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The Homeobox Gene *GBX2*, a Target of the *myb* Oncogene, Mediates Autocrine Growth and Monocyte Differentiation

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Summary

The homeobox gene *GBX2* was identified as a target gene of the v-Myb oncoprotein encoded by the avian myeloblastosis virus (AMV). *GBX2* activation by c-Myb requires signal transduction emanating from the cell surface while the leukemogenic AMV v-Myb constitutively induces the *GBX2* gene. Mutations in the DNA binding domain of AMV-Myb render it independent of signaling events and concomitantly abrogate the collaboration between Myb and CCAAT Enhancer Binding Proteins (C/EBP), which are involved in granulocyte differentiation. Ectopic expression of *GBX2* in growth factor–dependent myeloblasts induces monocytic features and independence from exogenous cytokines, reflecting distinct features of AMV–transformed cells. Our results suggest that Myb or factors it interacts with contribute to hematopoietic lineage choice and differentiation in a signal transduction–dependent fashion.

Introduction

The c-*myb* gene encodes a transcription factor that regulates the growth and differentiation of immature hematopoietic cells and that is required for mammalian hematopoiesis (Mucenski et al., 1991; Graf, 1992). Expression of c-*myb* is highest in proliferating, immature cells, and it is overexpressed in a variety of leukemias and solid tumors (Wolff, 1996). However, ectopic expression of c-*myb* is not sufficient to induce tumors in transgenic animals (Furuta et al., 1993), suggesting that activating mutations are required to reveal its oncogenic activity (Graf, 1992; Lipsick, 1996). Two different, independently isolated avian leukemia viruses have transduced v-*myb* oncogenes that encode mutated, activated Myb proteins and that induce acute leukemias in animals (Graf and Stehelin, 1982). Both viruses transform hematopoietic precursor cells in tissue culture, although each displays a unique transformation specificity within the hematopoietic system.

The E26 virus encodes a complex chimeric protein in which v-Myb is fused to a second oncoprotein, v-Ets. E26 causes a precursor type leukemia with prevailing erythroleukemic features and transforms multipotent and myeloid progenitors in vitro (Graf et al., 1992). Deletion of v-*ets* renders E26 nonleukemogenic in animals. However, the myeloid leukemogenicity and transforming activity of such v-*myb*–containing mutants can be revealed through concomitant expression of the chicken myelomonocytic growth factor (cMGF), a cytokine that stimulates the growth and survival of transformed myeloid cells and colony formation and differentiation of normal bone marrow cells (Leutz et al., 1984, 1989; Metz et al., 1991). Thus, myeloid cells transformed by the E26 version of v-*myb* fail to proliferate in animals because cMGF is limited, suggesting that the oncogene and the cytokine cooperate to induce leukemia.

In contrast to E26, the avian myeloblastosis virus (AMV) expresses only a truncated v-*myb* oncogene, which carries multiple point mutations, transforms monoblast-like cells, and induces a myeloid leukemia in animals. AMV-transformed "monoblasts," although responsive to cMGF, grow in a cMGF-independent manner and secrete small amounts of cMGF (Dini et al., 1995; A. L., unpublished data), suggesting that they grow in an autocrine fashion. While activation of the *cMGF* gene in macrophages involves the transcription factors AP1 and C/EBPβ (Sterneck et al., 1992b), cMGF expression in AMV-transformed cells has been linked to the AMV v-Myb DNA binding domain (DBD) (Introna et al., 1989; Dini et al., 1995). This suggests that the mutations acquired by AMV v-Myb play an essential role in determining the growth factor independence and hence the leukemogenicity of the transformed cells.

Here we describe that cMGF expression in AMV monoblasts is induced by *GBX2*, a homeobox gene that we identified as a target for AMV v-*myb*. Expression of *GBX2* in E26-transformed cells induces morphological and functional monocytic features and allows them to grow in the absence of exogenously added growth factor, thus reflecting the AMV phenotype. The protooncogene c-*myb* requires an activated system of protein kinases to induce GBX2. The latter requirement is unnecessary in AMV-transformed cells: the mutations in the v-Myb DNA binding domain override the need for cooperating protein kinases, allowing AMV v-Myb to constitutively activate GBX2 and simultanously to abrogate collaboration with CCAAT enhancer binding proteins (C/EBP), which are involved in granulocytic differentiation (Müller et al., 1995; Zhang et al., 1997). Our results identify GBX2 as an important downstream target of Myb and shed light on the mechanisms of growth control and lineage commitment in the hematopoietic system and the activation of the *myb* oncogene.

Results

The Homeodomain Protein GBX2 Binds the *cMGF* Promoter

One of the most striking features distinguishing AMV- and E26-transformed myeloid cells is that only the former exhibit autocrine growth capacity, caused by their ability to secrete small quantities of cMGF (Dini et al., 1995, and A. L., unpublished data). Since the *cMGF* promoter lacks binding sites for the Myb protein but nevertheless was activated by AMV but not by E26

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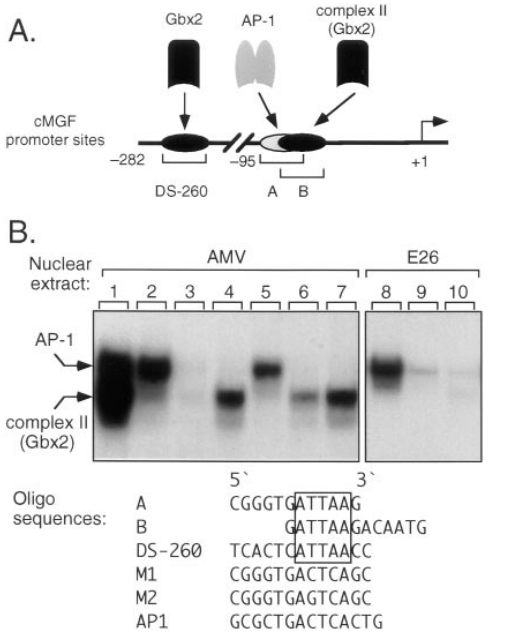

Figure 1. Nuclear Protein Binding to the cMGF Promoter A Site

(A) Scheme of the cMGF promoter and proteins binding to it.

(B) Nuclear extracts from AMV- or E26-transformed cells were incubated with labeled oligonucleotide corresponding to the cMGF promoter A site (lanes 1 and 8). Binding site competition analysis was perfomed with 50-fold molar excess of unlabeled competitor oligonucleotides: B site (lane 2), A site (lanes 3 and 9), AP1 site (lane 4 and 10), DS-260 (lane 5), M1 (lane 6), M2 (lane 7). Oligo sequences are shown below. Below, oligos A, B, and DS-260 correspond to cMGF promoter sequences as indicated in A. Oligos M1 and M2 are mutated versions of the cMGF promoter A site.

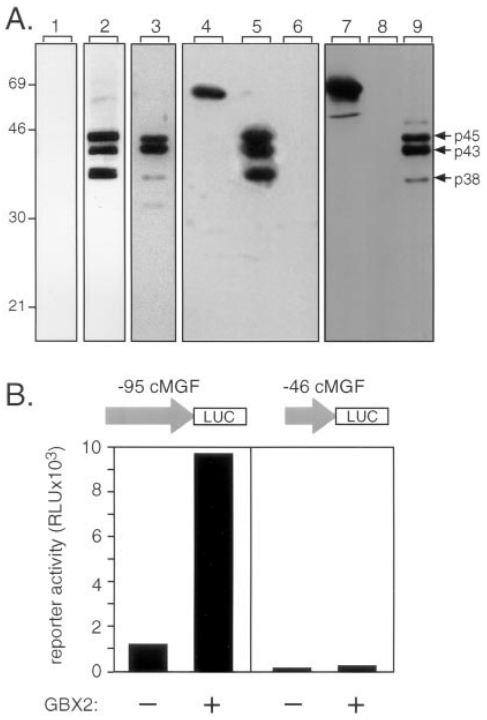

Figure 2. GBX2 Protein Expression and Transcriptional Activity

(A) Western blot and South-Western analysis of nuclear extracts. Immunoblots of nuclear extracts from BM2 cells (lanes 1 and 2) or Cos-7 cells transfected with a GBX2 expression vector (lane 3) using rabbit preimmune serum (lane 1) or antiserum raised against recombinant cGBX2 (lanes 2 and 3). South-Western analysis (lanes 4–9) using ^{32}P-labeled cMGF promoter A site as a probe. Recombinant, bacterially expressed GST-GBX2 as control (lanes 4 and 7), nuclear extracts of BM-2 (lane 5), REV lymphoblast (lane 6, negative control), vector-transfected Cos cells (lane 8), or Cos cells transfected with a GBX2 expression vector (lane 9).

(B) Reporter gene activation by GBX2. Luciferase expression from the −95cMGF promoter-luciferase construct or an A site deletion construct (−46cMGF) after transfection into QT6 fibroblasts along with or without a GBX2 expression vector as indicated. Bars represent means of duplicates.

v-Myb (data not shown), we hypothesized that AMV activates another transcription factor, which in turn induces *cMGF*.

We used an electrophoretic mobility shift assay to identify differences in cMGF promoter-binding proteins in nuclear extracts from AMV- and E26-transformed myeloblasts. As shown in Figure 1B, an oligonucleotide probe, which spanned the A site regulatory element centered around position −86 of the *cMGF* promoter (Sterneck et al., 1992b), shifted a novel, AMV-specific complex (lane 1) in addition to the previously identified AP1 complex present in both cell types (compare lanes 1 and 8). Competition experiments using canonical AP1 oligo (lane 2), the partially overlapping B site (lane 2), or DS-260, an upstream protein binding site of the *cMGF* promoter related to the A site (lane 5), suggested that a novel factor expressed in AMV-myeloblasts binds both to the A site and to a distal site at position −260 (data not shown) in the *cMGF* promoter. Finally, the use of mutated oligonucleotides revealed that an ATTA core sequence was essential for formation of the complex II but not for binding by AP1.

GBX2 Regulates the *cMGF* Gene

To identify the factor(s) that form complex II, we used a multimerized DS-260 oligonucleotide to screen a λgt11 cDNA expression library derived from AMV-transformed BM2 cells. We screened a total of 1.6 × 10^5 phages and isolated one positive clone that contained a 1.6 kb cDNA insert. Sequence analysis and data base search revealed an open reading frame able to encode a homeodomain protein closely related to GBX2. The highest protein sequence conservation is located in the homeodomain, which is identical in the chicken and mammalian proteins (data not shown).

The GBX2 coding region was expressed as a Glutathione-S-Transferase (GST) fusion protein in E. coli. The recombinant protein bound DNA with a specificity that was indistinguishable from that of native complex II (data not shown). GST-GBX2 was used as an antigen to prepare both rabbit polyclonal antisera and mouse monoclonal antibodies, and these antibodies revealed that the GBX2 protein expressed in transiently transfected Cos-7 cells was localized exclusively in nuclei (data not shown). To analyze GBX2 protein expression in more detail, we subjected nuclear extracts from AMV-transformed cells to Western blotting and to South-Western analyses (Figure 2A). Extracts from transfected

Cos-7 cells expressing the *GBX2* cDNA and Reticuloendotheliosis virus (REV)–transformed preB/T cells served as positive and negative controls, respectively. The *GBX2* cDNA encoded proteins of three different sizes (lane 3), migrating at approximately 45, 43, and 38 kDa (p45, p43, and p38, respectively), all of which were also detected in Western blots of AMV-transformed myeloblasts (lane 2). The same proteins were also detected in South-Western blot assays, using a radiolabled oligonucleotide probe derived from the *cMGF* promoter A site as a probe (lanes 5 and 9), suggesting that all three GBX2 forms are expressed in vivo, and all are able to bind specifically to DNA. To confirm that GBX2 can regulate the *cMGF* promoter, we examined whether ectopically expressed GBX2 can induce reporter expression. As shown in Figure 2B, GBX2 activated reporter expression from the cMGF minimal promoter when the GBX2 binding site was present, but not when it was deleted. Thus, GBX2 protein binds specifically to DNA and activates transcription from the *cMGF* promoter.

GBX2 Expression in Hematopoietic Cells

We used a semiquantitative RT-PCR procedure to analyze the expression of both *GBX2* and the closely related *GBX1* (also known as *cHox7*) genes in various chicken tissues as shown in Figure 3A. As expected, we found that both genes were expressed in brain, consistent with reports of murine *GBX* gene expression (Murtha et al., 1991; Bulfone et al., 1993). In addition, we found *GBX1* but not *GBX2* to be expressed in skeletal muscle. In contrast, *GBX2* but not *GBX1* was detected in all hematopoietic tissues examined including bone marrow, bursa of fabricius, liver, spleen, and thymus.

Next, we used Northern blot analysis to determine the expression of both *GBX* genes in various cell lines. Figure 3B shows expression of *GBX2* mRNA in blast-like cells of the myelomonocytic, erythroid, and lymphoid lineages. Reprobing the blot with a *GBX1*-specific probe did not reveal expression in any of the hematopoietic cell lines examined (data not shown). To determine whether hematopoietic expression of *GBX2* occurs in higher vertebrates, we examined various human hematopoietic cell lines (Figure 3C). The progenitor cell line K562 and the B-lymphocyte SKW64 line both expressed *GBX2*, while several others (Jurkat, Raji, Blin; data not shown) were negative or showed only negligible expression. Taken together, these data suggest that *GBX2* is expressed in several hematopoietic lineages in vertebrates.

GBX2 Is a *myb* Target Gene

To determine whether *GBX2* is a *myb* target gene, we took advantage of a macrophage cell line that harbors a chimeric estrogen receptor v-Myb fusion protein (v-MybER; 10.4HD11 cells) and that can be conditionally activated by estradiol (Burk and Klempnauer, 1991). As shown in Figure 4, estrogen treatment of 10.4HD11 cells rapidly induced expression of both *GBX2* and *mim-1*, the first *myb* target gene identified (Ness et al., 1989). To further explore the specificity of *GBX2* activation by v-mybER, cells were treated with estrogen or hydroxytamoxifen (OHT; Figure 4B), a synthetic estradiol antagonist that activates estrogen receptor fusion proteins but

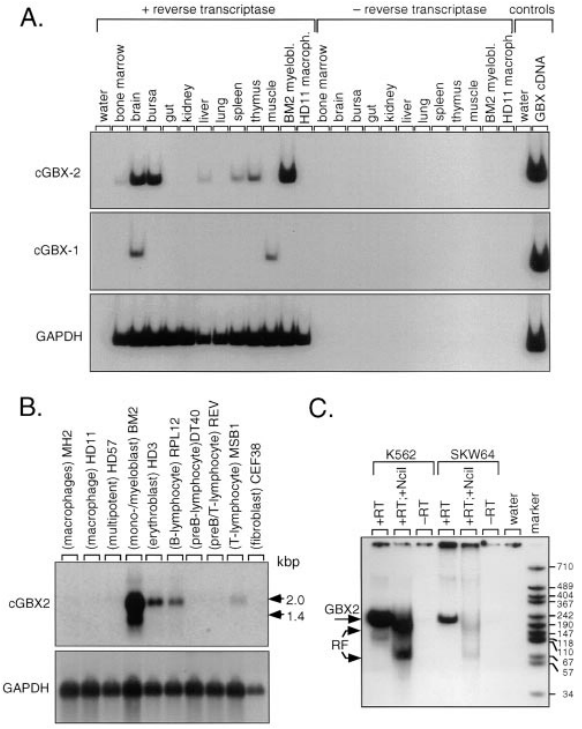

Figure 3. Expression Analysis of Chicken and Human *GBX* Genes

(A) RT-PCR assay of various tissues derived from 13-day-old chicken. RNA was extracted, reverse transcribed, and amplified using specific primers for GBX1, GBX2, and GAPDH, respectively. PCR fragments were labeled by inclusion of 0.5 μCi of [α^{32}P] dCTP into the reaction. PCR cycles (30 and 14) were performed to amplify GBX and GAPDH transcripts, respectively. Cycle number was in the linear amplification range for each of the transcripts.

(B) Northern analysis of polyA-enriched RNA from chicken cell lines as indicated using the 3′ untranslated GBX2 cDNA as a probe.

(C) RT-PCR analysis (30 cycles) of human hematopoietic cell lines using GBX2 specific primers. +RT, plus reverse transcriptase; −RT, without reverse transcriptase; +NciI, restriction enzyme digest as an additional control shows characteristic GBX2 restriction fragments (RF). Marker lane indicates fragment sizes in bp.

remains inactive on estrogen receptor targets. Activation of *GBX2* as well as *mim-1* occurred with both estrogen and OHT, largely excluding effects that might be caused by endogenous steroid hormone receptors or the ER moiety of the Myb-chimera. Furthermore, activation occured even when protein synthesis was blocked with cycloheximide (CHX) prior to hormone treatment (Figure 4B). In contrast, no *GBX2* or *mim*-1 gene activation was observed in CHX-treated 10.4HD11 cells in the absence of hormone or in estrogen/OHT-treated parental cells (Figure 4C). Taken together, these data suggest that *GBX2* is a direct target gene of the v-Myb protein.

The v-Myb protein encoded by AMV contains numerous point mutations that contribute to leukemogenicity and cMGF-independent growth and that affect the transformed cell phenotype (Introna et al., 1989; Dini et al., 1995). We therefore examined the structural requirements related to the ability of v-Myb to activate *GBX2* and *mim*-1 by transfecting a series of Myb mutants into HD11 macrophages that express both C/EBPα and C/EBPβ (A. Leutz, unpublished data). As shown in Figure

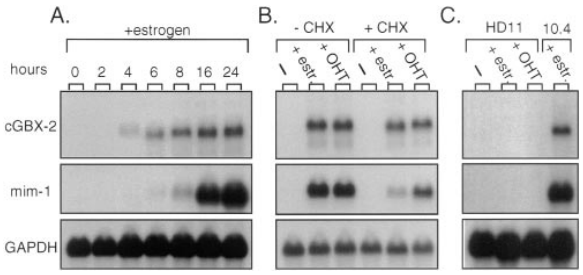

Figure 4. Activation of the *GBX2* Gene by a Chimeric Myb-Estrogen Receptor Fusion Protein

(A) The HD11–10.4 cell line, which harbors a recombinant protein encoding a portion of Myb fused to the hormone binding domain of the estrogen receptor, was treated with hormone. RNA was extracted after the indicated time. Blots with polyA-enriched RNA were sequentially probed with labeled GBX2, mim-1, and GAPDH fragments.

(B) HD11–10.4 cells were incubated with or without cycloheximide (CHX, 50 μg/ml) to block translation 15 min prior to the addition of estrogen (estr.) or hydroxytamoxifen (OHT). RNA was extracted after 5 hr and polyA-enriched RNA was subjected to the analysis.

(C) HD11 control showing that hormone treatment of the parental cells did not induce *GBX2* or *mim-1*.

5, only AMV v-*myb* induced *GBX2* expression, while all other Myb proteins with intact transactivation domains induced *mim*-1 but not *GBX2*. These findings agree with published results showing that E26 v-*myb* but not AMV v-*myb* activates *mim*-1 (Introna et al., 1989). These results suggest that AMV v-*myb* accumulated gain- as well as loss-of-function mutations, which led simultaneously to constitutive activation of GBX2 coupled with the loss to cooperate with C/EBP. To study this possibility further, we tested reciprocal exchange mutants between c-*myb* and AMV v-*myb*. As shown in Figure 5B and summarized in Figure 5C, the ability to induce *GBX2* and the inability to activate *mim-1* are both dependent on the AMV-specific point mutations in the v-Myb DNA binding domain and are independent of changes in the N- or C-terminal regions of Myb.

c-Myb Requires an Activated Signaling Pathway to Induce *GBX2*

Failure of c-*myb* to activate *GBX2* could reflect a change in target gene specificity of the AMV-myb version or a transient physiological condition that was not provided in our experiments shown in Figure 5. The observation that c-*myb* expressing v-*erb*B–transformed erythroblasts also expressed GBX2 (Figure 3B) suggested that an activated signal transduction pathway in conjunction with c-*myb* might be involved in *GBX2* activation. To examine this possibility, we took advantage of cells transformed by a recombinant retrovirus that harbored both, an E26-derived v-*myb* oncogene, and the human EGF receptor tyrosine kinase (Metz et al., 1991). Figure 6A shows that activation of the receptor tyrosine kinase by murine EGF can induce both *GBX2* and *cMGF* (Metz et al., 1991). To further explore a connection between c-*myb* and activated signaling pathways, we transfected polyoma middle T (POMT) or EJ-*ras* along with c-*myb* into HD11 cells or treated cells with phorbol ester to activate protein kinase C (PKC). As shown in Figure 6B,

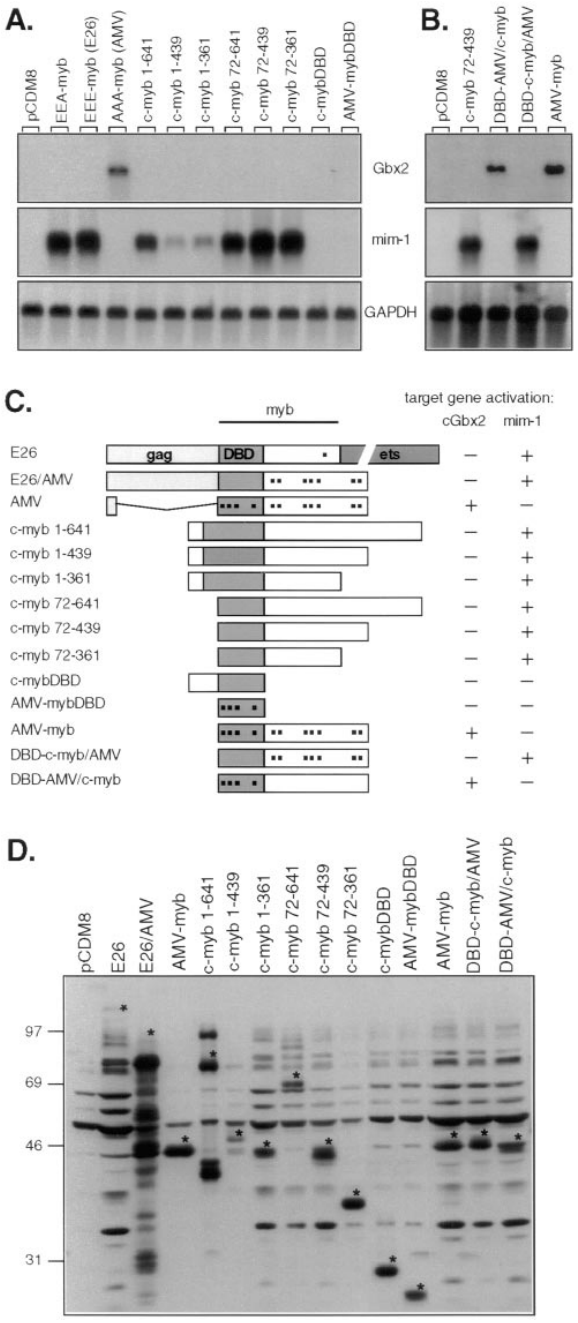

Figure 5. The DBD of v-*myb* Determines Activation of *GBX2* and *mim-1*

(A) Viral, cellular, or truncated Myb expression consructs were transiently transfected into the HD11 macrophage cell line. Cells were harvested after 36 hr, and polyA-enriched RNA was blotted and examined with GBX2, mim-1, and GAPDH specific probes.

(B) Truncated c-myb, AMV-myb, or domain swap mutants between AMV- and c-Myb were transfected and analyzed as described in (A) DBD-AMV/c-myb indicates that the DBD was derived from AMV-myb and the remainder from c-myb. DBDc-myb/AMV indicates that the DBD was derived from c-myb and the remainder from AMV.

(C) Summary of the constructs and results from (A) and (B).

(D) Protein expression controls of the constructs and transfections shown in (A) and (B).

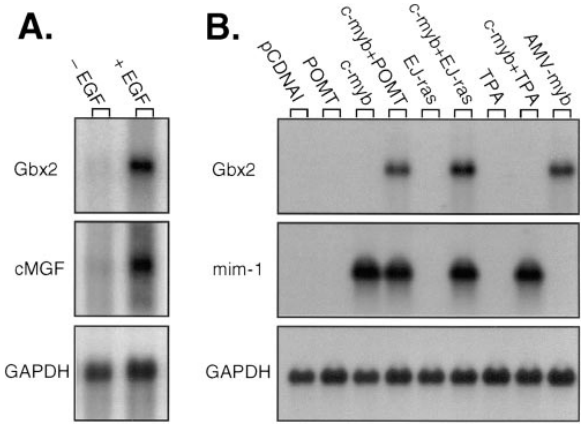

Figure 6. C-Myb Requires an Activated Signal Transduction Pathway to Induce *GBX2*

(A) Bone marrow cells transformed by a recombinant retrovirus encoding the v-*myb* moiety of the E26 virus plus the human EGF receptor tyrosine kinase were deprived of EGF for 12 hr or grown in the presence of EGF (50 ng/ml).

(B) The HD11 cell line was transfected with expression constructs encoding polyoma middle T (POMT) or EJ-*ras* alone or together with c-*myb*. Indicated cells were treated with phorbol ester (TPA) to activate PKC kinases 8 hr before harvest. RNA expression was monitored as in Figures 4 and 5.

GBX2 was activated when c-*myb* was cotransfected together with POMT or EJ-*ras* but not by POMT, EJ-*ras*, or c-*myb* alone or in conjunction with an activated PKC pathway. In contrast, activation of *mim-1* also required c-*myb* but occurred independently of cotransfected EJ-*ras*, POMT, or phorbol ester treatment through collaboration between Myb and C/EBP, which is abundantly expressed in HD11 cells (Sterneck et al., 1992b; Ness et al., 1993).

We conclude that c-Myb collaborates with, or is a target of, a signaling pathway originating at the cell surface to induce *GBX2*. The mutations in the DBD of the leukemic AMV v-Myb render it independent on such signals and, as a consequence, lead to the constitutive activation of *GBX2*. Simultaneously, the mutations in the AMV v-Myb DBD abrogate the collaboration between Myb and C/EBP, a hallmark of the granulocytic phenotype (Müller et al., 1995; Zhang et al., 1997).

Ectopic Expression of *GBX2* in Hematopietic Precursor Cells Alters Their Phenotype

Having shown that *GBX2* is a target gene of AMV v-Myb, a major question was whether ectopic expression of *GBX2* would alter the phenotype and cytokine dependence of myelomonocytic precursor cells. We therefore constructed a recombinant retrovirus that encodes *GBX2* in combination with the transforming v-*myb*-*ets* gene of the E26 virus (Figure 7A). Primary chicken bone marrow cells were infected with either the recombinant E26GBX2 virus, wild-type E26 or AMV viruses, or helper virus, and infected cells were seeded into semisolid tissue culture medium either in the presence or absence of cMGF. Table 1 shows that transformed colonies developed with both E26GBX2 and AMV but not with E26 virus in the absence of added cMGF. These results show that GBX2 enhanced the transformation potential of the E26 oncogene probably by causing at least partial independence from exogenously added cMGF.

Next, we characterized the phenotype of E26GBX2-transformed cells in more detail. We observed that E26GBX2-transformed colonies were generally more diffuse in comparison to the compact E26 myeloblast colonies (Figure 7A). Approximately 30%-50% of the E26GBX2-transformed colonies were without a sharp boundary, indicative of migratory monocytic cells, whereas $>$95% of the E26 colonies were extremely compact. When isolated and grown in liquid medium, E26GBX2-transformed cells were significantly larger than E26 myeloblasts (442 fl $\pm$ 75 fl versus 305 fl $\pm$ 19 fl; data from 12 clones each). Many E26GBX2-transformed cells grew as semiadherent cells, reminiscent of the AMV monoblast-like cells, while E26-transformed cells were always round and completely nonadherent (Figure 7C). To determine whether E26GBX2-transformed cells displayed monocytic features, we subjected them to phagocytosis assays and determined the expression of several cell surface markers. Figure 7C shows that E26GBX2- and AMV- but not E26-transformed cells actively phagocytosed bacteria and latex beads. All cell types expressed the general myeloid cell surface marker Myl51–2 (data not shown), but E26GBX2- and AMV-transformed cells expressed 5–10 fold more MHCII antigen on their surface (Ewert et al., 1984). These data show that E26GBX2 cells more closely resemble AMV-transformed monoblasts than E26-transformed myeloblasts.

When individual colonies of transformed cells obtained in the presence of cMGF were reseeded in liquid medium either with or without cMGF, 11 out of 12 E26GBX2 clones regrew in the absence of cMGF, while none of the E26 clones examined were able to grow in the absence of added cMGF (data not shown). This suggests that the E26-transformed cells remained completely cMGF-dependent, but that the E26GBX2-transformed cells no longer required exogenous cytokine addition for their growth or survival. To determine whether factor-independent growth was due to autocrine growth stimulation, we compared growth of both cell types under different conditions. First, pools of approximately 50 colonies from E26- or E26GBX2-infected bone marrow cultures produced in the presence of cMGF were expanded in liquid medium plus cMGF to eliminate selection for growth factor independence. The resulting cell populations were then reseeded, and their growth was monitored either in the presence or absence of cMGF (Figure 8A). The E26GBX2-transformed cells grew about twice as fast in the presence of exogenous cMGF as in its absence. In contrast, E26-transformed control cells were unable to proliferate at all in the absence of cMGF. As shown in Figure 8B, medium conditioned by E26GBX2- but not by E26-transformed cells contained a growth-promoting activity. Furthermore, growth of E26GBX2-transformed cells was inhibited by a cMGF-specific antiserum but not by an irrelevant serum (Figure 8C). Addition of excess, recombinant cMGF restored cell growth, indicating that inhibition was not mediated by antiserum toxicity (Adkins et al., 1984) (data not shown). We conclude that E26GBX2-transformed cells

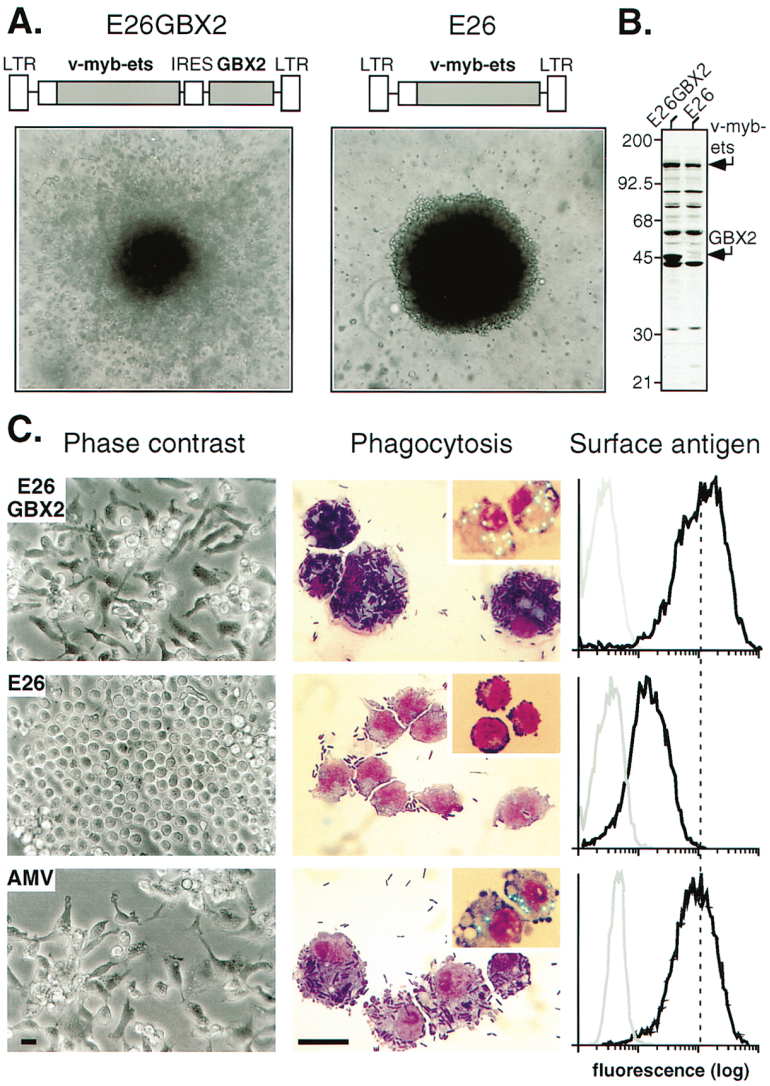

Figure 7. Morphologic, Functional, and Cytochemical Comparison between E26GBX2-, E26-, and AMV-Transformed Bone Marrow Cells

(A) Schematic presentation of the E26GBX2 and E26 viruses. LTR, long terminal repeats; IRES, internal ribosomal entry site. Below, morphology of E26 and E26GBX2 colonies. Bone marrow cells of freshly hatched chicken were infected and seeded into Methocel culture medium in the presence of cMGF. Micrographs of transformed colonies were taken 10 days postinfection.

(B) Protein expression in E26GBX2- and E26-transformed bone marrow cells. Arrows on the right indicate gag-myb-ets and GBX2 proteins as revealed by Western blotting of nuclear extracts using rabbit antiserum directed against Myb and a monoclonal mouse antibody directed against GBX2.

(C) Colonies from bone marrow transformation assays were isolated and cells grown in liquid medium. After 7 days, phase contrast micrographs (left panel) were taken from transformed E26GBX2 cells, E26 cells, or AMV cells as indicated. Cells were subjected to phagocytosis assays (middle panel) using bacteria or fluorescent latex beads (insert). Note that E26GBX2- and AMV- but not E26-transformed cells are phagocytic. Bar corresponds to 10 μm. Right panel, MHCII antigen expression revealed by cytofluorometry.

secrete cMGF (or an immunologically related cytokine), which stimulates the cells to grow through an autocrine mechanism. However, we cannot currently exclude expression of additional, yet unknown cytokines as indicated by partial neutralization by the cMGF antiserum of both growth (Figure 8C) and conditioned medium (data not shown) of E26GBX2-transformed cells.

To rule out the possibility that the observed differences between E26GBX2- and E26-transformed cells resulted from outgrowth of different target cells, we examined whether established E26 myeloblasts would change their phenotype after expression of GBX2. E26 myeloblasts superinfected with a GBX2-expressing helper-independent retrovirus (Hughes et al., 1987) became phagocytic and cMGF independent while no such changes were found in control virus infected or parental E26 cells (data not shown). Taken together, we conclude from these experiments that GBX2 induces a monoblast, AMV-like phenotype in myelomonocytic precursor cells.

Table 1. Growth of Transformed Colonies and Cytokine Dependence

Virus Strain		Colony Numbers[a] −cMGF	+cMGF	Ratio (−/+ cMGF)
E26	Exp. 1	7	228	0.03
	Exp. 2	25	344	0.07
E26GBX	Exp. 1	194	291	0.67
	Exp. 2	180	311	0.58
AMV	Exp. 1	457	510	0.90
	Exp. 2	610	650	0.94
RAV-2	Exp. 1	0	0	—
	Exp. 2	0	0	—

[a] Number of transformed colonies obtained after infection of 2×10^6 bone marrow cells.

Discussion

The v-*myb* oncogene of the avian myeloblastosis virus (AMV) transforms precursor cells of the monocytic lineage, which secrete the transcriptionally regulated cytokine cMGF in an autocrine fashion. We cloned the

chicken homeobox gene *GBX2* and identified it as a nuclear factor expressed in AMV monocytes that binds to and activates transcription from the *cMGF* promoter. Furthermore, we demonstrated that *GBX2* is a *myb* target gene and showed that ectopic expression of *GBX2* in myeloblasts mediates monocytic differentiation and growth factor independence, reflecting distinct features of the AMV v-*myb* oncogene.

The induction of *GBX2* by c-Myb requires an activated tyrosine kinase pathway originating at the cell surface. The dependence of Myb on signal transduction during *GBX2* activation is abrogated by mutations present in the AMV-Myb DBD. The same mutations abrogate the collaboration between Myb and C/EBP, which previously was identified as a bipartite switch controlling the expression of several differentiation-specific genes. Our data suggest that gain- and loss-of-function mutations in the DBD of Myb are simultaneously required to convert it into a leukemia gene and to determine the transformed phenotype.

Induction of the *GBX2* Gene Requires Myb in Conjunction with an Activated Signaling Pathway

The c-*myb* protooncogene is required for and controls adult hematopoiesis (Mucenski et al., 1991). When turned into an oncoprotein by mutation, it may constitutively exert regulatory functions, which c-Myb carries out in a strictly regulated fashion. We have shown that this is the case with Myb-induced *GBX2* expression. While c-Myb is dependent on signal transduction by Ras-like pathways to turn on *GBX2*, AMV v-*myb* turns on the same gene constitutively. Whether c-Myb is the direct target of kinase signaling or whether c-Myb collaborates with unknown proteins that represent such targets, however, remains to be determined.

Under normal conditions, activation of *GBX2* through Myb occurs through signals emanating at the cell surface. It will therefore be interesting to determine exactly which hematopoietic surface receptors transmit signals to induce *GBX2* expression and how signaling pathways regulate the transcriptional activity of Myb through its DBD. Currently, our results suggest that Ras-MAP-kinase pathways are involved although the mechanism remains to be elucidated.

None of the amino acids mutated in the AMV DBD either in the wild-type or the mutant configuration can serve as phosphorylation sites. Therefore, it seems likely that the activity of Myb is subject to regulation through allosteric modifications or through interacting proteins, which may or may not require adjacent *cis*-regulatory binding sites. Interestingly, the respective AMV point mutations are positioned on the surface of the Myb DBD that faces away from the DNA toward the solvent (Ogata et al., 1994). In the wild-type version, those positions are occupied by hydrophobic amino acids that might form a contiguous hydrophobic furrow. Such a hydrophobic surface structure is interrupted in AMV-myb. It therefore seems plausible that the DBD of Myb could serve as an interaction surface for one or several accessory protein(s) and that the AMV-myb mutations interfere with some of those interactions. It is tempting to speculate that hematopoietic transcription factors such as AML1, ets-1,-2, or PU.1 might be involved in such interactions, although preliminary experiments do not support this speculation.

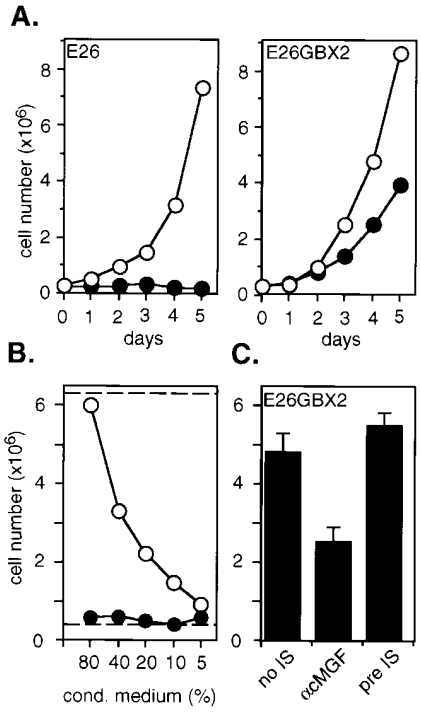

Figure 8. Growth Analysis of E26GBX2-Transformed Cells

(A) Growth curves. Cell numbers were determined by daily counting of E26- or E26GBX2-transformed cells grown in the presence (open circles) or absence of cMGF (filled circles).

(B) Growth factor production. Conditioned medium was harvested from E26GBX2 cells (open circles) or E26 myeloblasts (filled circles) maintained in the absence of cMGF for 48 hr. Conditioned media were then titrated on factor dependent E26 myeloblasts and cell numbers determined after 5 days.

(C) Autocrine growth. Growth inhibition induced by the cMGF antiserum on E26GBX2-transformed cells grown in the absence of cMGF. NoIS, no immune serum; αcMGF, cMGF antiserum; preIS, preimmune serum control.

Myb and the Regulation of a Homeobox Gene

Of particular importance is the finding that activated signaling pathways in conjunction with Myb induce the expression of a nonclustered homeobox gene. Since *GBX2* is involved in the regulation of other genes exemplified by the cMGF cytokine, our data also suggest that Myb acts as a master regulator in hematopoiesis by controlling the expression of transcription factors on a lower hierarchical level.

In the hematopoietic system, a number of homeobox genes might participate in the control of cell lineage commitment, proliferation, differentiation, and leukemia development (Kehrl, 1994; Shivdasani and Orkin, 1996). Certain members of the HOXB cluster are expressed in primitive hematopoietic cells, and overexpression of HOXB4 causes a selective expansion of this compartment (Sauvageau et al., 1995). Recently, three homeobox genes, *MEIS1*, *HOXA7*, and *HOXA9* were found upregulated in murine BXH2 leukemia induced by retroviral

insertion mutagenesis. Importantly, rearrangement and translocation of the *HOXA9* gene also occurs in human acute myeloid leukemia, establishing its role in hematopoietic transformation (Nakamura et al., 1996a, 1996b). Several other nonclustered homeodomain genes are also implicated in normal and dysregulated hematopoiesis, such as the *TCL-3* gene (*HOX11*), which is rearranged and up-regulated in rare cases of T-cell acute leukemia (Hatano et al., 1991; Kennedy et al., 1991), or the *MEIS1*-related *PBX1* gene, which is fused to the E2A transcription factor in pre-B cell lymphoblastic leukemias characterized by a t(1;19) rearrangement (Kamps et al., 1990; Kamps and Baltimore, 1993). Thus, the expression of individual genes or sets of homeobox genes in the hematopoietic system are clearly linked to proliferation and differentiation control.

It is noteworthy to mention that hematopoietic *GBX2* expression is not restricted to the monocytic lineage and was also not observed in more mature, v-*myc* transformed macrophages. It therefore appears that the differentiation activity and the expression of GBX2 is dynamically regulated, as it is the case with many other homeodomain proteins. Interestingly, both erythroid chicken HD3 cells and progenitor like human K562 cells express high levels of c-*myb*, activated tyrosine kinase oncogenes (v-*erb*B and bcr-abl, respectively), and GBX2. It will therefore be of particular interest to examine the functions of GBX2 in other hematopoietic lineages and to determine correlations between Myb, activated kinase oncogenes, and GBX2 expression in human leukemias. Clearly, the function of GBX2 in hematopoietic growth and differentiation control requires further examination, by deleting the gene in mice or by overexpressing GBX2 in distinct hematopoietic lineages, for example.

Is Myb Involved in Hematopoietic Lineage Commitment and Differentiation?

Constitutive activation of *GBX2* depends on the point mutations in the AMV v-Myb DBD, which determine the leukemogenicity and the phenotype of transformed cells (Introna et al., 1989). In this regard, it is interesting to compare our results with those obtained by others in two extensive surveys on the structure/function relationship of the v-Myb DBD (Introna et al., 1989; Dini et al., 1995). These studies showed that the monoblast phenotype and the growth factor production segregate with the AMV v-myb DBD configuration. Here we showed that the same region determines the collaboration with C/EBP proteins and the activation of the *GBX2* gene. It therefore appears that Myb together with C/EBP commits hematopoietic precursors toward the granulocytic lineage and that GBX2 dominantly determines the monocytic phenotype in myelomonocytic precursors. This assumption is supported by the data provided in this publication and by the observations that activation of a conditional C/EBPβ protein in E26 progenitor cells can induce eosinophilic differentiation (Müller et al., 1995) and that C/EBPα-deficient mice have no eosinophils or neutrophils (Zhang et al., 1997). We therefore propose that, in addition to its role in proliferation, Myb is involved in the commitment of hematopoietic lineages by regulating different sets of genes in response to extracellular signals and/or through interactions with different sets of transcription factors.

Experimental Procedures

Retroviral Constructs and Pseudotyping

The *GBX2* cDNA was inserted into an E26 IRES retrovirus construct and into RCAS-A (Hughes et al., 1987). Chicken embryo fibroblasts were transfected with recombinant and helper virus plasmid (RAV-2) and selected in G418 at 0.8 mg/ml as previously described (Metz et al., 1991). RCAS-A–transfected fibroblasts were grown without selection. Transgene expression was controlled by Western blotting after 10 days, and culture supernatants were stored at −70°C as a source of virus.

Chicken Tissues, Cell Lines, and Tissue Culture

Tissue was obtained from 13-day-old White Leghorn SPAFAS chicken. The HD3 cell line expressing v-*erb*B and the HD11 cell line expressing v-*myc* have been described (Beug et al., 1979, 1982). BM-2 is an AMV-transformed monoblast cell line (Moscovici and Gazzolo, 1982). MH2-transformed cells express v-*myc* and v-*mil* (Coll et al., 1983). HD57 cells are E26-transformed progenitors expressing the v-*myb-ets* oncogene (Graf et al., 1992). The cell lines MSB-1 and RPL-12 are T- and B-lymphoblasts, respectively, and were isolated from bird tumors (Beug et al., 1981). REV-NPB4 is a REV-transformed pre-B/pre-T-cell line expressing the v-*rel* oncogene (Beug et al., 1981). CEF38 is a fibroblast cell line (Kaaden, 1977). All cells were grown in Dulbecco's modified Eagle medium supplemented with 8% fetal bovine serum, 2% heat-inactivated chicken serum, 15 mM HEPES, penicillin, and streptomycin. Cells were maintained in 5% CO_2 atmosphere at 39°C.

In Vitro Transformation of Bone Marrow Cells

Bone marrow cells were prepared from 1- to 10-day-old chickens of the White Leghorn Spafas flock. Cells (2×10^6) were infected with 0.3 ml virus supernatant. The infected cells were seeded in DMEM suplemented with 0.8% methylcellulose in the presence and absence of recombinant cMGF (Leutz et al., 1989). After 8–10 days, transformed colonies were isolated and expanded in liquid culture. Superinfection (1×10^5 cells) with RCAS-A or RCASAGBX2 virus was performed 4 days later.

Cytokine Assays

Supernatants of cells were harvested, steril filtered, and titrated on E26 myeloblasts (Leutz et al., 1984). Briefly, E26 test cells were washed twice with DMEM to remove cMGF. Cells were then seeded into titrated supernatants in 96-well plates at 2×10^4 cells in 50 μl of medium. After 2 days, viable cells were counted. Autocrine growth was determined by adding anti-cMGF or preimmune serum at a concentration of 0.5% to the culture medium.

Phagocytosis, Cytochemical, and Immunocytochemical Methods

Cells were fed with bacteria or FITC-labeled latex beads at a 1:100 ratio for 1–2 hr as described earlier (Beug et al., 1979). Cytocentrifuge preparations of cells were stained with a Giemsa-like stain (Diff-Quick, Harleco). Cell suface antigen MHCII (Ewert et al., 1984) was analyzed by cytofluorometry. Subcellular localization of GBX2 was determined in transfected Cos-7 cells by indirect immunofloures-cence using the monoclonal anti-GBX2 antibody MC 4–2.2.

Synthetic Oligonucleotides

Concatemers of the following synthetic oligonucleotide pairs were used as binding site probes in the expression screening assay: distal site at −260 (decamer, upper strand), 5′-TCGACGGGTGATTAAGAC AATGAGGCAC-3′; (lower strand) 5′-TCGAGTGCCTCATTGTCTTAA TCACCCG-3′; proximal site at −90 (tetramer, upper strand), 5′-T CGA CGCGGGTTAATGAGTGACCAC-3′; (lower strand) 5′-TCGAGT GGTCACTCATTAACCCGCG-3′; Ap1 site (upper strand), 5′-TCGAG TGACTCAGCGCG-3′; (lower strand) 5′-TCGACGCGCTGAGTCAC-

3′. The following oligonucleotides were used as gene-specific primers for the RT-PCR reactions:

GBX2/5′ 5′-TCCAAGGCGGGGGAACCGTCG-3′
GBX2/3′ 5′-TGCTGGTGCTGACTCCTGAT-3′
GBX1/5′ 5′-AACCGCTCGGGAGAGCCCGTC-3′
GBX1/3′ 5′-TGCTGGTGCTGGCTGCGCAC-3′
GAPDH/5′ 5′-CCATGACAACTTTGGCATTG-3′
GAPDH/3′ 5′-TCCCCACAGCCTTAGCAG-3′
hGBX2/5′ 5′-AAGTACCTCTCCTTGACCGAG-3′
hGBX2/3′ 5′-GCTGACTTCTGATAGCGAACC-3′

Expression Screening and Isolation of *GBX2*

The chicken BM-2 cell cDNA library constructed in the expression vector λgt11 was obtained from A. Sippel (University of Freiburg). The library had an average insert size of approximately 1.5 kb. For lytic growth of bacteriophage l, the standard host strain Y1090 was used. Bacteriophage infection, plating, and screening were carried out according to published procedures (Singh et al., 1988; Vinson et al., 1988). In brief, bacteriophage fusion protein expression was induced after 5 hr at 42°C by overlaying with IPTG-impregnated nitrocellulose filters and overnight growth at 37°C. Plates were chilled, filters were lifted and immersed in HEPES binding buffer (25 mM HEPES [pH 7.9], 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT) supplemented with 8 M urea. After a renaturation step, filters were incubated for 2 hr in BLOTTO. Multimerized oligonucleotide probes were labeled by nick translation and incubated with filters for 4 hr in binding buffer. After four washes (a total of 30 min) in binding buffer containing 0.1% sodium pyrophosphate, filters were exposed at −70°C with intensifying screens. Phages were plaque purified and EcoRI inserts subcloned into Bluescript (Stratagene). A chicken *GBX1* genomic clone was derived from a genomic phage library screened with a GBX2 homeobox probe. Sequence analysis confirmed the close relationship of our GBX1 isolate with the published partial *chox7* sequence (Fainsod and Gruenbaum, 1989).

Expression Constructs

The insert of phage clone 30.1 was subcloned into the bacterial vector pGEX-1, and expression of glutathione-S-transferase/GBX2 fusion protein (Gst-GBX2) was induced by IPTG. Bacteria were harvested and proteins were extracted as described (Smith and Johnson, 1988). For eukaryotic expression, the phage 30.1 insert was subcloned into the pCDM8 expression vector.

RT-PCR

Total RNA was prepared from chicken tissue (Chomczynski and Sacchi, 1987). Total RNA (5 μg) was reverse transcribed using Superscript RNAseH (GIBCO/BRL). Of the resulting cDNA, 5% served as template for the PCR reactions in 50 μl of 1× *Taq* polymerase buffer (Pharmacia) in the presence of 200 μM dNTPs, 100 pmol of each primer, and 1 U *Taq* polymerase (Pharmacia) with the following profile: 5 min 95°C; 1 min 95°C, 1 min 55°C, 2 min 72°C for 18 (GAPDH) or 24 (GBX2, GBX1) cycles; and 10 min 72°C. Conditions for amplification of human *GBX2* cDNA were: 50 μM dNTPs, 2 U *Taq* polymerase, 100 pmol primers in 50 μl. To label the PCR products, each reaction contained 1 μCi of [α-^{32}P] dCTP (3000 mCi/mmol, Amersham). The samples were run on acrylamide DNA gels, dried, and exposed as described above.

Transient Transfections, Reporter Assay, and Resident Gene Activation Assay

Plasmids used for transfections were purified twice on CsCl gradients. QT6 and HD3 cells were transfected using DEAE-dextran as described previously (Sterneck et al., 1992a). Cos-7 cells were transfected with lipofectin (BRL). To monitor reporter gene activation, cells were harvested after 24 hr, whole cell lysates were prepared, and luciferase activity was determined as previously described (Sterneck et al., 1992a, 1992b). Activation of endogenous genes was determined as previously described (Ness et al., 1993; Kowenz-Leutz et al., 1994).

Antibodies

Mice and rabbits were immunized with bacterially derived Gst-GBX2 fusion protein. Preparation of cells producing monoclonal antibodies and purification of antibodies were done following standard procedures (Harlow and Lane, 1988).

Nucleic Acid Analysis

The *GBX2* cDNA was sequenced using dGTP, dITP, and 7-deaza-dGTP reactions in parallel. Poly(A)+ RNA was isolated using Dynabeads Oligo(dT)25 (Dynal). RNA was separated on 1% agarose-formaldehyde gels, transferred to nylon membranes, UV cross-linked, and baked at 80°C for 2 hr. The *GBX2*-specific DNA probe was isolated from phage 30.1 insert following HincII/ EcoRI digestion. The crossreacting GBX homeobox probe was a SstII and HincII fragment. Restriction fragments used as probes for *GAPDH*, *mim-1*, and *cMGF* have been described (Leutz et al., 1989; Ness et al., 1989). Probes were labeled by random priming and hybridized to membranes in QuickHyb (Stratagene) at 68°C for 2 hr. Filters were washed twice in 0.2 × SSC/0.1% SDS at 65°C for 15 min and exposed at −70°C. For rehybridization, filters were stripped for 10 min at 95°C in 0.05 × SSC/0.1% SDS.

Western and South-Western Analysis

Protein extracts were prepared by a mini nuclear extract procedure (Schreiber et al., 1989). For detection of GBX2 in hematopoietic cells, cells were lysed by Dounce homogenization. Proteins were subjected to reducing 12.5% SDS-PAGE and electrophoretically transferred onto PVDF membrane. Membranes were blocked with 5% nonfat dry milk in TBST (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.02% v/v Tween-20) for 2 hr and incubated overnight at 4°C with monoclonal antibodies at a concentration of 5 mg/ml in blocking solution. Blots were washed three times with TBST, one time in high-salt buffer (0.5 M NaCl, in TBST), and incubated with a horseradish peroxidase-coupled second antibody (goat anti-mouse) for 2 hr. Following three washes with TBST, immunoreactivity was monitored using a chemoluminescence system (ECL, Amersham). Southwestern analysis was performed as described (Singh et al., 1988). Multimerized oligonucleotide probes were labeled by nick translation. Unspecific competitor poly(dI-dC) was added to a final concentration of 5 μg/ml in HEPES binding buffer.

Electrophoretic Mobility Shift Assay

Double-stranded oligonuleotides were labeled with [α-^{32}P] dCTP (3000 mCi/mmol, Amersham) by fill-in reaction using Klenow DNA polymerase and purified by polyacrylamide gel electrophoresis. Nuclear extracts (0.5–1 μl) were incubated in a final volume of 10 μl with labeled fragment (0.25–1 ng) for 30 min on ice. For competition analysis, a 50-fold molar excess of cold oligonucleotides was used. The reaction mixture was separated on 5% polyacrylamide/bisacrylamide (29:1, w/w) gels at 25 mA at room temperature using 0.5× TBE as electophoretic running buffer.

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