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Functional antagonism between members of the *myb* family: B-*myb* inhibits v-*myb*-induced gene activation

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The oncogene v-*myb* and its cellular progenitor c-*myb* encode nuclear, DNA binding phosphoproteins that control the expression of certain target genes in immature hematopoietic cells. Here, we report the isolation of a *myb*-related chicken gene, chicken B-*myb*. We show that expression of B-*myb*, unlike that of c-*myb*, is not restricted to hematopoietic cells, suggesting that B-*myb* functions in a broader spectrum of cell types than c-*myb*. We have identified the authentic chicken B-*myb* protein as a nuclear protein of ~110 kDa. We show that the B-*myb* protein specifically recognizes v-*myb* binding sites *in vitro* and that binding is mediated by an N-terminally located DNA binding domain. Although B-*myb* protein recognizes *myb* binding sites, B-*myb* fails to transactivate several *myb*-responsive gene constructs as well as the endogenous *myb*-responsive gene *mim*-1. Instead, we find that B-*myb* represses v-*myb*- and c-*myb*-mediated activation of the *mim*-1 gene, most likely by competing with other *myb* proteins for binding sites. Our results raise the possibility that B-*myb* is an inhibitory member of the *myb* family.
Key words: chicken B-*myb*/*myb*-related gene/sequence specific DNA binding/transactivation

Introduction

The oncogene v-*myb* was originally defined by the chicken retroviruses AMV and E26 and is responsible for the transformation of myelomonocytic hematopoietic cells by these viruses (Moscovici and Gazzolo, 1982). The c-*myb* gene, from which v-*myb* is derived, is essential for the proliferation of hematopoietic precursor cells. C-*myb* expression is high in immature cells of all hematopoietic lineages and is turned off during their terminal differentiation (reviewed in Shen-Ong, 1990). Sustained expression of c-*myb* blocks differentiation of immature erythroid cells (Clarke *et al.*, 1988), whereas a block of c-*myb* expression reduces proliferation of precursor cells (Gewirtz and Calabretta, 1988). Mice lacking a functional c-*myb* gene suffer from severe defects in fetal hepatic hematopoiesis (Mucenski *et al.*, 1991). In contrast to hematopoietic cells, non-hematopoietic cells normally do not express c-*myb*.

V-*myb* and chicken c-*myb* encode nuclear, DNA binding phosphoproteins with molecular weights of 45 000 and 75 000, respectively (Boyle *et al.*, 1983; Klempnauer *et al.*, 1983; Moelling *et al.*; 1985; Klempnauer and Sippel, 1986, 1987). V-*myb* and c-*myb* proteins possess intrinsic DNA binding activity, specific for the sequence motif $PyAAC^{G}/_{T}G$ (Biedenkapp *et al.*, 1988), and the expression of gene constructs containing such binding sites is activated by v-*myb* or c-*myb* (Klempnauer *et al.*, 1989; Nishina *et al.*, 1989; Weston and Bishop, 1989; Ibanez and Lipsick, 1990). It is therefore assumed that *myb* proteins function as transcription factors. Consistent with such a role, a natural target gene for v-*myb*, the chicken *mim*-1 gene, has been identified in myeloid cells transformed by the E26 virus (Ness *et al.*, 1989).

Recently, two *myb*-related human genes, A-*myb* and B-*myb*, have been isolated (Nomura *et al.*, 1988). The predicted proteins encoded by these genes show strong amino acid sequence homology to the DNA binding domain of the c-*myb* protein, suggesting that these proteins form a family of DNA binding factors. We have isolated a *myb*-related gene from chicken, designated as chicken B-*myb*, since it encodes a protein that is highly related to the human B-*myb* protein. Expression of B-*myb*, unlike that of c-*myb*, is not restricted to hematopoietic cells, suggesting that B-*myb* functions in a broader spectrum of cells than c-*myb*. We have identified the authentic B-*myb* protein as a 110 000-M_r nuclear protein and analyzed its transactivation potential. Our results show that the B-*myb* protein does not activate promoters containing *myb* binding sites but that it inhibits transactivation mediated by another *myb* protein.

Results

Nucleotide sequence of chicken B-myb

A cDNA clone of a *myb*-related chicken gene was isolated from a cDNA library of AMV-transformed chicken myeloblasts by screening with a mixture of probes specific for human A-*myb* and B-*myb*. The nucleotide sequence of this clone (EMBL/GenBank/DDBJ accession number X67505) contains an open reading frame potentially encoding a protein of 686 amino acids with a calculated molecular weight of ~78 000. We have compared the chicken B-*myb* protein with the human B-*myb* protein and with the chicken c-*myb* protein (Figure 1A and B). The deduced protein is highly homologous to the predicted human B-*myb* protein, suggesting that we have cloned the chicken homolog of B-*myb*. While the B-*myb* proteins of both species show extensive homologies throughout their entire length, the sequences shared by the chicken B-*myb* and c-*myb* proteins are restricted to two parts of the proteins. The homology region located close to the N-terminus of the B-*myb* protein coincides with a 3-fold tandem repeat domain that functions as sequence specific DNA binding domain of v-*myb* and c-*myb* proteins (Klempnauer and Sippel, 1987; Howe *et al.*, 1990; Oehler *et al.*, 1990). The second, C-terminally located homology region has been first identified in a *myb*-related protein from *Drosophila melanogaster* (Peters *et al.*, 1987).

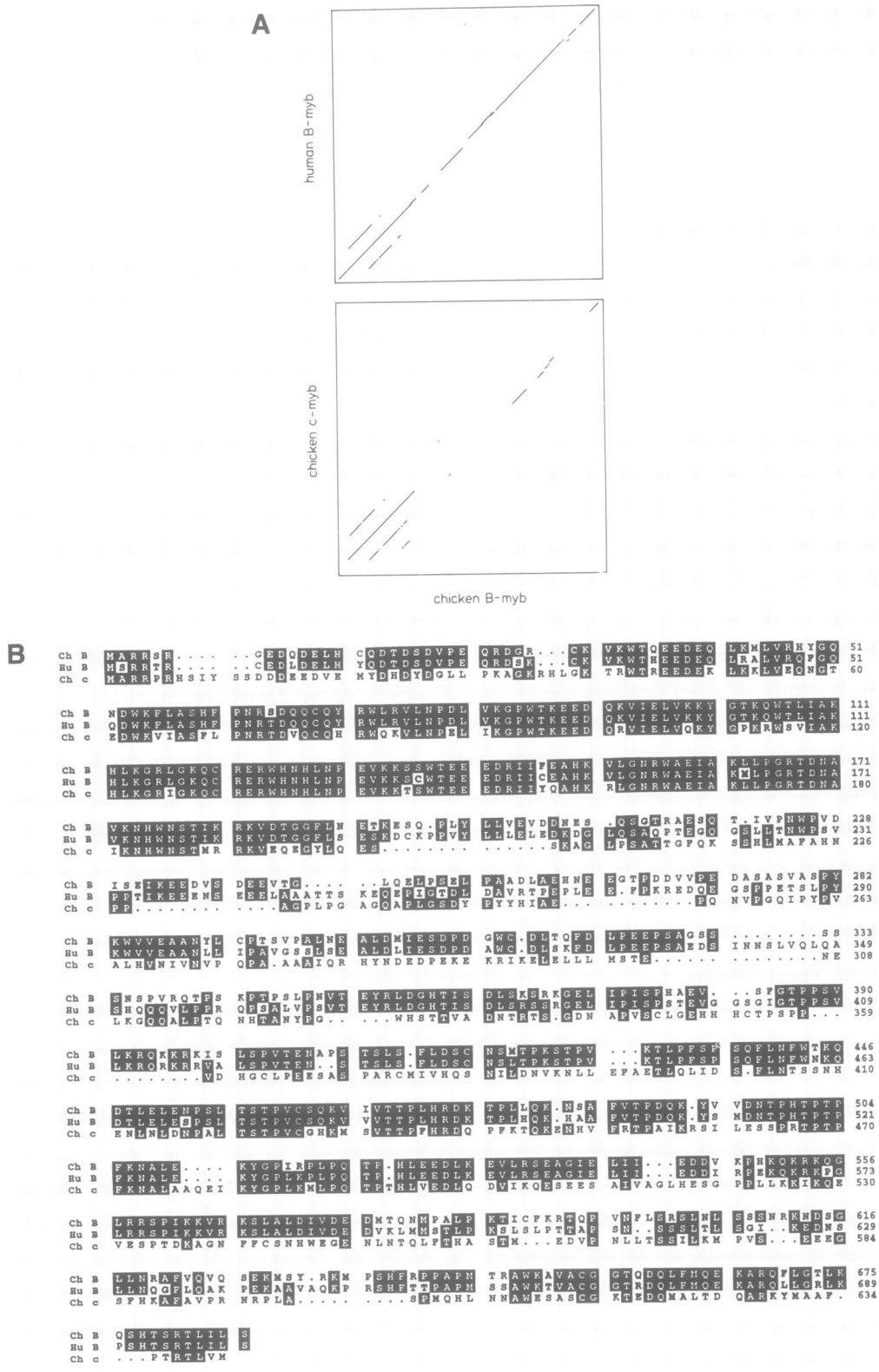

Fig. 1. Structure of the chicken B-*myb* protein. (**A**) Dot-matrix comparison of c-*myb* and B-*myb* amino acid sequences. The upper panel shows a schematic comparison of the human and chicken B-*myb* proteins. Amino acid sequence homologies are indicated by dots and lines. The lower panel shows a comparison of chicken c-*myb* and chicken B-*myb* proteins. (**B**) Amino acid sequence comparison of chicken B-*myb* (Ch B), chicken c-*myb* (Ch c) and human B-*myb* (Hu B) proteins. Positions of identity are shown in black.

As yet, a specific function has not been assigned to this domain. Interestingly, the acidic transactivation domain, identified in the v-*myb* and c-*myb* proteins (Weston and Bishop, 1989), is missing from the B-*myb* protein.

Identification of the chicken B-myb protein

We synthesized an N-terminal polypeptide of the chicken B-*myb* protein in *Escherichia coli* (see below) and immunized rabbits with the recombinant protein. The resulting antiserum

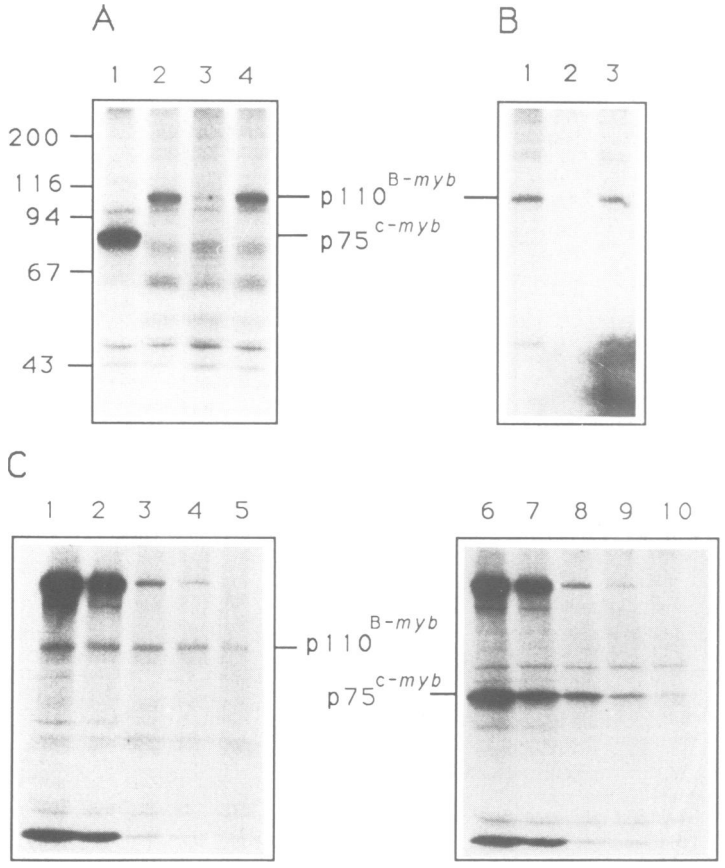

Fig. 2. Immunoprecipitation of the chicken B-*myb* protein. (**A**) Polyclonal rabbit serum specific for c-*myb* (lane 1) or B-*myb* (lanes 2–4) was used to immunoprecipitate proteins from extracts of [^{35}S]methionine-labeled AEV-transformed chicken erythroblasts. Lanes 3 and 4 show blocking experiments, in which the cell extract was supplemented with total bacterial protein of bacteria expressing (lane 3) or lacking (lane 4) recombinant B-*myb* protein. The positions of molecular weight markers (in kDa) and of p75$^{c\text{-}myb}$ and p110$^{B\text{-}myb}$ are indicated. (**B**) [^{35}S]methionine-labeled AEV-transformed chicken erythroblasts were analyzed by immunoprecipitation with B-*myb* specific antiserum without fractionation (lane 1) or after fractionation into cytoplasmic (lane 2) and nuclear (lane 3) fractions. Equivalent amounts of total cell extract or both fractions were used. (**C**) AEV-transformed chicken erythroblasts were radiolabeled with [^{35}S]methionine for 30 min and then transferred to growth medium lacking radioactive methionine and supplemented with 1 mM unlabeled methionine. Proteins were immunoprecipitated immediately (lanes 1 and 6) or after additional incubations of 1 h (lanes 2 and 7), 2 h (lanes 3 and 8), 3 h (lanes 4 and 9) or 4 h (lanes 5 and 10). Left and right panels show immunoprecipitations with B-*myb* and c-*myb* specific antisera, respectively. Bands corresponding to c-*myb* and B-*myb* proteins are marked, other bands represent proteins precipitated non-specifically.

immunoprecipitated a 110 000-M_r protein from extracts of AEV-transformed erythroblasts (Figure 2A). Precipitation of the 110 000-M_r protein, termed p110$^{B\text{-}myb}$, was specific since it could be blocked by an excess of bacterially expressed B-*myb* polypeptide. We note that the apparent molecular weight of the B-*myb* protein is considerably larger than its calculated molecular weight.

To investigate whether p110$^{B\text{-}myb}$ is a nuclear protein, we fractionated AEV-transformed chicken erythroblasts into nuclear and cytoplasmic fractions and immunoprecipitated the B-*myb* protein from both fractions. As illustrated in Figure 2B, p110$^{B\text{-}myb}$ was mainly present in the nuclear fraction.

C-*myb* and v-*myb* proteins are turned over rapidly *in vivo*. To investigate whether the B-*myb* protein is also unstable, we determined its half-life by the pulse–chase experiment illustrated in Figure 2C. Our results showed that the B-*myb* and c-*myb* proteins differ in their half-lives. While the c-*myb* protein has a half-life of ~1 h, p110$^{B\text{-}myb}$ was significantly more stable (half-life ~3–4 h).

Expression of B-myb in different cell types

High levels of c-*myb* expression are restricted to immature cells of various hematopoietic lineages. To investigate whether the B-*myb* expression pattern differs from that of c-*myb*, we analyzed B-*myb* and c-*myb* mRNA levels in several hematopoietic chicken cell lines and in chicken embryo fibroblasts. Figure 3A–C shows Northern blots of polyadenylated RNA from these cell lines hybridized to probes specific for B-*myb*, c-*myb* and GAPDH. In contrast to the vast differences in c-*myb* expression between these cells, all four cell types expressed similar levels of B-*myb* mRNA. The size of the major B-*myb* mRNA species was ~2.4 kb. A slightly smaller, minor transcript was usually detected; the identity of this RNA is unknown and was not investigated further. Our analysis showed that c-*myb* and B-*myb* are expressed independently of each other and that B-*myb* expression is not restricted to hematopoietic cells.

Sequence specific DNA binding by bacterially expressed B-myb protein

The presence of the N-terminal, 3-fold tandem repeat domain in the chicken B-*myb* protein suggested that it has sequence specific DNA binding activity. To study its DNA binding properties we expressed two N-terminal B-*myb* polypeptides in bacteria (Figure 4A and B). Expression vector p25/3B-*myb* encodes a polypeptide containing the first 229 N-terminal amino acids of the B-*myb* protein. pBM100 encodes a similar

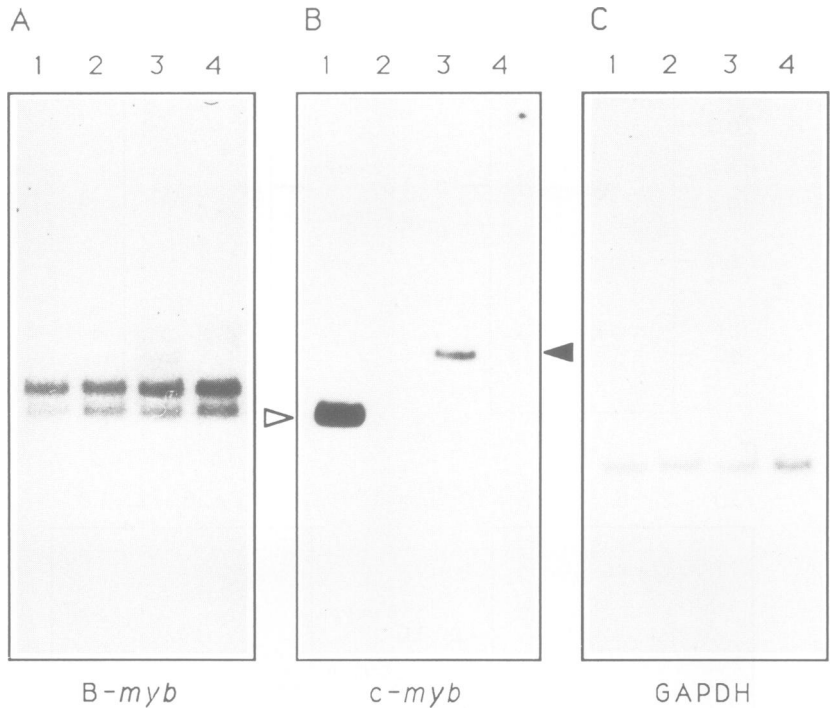

Fig. 3. Expression of B-*myb* mRNA in different cell types. (**A**)–(**C**) Northern blot of polyadenylated RNA from AMV-transformed chicken myeloblasts (lanes 1), MC29-transformed chicken macrophages (lanes 2), AEV-transformed chicken erythroblasts (lanes 3) and chicken embryo fibroblasts (lanes 4). The blot was hybridized sequentially to probes specific for B-*myb* (A), c-*myb* (B) or glyceraldehyde-phosphate dehydrogenase (C). In (B) v-*myb* and c-*myb* mRNAs are marked by open and closed arrows, respectively.

protein in which the first 62 N-terminal amino acids have been deleted. This deletion corresponds exactly to the N-terminal truncation of the v-*myb* protein of AMV and removes most of the first of the three tandem repeats present in the N-terminal repeat region. Both recombinant B-*myb* proteins carry 17 amino acids derived from the bacterial *trp* operon at the N-terminus.

To demonstrate sequence specific DNA binding activity of the bacterially expressed proteins, we performed gel retardation assays using radiolabeled oligonucleotides containing either a v-*myb* recognition motif (TAACGG) or a mutated version of this motif (TTTCGG). Figure 4C and D shows that both B-*myb* polypeptides bound to an oligonucleotide containing the v-*myb* binding site but not to the oligonucleotide containing the mutated *myb* binding motif. This experiment demonstrated that the N-terminus of the B-*myb* protein functions as a sequence-specific DNA binding domain whose sequence specificity is related to that of the v-*myb* protein. The experiment illustrated in Figure 4 also showed that deletion of most of the first of the three tandem repeats does not impair sequence-specific DNA binding.

To investigate whether v-*myb* and B-*myb* proteins bind to *myb* binding sites with similar affinities, we performed the titration experiments shown in Figure 5, using the *myb* binding motif 'A' from the chicken *mim*-1 promoter (Ness *et al.*, 1989). Quantification of the binding reactions showed that the dissociation constants of both proteins for this binding site were similar ($1-2\times10^{-9}$ and $2-3\times10^{-9}$ for the v-*myb* and B-*myb* proteins, respectively).

It has been claimed that the human B-*myb* protein exhibits a sequence specificity for DNA binding that is different from that of the human c-*myb* protein (Mizuguchi *et al.*, 1990). We have tested binding of the chicken B-*myb* protein to the binding site described by Mizuguchi *et al.* (1990) but have failed to detect specific binding of the chicken protein to this site (data not shown).

Transactivation by B-myb

To investigate whether chicken B-*myb* functions as a transactivator, we co-transfected B-*myb* and v-*myb* expression vectors with several different reporter gene constructs shown previously to be *myb*-inducible. Plasmid p3×ATk-Luc contains three copies of the *myb* binding motif 'A' from the chicken *mim*-1 gene (Ness *et al.*, 1989) fused to the HSV Tk promoter and the luciferase gene (De Wet *et al.*, 1987). p-81Tk-Luc (Nordeen, 1988) is a control plasmid lacking the *myb* binding sites. Plasmid p-240-Luc (Ness *et al.*, 1989) contains chicken *mim*-1 promoter sequences (from −240 to +150 bp) fused to the luciferase reporter gene. Figure 6 shows the results of co-transfections, using the chicken macrophage-like HD11 cell line. V-*myb* strongly activated the promoters containing *myb* binding sites (p3×ATk-Luc and p-240-Luc). Surprisingly, B-*myb* failed to activate p3×ATk-Luc above the level of the control plasmid and did not activate plasmid p-240-Luc. The level of expression of p3×ATk-Luc in the absence of exogenous v-*myb* or B-*myb* was similar to the level of expression of p-81Tk-Luc, suggesting that endogenous B-*myb*, which is expressed at low levels in HD11 cells (see below), does not activate the p3×ATk-Luc reporter gene. It is therefore unlikely that the failure of exogenous B-*myb* to activate this gene construct was due to it being already activated by the endogenous B-*myb*. Failure of B-*myb* to activate promoters containing *myb* binding sites is not peculiar to the HD11 cell line, since it was also observed when the quail fibroblast cell line QT6 or primary chicken embryo fibroblasts were

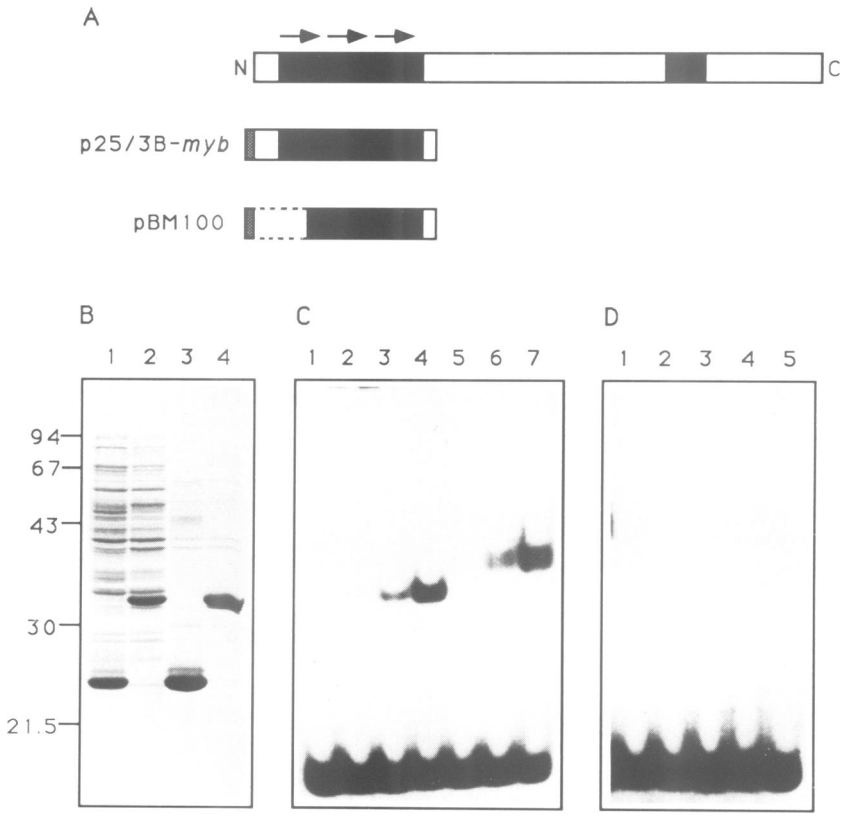

Fig. 4. Specific DNA binding by bacterially expressed B-*myb* protein. (**A**) Schematic diagram of chicken B-*myb* protein and two polypeptides encoded by bacterial expression vectors p25/3B-*myb* and pBM100. The two major conserved regions between B-*myb* and c-*myb* proteins are shown in black, the tandem repeats located at the N-terminus are marked by arrows. The N-termini of the bacterially expressed proteins are formed by 13 amino acids derived from vector sequences (stippled box). B-*myb* sequences missing in the pBM100 encoded protein are marked by broken lines. (**B**) Proteins of bacteria carrying pBM100 (lanes 1 and 3) or p25/3B-*myb* (lanes 2 and 4) and grown in the presence of IAA were fractionated in a 12% SDS–polyacrylamide gel and stained with Coomassie brilliant blue. Lanes 1 and 2: total bacterial protein from 25 μl of bacterial culture; lanes 3 and 4: insoluble bacterial protein from 75 μl of culture. (**C**) and (**D**) Electrophoretic mobility shift assays of recombinant B-*myb* polypeptides bound to a double-stranded oligonucleotide containing (C) or lacking (D) a v-*myb* binding site. In (C) the oligonucleotide was incubated without protein (lane 1), with ~10 (lane 2), 20 (lane 3) or 30 (lane 4) ng of the pBM100 encoded protein, or with ~10 (lane 5), 20 (lane 6) or 30 (lane 7) ng of the p25/3B-*myb* encoded polypeptide. In (D) the oligonucleotide was incubated in the absence of protein (lane 1) or in the presence of 20 (lane 2) or 30 (lane 3) ng of pBM100 encoded protein, or in the presence of 20 (lane 4) or 30 (lane 5) ng of p25/3B-*myb* encoded protein.

used for co-transfections (data not shown). To exclude the possibility that, for unknown reasons, fusion of *myb*-inducible promoters to the luciferase reporter gene is incompatible with transactivation by B-*myb*, we also performed co-transfections using a *myb* binding site containing promoter linked to the CAT gene. In this case B-*myb* again did not activate the promoter (data not shown), indicating that the inability of B-*myb* to activate a *myb* binding site containing promoter was independent of the reporter gene system used. The failure of B-*myb* to activate p3×ATk-Luc and p-240-Luc was not due to a lack of expression of B-*myb* protein in the transfected cells; similar levels of B-*myb* and v-*myb* proteins were detected by immunoprecipitation in cells transfected by our expression vectors. The level of expression of exogenous B-*myb* protein was far above the level of endogenous B-*myb* protein in these cells (data not shown).

To show that B-*myb* is also unable to activate the endogenous *mim*-1 gene, we transfected HD11 cells transiently with a B-*myb* expression vector and analyzed RNA from the transfected cells by Northern blotting. As illustrated in Figure 6D, B-*myb* failed to induce expression of the endogenous *mim*-1 gene. Taken together all these observations strongly suggest that B-*myb* is unable to transactivate promoters containing *myb* binding sites.

B-myb protein recognizes v-myb binding sites in vivo

The previous experiments have shown that B-*myb* does not activate promoters containing the *mim*-1 'A' *myb* binding site. Since B-*myb* protein binds to this site *in vitro*, we were interested to determine whether it recognizes this binding site also *in vivo*. To address this issue we fused the chicken B-*myb* protein to the potent activating region of the HSV VP16 protein (Triezenberg *et al.*, 1988) and then assessed the potential of the B-*myb*–VP16 hybrid protein to transactivate promoters containing *myb* binding sites. As illustrated in Figure 7, the plasmids p3×ATk-Luc and p-240-Luc were activated by the B-*myb*–VP16 fusion protein, whereas plasmid p-81Tk-Luc was not activated under the same conditions. Thus, by fusion with an activating region the B-*myb* protein was converted to a *myb* binding site dependent transactivator. Since the B-*myb*–VP16 fusion protein retains the B-*myb* DNA binding domain, we concluded that the B-*myb* protein recognizes the *mim*-1 'A' *myb* binding site *in vivo*.

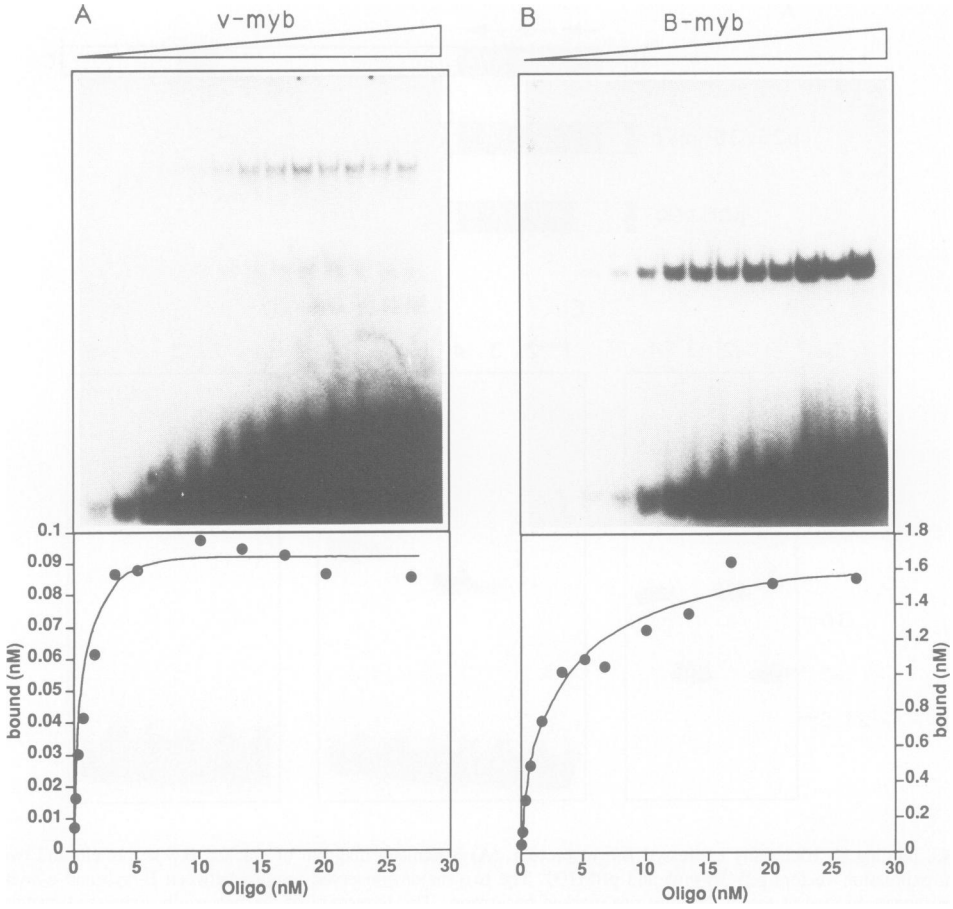

Fig. 5. Binding of recombinant v-*myb* and B-*myb* proteins to the *mim*-1 'A' *myb* binding site. Different amounts of radiolabeled oligonucleotide containing the *mim*-1 'A' *myb* binding site were subjected to electrophoretic mobility shift assays with a constant amount of bacterially expressed v-*myb* (**A**) or B-*myb* (**B**) protein. In the case of v-*myb*, full-length protein (encoded by pVM2101) was used; in the case of B-*myb*, the p25/3B-*myb* encoded protein encompassing the DNA binding domain of the B-myb protein, was used. Both bacterial proteins were purified from inclusion bodies as detailed in Materials and methods. The bottom panels show quantitative analyses of the binding reactions.

B-myb interferes with the activation of the mim-1 gene by v-myb and c-myb

The observation that the B-*myb* protein apparently binds to promoters containing *myb* binding sites, but does not activate them, suggested that B-*myb* might actually interfere with the activation of *myb* binding site containing promoters by other *myb* proteins. To investigate this possibility, we co-transfected plasmid p-240-Luc with both v-*myb* and B-*myb* expression vectors simultaneously. As shown in Figure 8A, activation of the *mim*-1 promoter by v-*myb* was inhibited by B-*myb*. A control experiment showed that the amount of v-*myb* protein in the transfected cells was not influenced by B-*myb* (Figure 8D). The extent of inhibition by B-*myb* was dependent on the relative doses of B-*myb* and v-*myb* expression vectors (Figure 8B), suggesting that the relative concentrations of both proteins determine whether or not the promoter is activated. In this context it is noteworthy that the B-*myb* protein has a longer half-life than the v-*myb* protein (see Figure 2); this might explain why relatively small amounts of B-*myb* expression vector were effective in inhibiting v-*myb* mediated transactivation. B-*myb* did not affect the basal expression (i.e. in the absence of exogenous v-*myb* protein) of the *mim*-1 promoter, suggesting that B-*myb* does not inhibit *mim*-1 promoter activity *per se*. Figure 8C illustrates that B-*myb* also inhibited activation of the *mim*-1 promoter by c-*myb*. The activation of the *mim*-1 promoter by c-*myb* was, however, considerably lower than the activation mediated by v-*myb*. We attribute this effect to the presence of a negatively acting domain in the c-*myb* protein, such as has been described for the mouse c-*myb* protein (Sakura *et al.*, 1989).

To investigate whether B-*myb* inhibits activation of the *mim*-1 gene in its natural context, we determined the effect of B-*myb* on *myb*-induced activation of the endogenous *mim*-1 gene. We transfected different combinations of expression vectors into HD11 cells and analyzed *mim*-1 expression by Northern blotting. As illustrated in Figure 9, B-*myb* interfered with the v-*myb* induced activation of the endogenous *mim*-1 gene. In similar experiments, activation of the endogenous *mim*-1 gene by c-*myb* was also inhibited by B-*myb* (data not shown). Thus, the behavior of the endogenous *mim*-1 gene supports the notion that B-*myb* suppresses *mim*-1 activation mediated by other *myb* proteins.

Inhibition of v-myb-mediated activation by B-myb involves competition for myb binding sites by v-myb and B-myb proteins

To substantiate the idea that B-*myb* suppresses *myb*-mediated transactivation by competing with other *myb* proteins for *myb* binding sites, we fused the v-*myb* and B-*myb* proteins to the

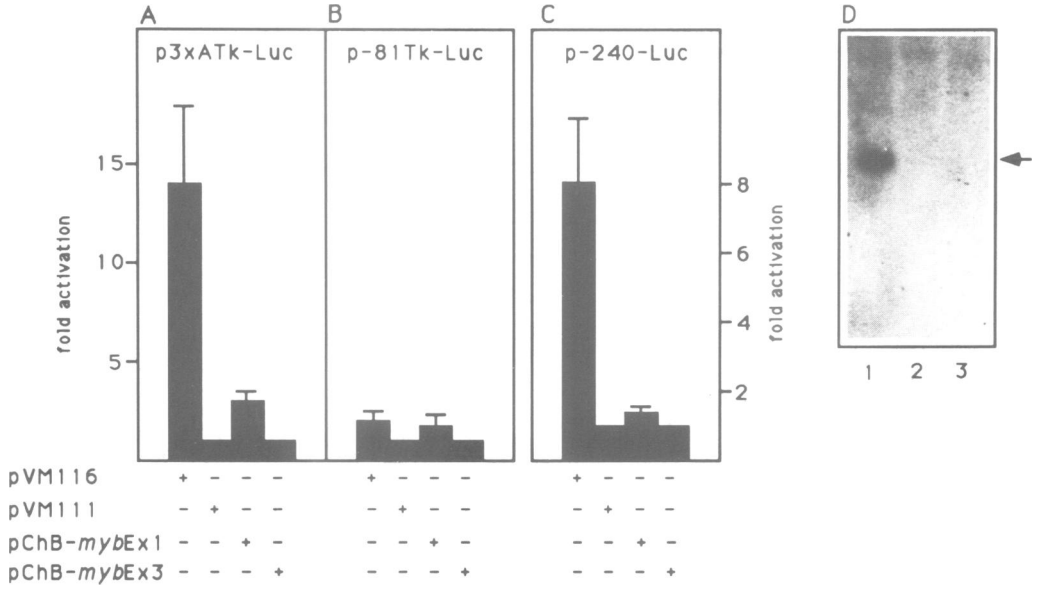

Fig. 6. Transactivation of different promoters by v-*myb* and B-*myb*. **(A)–(C)** HD11 cells were co-transfected with 5 μg of the indicated luciferase reporter plasmids, 10 μg of v-*myb* or B-*myb* expression vector as indicated below the columns, and 1 μg of pSV2CAT. Cells were harvested 24 h after transfection and analyzed for CAT and luciferase activity. Transfection efficiencies were normalized by analyzing the activity of the co-transfected pSV2CAT plasmid. The columns show the average activation factors of the luciferase reporter genes by v-*myb* and B-*myb*. The activity of each reporter gene in the absence of exogenous transactivator (i.e. in control transfections with the relevant frameshift vectors) was arbitrarily designated as 1. Thin lines show the standard deviations. **(D)** HD11 cells were transfected with 1 μg of pSV2B2 and 10 μg per cell culture dish of the following expression vectors: lane 1, pVM134; lane 2, pVM111; lane 3, pChB-*myb*Ex2 and 1 μg of pSV2B2. After 20 h polyadenylated RNA was prepared from the transfected cells and analyzed by Northern blotting for expression of the *mim*-1 gene. The amount of RNA loaded on to the gel was normalized with respect to transfection efficiencies, as estimated from the activity of the co-transfected luciferase plasmid. The integrity of the RNA was further checked by hybridization with a GAPDH specific probe (data not shown). The *mim*-1 RNA is marked by an arrow.

DNA binding domain of the yeast transactivator Gal4 and then determined the effect of these fusion proteins on the activity of a promoter containing Gal4 binding sites. pG5E1BCAT and pE1BCAT designate a pair of reporter plasmids both containing the adenovirus E1B promoter but differing by the presence or absence of Gal4 binding sites. Figure 10 illustrates the activity of the Gal4 binding site containing promoter construct relative the the promoter construct lacking Gal4 binding sites under different conditions. As expected, neither v-*myb* nor B-*myb* alone activated expression of the promoter containing Gal4 binding sites. Interestingly, Gal4–B-*myb* also failed to activate expression of pG5E1BCAT, whereas Gal4–v-*myb* activated this gene construct. This result again indicates that B-*myb* lacks transactivation potential. Activation mediated by Gal4–v-*myb* was completely blocked by Gal4–B-*myb*. By contrast, B-*myb* inhibited Gal4–v-*myb* mediated activation only slightly. This experiment suggests that the inhibition between B-*myb* and v-*myb* proteins results mainly from a competition of both proteins for the same binding site and that indirect inhibition (e.g. B-*myb* protein titrating out a factor required for activation by v-*myb*) does not play a major role.

Discussion

B-myb, a conserved member of the myb family, encodes a sequence specific DNA binding protein

There is now evidence for the existence of a large family of *myb*-related genes in many eukaryotic species, ranging from mammals to yeast, slime molds, and to higher plants (Gonda *et al.*, 1985; Gerondakis and Bishop, 1986; Katzen *et al.*, 1985; Slamon *et al.*, 1986; Paz-Ares *et al.*, 1987; Peters *et al.*, 1987; Marocco *et al.*, 1989; Tice-Baldwin *et al.*, 1989; Stober-Grässer *et al.*, 1992). Proteins encoded by these genes share an amino acid sequence of 50–55 residues which is tandemly repeated two or three times at the N-terminus of each protein and functions as a sequence-specific DNA binding domain (Klempnauer and Sippel, 1987; Biedenkapp *et al.*, 1988; Howe *et al.*, 1990; Oehler *et al.*, 1990; Frampton *et al.*, 1991). The novel gene described here encodes a protein that contains the characteristic *myb* repeat domain; in addition, a second region of homology, shared by all known *myb* proteins of vertebrates and of *Drosophila*, resides in the C-terminal half of the protein. We refer to the gene described here as chicken B-*myb*, since it encodes a protein whose amino acid sequence closely resembles that of the human B-*myb* protein. The isolation of this gene demonstrates that chickens possess multiple members of the *myb* family and that individual members of this gene family have been well conserved during evolution.

In vertebrates, c-*myb* is expressed at high levels predominantly in immature hematopoietic cells, for whose proliferation c-*myb* appears to be essential (Mucenski *et al.*, 1991). Outside of the hematopoietic system c-*myb* appears to play no major role. In contrast to c-*myb*, B-*myb* is expressed at significant levels in hematopoietic as well as in non-hematopoietic cells, suggesting the interesting possibility that B-*myb* plays a role in a broader spectrum of cell types than c-*myb*.

B-myb lacks transactivation potential

To address the possible function of B-*myb*, we have begun to study the B-*myb* protein in detail. B-*myb* encodes a

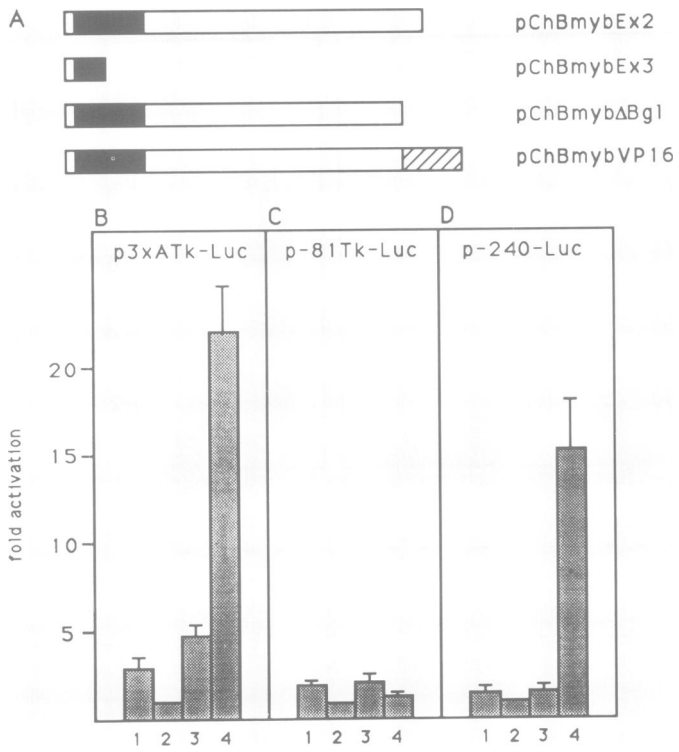

Fig. 7. Transactivation by a B-*myb*−VP16 fusion protein. (**A**) Schematic illustration of the proteins encoded by different B-*myb* expression vectors. Black and hatched bars represent the DNA binding domain and the VP16 transactivation domain, respectively. (**B**)−(**D**) HD11 cells were co-transfected with 3 μg of the indicated luciferase reporter plasmids, 5 μg of B-*myb* expression vectors and 1 μg of pSV2CAT per cell culture dish. The following B-*myb* expression vectors were used: column 1, pChB-*myb*Ex2; column 2, pChB-*myb*Ex3; column 3, pChB-*myb*ΔBgl; column 4, pChB-*myb*VP16. Cells were harvested 24 h after transfection. Transfection efficiencies were normalized with respect to the activity of the co-transfected pSV2CAT plasmid. The columns show the average activation factors of the different luciferase reporter genes by the indicated expression vectors. The activity of each reporter gene in the absence of exogenous transactivator (columns 2) was arbitrarily designated as 1. Thin lines indicate standard deviations.

M_r 110 000 nuclear protein that exhibits an extended half-life compared with v-*myb* and c-*myb* proteins. The repeat region of the B-*myb* protein, as in other members of the *myb* family, functions as a sequence specific DNA binding domain. As in the case of the c-*myb* protein, the integrity of the first of the three conserved repeats appears not to be important for specific DNA binding (Howe *et al.*, 1990; Oehler *et al.*, 1990). Our data suggest that the sequence specificities of the B-*myb* and v-*myb* proteins are similar to each other. While this work was in progress, Mizuguchi *et al.* (1990) have described a human B-*myb* specific binding site, using a β-galactosidase−human B-*myb* fusion protein. We and others (Howe and Watson, 1991) could not detect any binding of B-*myb* protein to this site. Our own efforts to identify binding sites that are differentially recognized by the B-*myb*, v-*myb* or c-*myb* proteins, have failed so far. Thus it remains open at present whether the different members of the *myb* family display distinct sequence preferences for specific DNA binding.

Despite its sequence specific DNA binding activity, B-*myb* is incapable of transactivating a variety of promoters containing *myb* binding sites, such as the *mim*-1 promoter, the HSV Tk promoter, the SV40 early promoter (data not shown), as well as the endogenous *mim*-1 gene. A possible explanation for this inability is suggested by the fact that the B-*myb* protein does not contain the acidic transactivation domain identified in v-*myb* and c-*myb* proteins (Weston and Bishop, 1989; Kalkbrenner *et al.*, 1990). That B-*myb* may indeed lack a functional transactivation domain is consistent with several observations. When fused to the heterologous Gal4 DNA binding domain, B-*myb* fails to activate a promoter containing Gal4 binding sites. Furthermore, when fused with a heterologous activating domain the B-*myb* protein was converted to a binding site dependent transactivator. The most compelling evidence for the inability of B-*myb* to function as a transactivator stems from the observation that B-*myb* inhibits *myb*-mediated activation of the *mim*-1 gene. Thus, in these experiments B-*myb* functions as an inhibitor rather than an activator of gene expression. Our experiments suggest that this inhibitory function is carried out by competition between B-*myb* protein and other *myb* proteins for the same binding sites.

Our findings contrast with previous reports, demonstrating transactivation of *myb* binding site containing artificial reporter gene constructs and of the human c-*myc* promoter by human B-*myb* (Mizuguchi *et al.*, 1990; Nakagoshi *et al.* 1992). At present we cannot explain this discrepancy. We note that the cell lines used for co-transfection by Mizuguchi *et al.* (1990) and Nakagoshi *et al.* (1992) differ from the cells used in this study. We cannot exclude the possibility that the ability of B-*myb* to transactivate is dependent on the cell type used for transfection. We also note that Mizuguchi *et al.* (1990) have observed only weak transactivation by B-*myb* and have not clearly demonstrated that this activation was dependent on *myb* binding sites present in the reporter genes. Furthermore, in case of the human c-*myc* promoter (Nakagoshi *et al.*, 1992) it was not shown that the *myb* binding sites present in the c-*myc* promoter are indeed

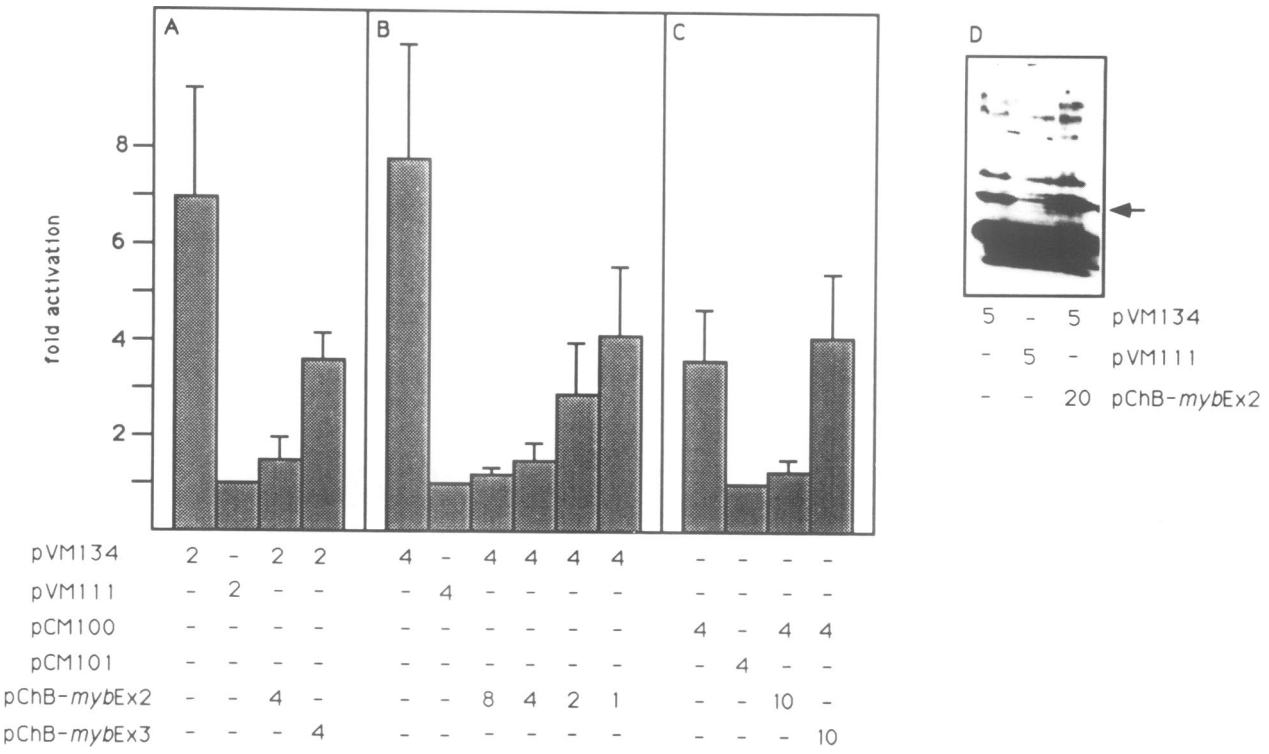

Fig. 8. B-*myb* inhibits v-*myb*- and c-*myb*-mediated activation of the *mim*-1 promoter. (**A**)–(**C**) HD11 cells were co-transfected with 3 μg of p-240-Luc, 1 μg of pSV2CAT and the indicated amounts (in μg) of expression vectors per cell culture dish. Cells were analyzed for CAT and luciferase activities 24 h after transfection. Transfection efficiencies were normalized with respect to the activity of the co-transfected pSV2CAT plasmid. The columns show the average activation of the reporter gene by the indicated combinations of expression vectors. The activity of the reporter gene in the absence of exogenous transactivator (the second column in each panel) was arbitrarily designated as 1. Thin lines show standard deviations. (**D**) HD11 cells were transfected with 3 μg of p-240-Luc, 1 μg pSV2CAT and the indicated amounts (in μg) of expression vectors per cell culture dish. 24 h later total nuclear protein of the transfected cells was analyzed by electrophoresis in a 10% SDS–polyacrylamide gel, followed by Western blotting with the *myb* specific monoclonal antibody *myb*2-37. The v-*myb* protein is marked by an arrow. The intense band below represents a cellular protein crossreacting with the antibody (Evan *et al.*, 1984).

responsible for transactivation by B-*myb*. Clearly, further work is required to resolve these discrepancies.

Is B-myb an inhibitory member of the myb family?

Although at present the function of B-*myb* remains obscure, our observations suggest that this function differs significantly from that of c-*myb*. It seems therefore unlikely that B-*myb* is simply a functional equivalent of c-*myb* in non-hematopoietic cells. Based on the results described here we favor the view that B-*myb* functions as an inhibitory member of the *myb* family. The existence of inhibitory factors in many transcription factor families seems to be a rather common phenomenon (Baeuerle and Baltimore, 1988; Baichwal and Tjian, 1990; Benezra *et al.*, 1990; Auwerx and Sassone-Corsi, 1991; Descombes and Schibler, 1991; Treacy *et al.*, 1991; Ron and Habener, 1992). Although in most cases the precise roles of these inhibitory factors are not known, their widespread occurrence suggests that regulation of gene expression frequently involves inhibitory interactions.

Our view that B-*myb* acts as an inhibitory member of the *myb* family is based mainly on the analysis of *mim*-1 expression. Although essentially none of the promoter constructs tested by us was ever activated by B-*myb*, we cannot completely rule out the possibility that certain, as yet unknown genes are activated by B-*myb*. Whether or not such B-*myb* inducible genes exist is therefore an important question for future work.

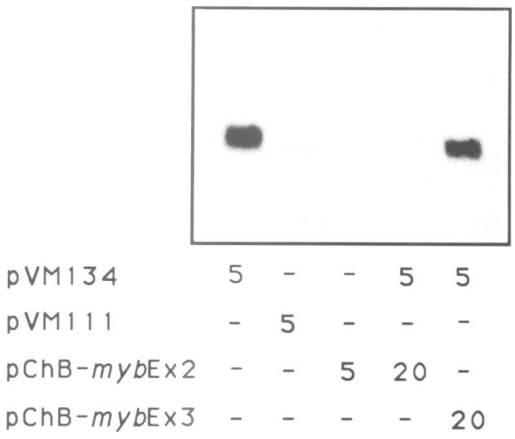

Fig. 9. B-*myb* inhibits activation of the endogenous *mim*-1 gene. HD11 cells were transfected with different expression vectors as indicated and with 1 μg of pSV2B2 per cell culture dish. After 20 h polyadenylated RNA was prepared from the transfected cells and analyzed by Northern blotting for expression of the *mim*-1 gene. The amount of RNA loaded on to the gel was normalized with respect to transfection efficiencies, as estimated from the activity of the co-transfected luciferase plasmid. The integrity of the RNA was checked by hybridization with a GAPDH specific probe (data not shown).

Materials and methods

Cells

The chicken cell lines BM2 (AMV-transformed myeloblasts), HD11 (MC29-transformed macrophages) and HD3 (AEV-transformed

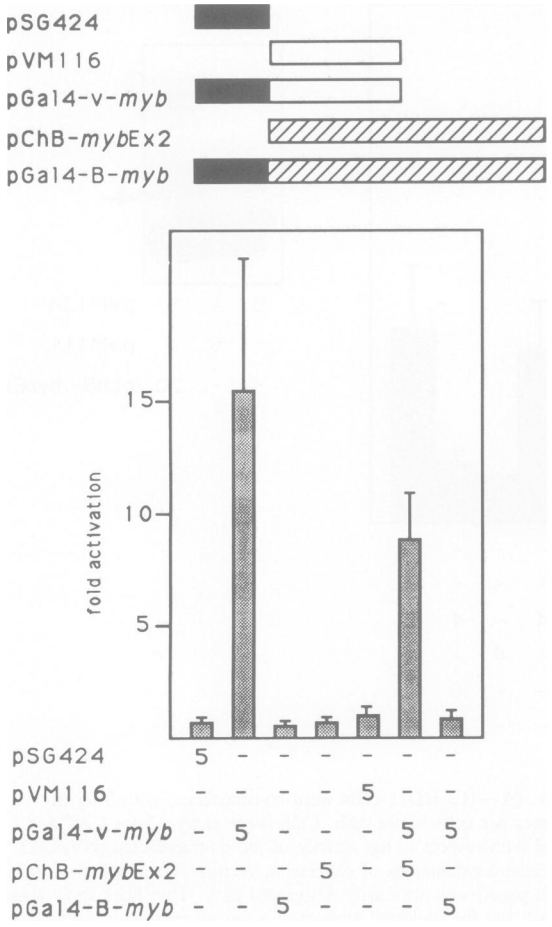

Fig. 10. Transactivation by Gal4−v-*myb* and Gal4−B-*myb* fusion proteins. **Top**. Schematic illustration of the proteins encoded by different expression vectors. Gal4, v-*myb* and B-*myb* sequences are shown as black, white and hatched bars, respectively. **Bottom**. QT6 cells were transfected with the indicated amounts (in μg) of expression plasmids per cell culture dish. Each combination of expression plasmids was co-transfected with 3 μg per cell culture dish of reporter plasmid pG5E1BCAT or, in a parallel transfection, with 3 μg of plasmid pE1BCAT. All transfections also included 1 μg pSV2B2 luciferase plasmid. Cells were analyzed for CAT and luciferase activities 20 h after transfection. Transfection efficiencies were normalized with respect to the activity of the pSV2B2 luciferase plasmid. The figure shows the activation of pG5E1BCAT relative to pE1BCAT by the different expression vectors. Thin lines show standard deviations.

erythroblasts) have been described (Klempnauer *et al.*, 1983; Burk and Klempnauer, 1991). QT6 is a line of chemically transformed quail fibroblasts (Moscovici *et al.*, 1977). Primary chicken embryo fibroblasts were obtained from Flow Laboratories.

Isolation and characterization of a chicken B-myb cDNA clone

A λgt11 cDNA library of BM2 cells (kindly provided by A.E.Sippel) was screened under reduced stringency with a mixed probe specific for the conserved regions of human A-*myb* and B-*myb* (kindly provided by N.Nomura). Insert fragments of positive phages were subcloned into the pBluescript vector and sequenced with the dideoxy chain termination technique (Sanger *et al.*, 1977).

Eukaryotic expression vectors

The v-*myb* expression vectors pVM116, pVM134 and the frameshift vector pVM111 have been described (Klempnauer *et al.*, 1989; Burk and Klempnauer, 1991). The c-*myb* expression vectors pCM100 and pCM101 were constructed as follows. A chicken c-*myb* cDNA clone (Rosson and Reddy, 1986) was first partially digested with nuclease *Bal*31 to remove most of the 3′ non-coding sequences present in this cDNA clone, thereby bringing a *Xba*I site, derived from the polylinker of the cloning vector, close to the translational stop codon of c-*myb*. Then the complete c-*myb* coding sequence was excised by digesting the DNA with *Not*I and *Xba*I. The *Not*I site is located immediately upstream of the initiator ATG. The *Not*I−*Xba*I fragment was then inserted between the *Eco*RI and *Xba*I sites of pVM130 (Klempnauer *et al.*, 1989), using an oligonucleotide linker (GAATTCTC-TAGAGCGGCCGC) to join the *Eco*RI and *Not*I sites. The resulting vector, pCM100, encodes a protein containing all c-*myb* amino acids and additionally 14 amino acids (MEAVIKNSRAAAAR) derived from the N-terminus of the retroviral *gag* protein and the linker. In pCM101, the c-*myb* coding region was inserted in the antisense orientation. This vector therefore does not encode c-*myb* protein. To construct the B-*myb* expression vector pChB-*myb*Ex1, a 2.4 kb *Nco*I−*Xba*I restriction fragment, containing the complete coding region of the chicken B-*myb* protein, was excised from plasmid pChB-*myb*240-3 and cloned between the *Eco*RI and *Xba*I sites of plasmid pVM130. The *Nco*I site overlaps with the translational start codon of the B-*myb* protein; the *Xba*I site resides in the polylinker of the pBluescript plasmid. The *Nco*I site was joined to the *Eco*RI site of the expression vector by an oligonucleotide linker, GAATTCCATGG. In the resulting plasmid, pChB-*myb*Ex1, the B-*myb* protein can be translated from its own ATG or, alternatively, from a spliced mRNA and the ATG of the retroviral *gag* gene, which is in-frame with the B-*myb* coding region. In this latter case the B-*myb* protein would contain eight additional N-terminal amino acids (MEAVIKNS), the first six of which are derived from the *gag* gene and the last two of which are derived from the linker. The plasmid pChB-*myb*Ex2 was derived from pChB-*myb*Ex1 by digesting with *Nco*I, filling-in the overhanging restriction ends and religation. The B-*myb* protein encoded by pChB-*myb*Ex2 is translated from its own ATG and does not contain additional N-terminal amino acids. The vector pChB-*myb*Ex3 is identical to pChB-*myb*Ex1, except that a frameshift mutation was introduced at the *Hin*dIII site within the B-*myb* DNA binding domain.

Plasmids pChB-*myb*ΔBgl and pChB-*myb*VP16 are derivatives of plasmid pChB-*myb*Ex2. pChB-*myb*ΔBgl was obtained by replacing B-*myb* coding sequences downstream of a *Bgl*II restriction site (located at position 1833 in Figure 1) by polylinker sequences (*Bam*HI−*Xba*I) of the pBluescript vector. In the protein encoded by this vector the 83 C-terminal amino acids of the B-*myb* protein are replaced by seven amino acids (TSSRAQL). In plasmid pChB-*myb*VP16 the same 83 C-terminal amino acids are replaced by the C-terminus of VP16 (amino acids 422−490, Triezenberg *et al.*, 1988).

To generate pGal4B-*myb* and pGal4v-*myb*, the 5′ ends of B-*myb* and v-*myb* were modified by PCR using the oligomers 5′CTGGATCCCCATGGCG-CGCCGCAG3′ (B-*myb*) and 5′CTGGATCCGGACAGATGTTCAGTG3′ (v-*myb*) as 5′ primers and 5′GGTCTAGATATCAACTGGCCAG3′ (B-*myb*) and 5′CTTCTAGAAGCCGGTGGTTGCCGAGGG3′ (v-*myb*) as internal primers. The modified 5′ ends, now containing *Bam*HI restriction sites immediately upstream of the start of the B-*myb* and v-*myb* coding regions, were then used to fuse the B-*myb* and v-*myb* coding regions to the Gal4 DNA binding domain, using the *Bam*HI site of pSG424 (Sadowski and Ptashne, 1989).

Bacterial expression vectors

To construct the bacterial expression vector p25/3B-*myb*, encoding an N-terminal B-*myb* polypeptide, two synthetic oligonucleotides (5′TAAGA-TCTGAATTCCATGGGCCGCCGCAGCC3′ and 5′GGTCTAGATATC-AACTGGCCAG3′) and the clone pChB-*myb*240-3 were subjected to the polymerase chain reaction to generate a DNA fragment from the 5′ end of the B-*myb* coding region. To construct the expression vector pBM100 the first of these primers was replaced by the oligonucleotide 5′TAAGATCT-GAATTCGAACCGCAGTGACCAGCAG3′, resulting in amplification of a DNA fragment whose 5′ end corresponds to the N-terminal truncation of the v-*myb* gene of avian myeloblastosis virus. Both DNA fragments were digested with *Bgl*II and *Xba*I and inserted between the *Bgl*II and *Xba*I sites of the v-*myb* expression vector pVM2028 (Klempnauer and Sippel, 1987), thus placing the B-*myb* coding sequences downstream of the *E.coli trp* promoter. In both cases, B-*myb* polypeptides are fused at the N-terminus to 17 amino acids (MLAIFVLKGSLDRDLNS) encoded by the trp operon and by linker sequences. In both cases translation is terminated by a TAG codon overlapping with the *Xba*I site. The bacterial expression vectors pVM2028, encoding a full-length v-*myb* protein has been described (Klempnauer and Sippel, 1987). The bacterial expression vector pVM2101 is identical to pVM2028 except that an *Nco*I−*Sal*I restriction fragment from the coding region of v-*myb* was replaced by the corresponding region from E26 virus. Recombinant *myb* proteins, all of which were obtained in insoluble form, were purified as described before (Klempnauer *et al.*, 1986; Klempnauer and Sippel, 1987). Polyclonal rabbit antisera were raised against the p25/3B-*myb* encoded polypeptide as described (Klempnauer *et al.*, 1986).

Northern blotting

Polyadenylated RNA was prepared and analyzed by Northern blotting as described (Klempnauer and Bishop, 1983).

Immunoprecipitation and cell fractionation

Cells were radiolabeled with [^{35}S]methionine (>800 Ci/mmol; Amersham) at a concentration of 100–300 μCi/ml. Extracts of labeled cells were prepared in 10 mM Tris–HCl pH 7.8, 50 mM NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS, usually at $1-3\times10^7$ cells/ml. Extract from 1 to 3×10^7 cells was used for each immunoprecipitation as described (Klempnauer *et al.*, 1983). In blocking experiments, 1 ml of extract (from 1 to 3×10^7 cells) was supplemented with total bacterial protein obtained by boiling bacteria from 100 μl of culture in 10 μl SDS sample buffer. C-*myb* protein was immunoprecipitated by rabbit serum raised against the v-*myb* protein (Klempnauer *et al.*, 1983). Cells were fractionated into nuclear and cytoplasmic fractions as described (Klempnauer and Sippel, 1986).

Western blotting

Western blotting was performed as described (Klempnauer *et al.*, 1986), using the monoclonal antibody *myb*2-37 (Evan *et al.*, 1984).

Gel retardation assays

To generate a double-stranded oligonucleotide containing a *myb* binding site, the single-stranded oligonucleotides 5'GATCCAGTAACGGTGT-AGCA3' and 5'GATCTGCTACACCGTTACTG3' were hybridized. Double-stranded oligonucleotides containing a mutated *myb* binding site were generated from the oligonucleotides 5'GATCCAGTTTCGGTGTAGCA3' and 5'GATCTGCTACACCGAAACTG3'. A double-stranded oligonucleotide containing the *mim*-1 'A' *myb* binding site (Ness *et al.*, 1989) was obtained by hybridizing the single strands 5'GACATTATAACGGT-TTTTTA3' and 5'GTAAAAAACCGTTATAATGT3'. Olignucleotides were radiolabeled by filling in the ends using the appropriate deoxynucleotide. Electrophoretic mobility shift experiments were performed as described (Oehler *et al.*, 1990).

Reporter genes, DNA transfection and CAT and luciferase assays

The reporter plasmids p-81Tk-Luc (Nordeen, 1989), p3×ATk-luc, p-240-Luc (Ness *et al.*, 1989), pG5E1BCAT, pE1BCAT (Lillie and Green, 1989), pSV2CAT and pSV2B2 (Klempnauer *et al.*, 1989) have all been described. DNA transfection was performed by the calcium phosphate co-precipitation method (Graham and van der Eb, 1973). Preparation of cell extracts, CAT and luciferase assays were performed as described (Klempnauer *et al*, 1989; De Wet *et al.*, 1987).

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