Oncogenic point mutations in the Myb DNA-binding domain alter the DNA-binding properties of Myb at a physiological target gene

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ABSTRACT

The oncoprotein v-Myb of avian myeloblastosis virus (AMV) transforms myelomonocytic cells by deregulating specific target genes. Previous work has shown that the oncogenic potential of v-Myb was activated by truncation of N- and C-terminal sequences of c-Myb and was further increased by amino acid substitutions in the DNA-binding domain and other parts of the protein. We have analyzed the activation of the chicken lysozyme gene which is strongly activated by c-Myb but not by its oncogenic counterpart v-Myb. We report that Myb acts on two different _cis_-regulatory elements, the promoter and an enhancer located upstream of the gene. Interestingly, the activation of the enhancer was abolished by the oncogenic amino acid substitutions. We demonstrated that a single Myb-binding site is responsible for the activation of the lysozyme enhancer by Myb and showed that the v-Myb protein of AMV was unable to bind to this site. Our data demonstrate for the first time that oncogenic activation of Myb alters its DNA-binding specificity at a physiological Myb target gene.

INTRODUCTION

Myb proteins constitute a family of related transcription factors that play important roles in the control of proliferation and differentiation of various cell types (1–3). The founding member of this family, v-Myb, was originally identified as the protein encoded by the retro-viral oncogene v-_myb_ of avian myeloblastosis virus (AMV) (4). v-_myb_ is a truncated and mutated derivative of the chicken c-_myb_ gene and is responsible for the ability of AMV to transform myeloid cells _in vivo_ and _in vitro_. c-_myb_ is not oncogenic but plays a key role in the development of the hematopoietic system. A large body of work has demonstrated that c-_myb_ is highly expressed in immature, proliferating hematopoietic cells and is down-regulated during their terminal differentiation (1–3). Constitutive expression of c-_myb_ blocks hematopoietic differentiation whereas disruption of the gene in mice leads to defects of multiple hematopoietic lineages and embryonic death (5). It is thought that c-_myb_ acts as a genetic switch in hematopoietic progenitor cells that controls alternative cell fates, such as proliferation, differentiation and apoptosis (6). v-_myb_ disrupts the normal control of these processes and thereby transforms cells of the myelomonocytic lineage.

v-Myb and c-Myb are nuclear DNA-binding proteins that recognize a specific sequence motif (7) and regulate the activity of promoters that contain this motif (8–11). By using different approaches, such as differential screening of cells transformed by a temperature-sensitive mutant of v-_myb_ (10), differential display of RNA from cells expressing estrogen-inducible or dominant-interfering versions of v-Myb or c-Myb (12–14) or microarray-based expression analyses (15,16) a substantial number of Myb-regulated genes have been identified. However, although a large number of potential Myb target genes are now known, the role of these genes in cell transformation and hematopoietic differentiation, and the molecular mechanisms by which Myb proteins regulate their expression, are only beginning to be understood.

Detailed studies on how Myb affects the expression of its targets have been performed only in a few cases. One of the most thoroughly studied Myb target gene is the chicken _mim-1_ gene (10). Because _mim-1_ is only transcribed in myelomonocytic cells and because it is induced to very high expression levels by Myb, _mim-1_ has been an attractive model to study how Myb activates target genes

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in a lineage-specific manner. Previous work has demonstrated that Myb cooperates with members of the CCAAT-box/enhancer-binding protein (C/EBP) family to activate *mim-1* expression (17,18). Furthermore, it was shown that c-Myb activates the *mim-1* gene by binding not only to the promoter but also to a myeloid-specific enhancer located upstream of the gene (19).

The oncogenic potential of v-Myb is primarily due to N- and C-terminal truncations that have occurred during transduction of *myb* sequences into the AMV genome (20). In addition to these truncations, v-Myb harbors several amino acid substitutions which have been acquired during the repeated passage of the virus in leukemic chickens and are scattered throughout the protein. It has been shown that these amino acid substitutions, some of which are located in the DNA-binding domain of the protein, strongly enhance the oncogenic potential of Myb (20). Furthermore, these substitutions have profound effects on the phenotype of the transformed cells and on which genes are activated by v-Myb. This has initially been observed with the *mim-1* and lysozyme genes, both of which are direct targets of c-Myb but cannot be activated by v-Myb as a result of amino acid substitutions in the DNA-binding domain of v-Myb (10,21). More recently a large number of genes have been identified whose regulation by Myb depends on the presence of some or all of the AMV-specific amino acid substitutions (16). On the molecular level, the amino acid substitutions within the DNA-binding domain of v-Myb appear to affect the activity of the protein in several ways. They were reported to disrupt a negative regulatory mechanism that is triggered by conformational changes catalyzed by the peptidyl-prolyl-isomerase Cyp-40 (22). In addition, it has been suggested that the amino acid substitutions have abrogated the ability of v-Myb to cooperate with the CCAAT-box/enhancer-binding protein C/EBPβ (23) and to interact with the aminoterminal tail of histone H3 and thereby facilitate histone tail acetylation (24). In case of the chicken *mim-1* gene we have shown that the substitutions affect the cooperation of the promoter of the gene with a Myb-inducible enhancer (19). Surprisingly, although several of the oncogenic amino acid substitutions are located within the DNA-binding domain, there is no evidence as yet that they affect the binding of the protein to physiological target sites.

Here, we have compared the regulation of the myeloid-specific chicken lysozyme gene by v-Myb and by a v-Myb variant lacking most of the oncogenic amino acid substitutions. Our work shows that Myb acts on two different *cis*-regulatory sequences of the lysozyme gene, the promoter and an enhancer located 2.7 kb upstream of the gene, and employs C/EBPα and PU.1 as cooperation partners. Interestingly, the oncogenic amino acid substitutions abolish the ability of v-Myb to stimulate the enhancer. We show that the activation of the enhancer by Myb depends on a single Myb-binding site whose recognition is abrogated by the oncogenic amino acid substitutions in the DNA-binding domain of v-Myb. Our data demonstrate for the first time that oncogenic activation of Myb results in altered DNA-binding properties of Myb at a physiological target gene.

MATERIALS AND METHODS

Cells

HD11 is a line of MC29 transformed chicken macrophages and was grown in basal Iscoves' medium supplemented with 8% fetal calf serum and 2% chicken serum. QT6 is a line of Japanese quail fibroblasts and was grown in basal Iscoves' medium supplemented with 8% fetal calf serum and 2% chicken serum (17). A doxycyclin-inducible expression vector for v-Myb was generated by subcloning the coding region for v-Myb from plasmid pCDE26v-myb (25) as a HindIII/XbaI DNA fragment into pCDNA4/TO/myc-His-A (Invitrogen). The resulting plasmid was then transfected together with pCDNA6-TR (which encodes the tet-repressor) into HD11 cells, followed by selection of stable transfectants in the presence of 750 µg/ml zeocin and 750 µg/ml blasticidin. Doxycyclin was omitted during the selection procedure to prevent v-Myb expression. Doubly-resistant stable cell clones were then analyzed by western blotting for doxycyclin-inducible expression of the v-Myb protein. Clone HD11-E was selected for further analysis.

Reporter genes, expression vectors and transfections

Chicken lysozyme reporter genes pCL2000 (containing lysozyme promoter sequences from −579 to +15), pCL2000-2.7, pTATA-2.7, pTATA-3.9, pTATA-6.1 and the pTATA vector have been described before (26). A truncated version of the enhancer (referred to as min-Enh) was generated by PCR using the primers 5′-CATAAGCTTGGCAGATGACTATGACTACT-3′ and 5′-CATGGATCCTCCTGCTTTGCAATTCAGAA-3′ followed by subcloning of the PCR fragment into pTATA via HindIII and BamHI sites. Point mutants of the −2.7 kb enhancer were generated by PCR in the plasmid pTATA-2.7 using the appropriate primers. In plasmid pTATA-2.7mut1 a Myb-binding site (underlined) was changed from TCTGCAGTTGGCAG to TCTGCAGCCGGCAG. In plasmid pTATA-2.7mut2 a Myb-binding site (underlined) was changed from TGAGGAACTAGCTG to TGAGGGACTAGCTG. In plasmid pTATA-2.7mut3 a Myb-binding site (underlined) was changed from ATTTCTGTTTGACCA to ATTTCTGGGTGACCC. In plasmid pTATA-2.7mut4 a Myb-binding site (underlined) was changed from GCAGGAGTTAGCGG to GCAGGAGTCAGCGG. In plasmid pTATA-2.7mutCEBP a C/EBP-binding site (underlined) was changed from TGGTATTTGGAAATAATA to TGGTACCTGGACCTAATA. All constructs were verified by sequencing. The β-galactosidase reporter gene pCMVβ was obtained from Clontech. An expression vector for AMV v-Myb (pCDAMVv-myb) has been described (19). pCDE26v-myb is a derivative of pCDAMVv-myb generated by replacing the v-*myb* coding region between the NcoI and SalI sites of v-*myb* with the corresponding sequence from c-*myb*. Expression vectors for chicken C/EBPα (pCDNA3-chC/EBPα), chicken C/EBPβ (pCDNA3-CCR) and PU.1 (pCDmPu.1) have been described before (25). DNA transfection was performed by calcium-phosphate

co-precipitation as described previously (17). The preparation of cell extracts, luciferase and β-galactosidase assays were performed as described (17).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as follows: approximately 10^8 cells were incubated for 10 min at room temperature with growth medium containing 1% formaldehyde and quenched by adding glycine (125 mM final concentration) and incubating for 5 min. After washing twice with ice-cold phosphate-buffered saline cells were suspended in RIPA buffer (10 mM Tris–HCl, pH 7.8; 50 mM NaCl; 1 mM PMSF; 0.5% sodium deoxycholate; 0.5% NP40; 0.1% SDS) and sonicated on ice (5 s pulsed intervals for 3 min). After centrifugation for 10 min at 14 000 r.p.m. the extract was pre-incubated with protein-A sepharose for 1 h at 4°C. The supernatant was then incubated with Myb-specific rabbit antisera raised against a bacterially expressed Myb protein lacking the DNA-binding domain (4) or against the DNA-binding domain of v-Myb (29), normal rabbit serum or no antiserum, overnight at 4°C on a rotating wheel. Samples were then incubated with protein-A sepharose for 1 h and washed in ELB buffer (50 mM Tris–HCl, pH 7.5; 120 mM NaCl; 1 mM EDTA; 6 mM EGTA; 15 mM sodium pyrophosphate; 20 mM sodium fluoride; 1 mM PMSF; 0.5% NP40) (three washes), with ELB buffer supplemented with 250 mM LiCl (two washes), with ELB buffer supplemented with 250 mM LiCl and 0.1% SDS (one wash) and two final washes with ELB buffer. The immunoprecipitates were eluted first with elution buffer 1 (100 mM NaHCO$_3$) and then with elution buffer 2 (10 mM Tris–HCl, 1 mM EDTA; 1% SDS; 10 mM DTT). The eluates were combined and after addition of Proteinase K (500 μg/ml final concentration) the DNA was reverse cross-linked by incubation for 12 h at 65°C. Finally, the immunoprecipitated DNA was extracted by phenol–chloroform, ethanol precipitated, resuspended in 50–100 μl of water and stored at −20°C. PCR was performed by using the following primers. Lysozyme promoter: 5′-CTGATGATGAACAATGGCTATG-3′ and 5′-CCCTTTGATTCCTCTGTTCTACA-3′, lysozyme −2.7 kb enhancer: 5′-CACTCCCACACTGAAACAA CA-3′ and 5′-TTTTTATCCTCTTCCTTGTAAGCAG-3′. PCR products were resolved on 2% agarose gels and stained with ethidium bromide.

Electrophoretic mobility shift assays

Pairs of complementary single-stranded oligonucleotides containing wild-type (wt) or mutated Myb-binding sites were annealed and used for gel retardation assays: MBS3wt: 5′-GGCTGGGAATATTTCTGTTTGACCAC CATGGAG-3′ and 5′-CTCCATGGTGGTCAAACAGA AATATTCCCAGC-3′; MBS3mut: 5′-GGCTGGGAAT ATTTCTGGGTGACCACCATGGAG-3′ and 5′-CTCC ATGGTGGTCACCCAGAAATATTCCCAGC-3′; mimA: 5′-GCTCTAAAAAACCGTTATAATGTACAGATATC TT-3′ and 5′-AAGATATCTGTACATTATAACGGTTT TTTAGAG-3′. After annealing, oligonucleotides were radiolabeled by filling in the ends using α^{32}P-dCTP and

Klenow polymerase. Bacterial v-Myb, using the expression vector pVM2028 and pVM2101, was prepared and binding experiments were performed as described (30). pVM2028 encodes the v-Myb protein of AMV and has been described (31). pVM2101 is a derivative of pVM2028 generated by replacing the v-*myb* coding region between the NcoI and SalI sites of v-*myb* with the corresponding sequence from c-*myb*. As a result, several of the amino acid substitutions harbored by the DNA-binding domain of AMV v-Myb were reverted to the sequences found in the DNA-binding domain of c-Myb.

RESULTS

Myb stimulates the activity of the promoter and an enhancer of the chicken lysozyme gene

To understand how Myb activates the expression of the chicken lysozyme gene we analyzed the influence of Myb on luciferase reporter genes containing the promoter or known *cis*-regulatory sequences of the chicken lysozyme gene. We used an expression vector encoding a modified version of v-Myb in which most of the AMV-specific amino acid substitutions had been reverted by replacing part of the coding region with the corresponding sequence of the c-*myb* gene. The structure of this modified v-Myb (referred to as v-Myb^{REV}) is shown schematically in Figure 1E. We have shown before that this modified version of v-Myb activates the endogenous chicken lysozyme gene, in contrast to v-Myb^{AMV}, which does not activate the gene (12). Reporter gene experiments were performed in the myelomonocytic cell line HD11 which lacks endogenous v-Myb or c-Myb. As shown in Figure 1A, v- Myb^{REV} strongly activated the lysozyme promoter and the reporter gene containing the −2.7 kb lysozyme enhancer. The −6.1 kb lysozyme enhancer was weakly activated and the −3.9 kb enhancer was not activated at all. We also performed experiments in parallel with the expression vector for v-Myb^{AMV}, reasoning that the direct comparison of the effects of both versions of v-Myb might provide clues about why the AMV version of v-Myb does not activate the lysozyme gene. As shown in Figure 1B, both Myb proteins displayed similar activities with the notable exception of the −2.7 kb enhancer which was not affected significantly by v-Myb^{AMV}. This difference was not due to different expression levels of both proteins (Figure 1D). Taken together, these experiments identified the promoter and the −2.7 kb enhancer as major Myb-responsive elements of the lysozyme gene. In addition, they demonstrated that the two versions of v-Myb display significantly different activities at the −2.7 kb lysozyme enhancer. These differences were also observed when co-transfections were performed in a fibroblast cell line, confirming that the presence of the amino acid substitutions in v-Myb^{AMV} has abolished the ability of the protein to stimulate the −2.7 kb enhancer (Figure 1C).

To confirm that Myb targets two different *cis*-acting regions of the lysozyme gene we performed chromatin immunoprecipitation experiments using a stable transfectant of the myelomonocytic HD11 cell line in which the
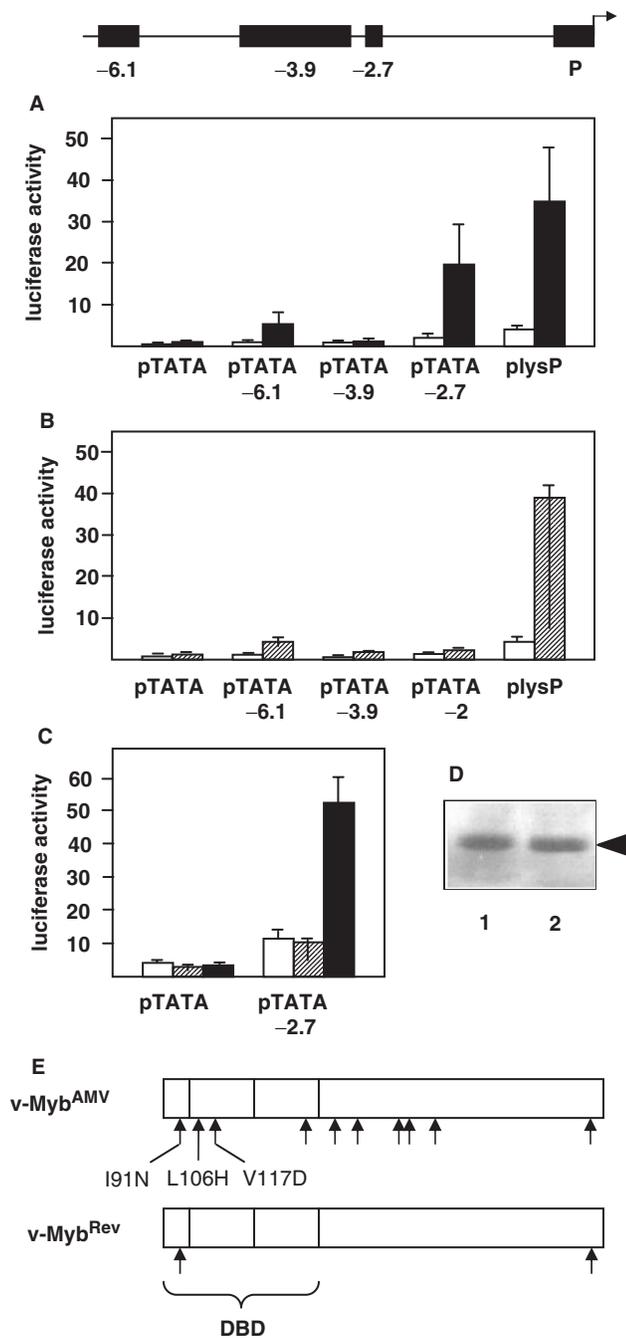
Figure 1. Myb-dependent activity of chicken lysozyme reporter genes. The lysozyme gene upstream region is shown schematically at the top. Black boxes indicate the promoter (P) or enhancer sequences (numbered boxes) that are covered by reporter genes. (**A**, **B**) HD11 cells were transfected with the indicated reporter genes and expression vector for v-Myb[REV] (black columns) or empty vector (white columns). To control the transfection efficiency cells were additionally transfected with the β-galactosidase plasmid pCMVβ. Cells were analyzed for luciferase and β-galactosidase activities 24 h after transfection. The columns show the average luciferase activity (arbitrary units) normalized to the β-galactosidase activity. Thin lines show standard deviations. Panel B shows an identical experiment except that expression vector for v-Myb[AMV] was used. (**C**) QT6 cells with the indicated reporter genes and expression vectors for v-Myb[AMV] (hatched columns), v-Myb[REV] (black columns) or empty expression vector (white columns). (**D**) Western blot of cells transfected with expression vectors for v-Myb[AMV] (lane 1) and v-Myb[REV] (lane 2) using Myb-specific

expression of v-Myb[REV] is induced by growing the cells in the presence of doxycyclin. Western blotting showed that the Myb protein was expressed in the presence but not in the absence of doxycyclin (Figure 2A). The chromatin immunoprecipitation experiment illustrated in Figure 2B confirmed the binding of Myb to the lysozyme promoter and the −2.7 kb enhancer following induction by doxycyclin. This experiment, therefore, supported the notion that Myb activates the lysozyme gene by targeting two different regions of the gene.

Myb synergizes with PU.1 and C/EBPα at the −2.7 kb lysozyme enhancer

The differential activity of v-Myb[REV] and v-Myb[AMV] at the −2.7 kb enhancer suggested that further analysis of the enhancer might provide a clue about why the AMV version of v-Myb does not activate the lysozyme gene. Several proteins have previously been implicated in binding to the −2.7 kb lysozyme enhancer, including Ets and C/EBP family members as well as an unknown protein binding to an 'AP-1-like' site (26,32,33). The protein binding to the Ets-binding site has been tentatively identified as PU.1 (26,33). To explore whether Myb cooperates with one of the known proteins that bind to the enhancer we performed co-transfection experiments with different combinations of expression vectors for Myb, PU.1, C/EBPα and C/EBPβ (Figure 3A). Herschlag and Johnson (34) defined synergism in transcriptional activation as existing when the effects of two factors are more than additive. To determine whether Myb is synergistic with any of the other factors we therefore compared the activity of the reporter gene, in the presence of each factor alone, to the activity observed when the same factors were expressed together. When transfected on its own, each of the factors was able to stimulate the activity of the enhancer to some extent. When expressed together, more than additive stimulation was observed when Myb was combined with C/EBPα or with PU.1, whereas merely additive stimulation was observed when Myb was combined with C/EBPβ. The degree of synergy is expressed in a quantitative manner by the numbers on top of the columns in Figure 3A, where a factor larger than one indicates more than additive effects. From these numbers it is apparent that C/EBPα and PU.1 synergize with Myb according to the definition of Herschlag and Johnson (34) whereas Myb and C/EBPβ are not synergistic. We also assessed the combined effects of C/EBPα and PU.1, and of Myb, C/EBPα and PU.1 on the enhancer (Figure 3B). Interestingly, C/EBPα and PU.1 did not act synergistically in the absence of Myb whereas the presence of Myb resulted in a very strong stimulation of the enhancer activity. Thus, it appears that Myb, C/EBPα and PU.1 act in concert to activate the lysozyme enhancer.

antiserum (28). The arrow marks the v-Myb protein. (**E**) Schematic illustration of the structure of v-Myb[AMV] and v-Myb[REV]. Arrows mark point mutations of v-Myb[AMV] relative to c-Myb. The three point mutation in the DNA-binding domain (DBD) that affects the ability of v-Myb to activate the lysozyme gene are highlighted with numbering referring to c-Myb.
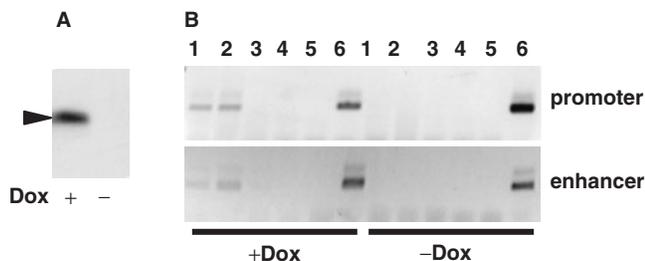
Figure 2. Chromatin-immunoprecipitation experiment. (**A**) Western blot analysis of Myb expression in HD11-E cells grown in the presence or absence of doxycyclin. The black arrow marks the v-Myb[REV] protein. (**B**) Chromatin immunoprecipitation experiment using HD11-E cells grown in the presence (left part) or absence (right part) of doxycyclin. Immunoprecipitation was carried out using two different Myb-specific antisera (lanes 1 and 2), non-immune serum (lane 3) or no antiserum (lane 4). Lane 5 shows no-template controls and lane 6 shows input controls. PCR primers were specific for the lysozyme gene promoter and the −2.7 kb enhancer. PCR products were analyzed by agarose gel electrophoresis.

C/EBPα and C/EBPβ have been shown to synergize with Myb in the context of other Myb-regulated genes, such as the *mim-1* and *tom-1* genes (17,35). However, pronounced differences in their ability to synergize with Myb have not been observed so far. We wondered whether the disparate behavior of both C/EBPs was due to subtle differences in their DNA-binding specificities. To address this issue we used expression vectors for recombinants of C/EBPα and C/EBPβ whose DNA-binding domains had been exchanged (see top of Figure 3C). The transactivation experiment illustrated in Figure 3C showed that the ability to synergize with Myb was dependent on the transactivation domain of C/EBPα. It is therefore unlikely that differences in DNA binding between C/EBPα and C/EBPβ are responsible for their different behavior. The differences in activity of C/EBPα and β at the −2.7 kb enhancer and the promoter of the lysozyme gene were reminiscent of previous work in which we had shown that ectopic expression of Myb and C/EBPα, but not of C/EBPβ, activated the endogenous lysozyme gene in chicken fibroblasts (17).

A single Myb-binding site is responsible for the activation of the −2.7 kb enhancer by Myb

To further understand the mechanism by which Myb activates the −2.7 kb lysozyme enhancer we examined the enhancer for Myb-binding sites which mediate the stimulation by Myb. As a first step we performed a transactivation experiment using a mutant of v-Myb[REV] which lacks DNA-binding activity. In this mutant a single amino acid residue (Asp186), which is directly involved in contacts with specific bases of the Myb recognition motif and has been shown to be crucial for the specific DNA-binding activity of Myb (36,37), was mutated to alanine. As shown in Figure 4A, the mutant Myb protein failed to stimulate the enhancer, consistent with the notion that one or several Myb-binding sites mediate the effect of Myb. We then examined the sequence of the enhancer for potential Myb-binding sites. There are four GTT- (or AAC- in the reverse orientation) motifs within the

enhancer sequence, which conform to the central core of the Myb-binding site. One of these motifs (designated as site 1 in Figure 4B) showed a good match to the Myb consensus site (PyAACT/GG) whereas the other sites were more distantly related. We mutated each of these motifs and examined the ability of v-Myb[REV] to activate the mutated enhancers. Figure 4B shows that only mutation of site 3 significantly reduced the stimulation of the enhancer by Myb. This suggested that binding site 3 plays a key role in the Myb-dependent activation of the enhancer. We also constructed a truncated version of the enhancer and found that its stimulation by v-Myb[REV] was essentially identical to that of the full-length enhancer (Figure 4C).

Myb-binding site 3 is situated between the Ets- and C/EBP-binding sites (see top of Figure 4B). We were therefore interested to know whether this site mediates the synergistic cooperation of Myb with PU.1 and C/EBPα. To address this we examined the effect of mutation of the Myb, PU.1 and C/EBP-binding sites on the ability of these transcription factors to synergize. We employed the MBS2 mutant to ascertain the role of the PU.1-binding site because mutation of Myb-binding site 2 had also destroyed the PU.1-binding site. Figure 5A shows that mutation of the PU.1-binding site abolished the ability of PU.1 to activate the enhancer and to synergize with Myb, confirming that this site mediates the effect of PU.1 on the enhancer. Mutation of Myb-binding site 3 had no effect on the ability of PU.1 to activate the enhancer in the absence of Myb but abrogated the synergistic effect of Myb and PU.1. Figure 5B shows that mutation of the C/EBP-binding site prevented C/EBPα from activating the enhancer and from synergizing with Myb, indicating that this site mediates the effect of C/EBPα on the enhancer. Mutation of Myb-binding site 3 did not affect the activation of the enhancer by C/EBPα but diminished the ability of Myb and C/EBPα to synergize. However, it did not completely abolish the synergy between Myb and C/EBPα, possibly because there was still residual binding of Myb to the mutant site. Taken together, the data presented in Figures 3–5 indicate that Myb activates the −2.7 kb lysozyme enhancer by binding to Myb-binding site 3 and cooperating with Pu.1 and C/EBPα bound to their cognate-binding sites.

Amino acid substitutions in the DNA-binding domain of the v-Myb protein of AMV abolishes binding of the protein to Myb-binding site 3 of the −2.7 kb lysozyme enhancer

The data illustrated in Figure 1 demonstrated that v-Myb[REV], in which most of the amino acid substitutions found in the v-Myb protein of AMV had been reverted, activated the −2.7 kb lysozyme enhancer, while v-Myb[AMV] was unable to do so. Since our work had shown Myb-binding site 3 to play a key role in the activation of the enhancer, we wondered whether or not v-Myb[AMV] was able to bind to this site. In the light of the current model of the Myb DNA-binding domain (37) a direct effect of the substitutions on DNA binding seemed unlikely because the amino acid residues that have been substituted in the DNA-binding domain of AMV v-Myb
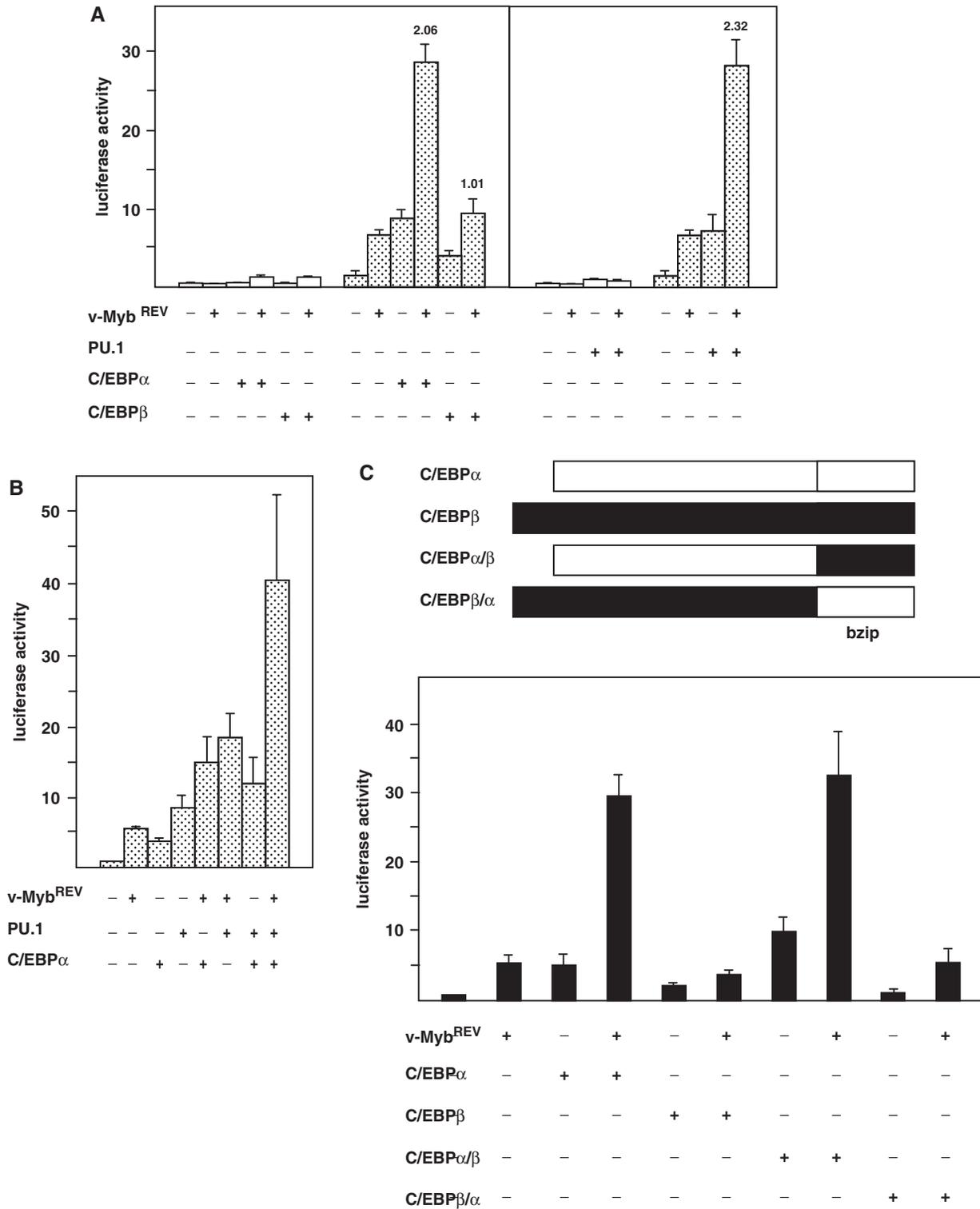
Figure 3. Synergistic activation of the −2.7 kb enhancer by Myb, C/EBP and PU.1. (**A**) QT6 cells were transfected with the reporter gene pTATA or pTATA-2.7 and different combinations of expression vectors for v-Myb[REV], C/EBPα, C/EBPβ and PU.1, as indicated at the bottom. To control the transfection efficiencies, cells were additionally transfected with the β-galactosidase plasmid pCMVβ. Cells were harvested 24 h after transfection and analyzed for luciferase and β-galactosidase activities. The columns show the average luciferase activity normalized to the β-galactosidase activity. Thin lines show standard deviations. The numbers above the columns indicate the extent of synergy between Myb and C/EBPα, C/EBPβ and PU.1, respectively. These numbers were determined by dividing the luciferase activity observed in the presence of both factors together by the sum of the luciferase activities observed for each factor alone. (**B**) QT6 cells were transfected with pTATA-2.7 and different combinations of expression vectors, as indicated at the bottom. Cells were analyzed as described in A. (**C**) C/EBPα, C/EBPβ and recombinant protein constructs are shown schematically at the top. QT6 cells were transfected with pTATA-2.7 and the expression vectors indicated below the columns. To control the transfection efficiencies, cells were additionally transfected with the β-galactosidase plasmid pCMVβ. Cells were analyzed as in (A).
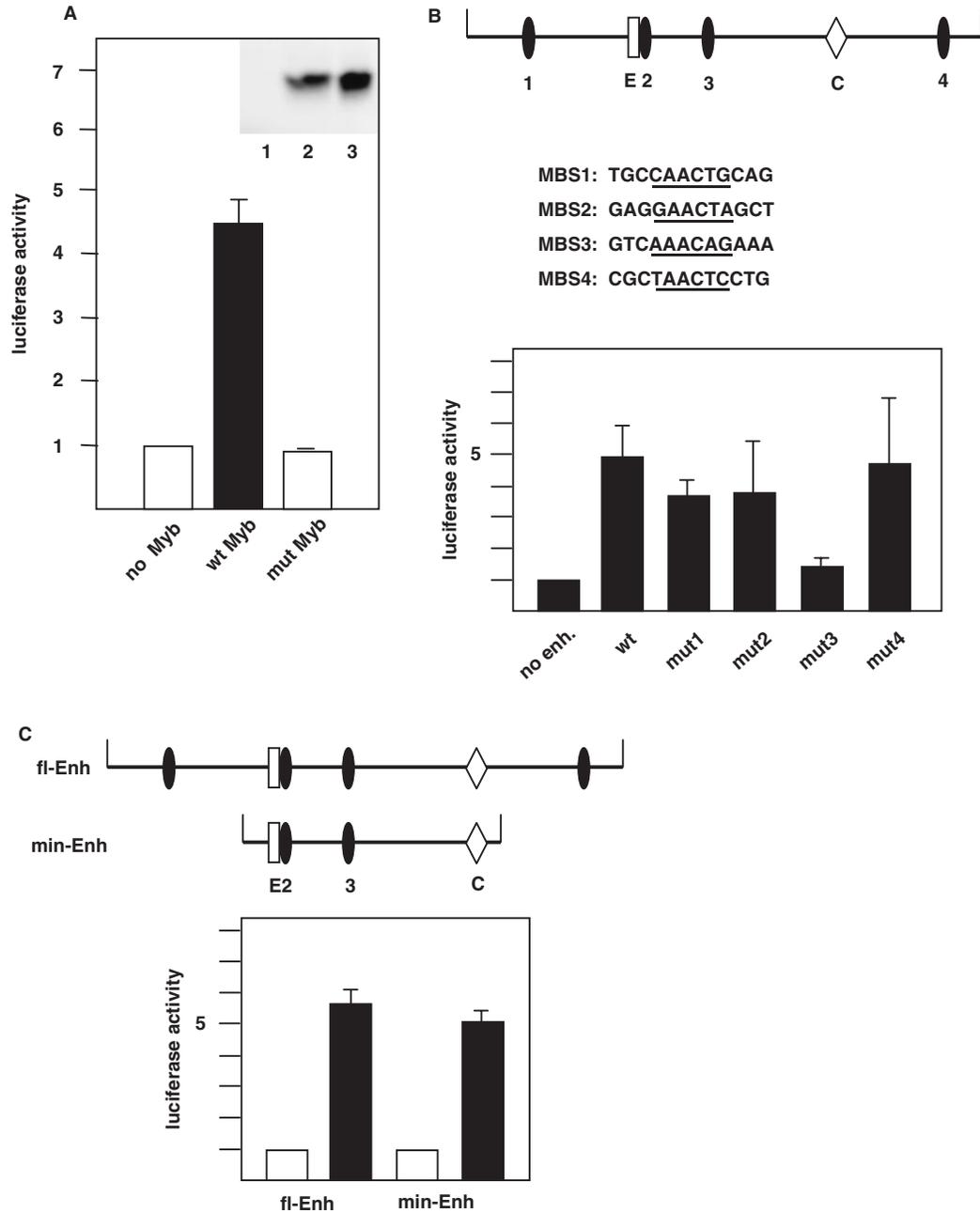
Figure 4. Identification of a functional Myb-binding site in the −2.7 kb enhancer. (**A**) QT6 cells were transfected with the reporter gene pTATA-2.7 and expression vector for v-Myb[REV] (wt-Myb), the N186A mutation of v-Myb[REV] (mut-Myb) or empty expression vector (no Myb), as indicated below the columns. To control the transfection efficiencies cells were additionally transfected with the β-galactosidase plasmid pCMVβ. Luciferase and β-galactosidase activities were determined 24 h after transfection. The luciferase activity was normalized to the β-galactosidase activity and is expressed in arbitrary units. The activity of the reporter gene in the absence of v-Myb was designated as 1. Thin lines show standard deviations. The insert at the top shows a western blot analysis of v-Myb expression in cells transfected with the same expression vectors. (**B**) The figure at the top illustrates the position of Ets (E), C/EBP (C) and potential binding sites for Myb (M) in the −2.7 kb enhancer. The sequences of the four potential Myb-binding sites are shown below. Results of reporter gene assays, performed in QT6 cells, are shown at the bottom. pTATA (no enh.), pTATA-2.7 (wt) or derivatives of this plasmid carrying point mutations in one of the four Myb-binding sites (mut1-4) were transfected with expression vector for v-Myb[REV]. Transfections were analyzed as in (A). (**C**) Reporter gene assays were performed in QT6 cells using the full-length or a truncated −2.7 kb enhancer construct. Cells were additionally transfected with expression vector for v-Myb[REV] (black bars) or empty expression vector (white bars). Transfections were analyzed as in (A).

point away from the bound DNA. On the other hand, by using artificial sites that were systematically altered in certain positions of the Myb consensus binding motif Brendeford *et al.* (38) have shown that the AMV-specific

amino acid substitutions indeed can have some effect on the DNA-binding activity of the Myb DNA-binding domain. To investigate whether the amino acid substitutions in the DNA-binding domain of AMV-v-Myb affect
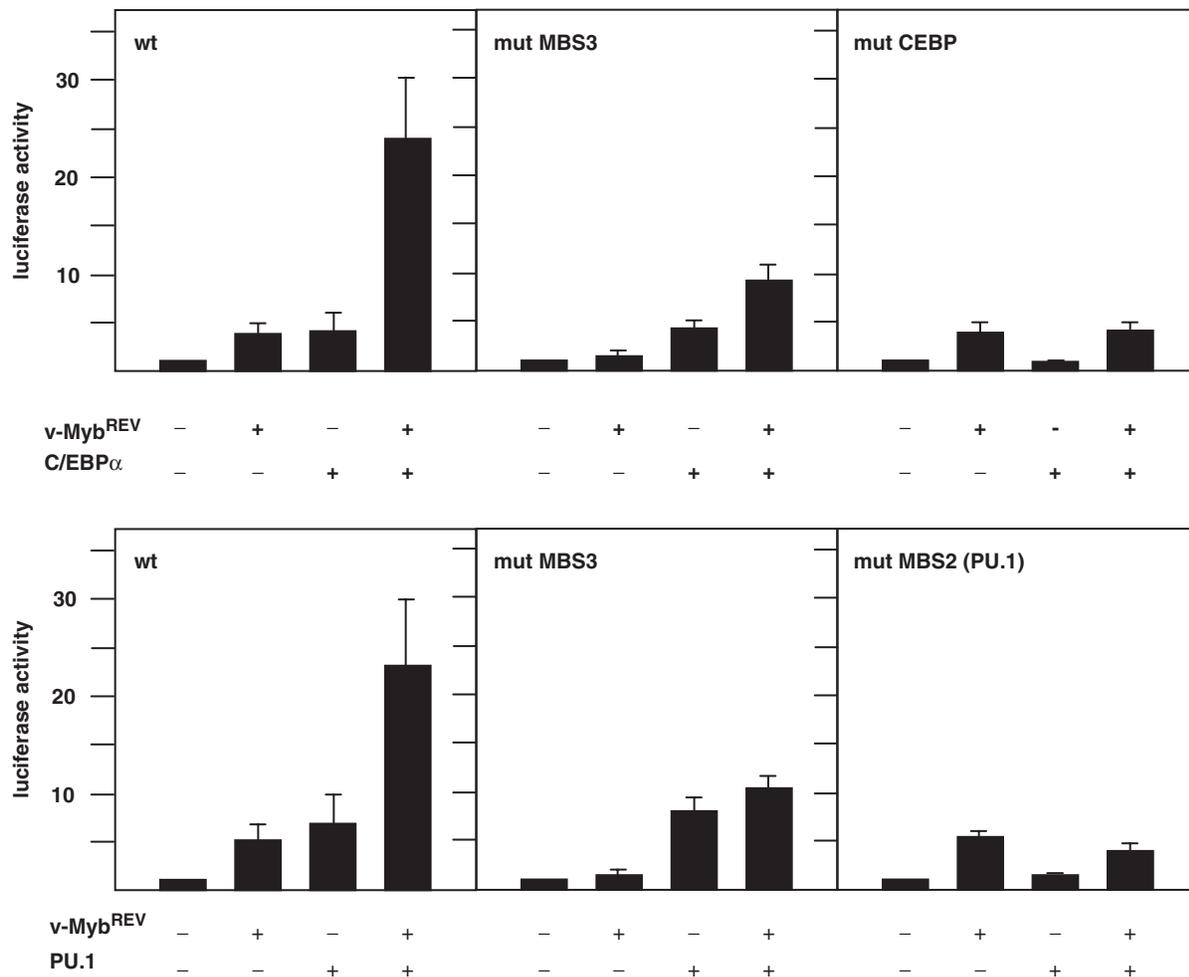
Figure 5. Myb-binding site 3 of the −2.7 kb enhancer mediates cooperation of Myb with PU.1 and C/EBPα. QT6 cells were transfected with the reporter genes indicted at the top and expression vectors for v-Myb[REV] and C/EBPα, as shown at the bottom. The luciferase activity was normalized with respect to the β-galactosidase activity and is expressed in arbitrary units. The activity of each reporter gene in the absence of v-Myb was designated as 1. Thin lines show standard deviations.

the ability to recognize binding site 3 of the −2.7 kb enhancer, we performed electrophoretic mobility shift assays using bacterially expressed v-Myb proteins containing or lacking AMV-specific amino acid substitutions within the DNA-binding domain. The experiment shown in Figure 6A demonstrated that bacterially expressed v-Myb, lacking AMV-specific amino acid substitutions within the DNA-binding domain, was indeed able to recognize Myb-binding site 3 *in vitro*. Binding to this site was weaker than to the Myb binding site A of the *mim-1* promoter, which was expected because binding site 3 of the lysozyme enhancer differs significantly from the Myb consensus binding motif. Mutation of binding site 3 abolished binding of Myb, consistent with the observation that mutation of this site abolished the activation of the enhancer by Myb. In the experiment illustrated in Figure 6B we compared the ability of both versions of Myb to bind to the *mim-1* A site and to Myb-binding site 3. Both proteins recognized the *mim-1* A site equally well; however, the v-Myb protein containing the AMV-specific amino acid substitutions was virtually unable to bind to

Myb-binding site 3. This observation clearly demonstrated that the amino acid substitutions in the DNA-binding domain of AMV v-Myb have affected the sequence specificity of the protein in such a way that it no longer recognizes Myb-binding site 3 of the lysozyme enhancer. This lack of binding provides a straightforward explanation for the inability of v-Myb[AMV] to activate the lysozyme enhancer.

DISCUSSION

For several reasons the chicken lysozyme gene is an interesting model system to explore the molecular mechanisms by which Myb activates the expression of its target genes. First, the transcriptional regulation of the lysozyme gene has been studied extensively before. Several *cis*-acting elements, including the promoter, several enhancers and a silencer have been characterized in detail and a number of transacting factors binding to these elements have been identified (26,27,32,39–43). Secondly, lysozyme gene expression is stimulated by
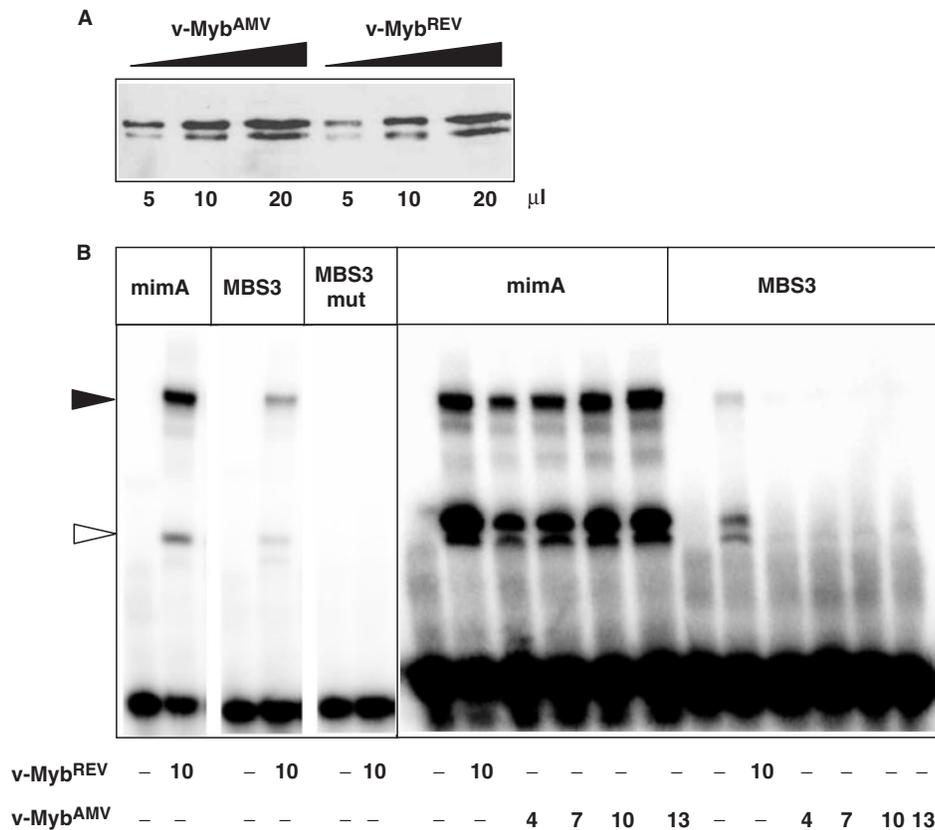
Figure 6. Electrophoretic mobility shift experiments. (**A**) Western blot analysis of the bacterially expressed v-Myb[REV] and v-Myb[AMV] proteins. The amounts (in μl) loaded onto the gel are shown at the bottom. Full-length Myb protein is marked by an arrow. (**B**) Binding reactions contained the radiolabeled double-stranded oligonucleotides shown and bacterially expressed Myb protein, as indicated above and below the lanes. The numbers below the lanes refer to the amounts (in μl) of protein added. Control reactions contained no Myb protein. Protein–DNA complexes were visualized by electrophoresis on native polyacrylamide gels followed by autoradiography. The black and white arrowheads mark complexes of full-length v-Myb and a proteolytic degradation product, respectively.

Myb to high levels only in the myelomonocytic lineage permitting it to address the mechanisms by which Myb activates a target gene in a lineage-specific manner. Thirdly, the lysozyme gene is activated by c-Myb and the Gag-Myb-Ets protein encoded by the E26 virus, but not by the highly oncogenic v-Myb protein of AMV (21). The inability of AMV to affect lysozyme gene expression has been attributed to the amino acid substitutions present in the DNA-binding domain of v-Myb. Analysis of the mechanisms underlying the activation of the lysozyme gene by Myb might therefore also provide further insight into the molecular function of the Myb DNA-binding domain and into how these oncogenic amino acid substitutions modulate the activity of the protein.

The data presented here provide novel insight into three major issues. First, our results strongly suggest that the activation of the lysozyme gene by Myb is not mediated by a single *cis*-acting element but by the concerted action of Myb on at least two *cis*-regulatory regions of the gene. Reporter gene assays as well as chromatin immunoprecipitation showed that Myb binds and activates the promoter and the myeloid-specific −2.7 kb enhancer of the lysozyme gene. This is highly reminiscent of two other Myb-regulated genes that we have studied recently,

the chicken *mim-1* and C/EBPβ genes. In both cases Myb has been shown to act on the promoter and, in addition, on a myeloid-specific enhancer located in the vicinity of the gene (19,44). A dual activation mechanism of Myb target genes, as exemplified by these genes, therefore seems to be a rather common phenomenon, at least for genes that are activated by Myb in the myelomonocytic lineage. The majority of studies that have addressed the mechanisms by which Myb regulates the expression of its targets have focused on the promoters of these genes; it is therefore possible that these studies so far have revealed only part of the complexity of gene regulation by Myb. It will be very interesting to explore if additional Myb-dependent *cis*-regulatory elements are also present in other Myb-regulated genes, particularly in genes whose activation by Myb is not restricted to the myelomonocytic lineage.

Secondly, our work provides insight into the lineage-specificity of the Myb-dependent activation of the lysozyme gene. We have identified C/EBPα and PU.1 as cooperation partners of Myb at the promoter and the −2.7 kb enhancer of the lysozyme gene. C/EBPs have been shown before to play important roles at the lysozyme promoter and the −2.7 kb enhancer, and PU.1 was shown

to bind to the −2.7 kb enhancer and to stimulate its activity (26,32,33). The cooperation of Myb with C/EBPα and PU.1, which are known to regulate the expression of numerous genes during myeloid differentiation, provides a straightforward explanation for the observation that Myb activates the lysozyme gene specifically in myeloid cells. An interesting aspect of our work is that PU.1 and C/EBPα apparently do not activate the lysozyme enhancer synergistically in the absence of Myb. However, when Myb is present synergy of all three factors is observed. This suggests that Myb which occupies a central position between C/EBPα and PU.1-binding sites orchestrates the function of these proteins. How this is done is not clear at present. It is possible that Myb directly interacts with these proteins or that a bridging protein such as p300/CBP is involved. Another interesting side aspect of our work concerns the cooperation of Myb with different members of the C/EBP family. Myb is able to synergize with different C/EBP factors; for example, the promoter of the *mim-1* gene is synergistically activated by Myb together with C/EBPα or C/EBPβ (17). However, transcriptional synergy at the lysozyme gene appears to be biased towards C/EBPα. This might explain our observation that ectopic expression of Myb and C/EBPα but not of Myb and C/EBPβ was sufficient to activate the expression of the endogenous lysozyme gene in chicken fibroblasts (17). The poor performance of C/EBPβ as a cooperation partner of Myb appears not to be due to inefficient binding of C/EBPβ, because C/EBPβ was clearly able to activate the lysozyme promoter and −2.7 kb enhancer on its own. Also, exchanging the DNA-binding domains between C/EBPα and C/EBPβ did not improve the ability of C/EBPβ to synergize with Myb. Apparently, the transactivation domain of C/EBPα is crucial for the cooperation with Myb in the context of the lysozyme enhancer and cannot be replaced by the transactivation domain of C/EBPβ. Clearly, further work is needed to understand why C/EBPβ performs only poorly as a cooperation partner of Myb at the lysozyme enhancer.

Thirdly, our work sheds new light on the role of the oncogenic amino acid substitutions in the v-Myb protein of AMV. Although truncation at either end of c-Myb was shown to be sufficient to activate its oncogenic potential the transforming capacity of the v-Myb protein of AMV has been significantly increased by a number of amino acid substitutions (20). These substitutions are located in the DNA-binding domain and other parts of the protein and have apparently arisen during the repeated passage of the virus in leukemic chickens, which were selected for the high oncogenic potential of the virus (20). Several studies addressing the impact of these mutations on Myb have suggested different aspects of the protein's function to be affected by these substitutions. For example, it has been demonstrated that the amino acid substitutions disrupt a negative regulatory mechanism that is based on conformational changes catalyzed by the peptidyl-prolyl-isomerase Cyp-40 (22), or ability of v-Myb to cooperate with the CCAAT-box/enhancer-binding protein C/EBPβ (23). More recently, the oncogenic amino acid substitutions were reported to affect the ability of Myb to interact with the amino terminal tail of histone H3 and to facilitate H3 acetylation (24). Furthermore, our own work has previously shown that the cooperation of the *mim-1* promoter and enhancer is affected by the substitutions (19). There is only one report which has implicated the amino acid substitutions in altered DNA-binding properties by showing that the AMV-specific amino acid substitutions result in less stable protein–DNA complexes when certain binding sites were used (38), however, since these experiments were performed *in vitro* using artificial binding sites it was not clear whether they are relevant for the activation of physiological Myb target genes. Our analysis of the lysozyme gene shows for the first time that the oncogenic amino acid substitutions in the Myb DNA-binding domain, affect the DNA-binding properties of the protein at a physiological target gene. It appears that the AMV-specific amino acid substitutions result in a subtle change of the sequence specificity of the Myb DNA-binding domain, which impedes the binding of v-Myb to the central Myb-binding site in the lysozyme enhancer. Our data clearly show that the lack of binding to the lysozyme enhancer is not due to a generally reduced DNA-binding activity, because binding to the Myb binding site from the *mim-1* promoter was unaffected. The amino acid substitutions of AMV v-Myb have been selected to increase the oncogenic potential of v-Myb; therefore, it appears likely that altered DNA binding contributes to the oncogenicity of v-Myb. Recently, Liu *et al.* (16) have identified a number of genes that are differentially regulated by Myb proteins that have been engineered to carry subsets of the AMV-specific amino acid substitutions. It will be interesting to explore whether altered DNA-binding properties also play a role in the differential activation of these genes by v-Myb and c-Myb. In a somewhat broader sense, it is surprising that the amino acid substitutions in the DNA-binding domain of v-Myb affect seemingly unrelated properties of v-Myb, such as DNA binding and various protein–protein interactions. This emphasizes the central role of this domain for Myb protein function.

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