

SHORT COMMUNICATION

v-Myb represses the transcription of *Ets-2*

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The v-Myb oncogene causes monoblastic leukemia and transforms only myelomonocytic cells in culture. The v-Myb protein is nuclear and binds to specific DNA sequences. To identify genes regulated by v-Myb, we utilized primary cells transformed by a retrovirus encoding a v-Myb-estrogen receptor (ER) fusion protein. The *Ets-2* gene was not expressed in v-Myb-ER transformed cells in the presence of estradiol, but was expressed within 4 h after estradiol withdrawal. The expression of *Ets-2* also increased dramatically following phorbol ester-induced differentiation of the v-Myb-transformed BM2 cell line. Conversely, *CRYP-alpha*, encoding a transmembrane tyrosine phosphatase, was expressed in the presence but not the absence of estradiol in v-Myb-ER transformed cells. *CRYP-alpha* was downregulated during the phorbol ester-induced differentiation of BM2 cells. Although *LIM-3* expression was estradiol-inducible in v-Myb-ER transformed monoblasts, *LIM-3* was expressed neither in primary yolk sac cells transformed by unfused v-Myb nor in BM2 cells. We conclude that although v-Myb has been intensively studied as a transcriptional activator, v-Myb can repress biologically relevant genes such as *Ets-2*, which promotes macrophage differentiation. In addition, we have shown that some genes that are regulated by a v-Myb-ER fusion protein may not be relevant to the biological function of the unfused v-Myb protein.

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The v-Myb oncogene of the avian myeloblastosis virus is unusual in that it causes only monoblastic leukemia in chickens and transforms only myelomonocytic cells in culture (Lipsick and Wang, 1999). Studies from a number of different laboratories have shown that the v-Myb protein is nuclear, binds to specific DNA sequences as a monomer, and can activate the expression of reporter genes (Boyle *et al.*, 1984; Klempnauer *et al.*, 1984; Biedenkapp *et al.*, 1988; Ibanez *et al.*, 1988; Garcia *et al.*, 1991). In addition, both v-Myb and its

normal cellular homologue c-Myb have been shown to be capable of activating gene expression in a variety of cell types (Ness *et al.*, 1989; Nishina *et al.*, 1989; Weston and Bishop, 1989; Ibanez and Lipsick, 1990; Nakagoshi *et al.*, 1992; Siu *et al.*, 1992; Cogswell *et al.*, 1993; Ku *et al.*, 1993; Melotti and Calabretta, 1994; Burk *et al.*, 1997; Worpenberg *et al.*, 1997; Schlichter *et al.*, 2001; Chen *et al.*, 2002; Bartley *et al.*, 2003; Rushton *et al.*, 2003; Braas *et al.*, 2004; Lang *et al.*, 2005; Liu *et al.*, 2006).

To attempt to identify biologically relevant Myb-regulated genes, we previously described a system in which primary chicken yolk sac cells could be oncogenically transformed in an estrogen-dependent fashion by a retrovirus that encoded a fusion of AMV v-Myb and the hormone-binding domain of the human estrogen receptor (Engelke *et al.*, 1997). We found that in the absence of estrogen, these transformed cells predominantly differentiated into multinucleated giant cells. By modifying the culture conditions with the addition of recombinant chicken myeloid growth factor (Leutz *et al.*, 1984), a G-CSF-related cytokine, we were able to grow relatively large numbers of primary v-Myb-ER transformed monoblasts that could be differentiated into mononuclear macrophages by the simple withdrawal of estradiol (Figure 1). The presence of macrophage differentiation was readily apparent within 24 h of hormone withdrawal and by 48 h virtually all cells in the culture showed features of macrophage differentiation including increased adherence to substrate, the induction of phagocytic vacuoles, and cessation of proliferation.

In order to identify genes that might be directly regulated by v-Myb, we extracted mRNA from v-Myb-ER transformed primary monoblasts maintained in estradiol and from sister cultures four hours after the withdrawal of estradiol. To determine which technique might be most useful for differential analysis of these mRNA populations, we conducted pilot studies on the v-Myb transformed BM2 cells line which can be induced to differentiate into mature macrophage following treatment with phorbol ester (Pessano *et al.*, 1979; Moscovici *et al.*, 1982). We found that in our hands, representation display analysis (RDA) (Hubank and Schatz, 1994) was quite reproducible and readily identified both known and unknown phorbol-inducible genes including collagenase, interleukin 8, ATP synthase, and macrophage inflammatory protein, all of which were verified by Northern blotting (Supplemental Data, Figure S1). We therefore utilized the RDA

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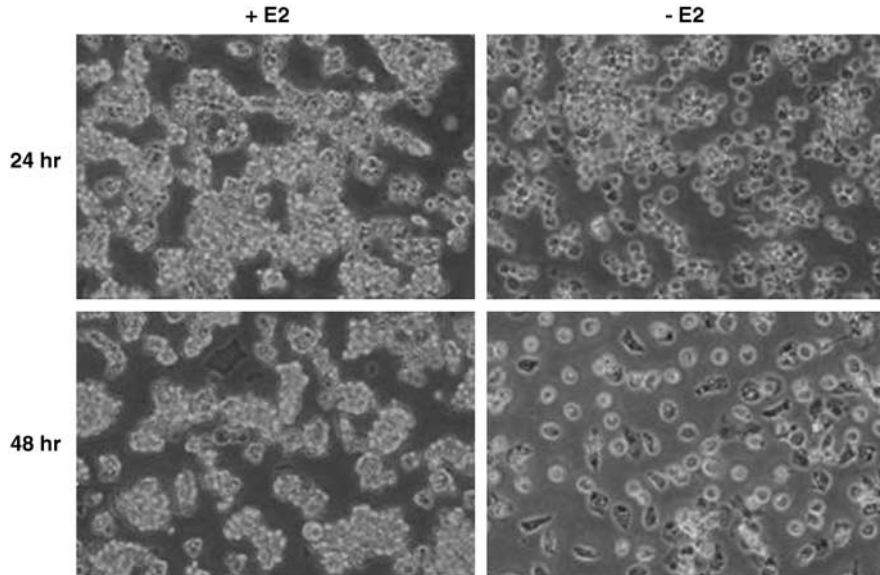


Figure 1 Macrophage Differentiation of v-Myb-ER Transformed Primary Yolk Sac Cells. Chick embryonic yolk sac myeloid cells (13 days) were harvested and infected with a selectable avian retrovirus encoding a v-Myb-ER fusion protein (N-dGEE) as previously described (Ibanez and Lipsick, 1988; Engelke *et al.*, 1997), and then cultured in the presence of estradiol (1 μ M) and recombinant cMGF (Leutz *et al.*, 1989). Sister cultures were maintained in the presence (+E2) or absence (-E2) of estradiol and phase contrast photomicrographs were taken at 24 and 48 h after withdrawal of estradiol.

method with three rounds of differential amplification of cDNA from v-Myb-ER transformed primary monoblasts before versus 4 h after withdrawal of estradiol (Figure 2). Subtraction was performed in both directions in order to identify genes whose expression was enriched following the withdrawal of estradiol, as well as those genes whose expression was enriched in the presence of estradiol.

Each visible ethidium bromide stained band from the third round of differential amplification was excised, extracted, molecularly cloned, sequenced and analyzed by a BLAST search of the NCBI databases. A number of genes were found to be significantly enriched in each pool (Table 1). We then used RNase protection assays as a further screen to identify those genes that were differentially regulated by four hours of estradiol withdrawal in v-Myb-ER transformed primary cells. We chose to focus on two genes that were repressed by v-Myb-ER in that their expression increased dramatically after withdrawal of estradiol (*ets-2* and *chemokine ah221*), and on two genes that were induced by v-Myb-ER (*CYRP-alpha* tyrosine phosphatase and *lim-3*) (Figure 3). In order to better understand the biological relevance of these genes to the transformed phenotype, we performed RNase protection assays in two additional systems. First, we compared gene expression in primary cells transformed by v-Myb without an estrogen receptor fusion. As predicted, these cells do not differentiate in response to estradiol. Second, we compared gene expression in the v-Myb transformed BM2 cell line before and after four hours of treatment with the differentiation-inducing agent phorbol ester.

Three classes of genes were identified by these experiments. Perhaps most interesting is our observation that *ets-2*, which itself encodes a DNA-binding

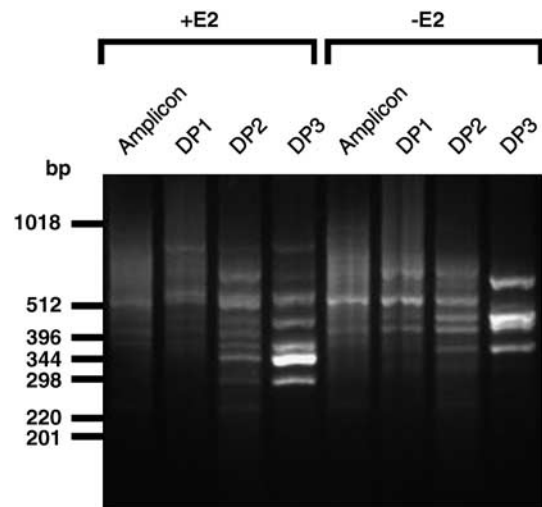


Figure 2 Representational difference analysis of primary yolk sac cells in the presence or absence of estradiol. Messenger RNA was harvested from sister cultures of v-Myb-ER transformed primary yolk sac cells maintained in the presence of estradiol and at 4 h after withdrawal of estradiol. RDA analysis was performed as previously described (Lisitsyn *et al.*, 1993; Hubank and Schatz, 1994) in order to enrich for genes expressed in the presence of estradiol (+E2) or in the absence of estradiol (-E2). Data shown are from a representative ethidium bromide-stained agarose gel electrophoresis of the initial amplicon DNAs and of the difference products (DP) after three sequential rounds of RDA. Data in Table 1 were obtained from molecular clones derived from bands in the DP3 lanes.

transcription factor, appears to be repressed by v-Myb. In particular, *ets-2* expression was absent in v-Myb-ER transformed primary cells but was induced within four hours after withdrawal of estradiol. Consistent with

Table 1 NCBI blast analysis of V-Myb-ER RDA results

Clone	Gene name	Genbank ID	E Value
<i>Forward RDA SEQS (n = 36)</i>			
f0611mf	Chaperonin containing TCP1, subunit 2 (CCT2)	NM-001012533	0
f0605mf	ChEST1003e21	CR390061	0
f0510mf	Coagulation factor XIII, A1	NM_204685.1	0
f0101mf	Ferritin heavy polypeptide (FTH1)	NM_205086	1.00E-107
f0102mf	Ferritin heavy polypeptide (FTH1)	NM_205086	1.00E-107
f0104mf	Ferritin heavy polypeptide (FTH1)	NM_205086	1.00E-107
f3406mf	Ferritin heavy polypeptide (FTH1)	NM_205086	1.00E-107
f0506mf	Glutaminyl-tRNA synthetase	NM_001012782	7.00E-102
f0103mf	LIM-3	L35570.1	2.00E-110
f0109mf	LIM-3	L35570.1	1.00E-114
f0204mf	LIM-3	L35570.1	1.00E-133
f0205mf	LIM-3	L35570.1	1.00E-133
f3413mf	LIM-3	L35570.1	2.00E-138
f3414mf	LIM-3	L35570.1	4.00E-143
f0802mf	MSH6 DNA mismatch repair protein	XM_419358.11	7.00E-126
f0501mf	No good match in GENBANK		
f0503mf	No good match in GENBANK		
f0507mf	No good match in GENBANK		
f0604mf	No good match in GENBANK		
f0607mf	No good match in GENBANK		
f0706mf	No good match in GENBANK		
f0804mf	No good match in GENBANK		
f3401mf	No good match in GENBANK		
f3403mf	No good match in GENBANK		
f3421mf	No good match in GENBANK		
f0702mf	Novel protein conserved in humans	XM_417601.1	9.30E-176
f0502mf	PTP CRYPalpha	NM_205407.1	0.00E+00
f0511mf	PTP CRYPalpha	NM_205407.1	1.00E-160
f0801mf	RAV-0 Gag	M73497.1	0
f0512mf	SEC23 interacting protein p125	XM_424389	5.00E-158
f3405mf	SEC23 interacting protein p125	XM_424389	2.00E-98
f3408mf	SEC23 interacting protein p125	XM_424389	3.00E-162
f3419mf	SEC23 interacting protein p125	XM_424389	5.00E-115
f0110mf	Similar to beta actin	XM_424240.1	7.00E-113
f0709mf	WD40 repeat protein	AJ719681	0
f0606mf	Weakly similar to Mgc4	NM_001030628.1	0.14
<i>Reverse RDA SEQS (n = 42)</i>			
r0108mf	28S ribosomal RNA gene	DQ018756.1	2.00E-130
r0104mf	ATPase	XM_417622	2.00E-67
r0110mf	CAMRA-1/CARD11 MAGUK	AJ851540	1.00E-126
r0410mf	Caspase recruitment domain CARD9	XM_425329.1	0.00E+00
r0407mf	Cathepsin S	NM_001031345.1	0.00E+00
r0701mf	Chemokine ah221	AY037860.1	0.00E+00
r0702mf	Chemokine ah221	AY037860.1	2.00E-129
r0601mf	Chemokine SCYA4	AF146730	0.00E+00
r0111mf	Coagulation factor C homolog, cochlin	NM_204937	1.00E-96
r0105mf	Coagulation factor XIII A1	NM_204685.1	2.00E-111
r0109mf	Coagulation factor XIII A1	NM_204685.1	3.00E-112
r0013mf	Coagulation factor XIII A1	NM_204685.1	2.00E-107
r0014mf	Coagulation factor XIII A1	NM_204685.1	2.00E-104
r0303mf	Coagulation factor XIII A1	NM_204685.1	8.00E-103
r0501mf	Coagulation factor XIII A1	NM_204685.1	0.00E+00
r0506mf	Coagulation factor XIII A1	NM_204685.1	0.00E+00
r0510mf	Coagulation factor XIII A1	NM_204685.1	1.00E-142
r0602mf	Coagulation factor XIII A1	NM_204685.1	0.00E+00
r0603mf	Coagulation factor XIII A1	NM_204685.1	0.00E+00
r0605mf	Coagulation factor XIII A1	NM_204685.1	7.00E-89
r0107mf	DMRT1 isoform e	XM_418817.1	2.00E-123
r0402mf	Erythrocyte band 4.1 like 3	NM_419142.1	9.00E-92
r0401mf	Ets-2	NM_205312.1	0.00E+00
r0505mf	Ets-2	NM_205312.1	0.00E+00
r0607mf	Ets-2	NM_205312.1	0.00E+00
r0106mf	Ferritin heavy polypeptide	NM_204685.1	2.00E-108
r0102mf	Glutamate transporter	XM_41878	2.00E-136
r0506mr	Glutaminyl tRNA synthetase	NM_001012782.1	2.00E-168
r0207mf	Hsc70	NM_205003.1	1.00E-136
r0301mf	Macrophage mannose receptor	XM_418617.1	1.00E-36
r0308mf	macrophage mannose receptor	XM_418617.1	1.00E-38

Table 1 (continued)

Clone	Gene name	Genbank ID	E Value
r0705f	Myosin heavy chain MYH9	NM_205477.1	0.00E+00
r0202mf	No good match in GENBANK		
r0206mf	No good match in GENBANK		
r0306mf	No good match in GENBANK		
r0404mf	No good match in GENBANK		
r0411mf	No good match in GENBANK		
r0508mf	No good match in GENBANK		
r0405mf	Proviral integration site 1- ser thr kinase	NM_419260.1	8.00E-23
r0208mf	Ribonuclease RSFR	NM_001007942.1	8.00E-135
r0103mf	RT POL-like protein	XM_417689	5.00E-12
r0205mf	Vimentin	XM_418622	9.00E-145

'E value' indicates the "expectation value" from our BLAST analyses. [<http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/glossary2.html>].

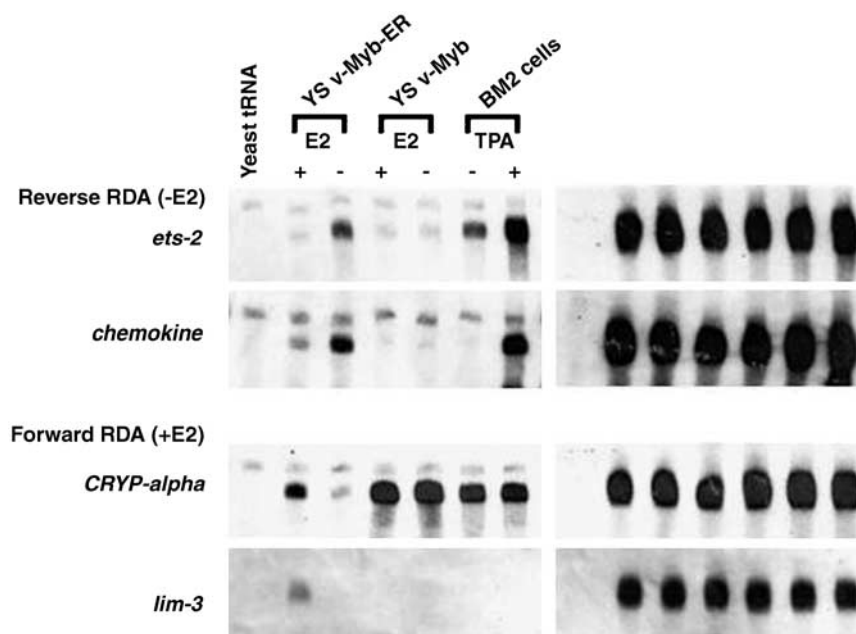


Figure 3 Differential expression of v-Myb-ER regulated genes. RNase protection was used to quantify unamplified mRNA for the indicated genes in three different experimental systems: primary yolk sac cells transformed by a v-Myb-ER encoding virus, primary yolk sac cells transformed by a v-Myb encoding virus, and the v-Myb-transformed BM2 cell line. RNA was isolated from primary cells cultured in the presence of estradiol (+E2) or 4 h after withdrawal of estradiol (-E2). RNA was isolated from BM2 cells in the absence of phorbol ester (-TPA) or four hours after treatment with this inducer of differentiation (+TPA). Yeast tRNA was used as a negative control for RNase protection. The panels on the right are controls in which a GAPDH RNA probe was used to measure RNase protection by the same RNA samples indicated in the left panel.

these observations, *ets-2* expression was absent in v-Myb transformed primary cells in the presence or absence of estradiol. In addition, the levels of *ets-2* increased dramatically in BM2 cells upon treatment with phorbol ester, an inducer of macrophage differentiation. Previous work has shown that Myb proteins are capable of repressing the transcription of transfected reporter genes. Our results now identify *ets-2* as a physiologically relevant target for repression by v-Myb. Our screen also identified chemokine *ah221* as a gene repressed by v-Myb-ER and v-Myb in primary leukemic cells and in the BM2 cell line (Figure 3).

Several lines of additional evidence imply that *ets-2* is directly regulated by v-Myb and that repression of *ets-2*

expression contributes to the highly virulent leukemogenic phenotype that was selected during the passage of AMV from chicken to chicken for many years. First, the myeloid-specific regulatory sequences of *ets-2* contain Myb-binding sites that have been conserved during the evolution of human and chicken (Begue *et al.*, 1997). Second, chromatin immunoprecipitation experiments using anti-Myb specific antibodies demonstrated that the v-Myb protein was directly bound to the *ets-2* promoter in v-Myb-transformed BM2 cells (Figure 4). Third, the ectopic expression of *ets-2* induces differentiation in a murine myeloid leukemia cell line (Aperlo *et al.*, 1996). Fourth, a hypomorphic allele of *ets-2* greatly reduced myeloid proliferation and inflammation

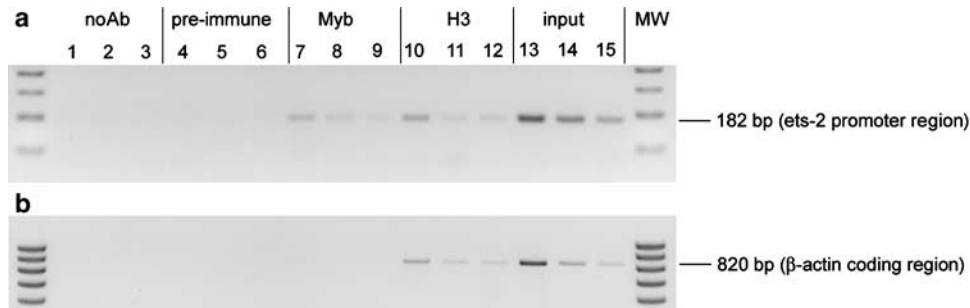


Figure 4 v-Myb protein is bound to the Ets-2 promoter in v-Myb transformed BM2 cells. Chromatin was isolated from formaldehyde crosslinked nuclei of BM2 cells, sheared by sonication, subjected to immunoprecipitation using the indicated antibodies, then analysed by PCR and gel electrophoresis following twofold serial dilution of precipitated material following the reversal of the crosslinks. Myb protein was immunoprecipitated using the polyclonal rabbit anti-BP2 and BP7 antibodies (Garcia *et al.*, 1991). ChIP samples with no antibody and with preimmune serum were used as negative controls, and ChIP with anti-histone H3 antibody (Upstate) was used as a positive control. Shown are PCR products using primers for the *ets-2* promoter region and β -actin coding region with twofolds of serial dilutions of ChIP-bound or input DNA templates. Templates used for negative control and BP2/BP7-ChIP sample were, respectively, 2% (lane 1, 4 and 7), 1% (lane 2, 5 and 8) and 0.5% (lane 3, 6 and 9) of total bound DNA, for H3-ChIP samples were 0.2% (lane 10), 0.1% (lane 11) and 0.05% (lane 12) of total bound DNA. Input represented 0.004% (lane 13), 0.002% (lane 14) and 0.001% (lane 15) of total input DNA. Forward and reverse primers for PCR were as follows: *ets2*-5: 5'-TGGCTTAGGAGAAATTGCTTG-3' (chicken genome 1, contig No. NW 060224, 6631362-6631383), *ets2*-3: 5'-CGGCTAGAGTGTGGCAAGTT-3' (chicken genome 1, contig No. NW 060224, 6631525-6631543), β -actin-5: 5'-GCACCACACTTTCTACAATGA-3' and β -actin-3: 5'-AGATGTGGATCAGCAAGCAG-3'. PCR reactions were performed for 37 cycles.

in the *motheaten-viable* (*me-v*) mouse model that is caused by germline mutation of the hematopoietic cell tyrosine phosphatase (*Hcph/SHP1*) (Wei *et al.*, 2004).

The observation that *ets-2* is expressed to some degree in undifferentiated BM2 cells is consistent with previous work showing that BM2 cells are not a reliable model for primary v-Myb-transformed monoblasts. For example, expression of the *mim-1* gene has never been detected in primary cells transformed by AMV v-Myb, but is readily detected in undifferentiated BM2 cells (Dini *et al.*, 1995; Ness *et al.*, 1989). In this regard, we created a variant of the BM2 cell line containing a tetracycline-regulated *ets-2* cDNA but were unable to detect any significant changes in proliferation, differentiation, or survival following induction of additional *ets-2* expression (data not shown).

Interestingly, the *c-myb* proto-oncogene has been independently transduced in two different avian acute leukemia viruses (Lipsick and Wang, 1999). AMV encodes v-Myb, a doubly truncated form of the normal c-Myb protein that also contains 10 amino-acid substitutions, which also contribute to leukemogenesis. In contrast, the E26 leukemia virus encodes a fusion protein in which a smaller portion of the c-Myb protein with a single amino acid substitution is fused directly to a large segment of the c-Ets-1 transcription factor. Previous studies have shown that the portion of c-Myb present in E26 is at best weakly oncogenic (Metz and Graf, 1991). Furthermore, the passage *in vivo* of a non-leukemogenic virus that encodes separate E26 Myb and c-Ets-1 proteins results in the selection for leukemogenic viruses in which deletions recreate a Myb-Ets fusion protein. The results presented above suggest that this fusion may be under selection for its ability to repress targets of both Myb and Ets transcription factors, perhaps because E26 Myb itself is not an efficient repressor of *ets-2* transcription.

A second class of gene identified in our screen is represented by *CYRP-alpha*, which encodes a transmembrane tyrosine phosphatase. This gene is expressed in primary monoblasts transformed by v-Myb-ER in the presence of estradiol, but is dramatically downregulated within four hours of withdrawal of estradiol (Figure 3). *CYRP-alpha* is also expressed in primary monoblasts transformed by unfused v-Myb, in either the presence or absence of estradiol. These results demonstrate that v-Myb-ER rather than endogenous ER is responsible for the activation of *CYRP-alpha* in v-Myb-ER transformed monoblasts. In addition, *CYRP-alpha* was highly expressed in the BM2 cell line. However, phorbol ester treatment did not inhibit *CYRP-alpha* expression in BM2 cells, implying that the down-regulation of this gene is not required for macrophage differentiation. We note that unlike primary leukemic cells, phorbol ester treatment of BM2 cells is readily reversible. These results therefore suggest that genes critical for cell proliferation may be deregulated in this cell line by additional mutations and/or epigenetic alterations.

A third class of gene identified in our screen is represented by *lim-3*, which encodes a homeobox-containing transcription factor critical for neural development. *lim-3* is expressed in v-Myb-ER transformed primary monoblasts in the presence of estradiol, but is rapidly down-regulated after withdrawal of estradiol in a fashion similar to *CYRP-alpha*. Surprisingly, no *lim-3* expression was detected in primary monoblasts transformed by v-Myb itself or in the BM2 cell line. These results imply that activation of *lim-3* expression by the v-Myb-ER fusion protein represents a neomorphic phenotype caused by fusion of the estrogen receptor hormone-binding domain.

In order to identify genes directly regulated by v-Myb and c-Myb, a variety of experimental approaches have been used. The first Myb-regulated gene, *mim-1*, was

identified in a differential cDNA library screen of primary cells transformed by a temperature sensitive mutant of the E26 virus, which encodes a tripartite Gag-Myb-Ets fusion protein (Ness *et al.*, 1989). However, although *mim-1* is abundantly expressed in differentiated myeloid cells, this gene was not expressed in monoblasts transformed by AMV v-Myb, nor were other Myb-regulated genes identified in this screen.

In an approach similar to that which we have taken here, a hybrid AMV/E26 v-Myb protein was fused to the hormone-binding domain of the human estrogen receptor and introduced into an established v-Myb-transformed macrophage cell line (Burk and Klempnauer, 1991). The utility of this system has been proven by the identification of a number of Myb-regulated genes (Burk *et al.*, 1997; Worpenberg *et al.*, 1997; Schlichter *et al.*, 2001; Braas *et al.*, 2004). A drawback of this system is that the cells of interest are already transformed by the v-Myb oncogene and are therefore not dependent upon the v-Myb-ER fusion protein for growth or survival. Myb-regulated genes have also been identified in murine cell lines transformed by a truncated c-Myb-ER fusion protein (Ferrao *et al.*, 1997; Bartley *et al.*, 2003). An advantage of this system is that these cells are dependent both upon Myb and upon exogenous growth factors for their proliferation and survival.

An alternative approach has been to create dominant interfering forms of Myb proteins by the fusion of the c-Myb DNA-binding domain, the transcriptional repressor domain from the *Drosophila* Engrailed protein, and the human estrogen receptor which were then expressed in established hematopoietic cell lines (Taylor *et al.*, 1996; White and Weston, 2000). This system has recently been used in conjunction with a subtractive cDNA cloning method to identify a number of Myb-regulated genes (Lang *et al.*, 2005). One disadvantage of this system is that one cannot distinguish among inhibition of different Myb proteins (e.g. c-Myb versus

B-Myb). A similar method has been described in which a Myb-Engrailed fusion protein has been inducibly expressed in a transformed erythroleukemia cell line under control of the metallothionein promoter (Chen *et al.*, 2002).

Recently, another approach has been described in which established cell lines of various lineages are infected with adenovirus vectors that express a variety of different wild-type and mutant Myb proteins and then gene expression has been analysed by microarray (Rushton *et al.*, 2003; Lei *et al.*, 2004; Liu *et al.*, 2006). The most remarkable finding of these studies has been that the panel of Myb-regulated identified in such experiments is highly dependent upon the specific Myb protein expressed as well as the specific cell line used for adenoviral infection. In this regard, those genes we have identified as being directly regulated by a v-Myb-ER fusion protein in primary transformed chicken monoblasts do not appear to have been identified in several other screens in which Myb proteins were expressed in various other cell types (Rushton *et al.*, 2003; Lang *et al.*, 2005; Liu *et al.*, 2006).

In summary, we have shown that in addition to its well-characterized role as an activator of gene expression, the v-Myb oncoprotein represses physiologically important genes including *ets-2*. We have also shown that v-Myb-ER fusion proteins can regulate genes not regulated by v-Myb itself. Therefore, caution must be used in interpreting the results of experiments in which fusions of the estrogen receptor hormone-binding domain and other regulatory domains have been used to identify genes regulated by Myb and other DNA-binding proteins.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).