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Mutations in the DNA-Binding and Transcriptional Activation Domains of v-Myb Cooperate in Transformation

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The v-Myb protein encoded by avian myeloblastosis virus causes oncogenic transformation of monoblastic cells committed to the monocyte/macrophage lineage. v-Myb is a doubly truncated form of its normal cellular counterpart, c-Myb. In addition to its N- and C-terminal deletions, v-Myb contains a number of amino acid substitutions relative to c-Myb. We have previously shown that neither overexpression of c-Myb nor introduction of these amino acid substitutions into c-Myb is sufficient for transformation of myelomonocytic cells. However, a doubly truncated form of c-Myb which lacked these substitutions transformed myeloblastic cells that appeared to be committed to the granulocytic pathway. We demonstrate here that mutations in both the DNA-binding and transcriptional activation domains of v-Myb are required for transformation of rapidly growing monoblasts rather than more slowly growing myeloblasts. These rapidly growing monoblasts do not express *mim-1*, a target gene for the Gag-Myb-Ets protein of E26 leukemia virus, or C/EBP proteins which cooperate with Myb to activate *mim-1* expression. Furthermore, v-Myb proteins which contain both sets of these mutations are weaker transcriptional activators relative to proteins which lack these mutations. These results support a model in which amino acid substitutions in v-Myb have been selected for their ability to activate only a subset of those genes which can be activated by a doubly truncated form of c-Myb. In particular, *mim-1* appears to represent a class of genes whose expression was selected against during the development of an increasingly virulent strain of avian myeloblastosis virus by passage in animals.

The v-*myb* oncogene of avian myeloblastosis virus (AMV) causes acute monoblastic leukemia in chickens and transforms myelomonocytic cells in vitro (27). The normal c-*myb* gene from which v-*myb* arose is expressed at high levels in immature hematopoietic cells of all lineages and is down-regulated as these cells differentiate (7, 9, 13, 42). Homozygous disruption of c-*myb* in a mouse causes death at day 15 of gestation because of a block in fetal hematopoiesis (30). The 48-kDa v-Myb protein of AMV is truncated at both its N and C termini relative to the normal 75-kDa c-Myb protein. In addition to these terminal deletions, v-Myb of AMV contains a number of amino acid substitutions relative to c-Myb. Both v-Myb and c-Myb are located in the cell nucleus, bind to a specific DNA sequence (PyAACT/GG), and can regulate the transcription of other genes (for a review, see reference 24).

Our laboratory has previously shown that neither constitutive expression of c-Myb nor introduction of the amino acid substitutions of v-Myb into c-Myb is sufficient for transformation of myelomonocytic cells (15). N-terminal truncation of chicken c-Myb strongly activated its oncogenic potential, whereas C-terminal truncation resulted in a weakly transformed phenotype which required the presence of exogenous growth factors (10, 15). Both of these singly truncated proteins caused the transformation of cells with an immature granulocytic phenotype (promyelocyte). Simultaneous truncation of both the N and C termini of c-Myb in the absence of the amino acid substitutions of v-Myb resulted in transformation of cells with an even more immature granulocytic phenotype (myeloblast). In contrast, the doubly truncated and multiply substituted v-Myb protein of AMV resulted in transformation of cells with an immature monocyte/macrophage phenotype (monoblast) (15, 40). The only difference between these two latter transforming proteins was the presence or absence of nine virally introduced amino acid substitutions. These amino acid substitutions can be placed into three groups on the basis of the functional domains in which they occur: four substitutions occur within the highly conserved DNA-binding domain, two substitutions occur within a proline-rich putative hinge region, and three substitutions occur within a domain required for transcriptional activation (Fig. 1).

Others have previously shown that reversion of single amino acid substitutions within the v-Myb DNA-binding domain results in transformation of promyelocytic cells rather than monoblasts (19). In addition, the E26 virus, which encodes a 135-kDa Gag-Myb-Ets fusion protein, transforms precursors of both erythroid and myelomonocytic cells (14, 29). The Myb portion of E26 transforming protein contains a more drastically truncated segment of c-Myb than v-Myb of AMV as well as a single amino acid substitution in Myb which differs from those in v-Myb of AMV (33). A temperature-sensitive mutant of E26 was used to isolate a Myb-inducible myeloid gene (*mim-1*) (32). This gene is expressed in myeloid cells transformed by E26 but not in monoblastic cells transformed by AMV or in erythroid cells transformed by E26. Activation of *mim-1* by various Myb proteins appears to require cooperation with proteins of the C/EBP family, including NF-M, a chicken homolog of C/EBP-β (4, 31). Interestingly, *mim-1* was expressed in myelocytic cells transformed by singly and doubly truncated forms of c-Myb which lacked all of the amino acid substitutions of v-Myb (15).

In order to understand the role of the amino acid substitutions of v-Myb in transformation by and evolution of AMV, we have now constructed and biologically tested a series of hybrid v-Myb-like proteins which contain various constellations of these substitutions. We found that mutations in both the DNA-binding domain and transactivation domain were required for proteins to be highly oncogenic. The presence of both sets of

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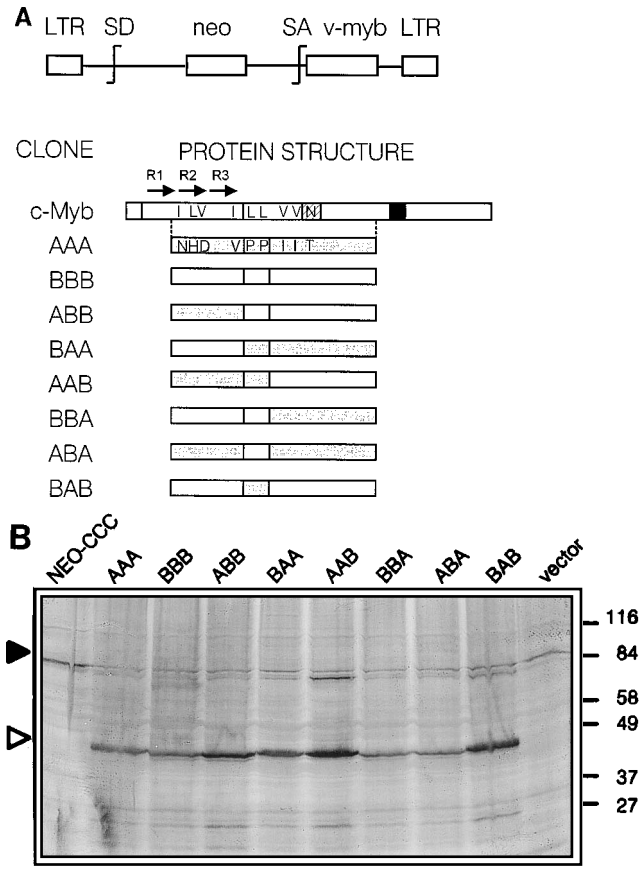

FIG. 1. Schematic of Myb proteins and immunoblot analysis. (A) Structure of wild-type and mutant Myb proteins. R1 to R3 indicate the highly conserved repeats (arrows) within the c-Myb DNA-binding domain. The hatched box indicates a domain that is required for transcriptional activation. The black box indicates a second more C-terminal domain of approximately 30 amino acids that is highly conserved among vertebrate and *Drosophila* Myb proteins. The v-Myb protein (AAA) is truncated at both the N terminus and C terminus relative to c-Myb. The form of v-Myb used in these studies has nine amino acid substitutions indicated by the one-letter amino acid code and by shading. The wild-type amino acid that appears in c-Myb at that position is indicated on the c-Myb protein structure. The BBB mutant is back mutated to resemble c-Myb at each of these nine residues and is not shaded to indicate that fact. Each subsequent mutant is shaded to indicate that part of the protein in which virus mutations are retained. (B) Proteins produced by *neo-myb* proviruses. *neo-myb* proviruses that encoded the proteins diagrammed in panel A were cotransfected into QT6 cells with DNA of the MAV-1 helper virus and selected for G418 resistance. Culture supernatants from pooled transfected colonies were used to infect fresh QT6 cells that were selected for G418 resistance again. Pooled, infected colonies were used as producer cell lines for subsequent transformation studies and analyzed for Myb protein expression by SDS-PAGE and immunoblotting with an anti-Myb monoclonal antibody. The closed triangle indicates the migration of c-Myb, whereas the open triangle indicates the approximate migration of v-Myb (AAA) and other doubly truncated Myb proteins. Vector, cells infected with provirus that contained no *myb* gene. The relative mobilities of coelectrophoresed prestained protein molecular mass markers (in kilodaltons) are indicated on the right.

these activated mutations correlated with rapid growth in cell culture, cell-cell adhesion, the absence of C/EBP and *mim-1* expression, and a general decrease in transcriptional activation by Myb protein.

MATERIALS AND METHODS

Plasmid constructions. DNA restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). Oligonucleotides were made on an Applied Biosystems (Foster City, Calif.) model 380B synthesizer. Recombinant DNA manipulations were carried out by using standard techniques (36). Retrovirus constructions that express c-Myb have previously been described (15). N-dGE (AAA [AMV v-Myb at all three positions]) is a derivative of previously described plasmid NEO-MYB-dGE (17) in which a segment of DNA, including the plasmid backbone and part of the chicken genomic DNA flanking the provirus, was deleted. In addition, the vector was further modified so that unique *Kpn*I and *Cla*I restriction sites flanked the v-*myb* coding region. The N-CLA vector was constructed first by digesting vector NEO-MAV-CLA (17) with *Eco*RI, which yields a fragment that contains simian virus 40 ori, myeloblastosis-associated virus (MAV) long terminal repeats, and the *neo* gene, to which *Not*I linkers were added. This provirus fragment was then ligated to plasmid pSVOd (26) which had been previously digested with *Eco*RI and *Ava*I and to which *Not*I linkers had been ligated. N-dGE was constructed from N-CLA by using the *neo-myb Bam*HI-to-*Cla*I fragment of NEO-MYB-dGE (17) after the upstream *Cla*I site had been destroyed.

NdGE-NM (no mutation) and BBB (back mutated to c-Myb at all three positions) were constructed by inserting the *Age*I-to-*Bsu*36I c*myb* fragment of NEO-CCC (15) into MT7-MYB-dGE (39). To exchange mutations in the DNA-binding domain, existing *Kpn*I and *Bst*EII sites were used to transfer fragments between MT7-dGE and MT7-dGE-NM. To exchange the two amino acid substitutions in the middle of v-Myb protein, existing *Bst*EII and *Sma*I sites were used. To exchange the three amino acid substitutions in the transactivation domain, the *Sma*I site in *myb* and the 3′ *Cla*I sites in vectors were used. All mutant genes were then inserted into the provirus vector N-dGE by a *Kpn*I-to-*Cla*I digest and exchange and were verified by sequence analysis, and restriction site polymorphisms which accompanied a mutation within each domain were analyzed. A plasmid that expressed rat C/EBP-β under the control of the murine sarcoma virus long terminal repeat was obtained from Steven McKnight (Tularik, South San Francisco, Calif.) and has been previously described (5). An isogenic control vector was constructed by excising the C/EBP-β gene with flanking restriction enzymes and religating.

Reporter genes. The EW5(−) chloramphenicol acetyltransferase (CAT) has five wild-type Myb binding sites derived from the A site of the *mim-1* promoter and has been previously described (8). The *mim-1* CAT and luciferase (DE-luc) reporter genes include the proximal promoter of the *mim-1* gene and have been previously described (10, 32).

Cells and media. Quail QT6 fibroblasts were grown in Dulbecco's modified essential medium supplemented with 5% fetal calf serum, 4.5 g of glucose per liter, 1× nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg of streptomycin per ml, and 100 U of penicillin per ml in a humidified 10% CO_2–90% air incubator at 37°C. Yolk sac cells were grown in Iscove's medium supplemented with 10% fetal calf serum, 5% chicken serum heat inactivated for 1 h at 56°C, 4.5 g of glucose per liter, 1× nonessential amino acids, 1× minimal essential medium vitamins, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg of streptomycin per ml, and 100 U of penicillin per ml in a humidified 5% CO_2–95% air incubator at 37°C. The cell lines BM-2 and MSB-1 have previously been described (1, 28).

DNA transfections and immunoblotting. Transient transfections into quail QT6 fibroblasts were performed by a modification of the calcium phosphate procedure (6, 18). Activator plasmids (3 to 5 μg) were cotransfected with reporter DNA (1 μg), tRNA (5.5 μg), and 0.5 μg of a plasmid that expressed β-galactosidase from the cytomegalovirus promoter as an internal control for transfection efficiency. Half of the cells from each transfection plate were scraped in phosphate-buffered saline and then solubilized by boiling for 4 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dyes. Normalized volumes of each were subjected to SDS-PAGE on 10% polyacrylamide gels, and proteins were transferred to nitrocellulose (BA-S 83; Schleicher & Schuell, Keene, N.H.). Transiently expressed Myb and C/EBP-β proteins were detected with monoclonal antibodies anti-Myb-2.2 and −2.7 (11) or rabbit antiserum raised against C/EBP, which was kindly provided by Steven McKnight. Blots were developed by incubation with anti-mouse or -rabbit immunoglobulin G-conjugated alkaline phosphatase (Promega, Madison, Wis.) and 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium.

Transformation and growth factor assays. Each Myb protein was assayed for transformation by cocultivation of virus-infected QT6 cells with hematopoietic cells from chicken embryonic yolk sacs. QT6 cells were first cotransfected with the replication-defective *neo-myb* virus and DNA of a cloned MAV-1 helper virus. G418-resistant colonies were then pooled and used as a source of virus to infect fresh QT6 cells (23). Myeloid cells from a 12-day-old embryonic yolk sac were infected with different viruses by cocultivation for 24 h with adherent, mitomycin-treated, G418-resistant, virally infected QT6 cells (17, 22). On day 5, aliquots of each culture were seeded into 0.8% Methocel and monitored for colony outgrowth. Replicate assays were the products of independent initial DNA transfections.

To determine the growth factor dependence of transformed cells, conditioned media were collected from non-virus-producing, v-Myb-transformed cells (BM-2) or yolk sac cells transformed by BBB. Cells transformed by BBB were seeded into fresh media that contained different concentrations of these conditioned media, and cell counts were determined 4 days later. Anti-chicken myeloid growth factor (anti-cMGF) serum at a 1:100 dilution or an irrelevant antiserum (anti-Mim-1) at the same dilution was added to some of these cultures at the time of seeding.

DNA transfections for CAT and luciferase activities. Transient transfections into QT6 quail fibroblasts were performed as described above. The phase extraction method was employed to determine the CAT activities in normalized volumes of extract as previously described (37). All experiments were performed at least twice, and the means of all experiments with standard deviations are shown. Appropriate dilutions of cell extracts were assayed in those cases for which the level of CAT activity indicated that the substrate was limiting. A background value, generally 100 to 300 cpm, was obtained by extracting CAT assay mixture to which no cell extract had been added. All data for activities shown in figures have been corrected for background, and actual counts ranged between 1,000 and 25,000 cpm. Extracts generated by luciferase reporter genes were prepared and tested for luciferase activity with a luminometer.

Animal cell extracts. Nuclear extracts were prepared as described by Andrews and Faller (2). Quantitation of total protein was determined by bicinchoninic acid assay according to the manufacturer's instructions (catalog no. 23225; Pierce). Transfection efficiency and Myb expression were assessed as described above by using β-galactosidase activity and immunoblotting, respectively.

Bacterial expression of Myb proteins. v-Myb and its mutants were expressed in *Escherichia coli* by using the pMT7-derived plasmids described above. Each plasmid was introduced into the BL21(DE3)(LysS) host strain kindly provided by F. W. Studier (41). Protein expression was induced by the addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when bacterial growth had reached an optical density at 600 nm of 0.4 to 0.7. Incubation was continued for 5 h at 37°C. Then bacteria were resuspended in a urea buffer, centrifuged to remove insoluble material, and diluted 1:20 into electrophoretic mobility shift assay (EMSA) buffer as previously described (12). Samples were quantitated by bicinchoninic acid assay to ensure that equivalent amounts of total protein were loaded for immunoblotting.

EMSAs. These assays for protein-DNA interaction were performed essentially as previously described (12), except that EMSAs performed with animal cell extracts were done in the presence of 4% Ficoll 400 (Sigma) and 50 ng (each) of poly(dI-dC), calf thymus, and salmon sperm DNA per μl. Gels were dried on DE81 chromatography paper (catalog no. 3658-915; Whatman) to prevent loss of probe during gel drying. The *mim-1* site A probe was prepared by annealing the synthetic oligonucleotide 26-mers

5′-dTCGAGCTAAAAAACCGTTATAATGTG
CGATTTTTTGGCAATATTACACAGCT-5′

and was radiolabelled by end filling with the Klenow fragment of DNA polymerase I as previously described (12).

RESULTS

Construction, expression, and oncogenic activities of v-Myb mutants. We have previously demonstrated that v-Myb and doubly truncated c-Myb without the amino acid substitutions of v-Myb can cause leukemic transformation of primary hematopoietic cells in culture (40). However, v-Myb (AAA) transforms approximately 10-fold better than doubly truncated c-Myb (BBB). The only differences between these two proteins are nine amino acid substitutions due to mutations that AMV presumably acquired during repeated passage in animals (10, 15) (Fig. 2A). Two additional amino acid substitutions at the C terminus of AMV v-Myb occur in a region that can be deleted without altering transforming activity (22). Therefore, we used a protein that lacked this C-terminal region as well as the *gag*- and *env*-encoded termini of v-Myb of AMV as the starting point for our studies (17).

Our goal was to determine which of these nine amino acid substitutions were responsible for the difference in transformation between AAA and BBB. Four of these amino acid substitutions map to the DNA-binding domain, two residues map to a putative proline-rich hinge region, and three residues fall within the transcriptional activation domain (8a). In order to determine the consequences of these virally introduced mutations within each of these three major regions of v-Myb, we constructed a series of eight retroviruses to express all possible permutations of these three domains (Fig. 1A).

To test which v-Myb mutants transformed hematopoietic cells in vitro, each *neo-myb* virus was cotransfected with replication-competent MAV-1 helper virus into avian QT6 cells to yield a producer cell line. Transfected cells were selected for G418 resistance and passaged several times. Then the same amount (1 ml) of virus-bearing supernatant was recovered and used to infect fresh QT6 fibroblasts selected for G418 resistance. Approximately 100 resistant foci per plate were obtained, indicating a similar titer for each cell line tested. Then resistant, infected cell lines were passaged several times, and similar levels of Myb proteins of the expected molecular weights were observed, indicating that no major rearrangements had occurred (Fig. 1B). This demonstrated the infectivity and stable integration of each *neo-myb* virus and production of each Myb protein, regardless of its transforming ability.

All of the resulting viruses were analyzed for the ability to promote growth of hematopoietic cells in semisolid media (0.8% Methocel) and liquid culture. Hematopoietic cells from 12-day-old chicken embryonic yolk sacs were infected by cocultivation with producer cell lines. On the following day, infected hematopoietic cells were transferred to new dishes and monitored daily. Cells from two independent infections were cocultivated, and on day 5, cells were seeded into Methocel. Cultures were monitored, and at 3 weeks postcocultivation, colonies were enumerated (Fig. 2A). As predicted, cells incubated with uninfected QT6 cells, cells incubated with provirus that contained no *myb* gene, and cells infected with c-Myb-encoding virus were unable to support colony growth or cellular outgrowth in liquid culture under these conditions. We had previously shown that N-terminal truncation of the first repeat of c-Myb oncogenically activates the protein (10). Therefore, it was not surprising that since all of the doubly truncated mutants tested have deletions of the first repeat, all transformed at some level. However, there was a wide range of transformation efficiency.

Only those two proteins with amino acid substitutions in both the DNA-binding domain and the transactivation domain (AAA and ABA) strongly transformed (i.e., greater than 10-fold-more colonies than BBB). No other combination of two mutant domains of v-Myb nor any mutant domain alone was sufficient for strong transformation. Among this panel of mutants, a similar relative transformation efficiency was determined by monitoring the growth rates of transformed cells in liquid culture (Fig. 2B). These results demonstrate that mutations in both the DNA-binding and transactivation domains of v-Myb cooperate for high-efficiency transformation.

Cells transformed by AAA and ABA, in addition to their growth advantage, had a high tendency to aggregate, similar to that observed with the AMV-transformed BM-2 cell line (Fig. 3). The degrees of aggregation among Myb-transformed primary cells varied from very little in BBB- and BAB-transformed cells to large aggregates of over 100 cells per aggregate in AAA- and ABA-transformed cells. This aggregation correlates with rapid growth, possibly by increasing local concentrations of autocrine growth factors. Cells transformed by v-Myb proteins that lack amino acid substitutions within the DNA-binding domain do not exhibit much aggregation. Therefore, this aggregation phenomenon correlates with the presence of amino acid substitutions within the v-Myb DNA-binding domain.

In an attempt to further understand the growth advantage of cells transformed by mutants such as AAA and ABA, we assayed cMGF expression by immunoblot analysis. We noted an immunoreactive protein in AAA- and ABA-transformed cells that comigrated with a protein found in v-Myb-transformed BM-2 cells but was not present in extracts from cells that grew poorly (BBB and BAB) (data not shown). To determine if medium from cells that grew well and expressed an immunoreactive band of the correct size for cMGF was rich with this factor, we took conditioned medium from the AMV-transformed BM-2 cell line, a virus nonproducer, and medium from weakly transformed BBB cells and assayed to see if either

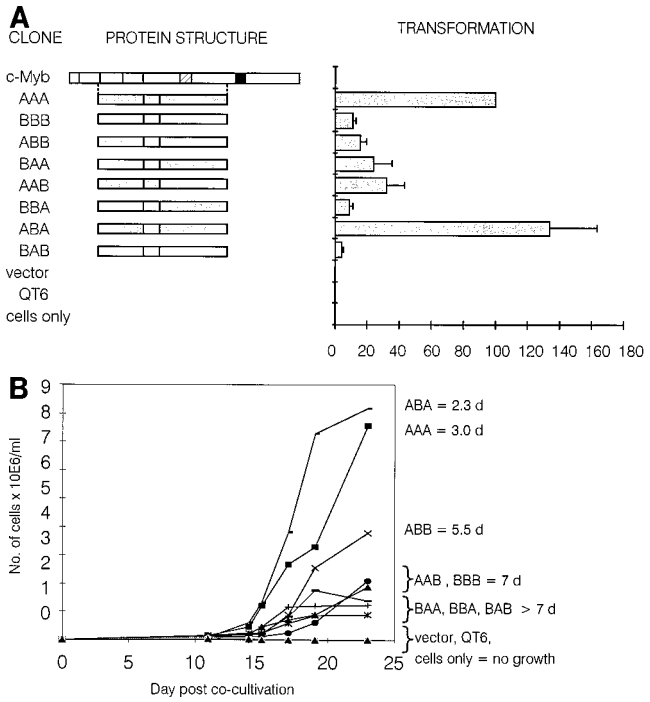

FIG. 2. Transformation by Myb mutants. (A) The cell lines analyzed in Fig. 1 were treated with mitomycin and then used to infect 12-day-old embryonic yolk sac cells by cocultivation. The numbers of colonies in Methocel were determined 3 weeks after the same numbers of cells were seeded at day 5 postinfection. Data are averages of two independent experiments normalized to v-Myb, with v-Myb having a transformation efficiency of 100 U and generating 364 (experiment 1) and 776 (experiment 2) colonies per 5-ml culture. Vector, *neo* virus with no *myb* gene. QT6, cocultivation with uninfected QT6 cells. Cells only, results for an equal number of hematopoietic cells after seeding Methocel without any cocultivation. (B) The transformation efficiency of each v-Myb mutant was analyzed in liquid culture as well; approximate population doubling times in days (d) are indicated.

medium enhanced the growth of BBB-transformed cells. Only medium from BM-2 cells enhanced growth. Moreover, this enhancement of growth was blocked by antiserum to cMGF but not by an irrelevant antiserum (Fig. 4A).

We previously showed that different alterations in c-Myb protein resulted in transformation of different types of myelomonocytic cells (15). However, cytocentrifugation of cells transformed by our new series of doubly truncated Myb proteins did not show any marked differences in morphology (data not shown). Therefore, we used fluorescence-activated cell sorter analysis to assay for the presence of the IC3 cell surface antigen, previously described as a marker for chicken granulocytes (25). We detected IC3 on the surfaces of cells transformed by BBB and BAB but not AAA or ABA (Fig. 4B). These data suggest that the much more rapidly growing AAA- and ABA-transformed cells are not committed to the granulocytic lineage, in contrast to the more slowly growing BBB- and BAB-transformed cells.

Relative DNA binding does not correlate with transformation efficiency. We previously determined that transforming Myb proteins with N-terminal truncations bound less well to DNA than nontransforming Myb proteins with all three N-terminal repeats (10). In addition, we noted higher-affinity binding of the purified v-Myb DNA-binding domain relative to the same domain with all four amino acid mutations back mutated to the wild type. We therefore wished to determine if the spectrum of oncogenic differences in our series of v-Myb mutants could be explained by differences in DNA binding. For this purpose, each mutant v-Myb protein was expressed in *E. coli* by using the inducible T7 RNA polymerase system (41). First, protein lysates were normalized for Myb protein content by immunoblotting (Fig. 5A). Normalized lysates were used to test the binding of each Myb protein in an EMSA with an oligonucleotide which contained one strong Myb binding site from the *mim-1* promoter (32) (Fig. 5B). The presence of Myb in the protein-DNA complex was confirmed in each case by alteration of the complex with anti-Myb antiserum but not preimmune serum. Although some apparent differences in relative affinity for DNA were observed, there was no correlation between the efficiency of leukemic transformation by these proteins and the strength of their DNA binding. Interestingly, although we had shown that the mutations of v-Myb increase the affinity of the isolated DNA-binding domain for its recognition site (10), in the context of the entire v-Myb protein, this difference was modified considerably by the presence or absence of other mutations within the protein (compare AAA and BBB, for example).

Proteins are often modified differently in bacteria than in animal cells; therefore, we also wished to assay the relative DNA binding by our panel of mutant proteins in animal cells. For example, an asparagine in c-Myb has been replaced by a threonine in v-Myb (Fig. 1); this particular amino acid is often phosphorylated in animal cells but rarely in bacteria. In order to produce sufficient protein in animal cells for biochemical analysis, provirus DNAs that encoded each Myb protein were transfected into avian fibroblasts. Immunoblot analysis of fractionated cells demonstrated that each mutant protein was expressed at similarly high levels and localized to the nuclei of cells transfected with *myb* expression vectors but not with a control vector (Fig. 6A). Interestingly, differences in mobility during SDS-PAGE were observed among proteins. In particular, the AAA, BAA, AAB, and BAB proteins migrated more slowly than BBB, ABB, BBA, and ABA. These data imply that altered mobility correlates with the presence of two proline substitutions in the middle hinge region of v-Myb and strongly suggest that this altered mobility reflects an altered protein structure as well. However, the presence or absence of these two prolines did not clearly correlate with transformation efficiency (Fig. 2).

To determine if these different Myb mutant proteins have different binding activities when produced in animal cells, we tested each nuclear extract in an EMSA with the same oligonucleotide described above (Fig. 6B). Similar protein-DNA complexes were present in all cells which expressed Myb proteins but were not present in cells transfected with a control expression vector. To confirm that Myb proteins were present in shifted complexes, each extract was incubated with Myb antibody and the resulting complexes displayed a prominent supershift. In contrast, preimmune sera had no effect on the mobilities of protein-DNA complexes. The abundance of protein-DNA complexes correlated well with the abundance of Myb proteins detected by immunoblotting, indicating no great differences in affinity for DNA among the different proteins. From these data, we concluded that differential transformation among the mutant proteins was unlikely to be due to differences in relative DNA binding to the *mim-1* A site. However, it remains possible that different results may have been obtained with a different Myb binding site.

Transactivation of a model reporter gene does not correlate with transformation efficiency. Because strength of DNA binding did not correlate with transformation efficiency, we next wished to determine whether oncogenic potential correlated with transcriptional transactivation by each protein. The panel

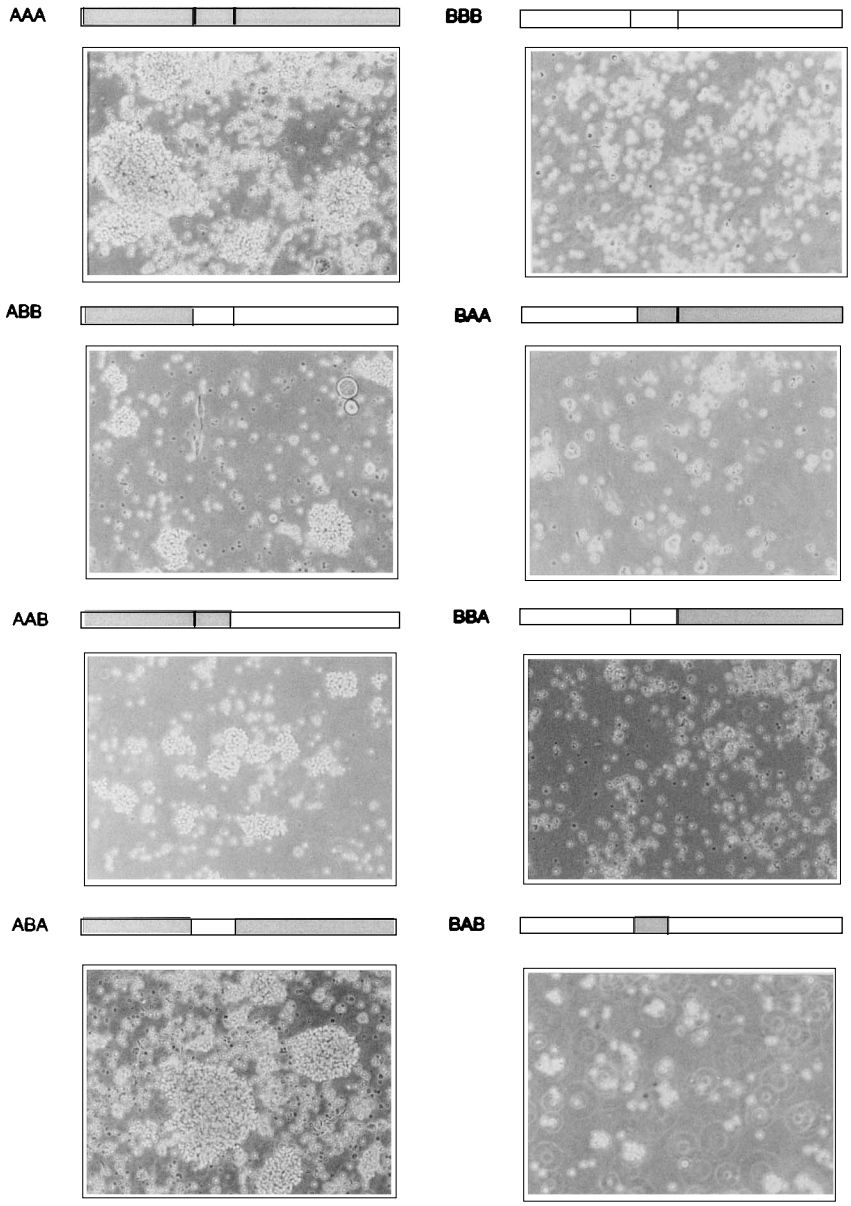

FIG. 3. Growth pattern of Myb-transformed cells. Hematopoietic cells transformed by each v-Myb mutant exhibited various degrees of cell-cell contact, from virtually no aggregation (BBB) to large aggregates of cells (AAA). Transformed cells were maintained in liquid culture and photographed at 3 weeks. A schematic of each transforming protein is illustrated above the corresponding photograph as described in the legend to Fig. 1. Magnification, ×100.

of v-Myb mutants was therefore challenged with two different Myb response element reporter genes and assayed for transcriptional activation in a transient transfection assay (Fig. 7A). The EW5(−) CAT reporter contains a minimal TATA box promoter from the adenovirus E1B gene prefaced by five tandem copies of the highest-affinity Myb binding site of the *mim-1* promoter (8). The *mim-1* CAT reporter contains a naturally occurring promoter with three Myb binding sites (A, B, and C) of various affinities derived from the endogenous Myb-inducible *mim-1* gene (32). Provirus DNAs which expressed each Myb mutant as an activator were cotransfected with either of these reporter genes, and cell lysates were assayed for CAT activity. Proteins BBB and BAB, which weakly transformed, were the strongest transcriptional activators of both test genes (Fig. 7B). Conversely, proteins AAA and ABA, which strongly transformed, were among the weakest transcriptional activators of both test genes. However, proteins ABB, BAA, and BBA were weak in both transformation and transcriptional activation assays. We concluded that although strongly transforming proteins were clearly among the weakest transcriptional activators, weak transcriptional activation alone was not a good predictor of high-efficiency transformation.

Cotransactivation with C/EBP-β correlates with weak transformation by Myb proteins. Previous studies have demonstrated that v-Myb and c-Myb can synergize with NF-M, the chicken homolog of C/EBP-β, to activate the endogenous *mim-1* gene (4, 31). We therefore sought to determine if the differences in transforming potential of our series of Myb proteins were related to their abilities to cooperate with C/EBP proteins in transcriptional activation. Each *myb* provirus was

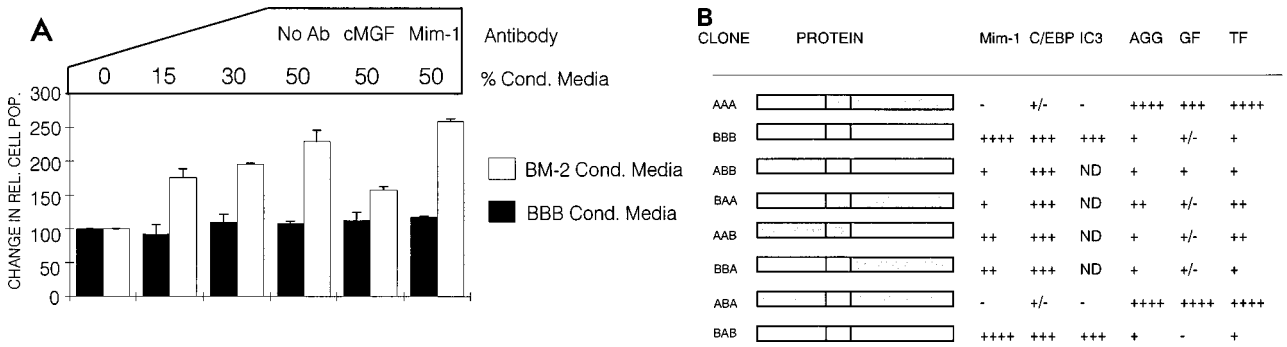

FIG. 4. Growth factor dependence of transformed cells. (A) Two established populations of BBB-transformed cells derived from separate experiments were incubated in parallel with either conditioned (cond.) media from the highly transformed v-Myb nonproducer BM-2 cell line or weakly transformed BBB cells themselves. Data are averages of two experiments with standard deviations. (B) Summary of assayed markers, including the mature granulocyte marker IC3, aggregation in liquid culture (AGG), growth factor production (GF), and transformation efficiency (TF) (see text for details). ND, not done. −, not detectable; +/−, barely detectable; + to ++++, detectable in increasing degrees.

cotransfected into avian fibroblasts with an expression vector which produces exogenous C/EBP-β and with a reporter gene which contained the *mim-1* promoter driving firefly luciferase cDNA (Fig. 8A). Among doubly truncated Myb proteins, AAA and ABA were markedly less efficient at synergizing with C/EBP-β protein in transactivation of the *mim-1* promoter.

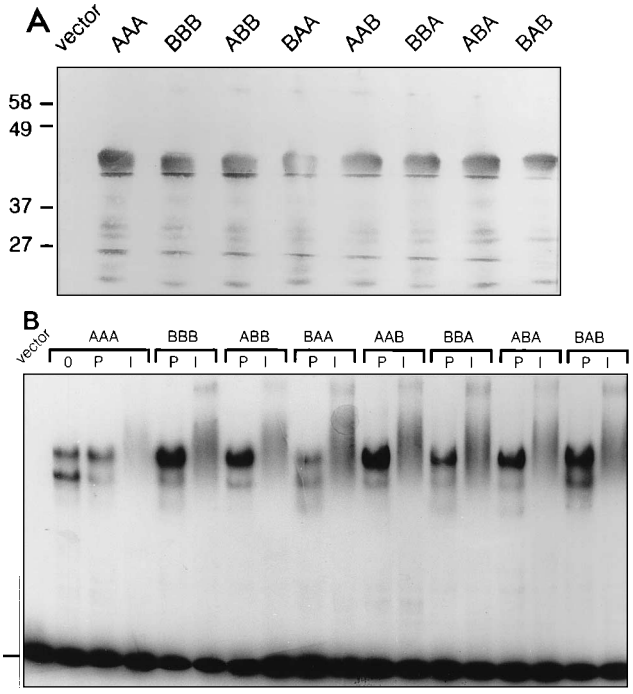

FIG. 5. DNA binding activities of v-Myb mutants expressed in *E. coli*. (A) Each v-Myb mutant was expressed in *E. coli* by using the T7 RNA polymerase system. Equal amounts of total protein were loaded in each lane, and Myb proteins were detected by SDS-PAGE and immunoblotting. Vector, an *E. coli* extract derived from cells that contained a T7 vector with no *myb* gene. The relative mobilities of coelectrophoresed prestained protein molecular mass markers (in kilodaltons) are indicated on the left. (B) Each v-Myb mutant was analyzed by EMSA for its ability to bind an oligonucleotide that contained a single Myb binding site derived from the *mim-1* promoter. The same extracts used in panel A were incubated with probe alone (0), probe and preimmune sera (P), or probe and immune sera (I). The line segment on the lower left indicates the migration of free probe. Vector extract was incubated with probe alone.

Interestingly, these same two proteins in the series had high transformation efficiencies. Immunoblots of the same cell extracts used for luciferase assays were performed to determine the levels of Myb and C/EBP proteins (Fig. 8B). These proteins were detected only in cells into which appropriate DNAs had been transfected. Furthermore, the levels of Myb and C/EBP proteins did not vary significantly among different cells, indicating that the observed differences in cotransactivation were not due to differences in protein stability or to cross-regulation of expression vectors by their protein products. These results suggest that the abilities of various doubly truncated Myb proteins to cooperate with C/EBP-β correlate inversely with their efficiencies of transformation.

High-efficiency transformation by v-Myb correlates with the absence of Mim-1 and C/EBP proteins. We next wished to determine if the observed differences among Myb proteins in cotransactivation of the *mim-1* promoter with C/EBP were mirrored in the phenotypes of transformed myelomonocytic cells. Therefore, we determined the levels of Myb, Mim-1, and C/EBP proteins in the primary hematopoietic cells transformed by each of the doubly truncated Myb proteins (Fig. 9). Anti-Myb antibodies demonstrated the presence of doubly truncated Myb proteins in each transformed cell type, as expected. Antisera raised against Mim-1 detected this 30-kDa protein in cells transformed by all of the doubly truncated Myb proteins except AAA and ABA, the two proteins with high transformation efficiencies. Unexpectedly, the AMV-transformed BM-2 cell line also had detectable levels of Mim-1. Antiserum raised against rat C/EBP-β was able to detect a protein with the appropriate relative mobility in the AMV-transformed BM-2 cell line which expresses NF-M (chicken C/EBP-β) but not in the Marek's disease virus-transformed MSB-1 T-cell line which does not express NF-M (data not shown). This antiserum detected C/EBP-related proteins in cells transformed by all of the doubly truncated Myb proteins except AAA and ABA. These results demonstrate that the presence of C/EBP strongly correlates with *mim-1* expression in vivo. Furthermore, the amino acid substitutions in the DNA-binding and transcriptional activation domains of v-Myb are required to prevent expression of C/EBP and Mim-1 in primary transformed cells. Finally, lack of expression of C/EBP and Mim-1 correlates with high-efficiency transformation by Myb proteins that carry these two mutant domains (AAA and ABA).

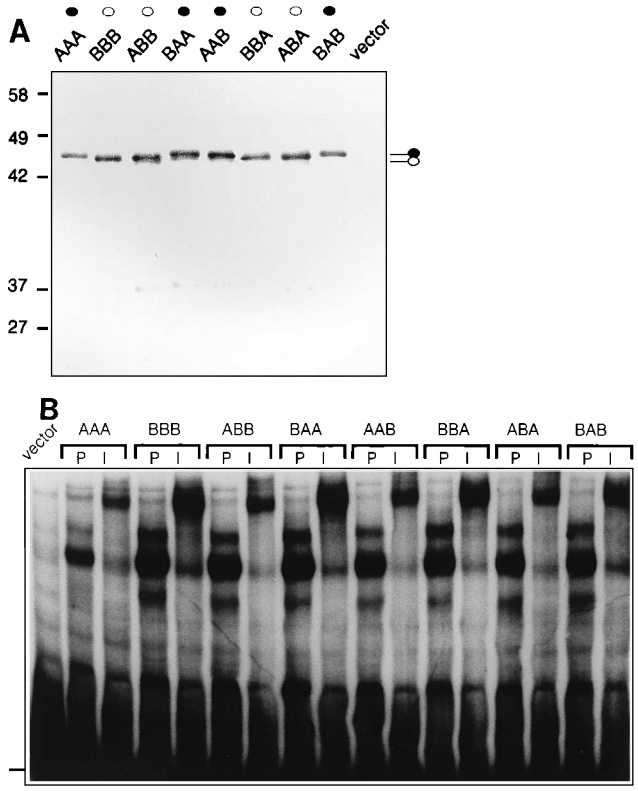

FIG. 6. DNA binding activities of v-Myb mutants expressed in animal cells. (A) Each v-Myb mutant provirus was transfected into QT6 cells. Nuclear extracts were prepared 2 days later, and Myb protein content was normalized by immunoblot analysis with an anti-Myb monoclonal antibody. Vector, extracts generated from QT6 cells transfected with the same amount of provirus DNA that lacked the *myb* gene. Each Myb protein with two virally introduced proline mutations in the central hinge region of the protein (XAX) was slightly retarded in its electrophoretic mobility, as indicated on the right by a closed circle and a matching circle above the corresponding lane. Mutants without these mutations migrate slightly faster and are indicated by open circles. The relative mobilities of coelectrophoresed prestained protein molecular mass markers (in kilodaltons) are indicated on the left. (B) Myb proteins from transfected QT6 cell nuclear extracts analyzed in panel A were compared by EMSA for the ability to bind an oligonucleotide that contained a single Myb binding site derived from the *mim-1* promoter. Extracts were incubated with probe and preimmune sera (P) or probe and immune sera (I). The line segment on the lower left indicates the migration of free probe.

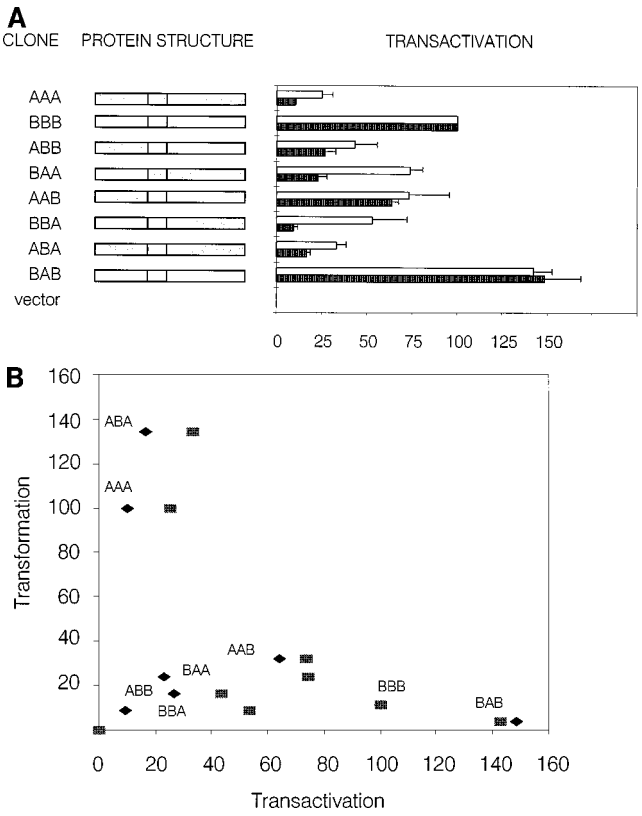

FIG. 7. Transactivation by Myb mutants. (A) Each Myb protein was tested for its ability to activate transcription from a CAT reporter plasmid prefaced by either the *mim-1* promoter (open bars) or five tandem copies of a high-affinity Myb binding site and a miminal TATA box (EW5) (solid bars). Activities shown are relative to that of activator BBB, which was assigned a value of 100 U. Data are averages of two experiments, with error bars indicating standard deviations. The schematics of protein structure are the same as in Fig. 1A. For the *mim-1* and EW5 promoters, 100 U was approximately 3,000 and 12,000 cpm, respectively. All Myb proteins were expressed at equivalent levels, as analyzed by immunoblot analysis (data not shown). (B) Transformation data for all mutants in Fig. 2A were plotted against transactivation data in Fig. 5A for both the *mim-1* promoter (rectangles) and the EW5 promoter (diamonds). Mutant BBB data points overlap. Vector alone is plotted at 0.

DISCUSSION

We have shown that the amino acid substitutions within the DNA-binding and transcriptional activation domains of v-Myb are required for high-efficiency transformation of primary myelomonocytic cells in culture. Such strong transformation correlated with lack of expression of C/EBP and *mim-1* in transformed cells. *mim-1* was isolated in a differential cDNA screen of myeloid cells transformed by a temperature-sensitive E26 virus which produces a Gag-Myb-Ets fusion protein (32). However, *mim-1* was not expressed in erythroblasts transformed by the same virus or in monoblasts transformed by AMV. The Myb portion of E26 transforming protein has a single amino acid substitution relative to the homologous region of c-Myb, and this substitution differs from those found in AMV v-Myb (33). Single amino acid reversions within the DNA-binding domain of AMV v-Myb can also result in expression of *mim-1* in transformed cells (19).

The results presented here suggest that the amino acid substitutions within AMV v-Myb which occurred during repeated passage of virus in leukemic chickens were selected for the ability to preclude expression of C/EBP and Mim-1 in transformed cells. Therefore, although *mim-1* is clearly a Myb-inducible gene, its protein product is almost certainly not required for oncogenic transformation. Rather, *mim-1* appears to be part of a differentiation program normally induced by c-Myb during granulocytic differentiation. The fact that v-Myb was selected for its failure to activate this program is consistent with our model that v-Myb regulates only a subset of those genes normally regulated by c-Myb (10).

Originally we determined that oncogenic activation caused by N-terminal deletion of the first repeat of c-Myb correlated with a decrease in DNA binding. We have now examined the relative DNA binding of a panel of mutants of v-Myb that are all deleted within the first repeat. Predictably, they all do not bind as well as proteins with intact N termini, and yet among themselves, the panel of mutants bind similarly, presenting nevertheless a wide range of oncogenicity. This suggests that DNA binding itself is essentially unchanged and that different protein-protein contacts are likely to be responsible for the different biological activities of these proteins. In support of this idea, we have shown that either of two conserved repeats

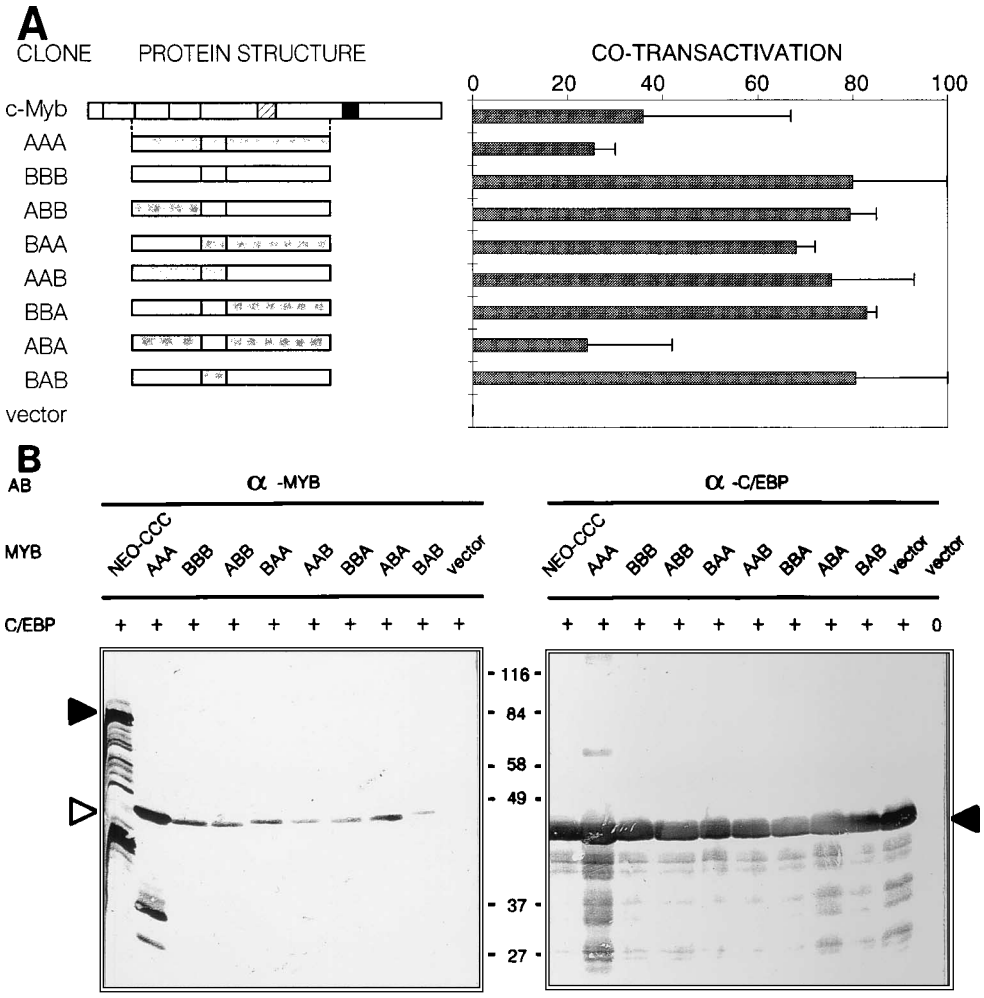

FIG. 8. Cotransactivation by Myb mutants and C/EBP-β. (A) Each Myb protein was tested for its ability to coactivate transcription from a reporter plasmid with the *mim-1* promoter in the presence of the same amount of cotransfected C/EBP-β (3.0 μg). Data are averages of two experiments with standard deviations. Activities shown are relative to that of the strongest activator in each experiment, which was assigned a value of 100 U. C/EBP-β alone could activate by approximately 20 U; therefore, this value was subtracted so that vector activity equaled 0. The schematics of protein structure are the same as in Fig. 1A. For the *mim-1* promoter, 100 U was approximately 1,000 light units. (B) The same extracts analyzed for Myb and C/EBP-β coactivation of the *mim-1* promoter were analyzed for protein expression by immunoblotting. The antibody (AB) used, the type of Myb (MYB) cotransfected, and the presence (+) or absence (0) of cotransfected C/EBP-β are indicated. The black triangle indicates the approximate migration of c-Myb, the open triangle indicates the migration of v-Myb and v-Myb mutants, and the gray triangle indicates the migration of C/EBP-β.

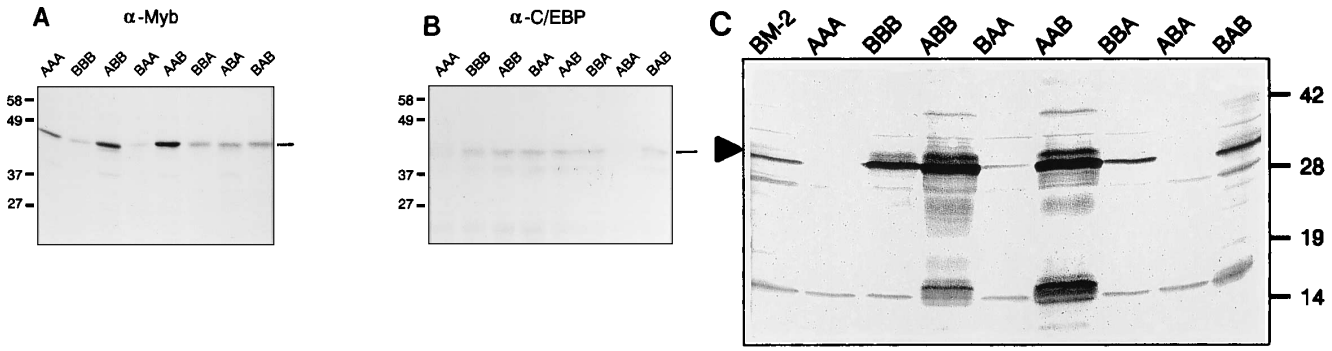

FIG. 9. Expression of C/EBP and Mim-1 in transformed cells. Hematopoietic cells transformed by each v-Myb mutant were analyzed for Myb (A), C/EBP-related proteins (B), and Mim-1 (C) by immunoblotting. A line segment to the right of a panel (A and B) or a closed triangle to the left (C) indicates the approximate migration of immunoreactive proteins. The relative mobilities of coelectrophoresed prestained protein molecular mass markers (in kilodaltons) are indicated on the left (A and B) or right (C).

of the *Drosophila* Myb DNA-binding domain (but not both) could be exchanged into chicken v-Myb and still result in a transforming protein (3). Interestingly, all of these chimeric proteins bound DNA equally well, although some completely failed to transform while others transformed at levels similar to those of wild-type v-Myb. These data suggest that the specific protein-protein contacts made by v-Myb are critical for initiating patterns of gene expression involved in proliferation and differentiation.

We previously showed that deletion of the N terminus of c-Myb strongly activates its oncogenic potential, whereas deletion of the C terminus activates only weakly (10, 15). Introduction of the amino acid substitutions of v-Myb into c-Myb was not sufficient for transformation. Furthermore, introduction of these v-Myb substitutions into singly truncated c-Myb proteins abrogated their transforming abilities. These results suggest a route by which the v-*myb* oncogene of AMV has evolved between its initial isolation in 1941 and its molecular cloning in 1980 (16, 38). Deletion of the N terminus is likely to have occurred first because it is a more oncogenic deletion than a C-terminal deletion, at least in culture, and because mutagenesis of the 5′ end of the c-*myb* locus by retrovirus insertion has been shown to occur in chickens (20, 34). C-terminal truncation after initial N-terminal truncation would clearly have improved the transformation efficiency of this virus (15). Such a second truncation results in the shift of transformed cells to a more immature myelocytic phenotype. Repeated passage of virus in vivo selected for increased virulence, which resulted in a decrease in the average incubation period (from 138 days for the 1st passage to 18 days by the 16th passage) (16). It appears to be likely that this resulted in the amino acid substitutions which were the final step in the evolution of v-*myb*. These amino acid substitutions likely occurred after the two truncations because as noted above, they are collectively incompatible with transformation by singly truncated Myb proteins.

One unexpected result was that the AMV-transformed BM-2 cell line expressed both C/EBP and Mim-1, whereas primary monoblasts transformed by wild-type v-Myb protein did not. In addition, BM-2 cells can be induced very rapidly to differentiate into adherent macrophages in response to tetradecanoyl phorbol acetate, whereas we have found that primary hematopoietic cells transformed by v-Myb (AAA), but not by back-mutated BBB protein, are much more resistant to tetradecanoyl phorbol acetate (data not shown). This suggested to us that the v-*myb* gene within the single AMV provirus within the BM-2 cell genome harbors a previously unrecognized mutation relative to v-*myb* isolated from primary leukemic chicken cells (21, 35). However, resequencing of v-*myb* genes from BM-2 cells did not reveal any unexpected mutations. These results suggest that BM-2 cells have sustained additional mutations, relative to primary AMV-transformed cells, which result in expression of C/EBP and Mim-1 and an altered response to tetradecanoyl phorbol acetate. Furthermore, they underscore the differences inherent in using established cell lines rather than transformation of primary cell cultures in order to understand the transformation process.

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