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Estrogen-dependent alterations in differentiation state of myeloid cells caused by a v-*myb*/estrogen receptor fusion protein

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The oncogene v-*myb* and its cellular progenitor c-*myb* encode nuclear, DNA binding phosphoproteins that are thought to regulate the expression of *myb*-responsive genes during myeloid differentiation. To identify such *myb*-regulated genes, and to explore the mechanisms by which v-*myb* affects their expression, we have established a conditional expression system for v-*myb*. We have converted the v-*myb* protein to an estrogen-inducible transactivator by fusing the protein to the hormone binding domain of the human estrogen receptor. Expression of the chimeric protein in a chicken macrophage cell-line causes estrogen-dependent, reversible changes in the differentiation state as well as alterations in the gene expression program of the cells. We have used this estrogen-dependent v-*myb* expression system to identify a novel v-*myb* regulated gene.

Key words: estrogen-dependent expression system/*myb*-regulated genes/v-*myb* oncogene

Introduction

The retroviral oncogene v-*myb*, originally defined by the chicken retroviruses AMV and E26, transforms myelomonocytic hematopoietic cells *in vivo* and *in vitro* (Moscovici and Gazzolo, 1982). The progenitor of v-*myb*, c-*myb*, is expressed at high levels in immature cells of various hematopoietic lineages (Westin *et al.*, 1982; Craig and Block, 1984; Gonda and Metcalf, 1984). Sustained expression of c-*myb* blocks differentiation of immature erythroid cells (Clarke *et al.*, 1988), whereas a reduction of c-*myb* expression blocks the proliferation of precursor cells (Gewirtz and Calabretta, 1988), suggesting that c-*myb* plays a role in controlling proliferation and differentiation of hematopoietic cells.

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). The v-*myb* protein binds specifically to the sequence motif PyAAC$^{G}/_{T}$G (Biedenkapp *et al.*, 1988). The domain of v-*myb* protein responsible for sequence-specific DNA binding resides within an N-terminal located repeat region that has been highly conserved during evolution (Klempnauer and Sippel, 1987).

There is strong evidence that *myb* proteins act as transcriptional activators. Artificial promoter constructs containing *myb* binding sites are activated by *myb* (Klempnauer *et al.*, 1989; Ness *et al.*, 1989; Nishina *et al.*, 1989; Weston and Bishop, 1989; Ibanez and Lipsick, 1990). Moreover, a temperature-sensitive mutant of the v-*myb*-containing E26 virus has been used to identify a *myb*-regulated gene, designated as *mim*-1, whose promoter region contains three v-*myb* binding sites (Ness *et al.*, 1989).

To study the expression of genes that are controlled by v-*myb*, we have constructed a conditional v-*myb* expression system by fusing the v-*myb* protein to the hormone binding domain of the human estrogen receptor. Here, we describe and characterize this estrogen-dependent v-*myb* expression system and report the identification of a novel *myb*-regulated gene

Results

Hormone-dependent activation of myb-inducible genes by v-myb/ER fusion proteins

Figure 1A illustrates the structure of chimeric v-*myb*/ER proteins generated in this study. pE*myb*ER encodes a protein in which the estrogen binding domain of the human estrogen receptor was fused to the C-terminus of the v-*myb* protein of AMV. pEE26*myb*ER encodes a similar protein, in which a large portion of the DNA binding domain has been replaced by equivalent sequences from the v-*myb* protein of E26. The proteins encoded by pE*myb*ERΔ and pEE26*myb*ERΔ carry deletions of an acidic transactivation domain identified previously (Weston and Bishop, 1989). All expression vectors yielded similar levels of proteins that had the expected sizes and reacted with a *myb*-specific monoclonal antibody (Figure 1B). The *myb*/ER fusion proteins were equally distributed between the nucleus and the cytoplasm in the absence of estrogen and acquired a predominantly nuclear location in the presence of estrogen (Figure 1B).

To determine whether *myb*/ER fusion proteins act as hormone-dependent transactivators, we co-transfected pE*myb*ER and pEE26*myb*ER with a *myb*-responsive reporter gene into QT6 cells. The transfected cells showed hormone-dependent activation of the reporter gene, similar in its extent to the activation of the same gene construct by the unfused v-*myb* protein (Figure 2). Thus, it appeared that v-*myb*/ER fusion proteins were estrogen dependent for transcriptional activation of a transiently transfected reporter gene.

To investigate whether a gene residing in the genome was also activated in a hormone-dependent manner, we studied the effect of estrogen on the endogenous *mim*-1 gene in HD11 cells expressing v-*myb*/ER fusion protein. HD11 is a line of MC29-transformed chicken macrophage-like cells (Leutz *et al.*, 1984), that lack endogenous estrogen receptor and therefore cannot activate estrogen-responsive genes (A.Hecht, personal communication). HD11 cells also lack *mim*-1 expression, however, *mim*-1 can be activated in these cells by v-*myb* (Ness *et al.*, 1989). The Northern blot shown in Figure 3 confirmed that the E26 version of v-*myb* (pVM134) activated the *mim*-1 gene, whereas the v-*myb* gene of AMV (pVM116) failed to do so, as expected from

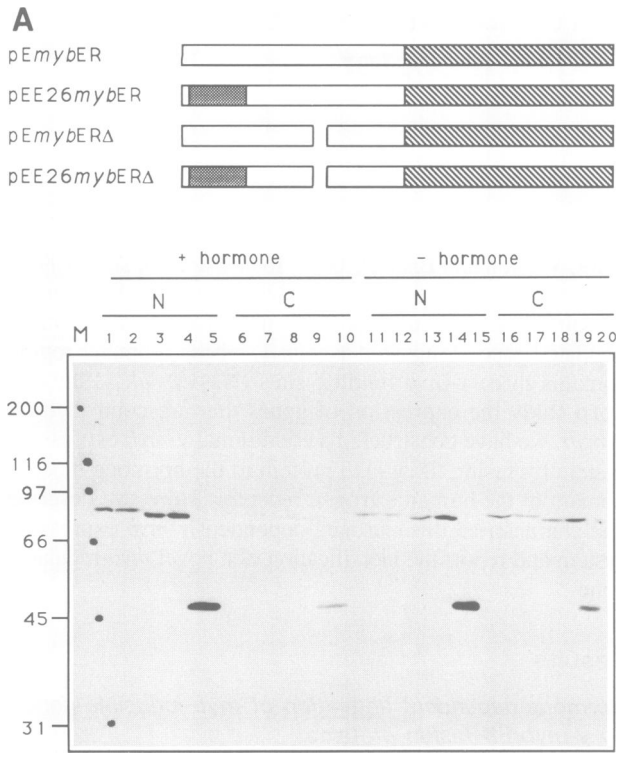

Fig. 1. Expression of v-myb/ER fusion proteins. (**A**) Structure of *myb* proteins encoded by expression vectors used in this study. White and stippled boxes represent v-*myb* sequences derived from AMV and E26 virus, respectively. Hatched boxes mark the estrogen binding domain. Deleted sequences are represented by gaps. (**B**) Expression of *myb* proteins in QT6 cells. Cells transfected with 20 μg of the plasmids pE*myb*ER (lanes 1, 6, 11 and 16), pEE26*myb*ER (lanes 2, 7, 12 and 17), pEE26*myb*ERΔ (lanes 3, 8, 13 and 18), pE*myb*ERΔ (lanes 4, 9, 14 and 19) or pVM116 (lanes 5, 10, 15 and 20) and 2 μg of the pSV2B2 luciferase plasmid were grown with (+ hormone) or without (− hormone) 2 μM β-estradiol for 20 h. The transfection efficiencies were normalized by analyzing luciferase activity. The cells were fractionated into nuclear (N) and cytoplasmic (C) fractions. Proteins were analyzed by Western blotting with the *myb*-specific monoclonal antibody CB100-18. The sizes (in kDa) and positions of molecular weight markers are indicated on the left.

previous work (Ness *et al.*, 1989; Introna *et al.*, 1990). Transfection of HD11 cells with pEE26*myb*ER resulted in estrogen-dependent activation of *mim*-1, whereas transfection with pE*myb*ER or with pEE26*myb*ERΔ failed to induce *mim*-1 expression. We concluded that the E26 v-*myb*/ER fusion protein activates an endogenous gene in a hormone-dependent manner. The specificity of *mim*-1 activation, exhibited by the AMV and E26 versions of v-*myb*, was not altered by the hormone binding domain. Our results also show that the v-*myb* transactivation domain was required for the activation of the *mim*-1 gene.

Reversible, estrogen-induced changes in the differentiation state of a chicken macrophage cell line stably expressing v-myb/ER fusion protein

Expression of v-*myb* in myeloid cells causes them to acquire an immature phenotype (Beug *et al.*, 1987; Ness *et al.*, 1987). To investigate whether the v-*myb*/ER fusion protein affects the differentiation of myeloid cells in a hormone-dependent manner, we stably integrated expression vector pEE26*myb*ER into HD11 cells using G418 selection. We screened G418-resistant cell clones for the amount of

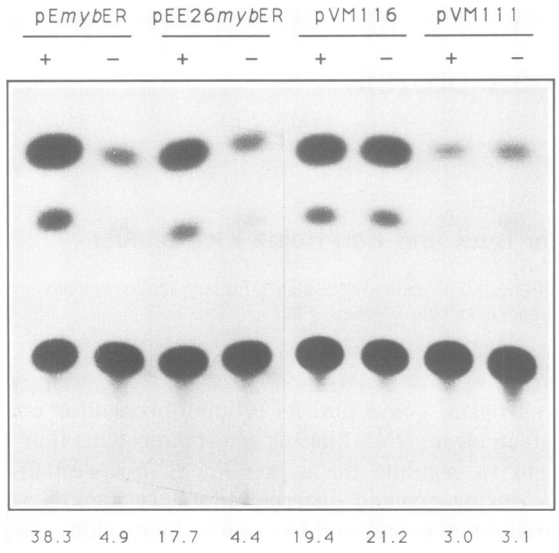

Fig. 2. Estrogen-dependent activation of a *myb*-responsive reporter gene. QT6 cells were co-transfected with 5 μg of the *myb*-responsive reporter plasmid p203-2, 20 μg of the indicated *myb* expression vectors and 2 μg of the pSV2B2 luciferase plasmid per cell culture plate. 14 h later the cells from each dish were divided into two aliquots and cultured further with (+) or without (−) 2 μM β-estradiol. 38 h after transfection cells were harvested and analyzed for CAT and luciferase activity. Transfection efficiencies were standardized with respect to luciferase activity. Numbers below the lanes indicate the percentage of conversion of [^{14}C]chloramphenicol to acetylated derivatives.

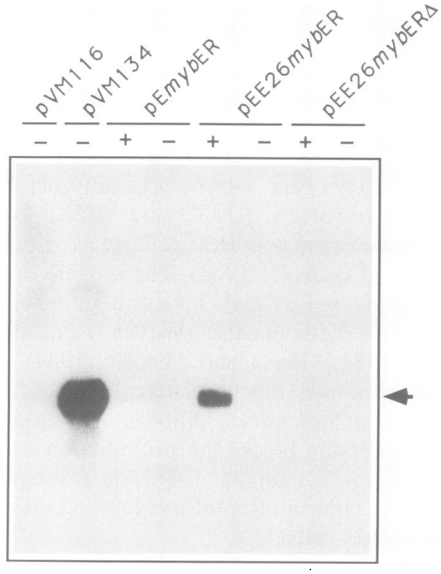

Fig. 3. Estrogen-dependent activation of the chicken *mim*-1 gene. HD11 cells were transfected with 20 μg per plate of the indicated *myb* expression vectors and 2 μg per plate of the pSV2B2 luciferase plasmid. Cells transfected with plasmids encoding v-*myb*/ER fusion proteins were divided into two aliquots 14 h after transfection and cultivated further with (+) or without (−) 2 μM β-estradiol. 38 h after transfection polyadenylated RNA was isolated and *mim*-1 expression was analyzed by Northern blotting. Transfection efficiencies were normalized by analyzing the activity of the pSV2B2 luciferase plasmid. The arrow marks the position of *mim*-1 RNA.

myb/ER fusion protein by Western blotting and selected one clone, designated as 10.4, for further analysis. The level of v-*myb*/ER protein in this clone was similar to the level of

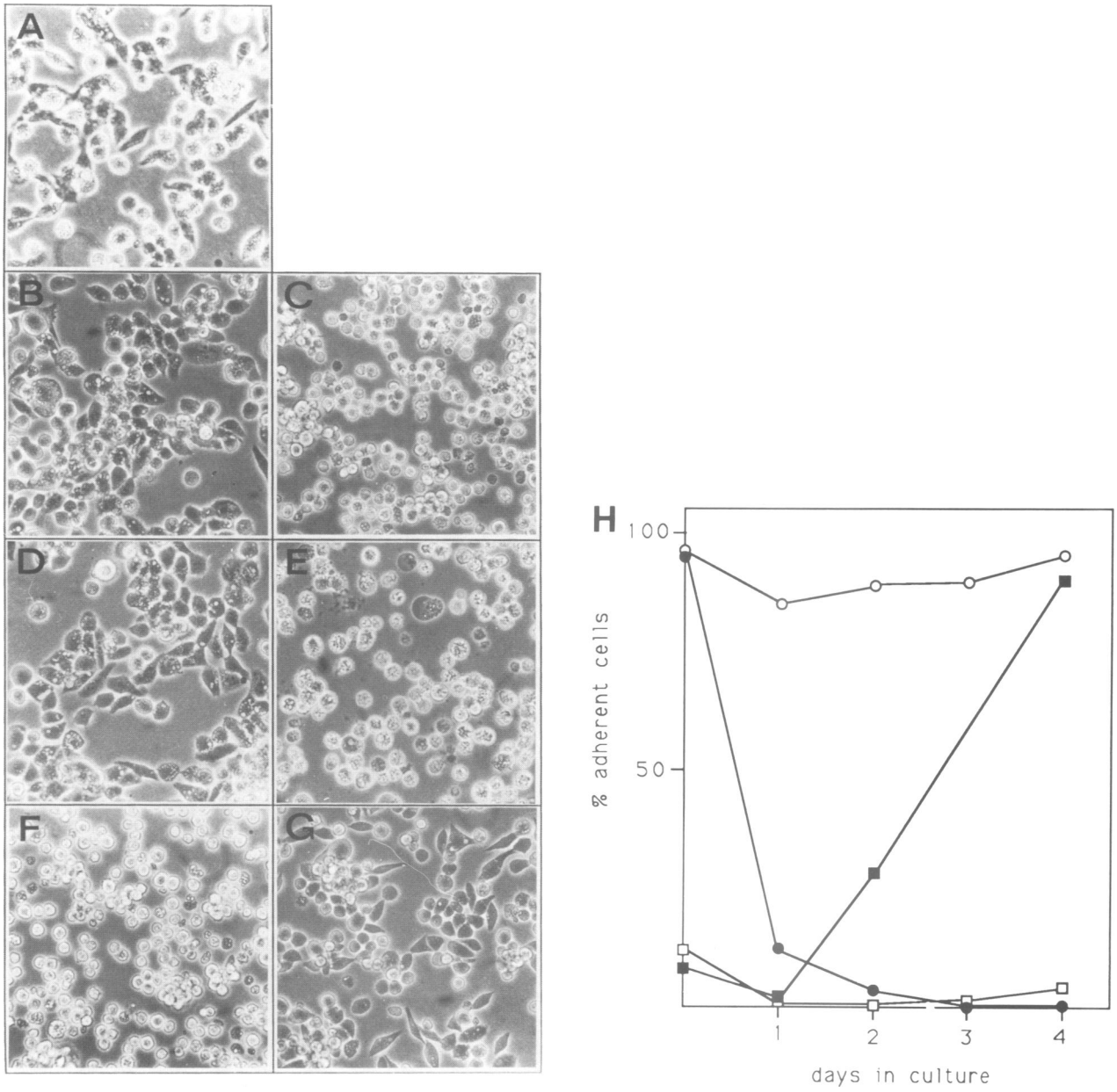

Fig. 4. Effect of v-*myb*/ER fusion proteins on cell morphology and adherence. (A–G) Photographs of HD11 cells (**A**) and of 10.4 cells growing permanently in the absence (**B** and **D**) or for 2 weeks in the presence (**C** and **F**) of 2 μM β-estradiol. Panel **E** shows 10.4 cells grown in the presence of hormone for 1 day. Panel **G** shows 10.4 cells first grown for 2 weeks in the presence of hormone followed by cultivation in the absence of hormone for 2 days. (**H**) Adherence properties of 10.4 cells. Cells grown permanently in the absence of hormone were cultivated further for the indicated periods of time without (empty circles) or with (filled circles) 2 μM β-estradiol. Cells grown for two weeks in the presence of 2 μM β-estradiol (squares) were cultivated further for the indicated periods of time without hormone (filled squares) or with 2 μM β-estradiol (empty squares). Only viable cells were included in the analysis.

v-*myb* protein in AMV-transformed myeloblasts (data not shown).

As illustrated in Figure 4 and Table I, 10.4 cells showed estrogen-dependent changes in their differentiation phenotype. Without hormone 10.4 cells, like HD11 cells, grew adherent and most cells had a flattened appearance and showed phagocytosis. In the presence of hormone, virtually all cells acquired a rounded appearance, grew in suspension and did not show phagocytosis. Addition of estrogen to HD11 cells had no effect, indicating that these alterations were mediated by the *myb*/ER protein. Thus it appeared that the v-*myb*/ER fusion protein induces an immature state of myeloid differentiation in a hormone-dependent manner.

Since the 10.4 clone might represent an unusual variant of HD11 cells we analyzed other subclones expressing v-*myb*/ER fusion protein. These clones also showed estrogen-dependent alterations in differentiation state that were very similar to those described for 10.4 cells (data not shown). In a parallel study we generated subclones of HD11 cells stably expressing a fusion protein of v-*myb* and the hormone binding domain of the rat glucocorticoid receptor. These clones also showed hormone-dependent changes in differentiation state, similar to those described above, except that in this case dexamethasone was required to induce these alterations while estrogen had no effect. Conversely, dexamethasone had no effect on 10.4 cells (data not shown).

Table I. Effect of estrogen on phagocytotic activity

Cells	Hormone	% Positive cells[a]
10.4	−	80
10.4	+	2
10.4	+/−[b]	77
HD11	−	95
HD11	+	95

[a] Cells having ingested more than five latex beads were considered positive. At least 200 cells were counted.
[b] Cells were first grown for 2 weeks in the presence of hormone and then cultivated in the absence of hormone for one additional week.

Thus, the effects of estrogen on 10.4 cells were not unique to this subclone. That a different hormone was required to induce phenotypic effects when different fusion proteins were expressed, strongly argues that the hormone-dependent effects on differentiation were indeed mediated by the chimeric proteins and were not due to selection of rare variants of HD11 cells.

To investigate how rapid phenotypic changes occurred in 10.4 cells we performed time course experiments. We found that already 1 day after addition of estrogen most cells had lost adherence (Figure 4D and E). However, between 30% and 50% of the cells died subsequently. When grown continuously with estrogen, the cells remained viable, indicating that the *myb*/ER fusion protein was not toxic *per se*. Apparently, the transition from adherent to non-adherent growth is accompanied by cell death. Interestingly, a similar fraction of cells died when myeloid cells, transformed by a temperature-sensitive mutant of the E26 virus, were shifted from non-permissive to permissive temperature (Beug *et al.*, 1987). The phenotypic effects induced by estrogen were fully reversible after removal of estrogen (Figure 4F−H and Table I). In this case there was no cell death. The reversibility of the phenotypic changes suggests that we have not selected subpopulations of cells having altered properties.

Activation of myb-inducible genes in a chicken macrophage cell line stably expressing v-myb/ER fusion protein

We next analyzed the effect of estrogen on known *myb*-inducible genes in 10.4 cells. *Mim*-1 expression was strongly induced by estrogen in 10.4 cells, but not in HD11 cells (Figure 5). On longer exposures of the Northern blot shown in Figure 5, *mim*-1 activation was evident even after a 2 h treatment with estrogen. The rapid induction of *mim*-1 RNA was consistent with the notion that v-*myb* directly activates *mim*-1 (Ness *et al.*, 1989).

Expression of the chicken lysozyme gene, recently found to be *myb*-regulated (Introna *et al.*, 1990), was also induced by estrogen in 10.4 cells, but not in HD11 cells (Figure 5). The lysozyme gene was also activated rapidly after addition of estrogen, suggesting that it might be regulated directly by v-*myb*. To explore this possibility, we analyzed the induction of *mim*-1 and lysozyme gene expression in the presence of 50 μg/ml cycloheximide. Control experiments showed that this concentration of cycloheximide inhibited protein synthesis virtually completely (data not shown). Expression of *mim*-1 and the lysozyme gene was induced by estrogen even in cycloheximide-treated cells, indicating that the activation of these genes was a direct effect of v-*myb* (Figure 6). In both cases, the level of expression was

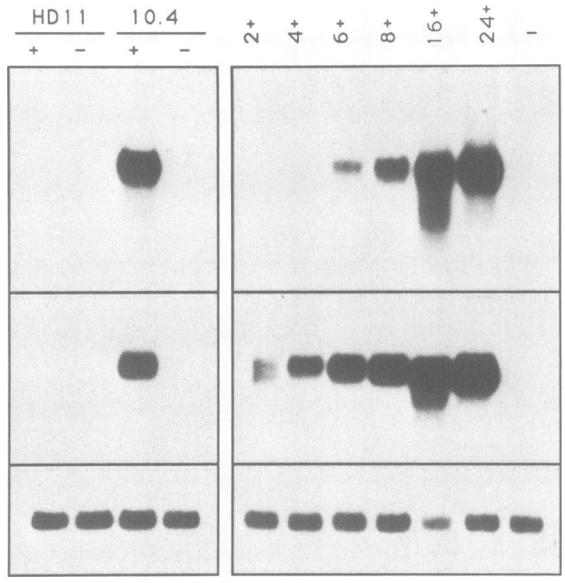

Fig. 5. Induction of *mim*-1 and lysozyme gene expression by estrogen in cells stably expressing v-*myb*/ER fusion protein. **Left panel**: HD11 cells or 10.4 cells were grown with (+) or without (−) 2 μM β-estradiol for 24 h. Polyadenylated RNA from these cells was then analyzed by Northern blotting and hybridized sequentially to probes specific for *mim*-1 (top), chicken lysozyme (middle) or GAPDH (bottom). **Right panel**: 10.4 cells were grown for 24 h without hormone (−) or for the indicated times with 2 μM β-estradiol. Polyadenylated RNA was analyzed as described above.

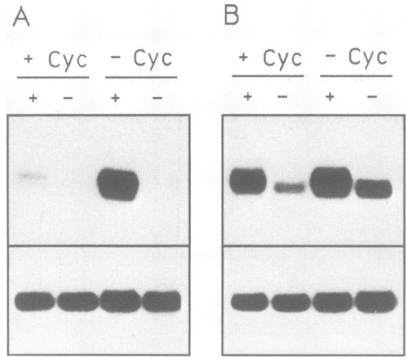

Fig. 6. Induction of *mim*-1 and lysozyme gene expression in the presence of cycloheximide. (**A**) 10.4 cells were grown in the presence (+Cyc) or absence (−Cyc) of 50 μg/ml cycloheximide either with (+) or without (−) 2 μM β-estradiol. The hormone was added to the cultures 15 min later than cycloheximide. Cells were harvested 5 h after addition of hormone. Polyadenylated RNA prepared from these cells was analyzed by Northern blotting. The same blots were hybridized sequentially to a *mim*-1 probe (top) and a GAPDH probe (bottom). (**B**) 10.4 cells were treated as in A, except that the cells were harvested 4 h after addition of the hormone. Northern blots were hybridized sequentially to probes specific for the chicken lysozyme gene (top) or GAPDH (bottom).

decreased after inhibition of protein synthesis. This reduction could be due to a decrease in the level of the v-*myb*/ER fusion protein during cycloheximide treatment (data not shown).

Identification of a novel myb-regulated gene

To assess the usefulness of the estrogen-inducible v-*myb* expression system for the identification of novel v-*myb*-regulated genes, we first investigated how selective the

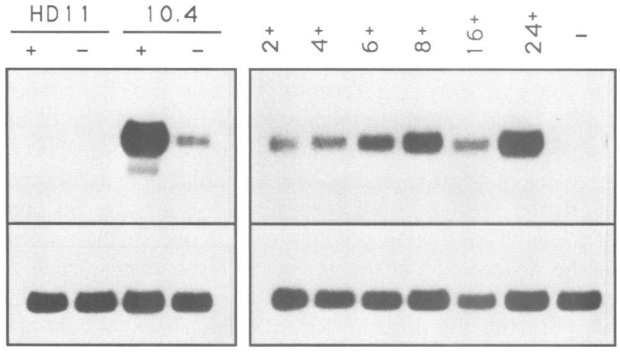

Fig. 7. Activation of the MD-1 gene. **Left panel**: HD11 cells or 10.4 cells were grown with (+) or without (−) 2 μM β-estradiol for 24 h. Polyadenylated RNA prepared from these cells was then analyzed by Northern blotting. The same blot was hybridized sequentially with probes specific for MD-1 (top) or GAPDH (bottom). **Right panel**: 10.4 cells were grown for 24 h in the absence of hormone (−) or for the indicated periods of time in the presence of 2 μM β-estradiol. Polyadenylated RNA from these cells was then analyzed as described above.

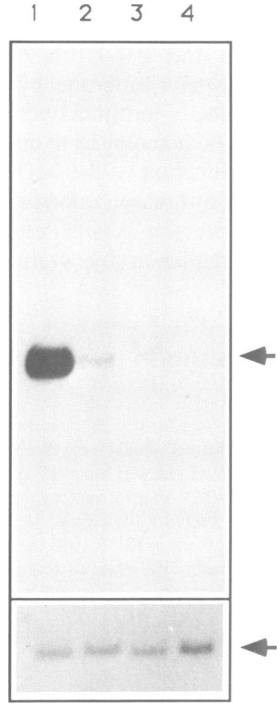

Fig. 8. Expression of MD-1 in different chicken cell lines. Polyadenylated RNA prepared from the BM2 cell line of AMV-transformed myeloblasts (lane 1), HD11 cells (lane 2), AEV-transformed erythroblasts (lane 3) or primary chicken embryo fibroblasts (lane 4) was analyzed by Northern blotting. The blot was hybridized sequentially with probes specific for MD-1 (top) or GAPDH (bottom). The MD-1 and GAPDH transcripts are marked by arrows.

activation of cellular genes by estrogen was. We used clones that were randomly selected from a cDNA library of AMV-transformed myeloblasts to probe Northern blots of 10.4 cells grown with or without estrogen. By screening 400 cDNA clones we found two clones that hybridized to estrogen-induced RNA species. One of them was identical to *mim*-1, while the other clone, referred to as MD-1, did not hybridize to probes specific for *mim*-1 or the lysozyme gene. The vast majority of clones hybridized to RNA species that were not affected by estrogen.

Since the MD-1 clone appeared to represent a novel *myb*-regulated gene, we analyzed it in more detail. After addition of estrogen, MD-1 expression was increased in 10.4 cells, but not in HD11 cells, indicating that this induction was mediated by the *myb*/ER fusion protein (Figure 7). MD-1 expression was induced rapidly by the hormone, however, the level of MD-1 RNA was not increased in the presence of cycloheximide (data not shown). This suggests that v-*myb* does not induce the MD-1 gene directly or that, in addition to v-*myb* protein, a second labile protein is required to activate MD-1.

Figure 8 shows a Northern blot analysis of MD-1 expression in several chicken cells. AMV-transformed myeloblasts contain much higher levels of the MD-1 RNA than HD11 cells, suggesting that the induction of the MD-1 gene in 10.4 cells is not specific to the *myb*/ER fusion protein. AEV-transformed erythroblasts and chicken embryo fibroblasts do not express MD-1, suggesting that in addition to being *myb*-inducible, MD-1 expression is cell-type dependent.

Figure 9 illustrates the nucleotide sequence and the deduced amino acid sequence of the MD-1 cDNA clone. This clone seems to be of almost full length, since it is only slightly shorter than the MD-1 RNA (~1.3−1.4 kb). The MD-1 sequence exhibits an open reading frame potentially encoding a protein of 160 amino acids. The predicted MD-1 protein contains a cluster of hydrophobic amino acids close to its N-terminus, suggesting that it might be secreted through the endoplasmic reticulum. Otherwise, the predicted protein is not homologous to other known proteins.

Discussion

It is generally believed that c-*myb* plays an important role during proliferation and differentiation of most hematopoietic cells. The v-*myb* oncogene transforms myelomonocytic cells and induces them to acquire an immature state of differentiation. It has been shown that v-*myb* even causes myeloid cells of more mature stages of differentiation to 'dedifferentiate' to an immature state, suggesting that v-*myb* affects a switch during myeloid differentiation. Since v-*myb* encodes a transcriptional activator, it appears an attractive hypothesis that v-*myb* affects myeloid differentiation by controlling certain, as yet mostly unknown, target genes.

To study the regulation of genes by v-*myb* during myeloid differentiation, we have established a conditional v-*myb* expression system. The strategy used to construct this system is based on the observation that the hormone binding domains of steroid receptors exhibit hormone-dependent inactivation functions and, when fused to heterologous proteins, can confer hormone-dependence on these proteins. This strategy has been used before to generate hormone-dependent variants of the adenovirus EIA protein (Picard *et al.*, 1988), human c-*myc* (Eilers *et al.*, 1989), mouse c-*fos* (Superti-Furga *et al.*, 1991) and the transcription factor C/EBP (Umek *et al.*, 1991) and therefore might be applicable to many transcription factors.

In the case of *myb*, temperature-sensitive mutants of the E26 virus have been described (Beug *et al.*, 1984), however, unlike the conditional system described here, these mutants exert their conditional effect in the context of the

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1        CGGTGCAACCATGAAGACATTGAATGTTCTCGCTCTCGTCTTAGTCCTGCTTTGC
                   M  K  T  L  N  V  L  A  L  V  L  V  L  L  C

56   ATCAATGCCAGCACAGAGTGGCCTACACACACAGTCTGCAAGGAGGAAAACTTGGAGATA
      I  N  A  S  T  E  W  P  T  H  T  V  C  K  E  E  N  L  E  I

116  TATTACAAAAGCTGTGATCCCCAGCAAGACTTTGCTTTCAGCATTGACCGTTGTTCAGAT
      Y  Y  K  S  C  D  P  Q  Q  D  F  A  F  S  I  D  R  C  S  D

176  GTCACAACCCACACCTTTGACATCAGAGCTGCAATGGTCCTAAGACAAAGCATCAAGGAA
      V  T  T  H  T  F  D  I  R  A  A  M  V  L  R  Q  S  I  K  E

236  CTGTATGCCAAGGTTGATCTGATCATAAATGGGAAGACTGTCTTAAGCTACTCAGAGACA
      L  Y  A  K  V  D  L  I  I  N  G  K  T  V  L  S  Y  S  E  T

296  CTCTGTGGACCAGGCCTTTCTAAGCTAATTTTCTGTGGAAAGAAGAAAGGAGAACATCTC
      L  C  G  P  G  L  S  K  L  I  F  C  G  K  K  K  G  E  H  L

356  TACTATGAGGGACCAATCACACTGGGAATCAAAGAAATCCCACAGGGAGATTACACTATC
      Y  Y  E  G  P  I  T  L  G  I  K  E  I  P  Q  G  D  Y  T  I

416  ACAGCAAGGCTGACTAACGAAGATCGCGCCACTGTTGCTTGTGCTGATTTTACCGTGAAA
      T  A  R  L  T  N  E  D  R  A  T  V  A  C  A  D  F  T  V  K

476  AATTATTTAGATTATTAAGCAAAACAACGCACTCGGTCCGACTCCCTTAAAACTACAGAT
      N  Y  L  D  Y

536  TCCTAAAACTATTCAAGCCCAGTGAGCTGCTTGCATGCTTCAGTGATTCTGAAGGAAAGA

596  TCTCCCGCACGGTGGTTCTGATGCTGTTCCTCTTCGTAATTCACTTTTTTGGAGAAGTCA

656  CTAGGCCCTACCCTCTAGTGGTAATTTTATCTCCAAATGCACTCTGTAGCCCACTTTTCG

716  CTTTTAATATATACAGCTGCAAATAGAAAGTATTTGATACCAACATTCTCATCTCAGGAT

776  GAAAATAGTACAAAGCAGAAGAGGCGAGAGCCAAAACAGATTTTTGCAGTAAGCTATGGA

836  GGTATCCATTTCTAACACAAGCTAAAGAAGATTGTCATATGTATTATGCAGTTATAGCAC

896  TCAACATTTTCAGTTTTTCACAAGGCCTGTTTGGAGCCTCCATTGGTATAAATTTTGTTG

956  TAACCACAGAACAAAGACCAAATAGGATGAACATGGCTCCATGTTCAGTCACTCTATTCA

1016 TATCATTTAAGTTTTCATGATTCTTCTTGTATATTTTTTTTTATTCTTTAATGTTTACAG

1076 TGATGTGAGAATCCTTTTGTTTAAGCTACATGCTGTTCCCGCTTGTCAATAAATCTGCAA

1136 GAAAAAAAAAAAAAAAAAAAA
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Fig. 9. Nucleotide sequence of the MD-1 cDNA clone. The putative ATG start codon and the TAA stop codon terminating the longest open reading frame of the MD-1 cDNA clone are underlined. The deduced amino acid sequence of the putative MD-1 protein is shown. (EMBL/GenBank/DDBJ accession number X60450).

gag−myb−ets fusion protein encoded by E26. Although these mutants have been valuable in the identification of the *mim*-1 gene (Ness *et al.*, 1989), the estrogen-dependent v-*myb* expression system has several possible advantages. When this system is introduced into cells lacking endogenous estrogen receptor, administration of estrogen will not lead to unwanted side effects, such as the activation of heat shock genes in temperature-shift experiments. Furthermore, the *myb*/ER protein can be activated by estrogen in the absence of new protein synthesis. This allows assessment of whether a given target gene is regulated by *myb* directly, as we have demonstrated here for *mim*-1 and the chicken lysozyme gene. Direct regulation of *mim*-1 by v-*myb* is consistent with the suggestion that the v-*myb* protein interacts with *myb* binding sites in the *mim*-1 promoter and thereby activates the gene (Ness *et al.*, 1989). That the lysozyme gene is also regulated directly by v-*myb* was unexpected, since no *myb* binding sites have been identified in the promoter of this gene. Thus, either v-*myb* activates the lysozyme gene from binding sites located further upstream of the promoter, or by a different mechanism. It will be of interest to explore the activation of the lysozyme gene by v-*myb* in more detail.

It was beyond the scope of this work to investigate in detail the mechanism of hormone-dependent modulation of the activity of the v-*myb*/ER fusion proteins. A hormone-dependent transit of the protein to the nucleus, as described for the glucocorticoid receptor (Picard and Yamamoto, 1987), could contribute to the estrogen dependence of the v-*myb*/ER fusion proteins, because the nuclear transport of the v-*myb*/ER fusion proteins was less efficient in the absence of estrogen than in its presence. Since considerable fractions of the *myb*/ER fusion proteins were found in the nucleus in the absence of hormone, other mechanisms, such as estrogen-dependent binding of the hsp90 protein to the hormone binding domain (Picard *et al.*, 1988), might also be responsible for the hormone dependence of the v-*myb*/ER fusion protein.

In an attempt to assess the complexity of the estrogen-induced changes in gene expression in HD11 cells stably expressing v-*myb*/ER fusion protein, we have discovered a novel *myb*-regulated gene, referred to as MD-1. The fact that we have found this gene by screening relatively few cDNA clones suggests the existence of a considerable number of *myb*-regulated genes. The predicted MD-1 protein carries a hydrophobic region at its N-terminus, suggesting that the MD-1 protein might be secreted through the endoplasmic reticulum. Since there is no second strongly hydrophobic domain, the MD-1 protein apparently is not anchored in the plasma membrane by a transmembrane domain. Except for the N-terminal hydrophobic region the MD-1 protein shows no homologies to other known proteins. Thus, the potential function of the MD-1 protein remains open at present. In any case, our results show that the conditional v-*myb* expression system described here is useful to identify genes regulated by v-*myb* during myeloid differentiation.

Materials and methods

Cells

QT6 is a line of chemically transformed japanese quail fibroblasts (Moscovici *et al.*, 1977). AEV-transformed chicken erythroblasts (HD3) and HD11, a line of MC29-transformed chicken macrophages, were obtained from T.Graf. BM2 is a line of AMV-transformed myeloblasts (Moscovici *et al.*, 1982). Primary chicken embryo fibroblasts were obtained from Flow Laboratories. Cells were grown in RPMI1640 medium supplemented with 10% tryptose phosphate broth, 5% fetal calf serum and 5% chicken serum (BM2 cells), or in RPMI1640 medium supplemented with 8% fetal calf serum and 2% chicken serum (HD3 cells), or in Iscove's modified Dulbecco's medium supplemented with 8% fetal calf serum and 2% chicken serum (all other cells) in 5% CO_2 at 37°C. Cells expressing v-*myb*/ER fusion protein were grown in the same medium lacking phenol red. Serum was treated with activated charcoal to remove endogenous hormone. β-estradiol was dissolved at 2 mM in ethanol and added to cultures at 2 μM. Control cultures received an equivalent amount of ethanol. Adherence of cells was analyzed as described (Beug *et al.*, 1984). Phagocytotic activity was determined by incubating cells with fluorescent latex beads (approximate diameter 1 μm, Polysciences) for 2 h at 37°C, washing them, incubating them for an additional 1 h at 37°C and analyzing them in a fluorescent microscope. Adherent cells were trypsinized before they were incubated with latex beads.

Reporter plasmids

The reporter plasmid p203−2, containing the SV40 early promoter and five copies of a synthetic *myb* binding site was constructed as follows. Five copies of a double stranded oligonucleotide, prepared from complementary oligonucleotides GATCCAGTAACGGTGTAGCA and GATCTGCT-ACACCGTTACTG, were cloned in tandem into the *Bam*HI site of pBluescriptSKM13−. A *Bam*HI−*Bgl*II fragment from pA10CAT2 (Laimins *et al.*, 1982), containing the SV40 early promoter, the CAT gene and a polyadenylation signal, was then inserted into the *Bam*HI site adjacent to the *myb* binding sites. The pSV2B2 luciferase reporter plasmid has been described (Klempnauer *et al.*, 1989).

Construction of v-myb/ER expression vectors

The v-*myb* expression vectors pVM116 (containing the full-length v-*myb* gene of AMV and the neomycin-resistance gene) and pVM111 (containing a frame-shift version of the v-*myb* gene of AMV and the neomycin-resistance gene) have been described (Klempnauer *et al.*, 1989). pVM134 is a derivative of pVM116 in which v-*myb* coding sequences, located between an *Nco*I site and a *Sal*I site, have been replaced by equivalent sequences from the v-*myb* gene of E26. Plasmid L2.62 is a derivative of pVM116, in which sequences encoding the last 18 C-terminal amino acids of the v-*myb* protein have been deleted and replaced by the sequence GATAAGCTT. Note that this sequence contains a *Hin*dIII site used subsequently for cloning (see below). Plasmid DIN.1 is a derivative of pVM116 in which amino acids 207–234 of the v-*myb* protein (the numbering of residues is the same as in Klempnauer *et al.*, 1982) have been replaced by amino acids ISLS (amino acids are named by single-letter amino acid code). All v-*myb*/ER expression vectors described below are derivatives of pVM116. pVM116 was first digested with *Kpn*I and *Eco*RI. The *Kpn*I and *Eco*RI sites are located upstream of v-*myb* and within the v-*myb*, respectively (Klempnauer *et al.*, 1989). To construct pE*myb*ER, a *Kpn*I–*Hin*dIII fragment of plasmid L.2.62, containing the v-*myb* gene of AMV from which the last 18 amino acids had been removed (see above) was combined with a *Bam*HI–*Eco*RI fragment from plasmid pHE14 (Kumar *et al.*, 1986), encoding the hormone binding domain of the human estrogen receptor and a synthetic linker (AAGCTTAAGGATCC) to join the *Hin*dIII and *Bam*HI sites, and cloned between the *Kpn*I and *Eco*RI sites of plasmid pVM116. The resulting plasmid, pE*myb*ER, encodes a protein containing amino acids 1 to 364 of v-*myb*, followed by the amino acids DKLKDP and amino acids 282–595 of the human estrogen receptor. pEE26*myb*ER was constructed in the same way, except that plasmid L2.62 was modified first by exchanging v-*myb* sequences located between the *Kpn*I site and an *Eco*RI site (which resides at the 3′ end of the v-*myb* DNA binding domain) by equivalent sequences from plasmid pVM134. Thus, the only difference between the proteins encoded by pE*myb*ER and pEE26*myb*ER is that sequences between an *Nco*I site and an *Eco*RI site, which encode most of the v-*myb* DNA binding domain, are derived from AMV or E26, respectively. pE*myb*ERΔ was constructed as pE*myb*ER except that plasmid L2.62 was modified first by replacing v-*myb* sequences located between an *Nco*I site and *a Bal*I site (located close to the 5′ and 3′ ends of v-*myb*) by equivalent sequences from plasmid pDIN1. The protein encoded by pE*myb*ERΔ differs from that encoded by pE*myb*ER by a deletion of an acidic transactivation domain (Weston and Bishop, 1989). pEE26*myb*ERΔ was generated as pEE26*myb*ER except that the E26 version of plasmid L2.62 was modified further by exchanging v-*myb* sequences located between an *Eco*RI site (located immediately 3′ to the DNA binding domain) and a *Bal*I site (located close to the 3′ end of v-*myb*) were replaced by equivalent sequences from plasmid pDIN1. The protein encoded by pEE26*myb*ERΔ thus contains an internal deletion of v-*myb* sequences, but differs from the protein encoded by pE*myb*ERΔ in that the sequences encoding most of the DNA binding domain are derived from E26.

Transfections, CAT and luciferase assays, G418 selection

DNA was transfected by the calcium phosphate co-precipitation method (Graham and van der Eb, 1973), as modified by Theisen *et al.* (1986). Cell extracts were prepared by suspending the cells in 0.1 M potassium phosphate (pH 7.8), 1 mM dithioerythrol, freeze–thawing them three times, and spinning down debris. CAT and luciferase assays were performed as described (Theisen *et al.*, 1986; De Wet *et al.*, 1987). G418 selection was performed at a concentration of 400 μg/ml G418. Surviving cells were cultivated further in the presence of 200 μg/ml G418.

Western blotting

Western blotting and subcellular fractionation have been described before (Klempnauer *et al.*, 1989). *Myb* proteins were immunostained with the *myb*-specific monoclonal antibody CB100–18 (Klempnauer *et al.*, 1986).

RNA analysis

Northern blotting was performed as described (Klempnauer and Bishop, 1983). To generate radiolabeled probes from randomly selected cDNA clones single phage plaques of a λgt11 cDNA library of BM2 cells were picked, pools of 10 plaques each were then eluted in water and the insert fragments of the phage clones were amplified by PCR using phage-specific primers flanking the inserts. The amplified fragments were radiolabeled and used to hybridize Northern blots.

Nucleotide sequencing

Nucleotide sequencing was performed by the dideoxy chain termination technique (Sanger *et al.*, 1977).

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