target genes, only those Myb proteins that also interact with the H3 tail promote H3 acetylation in the vicinity. These results therefore indicate that (1) the c-Myb-R2 SANT domain is involved in H3 positioning in nucleosomes for subsequent acetylation, and (2) v-Myb is defective for this function and withdraws from assisting in the expression of genes required for cell differentiation.

Gene activation and Myb-mediated H3 acetylation

C/EBPs collaborate with c-Myb but not with v-Myb to activate myeloid genes (Ness et al. 1993; Kowenz-Leutz et al. 1997). The N terminus of C/EBPβ supplies the chromatin remodeling complex SWI/SNF that is essential for cell differentiation and for the activation of a set of granulocytic genes (Kowenz-Leutz and Leutz 1999; Pedersen et al. 2001). The DBD of c-Myb, but not the DBD of v-Myb, can interact with the bZip region of C/EBPβ when both transcription factors are bound to their *cis*-regulatory sites at a distance and loop out intervening DNA (Ness et al. 1993; Tahirov et al. 2002). We therefore wondered about the importance of the individual contributions of H3 tail acetylation by Myb, on one hand, and SWI/SNF recruitment by C/EBP, on the other hand. To address this question, several chimeric Myb mutants were constructed that combine viral and cellular Myb with the N terminus of C/EBPβ (Fig. 6A). We have previously shown that fusing the N-terminal SWI/SNF recruiting domain of C/EBPβ to Myb yields a chimeric transcription factor that forcefully recruits SWI/SNF and thus supersedes further C/EBPβ requirement to activate endogenous Myb target genes (Kowenz-Leutz and Leutz 1999). As shown by immunoprecipitation in Figure 6B, chimeras between the N terminus of C/EBPβ and c-Myb or v-Myb proteins may recruit the SWI/SNF complex as indicated through binding of brahma (hBrm), a core factor of the SWI/SNF complex (Fig. 6B, upper panel). Expression controls (Fig. 6B, lower panel) further showed similar expression of the constructs. ChIP assays (Fig. 6C) of the mim-1 promoter further revealed that both βN-c-myb and βN-v-myb chimeras may recruit brahma and p300 to the mim-1 promoter, suggesting that chromatin remodeling plus histone modification machineries are available at relevant genes. However, acetylation of H3 occurs only with Myb constructs that harbor wild-type Myb R2–SANT domains. Gene expression examined by Northern blotting (Fig. 6D) further revealed that activation of the endogenous mim-1 target gene, which essentially requires SWI/SNF, nevertheless remains entirely dependent on the ability of R2 to interact with H3 tails. These data suggest that both chromatin remodeling and histone acetylation may occur independently of each other but are required for the activation of endogenous genes. The data also suggest that the oncogenic R2 domain of v-myb represents a loss of function that defeats interaction with and acetylation of H3 tails to block activation of differentiation genes.

These results imply that Myb H3 tail interaction and acetylation is a bottleneck during the activation of differentiation genes by Myb transcription factors. The acetylation state of histones, however, is balanced by histone acetylases and histone deacetylases (HDACs). We therefore wondered whether the HDAC inhibitor Trichostatin A (TSA), which enhances H3 acetylation, would override repression of differentiation genes in v-myb-transformed hematopoietic cells. To test this possibility, chick bone marrow cells were infected with the classical leukemogenic AMV, and transformed myeloblasts were grown without and with TSA to monitor H3 acetylation and gene expression in primary leukemic cells. As shown in Figure 7A, both mim-1 and lysozyme genes were barely expressed in AMV-trans-

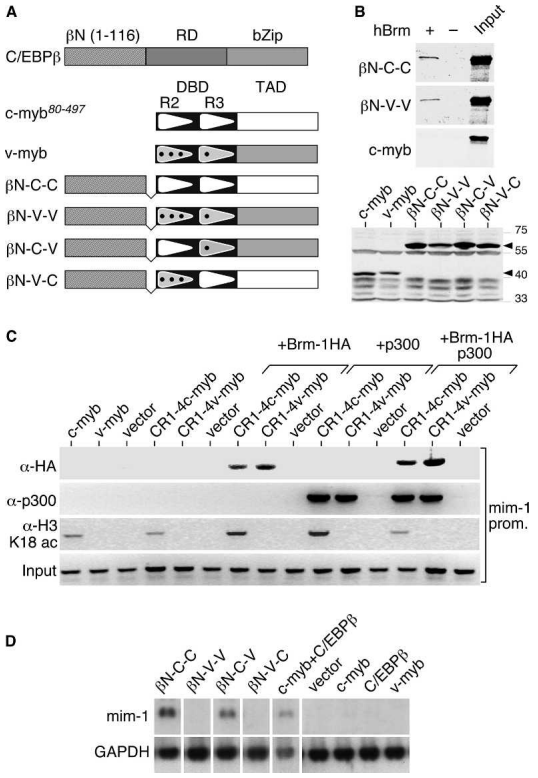

Figure 6. The Myb–DBD determines H3 tail acetylation and gene activation in chimeric C/EBPβ–Myb domain swap proteins. (*A*) Schematic representation of the constructs. C/EBPβ has an N-terminal SWI/SNF recruiting and transactivation domain (βN, amino acids 1–116), a central regulatory domain (RD) and a C-terminal b-Zip domain. The Myb–DBD is indicated as a black box. R2 and R3 Myb repeats are shown as triangles (white and gray triangles represent c-Myb and v-Myb DBD, respectively), and v-Myb mutations are indicated as black dots. The C-terminal transactivating fragment of c-Myb or v-Myb (TAD) are depicted as white and gray boxes. Chimeric constructs consist of the C/EBPβ N terminus (βN) fused in frame to Myb. Chimeric Myb proteins consisted of c-Myb (βN-C-C) or v-Myb (βN-V-V), or domain swaps (βN-C-V; βN-V-C) as indicated. (*B*, *upper* panel) Fusion proteins as indicated were translated in vitro in the presence of ^{35}S-methionine and mixed with or without (+/–) in vitro-translated hemagglutinine HA-tagged hBrm (without radioactive label), as indicated. Complexes were immunoprecipitated with a HA-specific antibody (αHA-IP, indicated on the *top*) and revealed by SDS-PAGE and fluorography. Input lanes represent 10% of material used for IP. (*Lower* panel) Cell lysate controls of SDS-PAGE separated and blotted proteins to show expression of chimeric proteins. (*C*) Expression vectors encoding chimeric proteins as indicated were transfected together with the coactivators or combination thereof as indicated on the *top*. ChIP was performed using antibodies to HA, p300, or acetylated H3 as indicated on the *left*. Coprecipitated DNA encompassing the mim-1 promoter was analyzed by PCR amplification. A negative image of an ethidium bromide-stained gel is shown. Input lanes represent 0.1% of material used for IP. (*D*) Expression vectors encoding chimeric proteins as indicated in *A* or controls (c-myb + C/EBPβ, vector, c-myb, C/EBPβ, v-myb) were transfected into QT6 fibroblasts. RNA was harvested after 24 h, and RNA blots were probed with *mim-1* and subsequently with GAPDH as internal control.

formed myeloblasts. Treatment with TSA, however, strongly enhanced the acetylation of H3 K18 and K23 and concomitantly strongly enhanced Myb-target gene expression. Controls showed equal loading of RNA blots, and v-Myb and C/EBPβ controls showed only minor effects of TSA on protein expression. Moreover, TSA treatment overcame the v-myb differentiation block and induced morphologic differentiation of self-renewing myeloblasts growing in suspension into adherent macrophages (Fig. 7B). Taken together, these data show that the differentiation block of the classical *v-myb* oncogene is linked to a defective epigenetic function of the oncoprotein.

Discussion

Here we describe a novel function of c-Myb: The DBD of c-Myb is able to bind to the N-terminal tail of histone H3 and to make it accessible for acetylation. Binding of the Myb–DBD to histones is specific for H3 and H3.3 tails and is a prerequisite for the activation of myeloid genes characteristically expressed in differentiating cells. The leukemogenic v-Myb version transduced by the classical AMV carries three amino acid substitutions in its DBD that disrupt H3 interaction, H3 tail acetylation, and, subsequently, the activation of chromatin embedded genes involved in cell differentiation. Our results show for the first time that the c-Myb–DBD carries a function that is characteristic for SANT domain proteins involved in histone acetylation and that v-Myb is deficient in cata-

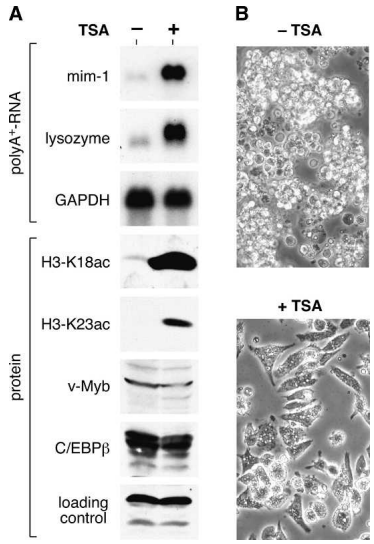

Figure 7. TSA overrides the v-myb differentiation block and activates histone acetylation and myeloid differentiation genes. (*A*) AMV-transformed myeloblasts were treated without or with TSA (+/– TSA) as indicated. Polyadenylated RNA was analyzed by Northern blotting, and cellular protein was analyzed by SDS-PAGE and Western blotting. (*B*) Micrographs of AMV-transformed myeloblasts. AMV-myeloblasts grow as suspension cells (–TSA) and acquire an adherent macrophage phenotype when treated with TSA (+TSA) for 48 h.

lyzing this covalent chromatin modification, linking defective chromatin remodeling by v-Myb to its tumorigenic potential.

Differential H3 tail acetylation by c-Myb and v-Myb

The results presented here show that the c-Myb–DBD interacts specifically with histone H3 and/or H3.3 tails. The physical interaction between H3 tail and c-Myb–DBD is required for acetylation of K18 and K23 and is a prerequisite for gene transcription. Our results show that acetylation of both sites occurs in a p300-dependent fashion. Although it has been shown that K18 and K23 are targets of acetylation by p300, we can not distinguish whether p300 itself or another associated histone acetyltransferase (HAT) modifies the N terminus of H3 in a Myb-dependent fashion. Regardless of the molecular details, it is conceivable that the interaction between Myb and the H3 tail positions the flexible H3 N terminus for its subsequent modification by histone modifying enzymes, including acetylation of K18 and K23.

Mutations in the DBD that render v-Myb leukemogenic abolish the interaction with H3 tails. As a consequence, H3 tails at mim-1 and lysozyme loci are not acetylated at K18 and K23 in the presence of v-Myb and both genes remain silent. Our data thus provide an explanation for how c-Myb participates in the modification of chromatin during activation of differentiation genes and argues for a dominant-negative role of its leukemic counterpart v-Myb. It is interesting to note that among the mutations accrued in v-Myb, only the three amino acid substitutions affecting R2 of the Myb DBD are required simultaneously to maintain its high leukemogenic potential. Any back mutation to the wild-type configuration in R2 diminishes the leukemogenicity, prolongs the latency, alters the phenotype of transformed cells, and restores the interaction with and the acetylation of the H3 tail. It has been shown that the three amino acid substitutions in R2 remove a hydrophobic patch on the surface of R2, and alter the packaging mode of the hydrophobic R2 core (Tahirov et al. 2002). It has also been suggested that the R2 surface might participate in the interaction between c-Myb and other coregulatory factors such as p100 and Cyp40 (Leverson and Ness 1998; Leverson et al. 1998), D-type cyclins (Ganter et al. 1998), HSF3 (Kanei-Ishii et al. 1997), or C/EBP (Tahirov et al. 2002). It will therefore be interesting to uncover the structural basis of the histone c-Myb interaction to further examine whether c-Myb interacts with other proteins and H3 simultaneously.

Differential interaction between C/EBPs and c-Myb or v-Myb

The c-Myb and v-Myb DBDs were previously shown to differentially interact with C/EBPs, a family of transactivators involved in hematopoietic lineage decision and in proliferation control. C/EBPs are required for the synergistic activation of myeloid genes and for myeloid cell maturation (Ness et al. 1993; Muller et al. 1995). Removal of the hydrophobic patch on R2 by the leukemogenic v-Myb mutations disables the interaction with the C-terminal bZip domain of C/EBPs. It was therefore speculated that failure of the v-Myb–DBD to interact with C/EBP might reflect the block in differentiation and the leukemogenic potential of v-Myb (Tahirov et al. 2002), a conclusion that is difficult to reconcile with biological results obtained with a C/EBP-binding-deficient v-Myb mutant (Introna et al. 1990). Results presented here suggest, however, that defective histone acetylation is a domineering effect of the v-Myb oncoprotein that, together with a defective C/EBP interaction, inhibits activation of differentiation genes.

This conclusion is derived from results obtained with chimeric proteins that fuse the transactivation domain of C/EBP with either c-Myb or v-Myb. We have shown previously that a chimeric c-Myb construct with the C/EBPβ N terminus fused to Myb takes over functions of the otherwise bipartite Myb–C/EBP switch: The chimeric protein recruits the SWI/SNF complex and thus no longer requires solitary C/EBPβ during mim-1 and lysozyme activation (Kowenz-Leutz and Leutz 1999). The transcriptional cooperation between Myb and C/EBP therefore occurs through functional domains on different transcription factors that can be combined into a single transcription factor. In fact, the fusion protein that contains c-Myb and the C/EBPβ N terminus becomes hyperactive for mim-1 transcription, compared with the regular C/EBP–Myb interaction. This suggests that cooperative events that occur between functional domains located on different transcription factors may become more efficient once the two proteins are in close proximity. It was therefore surprising to observe that a similar chimeric transcription factor that carries only the R2 SANT domain mutations of v-Myb entirely failed to activate target genes. This finding suggested that although all required components, such as the SWI/SNF and histone acetylation complexes, are recruited to target loci, acetylation of H3 tails remains essential and can not be bypassed by forced SWI/SNF recruitment.

The data also suggest that remodeling and acetylation at target loci may occur independently of each other and are simultaneously required during gene activation in our experimental setting. What remains to be determined is whether in normal hematopoiesis both events are temporally separated. Published data from others suggest that loci of myeloid genes are somehow primed early in hematopoietic precursor cells, before the onset of expression of differentiation genes. Yet, TSA treatment of precursors that do not express C/EBP leads to profound alterations at the –3.9-kb lysozyme enhancer without inducing lysozyme expression, whereas TSA treatment in cells that do express C/EBP also induced lysozyme gene expression (Kontaraki et al. 2000; Lefevre et al. 2001). However, how this relates exactly to acetylation of histones or remodeling of nucleosomes at rel-

evant sites has still to be addressed. Nevertheless, the data presented here and by others suggest that SWI/SNF-mediated remodeling at target sites becomes imperative for gene transcription in concert with H3 modifications and vice versa.

Gain and loss of functions of v-Myb

The inability of v-Myb to bind to H3 tail and to C/EBP lends support to the idea that the v-Myb–DBD mutations convert the protein into a dominant-negative transcription factor (Lipsick and Wang 1999). However, at least one target gene of Myb, termed GBX2, is activated constitutively by v-Myb but not by c-Myb. In contrast to myeloid differentiation genes that are coregulated by C/EBPs, activation of GBX2 depends on the three R2 mutations in v-Myb (Kowenz-Leutz et al. 1997). Accordingly, failure to bind to H3 and to acetylate H3 by v-Myb could still permit the activation of some genes. We have shown previously that GBX2 becomes activated by c-Myb once a receptor tyrosine kinase pathway (RTK) is concomitantly activated (Kowenz-Leutz et al. 1997). This raises the interesting possibility that during RTK signaling, c-Myb functions in H3 positioning, acetylation, or both might be transiently altered, reflecting a constitutive function of v-Myb. One possibility is that such Myb-target genes do not depend on Myb binding to *cis*-regulatory sites on DNA but on interactions between Myb and other transcription factors that transmit Myb functions unrelated to H3 tail acetylation (Schubert et al. 2004). Yet another possibility is that critical activities of Myb in proliferation might still require distinct Myb functions, such as p300 binding, but that these functions are nontranscriptional; such as, for example, during DNA replication (Beall et al. 2002). Although it is difficult to distinguish between these possibilities, our results suggest that the function of c-Myb during differentiation depends on H3 tail acetylation and that this functions becomes abrogated by R2 mutations in the oncogene. This conclusion is also supported by the result that inhibition of deacetylation, which leads to strongly enhanced, genome-wide H3 acetylation, could induce transcription of differentiation genes and myeloid differentiation of primary v-Myb-transformed myeloblasts, similar to what has been seen by others using an AMV-transformed cell line (Nemajerova et al. 2003).

Leukemogenic v-Myb has a defective SANT domain

The SANT domain was identified based on its similarity with Myb-repeats found in many proteins involved in chromatin regulation (Aasland et al. 1996). SANT domains are not only binding modules but are also involved in enzymatic reactions of several chromatin remodeling enzyme complexes. Examples are the yeast HAT complexes SAGA and ADA that possess a number of transcriptionally relevant subunits, including a trimeric module consisting of the HAT Gcn5, Ada3, and the SANT domain protein Ada2p (Grant et al. 1997). Mutational analysis revealed that the Ada2p SANT domain is required for Gcn5 interaction, for full HAT activity mediation in the SAGA complex, and for recognition of nucleosomal substrates (Boyer et al. 2002; Sterner et al. 2002). SANT domain functions were therefore suggested to include histone tail presentation, alignment of substrate and catalytic sites for chromatin acetylating enzymes, and modulation of enzyme functions (Boyer et al. 2002). SANT domain proteins are, however, also found in other chromatin remodeling complexes. The N-CoR corepressor of nuclear receptors is a SANT domain protein that interacts with and stimulates HDAC3 (Zhang et al. 2002). Another SANT domain protein, MTA2, is involved in the modulation of the enzymatic activity of the HDAC1 complex (Zhang et al. 1999). CoREST and Mta-lp proteins also form complexes with HDACs and stimulate their enzymatic activities (Humphrey et al. 2001; You et al. 2001). The SANT domain of the nuclear receptor/corepressors SMRT is a critical component of a deacetylase activation domain (DAD) that binds and activates HDAC3 as part of a histone interaction domain (HID). HID enhances repression by increasing the affinity of the DAD HDAC3 enzyme for its histone substrate. The SANT-containing HID preferentially binds to unacetylated histone tails, implying that the SANT domain participates in deciphering the histone code (Yu et al. 2003). Mutations in the SANT domain in the SWI/SNF-complex protein SWI3p abolish the activity of the complex in vivo (Boyer et al. 2002). Mutations that affect only the DNA binding of SWI3, however, do not abrogate its activity, suggesting that, in addition to DNA binding, additional functions of the SANT domain are essential (Boyer et al. 2002). In addition, the function of the chromatin remodeling RSC complex also depends on an intact SANT domain. These results suggest that the SANT domain is involved in the interaction with proteins recognizing various histone substrates and mediating successional modifications.

A salient feature of the Myb DBD is that its three SANT domains are tandemerized and that two of them, R2 and R3, form a composite DNA-binding module that is found in many Myb related proteins (Frampton et al. 1989; Tahirov et al. 2002). In this characteristic Myb-repeat structure, the respective third α helices of both SANT domains are positioned so as to form a kinked extended helix that interacts with the *cis*-regulatory site on DNA. Although the overall structure of SANT domains appears to be conserved (Grune et al. 2003), it has been suggested that SANT domain functions may vary with respect to surface charge and that Myb-SANT domains could be functionally divergent from other SANT domains (Boyer et al. 2004). Yet, results presented here show that the Myb–DBD couples histone substrate binding and catalysis with gene activation and that this essential feature is lost in the oncogenic v-Myb variant.

The three amino acid substitutions in the DBD of v-Myb act in concert to achieve a block in cell differentiation and an acute myeloblastosis (Introna et al. 1990). Although any back mutations affect both oncogenic functions, the D117V back mutation still fails to bind

C/EBP but regains H3 tail binding and exhibits an intermediate myeloblast/promyelocyte phenotype (Introna et al. 1990). This finding, together with data presented here, suggests that the Myb–C/EBP interaction is largely responsible for cell differentiation, whereas abrogation of H3 tail acetylation is crucial for the leukemogenic conversion of the oncoprotein.

Materials and methods

Plasmid constructs

A PCR fragment corresponding to the DBD of the avian c-Myb protein (amino acids 72–192) was generated and cloned in frame with the LexA DBD in the pBTM 116 vector (Clontech). Lysine amino acid 128 of the c-Myb DBD was mutated to methionine by site directed mutagenesis (QuickChange, Stratagene). PCR-generated fragments encoding human H3.3 amino acids 1–42 and 42–136, and human H3 amino acids 1–30 and 27–42 were inserted into BamH1/EcoR1 sites in GST expression vector pGEX4T-2 (Pharmacia). DNA fragments encoding human c-Myb DBDs were generated by PCR and inserted into the BamH1 site of pGEX4T-1 (Pharmacia) and into the BamH1 site of the vector pCDA3.1A (Invitrogen) for mutagenesis. Amino residues within the c-Myb DNA were mutated by site-directed mutagenesis (QuickChange, Stratagene). C/EBPβ–Myb fusion proteins were generated by cloning PCR-generated fragments derived from C/EBPβ (CR1–4: amino acids 1–113) equipped with optimized translation initiation sequences in frame to the c-Myb or v-Myb PCR derivatives in pCDM8 (Invitrogen). The AMV v-Myb back mutants were gifts from Thomas Graf (Albert Einstein College of Medicine, New York, NY) (Introna et al. 1990). The PCR products from the v-Myb constructs were inserted into pCDNA3 vector carrying three C-terminal repeats of Flag-tag sequences. The GST–H2A, GST–H2B, GST–H4, and GST–H3 fusing protein expressing vectors were gifts from Dr. A. Hecht (University of Freiburg, Freiburg, Germany) and were described before (Hecht et al. 1995). The p300 expression vector was gifts from Dr. R. Eckner (University of New Jersey, Newark, NJ) (Eckner et al. 1994).

Yeast two-hybrid screening

The L40 yeast strain was sequentially transformed with the pBTM116-Myb-K128M bait and a human bone marrow cDNA library in the pGAD10 vector (Clontech). The screening procedure was performed according to the manufacturer's protocol and as described (Ansieau and Leutz 2002).

In vitro transformation of bone marrow cells, tissue culture, and immunoprecipitation

Bone marrow cells were prepared, infected with wild-type and mutant c- and v-Myb chicken retroviruses as indicated. Transformed colonies were isolated and expanded in the presence of the cytokine cMGF in liquid culture as described (Kowenz-Leutz et al. 1997). For ChIP analysis and endogenous gene activation assays, 2×10^7 cells were analyzed before or after overnight treatment with 100 ng/mL TSA (Sigma). Hela and HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. QT6 fibroblasts cells were grown in DMEM supplemented with 2% chicken serum and 8% FBS. Cells were transfected by calcium phosphate precipitation as described. Cell extracts were prepared in 10 mM Tris-HCl (pH 7.9), 100 mM KCl, 2.5 mM MgCl2, 0.1% NP40, 10 µg/mL aprotinin, 2 µg/mL pepstatin, and 2 µg/mL leupeptin. For IP, protein G-Sepharose was preincubated with the respective antibodies and washed. Cell extracts were incubated with beads overnight at 4°C (30–50 µL of beads per 50 µL of extract) in a final volume of 1 mL buffer. Beads were washed, and bound proteins were resolved by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore). Immunoblots were developed using the ECL-enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

ChIP assay

ChIP was performed essentially as described (Mo and Dynan 2002). Briefly, for each experiment, four plates (150 mm in diameter) of cells were adjusted to 1% formaldehyde for 10 min, quenched with glycine (final concentration, 0.125 M), harvested, and lysed, and chromatin was sheared by sonification. Antibodies against acetylated histone H3 K18 and K23 were purchased from Upstate. Following IP, cross-links were reversed by heating for 5 h at 65°C. Nucleic acids were precipitated with ethanol and resuspended in 600 µL of water, and PCR analysis was performed (30 cycles: 1 min at 94°C, 2 min at 68°C; final extension for 10 min at 68°C). One microliter to 9 µL of DNA was incubated in the presence of 1 pmol of each primer, 1 mM dNTPs, 2 µL of dimethyl sulfoxide, and 0.5 U of *Taq* DNA polymerase in 10 mM Tris-Hcl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR products were analyzed on 2% agarose gels. The following PCR primers were used: for the mim-1 gene (–292 to +37), 5′-AAGCGTTAAGAAATGGTTCAACCAA-3′ and 5′-ACCAGGCTGAGCAGGGCGATCAGG-3′; for the lysozyme gene promoter (–346 to +37), 5′-GCCAAATTTGCATTGT CAGGAAATG-3′ and 5′-GACCTCATGTTGCCAGTGTCGT AC-3′; and for the lysozyme 3′ region, 5′-CATTTGCAAAT CACTGCAGTGTGTG-3′ and 5′-GTGAACAGTGGCATCCT GTAAACTAA-3′.

GST pull-down experiments

Single human recombinant histone proteins were purchased from Upstate. GST–Myb DBD and GST histone fusion proteins were expressed in bacteria and prepared according to standard procedures. The Myb DBD was in vitro-translated and ^{35}S-methionine-labeled (TNT kit, Promega) in the presence of 100 µg/mL each of aprotinin, pepstatin, and leupeptin. For GST–Myb DBD and recombinant human histone protein interactions studies, 2 µg of each histone proteine was incubated with 5 µg of GST Myb DBD coupled to gluthathione-Sepharose (10 mM Tris at pH 8.0, 150 mM NaCl, 1 mM MgCl, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreithol) for 3 h at 4°C. Beads were repeatedly washed in binding buffer; bound proteins were separated on 12% SDS-PAGE and detected by Coomassie staining. For GST–histone and Myb–DBD interaction pull-downs, 15 µL of in vitro translation cocktail was incubated with equal amounts of bacterially expressed GST fusion proteins coupled to glutathione-Sepharose overnight at 4°C. The beads were washed six times with NETN (20 mM Tris at pH 8, 1 mM EDTA, 50 mM NaCl, 0.5% NP40). Bound proteins were eluted in SDS-PAGE sample buffer, separated on 10% SDS-PAGE, and detected by fluorography.

Endogenous gene activation assay and Northern blot analysis

Total RNA was extracted using a guanidinium-isothiocyanate method. Polyadenylated RNA was isolated, separated on 1.2% formyldehyde-agarose gel, and blotted on nylon membranes; the

mRNA of differentiation specific genes was detected as described (Kowenz-Leutz et al. 1997).

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