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tom-1, a novel v-Myb target gene expressed in AMV- and E26-transformed myelomonocytic cells

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The retroviral oncogene v-*myb* is a mutated and truncated version of the c-*myb* proto-oncogene and encodes a transcription factor (v-Myb) that specifically transforms myelomonocytic cells. Two different variants of v-*myb*, transduced independently by the oncogenic chicken retroviruses AMV and E26, have been characterized. It is believed that both variants of v-Myb transform myelomonocytic cells by affecting the expression of specific genes; however, no target genes common to both oncogenic viruses have been identified. Here, we describe the identification of a novel v-Myb target gene, designated as *tom-1* (target of myb 1). The *tom-1* gene has two promoters, one of which is Myb-inducible. *tom-1* is expressed at elevated levels in AMV-transformed as well as in E26-transformed myeloid cells. We show that *tom-1* activation by v-Myb does not require *de novo* protein synthesis and that the Myb-inducible *tom-1* promoter contains a functional Myb binding site. Thus, *tom-1* is the first example of a direct target gene for both oncogenic forms of the v-*myb* gene. Further analysis of the Myb-inducible *tom-1* promoter shows that a C/EBP binding site is juxtaposed to the Myb binding site and that C/EBP is required for the Myb-dependent activation of the promoter. Together with previous work our results suggest that C/EBP may be a general cooperation partner for v-Myb in myelomonocytic cells.

Keywords: C/EBP/myelomonocytic cells/target gene/ *tom-1* gene/v-*myb* oncogene

Introduction

The oncogene v-*myb* of the avian myeloblastosis virus (AMV) and avian leukemia virus E26 encodes a transcription factor which is responsible for the transformation of myelomonocytic cells by both viruses (for review see Graf, 1992). v-*myb* is a structurally altered form of the chicken c-*myb* gene (Klempnauer *et al.*, 1982, 1983) which is highly expressed in most hematopoietic progenitor cells and is essential for the development of the hematopoietic system. The important role of c-*myb* in hematopoietic cells has been demonstrated by the observation that mice that lack a functional c-*myb* gene die during embryonic development from severe defects in fetal hepatic hematopoiesis (Mucenski *et al.*, 1991). c-*myb* is also expressed in certain non-hematopoietic cells, however, its role in these cells has not been determined (Thiele *et al.*, 1987; Desbiens *et al.*, 1991; Queva *et al.*, 1992; Plaza *et al.*, 1995; Sitzmann *et al.*, 1995).

The proteins encoded by v-*myb* and c-*myb* (referred to as v-Myb and c-Myb) bind to the sequence motif PyAAC$^{G}/_{T}$G (Biedenkapp *et al.*, 1988) and activate promoters containing such binding sites (Klempnauer *et al.*, 1989; Ness *et al.*, 1989; Nishina *et al.*, 1989; Weston and Bishop, 1989; Ibanez and Lipsick, 1990). Transformation of myelomonocytic cells by mutants of v-*myb* correlates with their ability to activate transcription of model reporter genes (Lane *et al.*, 1990; Frampton *et al.*, 1993), suggesting that cell transformation by v-*myb* depends on the activation of crucial target genes. However, so far only a few genes expressed in v-*myb* transformed myeloblasts, such as the *mim-1* gene (Ness *et al.*, 1989) or the lysozyme gene (Introna *et al.*, 1990; Burk and Klempnauer, 1991), have been identified as direct targets for v-Myb. Recently, it has been shown that v-Myb activates the *mim-1* gene by cooperating with members of the C/EBP transcription factor family (Burk *et al.*, 1993; Ness *et al.*, 1993) and that direct interaction of v-Myb and C/EBP is crucial for the cooperation of both factors (Mink *et al.*, 1996).

The *mim-1* and lysozyme genes are expressed at elevated levels only in E26-transformed but not in AMV-transformed myeloblasts (Ness *et al.*, 1989; Introna *et al.*, 1990), suggesting that the increased expression of these genes itself is not essential for v-*myb* induced cell transformation. Presumably, other as yet unknown genes are activated by both oncogenic forms of the *myb* gene and serve as crucial targets for v-Myb in myelomonocytic cells. We have used a myelomonocytic cell line expressing a conditional version of v-Myb to identify Myb-regulated genes. Here, we describe the isolation and preliminary characterization of *tom-1*, a novel direct v-Myb target gene which is activated by both versions of v-*myb*.

Results

Cloning of a novel v-Myb target gene by differential display

To identify v-Myb regulated genes we used a chicken macrophage cell line expressing an estrogen receptor–v-Myb fusion protein (Burk and Klempnauer, 1991). Estrogen treatment of this cell line (referred to as 10.4) leads to the activation of the known Myb-inducible genes, such as *mim-1* and the lysozyme gene (Burk and Klempnauer, 1991). 10.4 cells were grown for 24 h with or without estrogen and analyzed by differential display (Liang and Pardee, 1992). We obtained several differentially amplified DNA fragments, one of which hybridized

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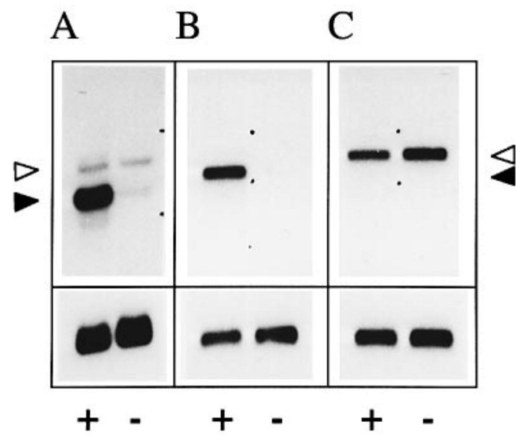

Fig. 1. Induction of *tom-1* expression by estrogen in cells stably expressing a v-Myb–ER fusion protein. Polyadenylated RNA from 10.4 cells grown with (+) or without (–) 2 μM β-estradiol for 24 hrs was analyzed by Northern blotting using the cloned differential display fragment (**A**) or *tom-1*A-specific (**B**) or *tom-1*B-specific (**C**) fragments as probes. See Figure 2 for the positions of these probe fragments. Control hybridizations of the blots with a S17-specific probe are shown in the bottom panels. The *tom-1*A and *tom-1*B RNAs are marked by black and white arrowheads, respectively.

to a 2.1 kb mRNA whose expression was strongly induced by estrogen treatment of 10.4 cells (Figure 1A). As shown in Figure 1A, the display fragment also hybridized to a second 3 kb mRNA whose expression was not affected by the hormone. Screening of a cDNA library of AMV-transformed myeloblasts using the display fragment yielded two classes of clones sharing ~1600 bp of identical sequence at their 3′ ends but containing different 5′ sequences. We prepared hybridization probes containing only specific sequences from the 5′ ends of both types of clones and hybridized them to Northern blots containing RNA from 10.4 cells grown with or without estrogen. As illustrated in Figure 1B and C, these specific probes detected either the *myb*-inducible 2.1 kb mRNA or the uninduced 3 kb mRNA. Further analysis of genomic clones (see below) showed that both RNAs were derived from a single gene having two different promoters, different 5′ exons but common 3′ exons. We have designated this novel *myb* regulated gene as *tom-1* (target of myb 1). For convenience, the 2.1 kb and 3 kb *tom-1* RNA species will be referred to as *tom-1*A and *tom-1*B RNA, respectively.

Figure 2A shows the deduced amino acid sequences of the longest open reading frames (ORFs) of the *tom-1*A and B transcripts. The *tom-1*B transcript contains an ORF potentially encoding a 515 amino acid protein. Since the longest of our cDNA clones derived from this transcript is ~200 bp shorter than the estimated size of the transcript, it is possible that this ORF lacks the authentic 5′ end. The *myb*-inducible *tom-1*A transcript contains an ORF potentially encoding a 147 amino acid protein. The carboxy-terminus of this predicted protein is identical to that of the protein encoded by the *tom-1*B transcript, but the amino-terminal sequences of both proteins are different. The structures of the two deduced proteins are shown schematically in Figure 2B. Homology searches have not revealed significant homologies of the putative *tom-1* proteins to other proteins. Unlike chicken lysozyme and the *mim-1* protein, both of which are secreted from

A

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Tom-1B  MDFLLGNPFSSPVGQRIERATDGSLRGEDWSLNMEICDIINETEEGPKDA   50

Tom-1B  FRAIKKRIVGNKNFHEVMLALTVLETCVKNCGHRFHILVASQDFVESVLV  100

Tom-1B  RTILPKNNPPAIVHDKVLTLIQSWADAFRSSPDLTGVVAVYEDLRRKGLE  150

Tom-1B  FPMTDLDMLSPIHTPRRSVYSSNSQSGQNSPAVNSPQQMESILHPVTLPS  200

Tom-1B  GRDTSSNVPITPTQEQIKKLRSELEVVNGNVKVMSEMLTELVPSQAETSD  250

Tom-1B  LELLQELNRTCRAMQQRVLELIPRVQHEQLTEELLLINDNLNNVFLRHER  300

Tom-1B  FERVRTGQPVKAPSEAENNLIDLRPSTPPAVRQPEVTNNLSSQLAGMTLG  350
Tom-1A                                                 MIC    3

Tom-1B  SRSVSAGLHSLDTSGKLEEEFDMFAVTRGSSLAEQRREVKYEDPQATKGL  400
                                              ............
Tom-1A  YSCKKGPGWRGNLMRASGRGFILLAWMDCFPQSAWIRWVKYEDPQATKGL   53

Tom-1B  AGALDARQQNTGAEESSASSDGAQLTNWMMRQGMVPVPQANFMEDIEKWL  450
        .............                     ................
Tom-1A  AGALDARQQNTGA---------------------VPVPQANFMEDIEKWL   82

Tom-1B  STDVGESEDGKGVTSEEFDKFLEERAKVADRLPTLSSSSAGTPVSPAAAS  500
        ..................................................
Tom-1A  STDVGESEDGKGVTSEEFDKFLEERAKVADRLPTLSSSSAGTPVSPAAAS  132

Tom-1B  RHQKQAKEDDAMFAL  515
        ...............
Tom-1A  RHQKQAKEDDAMFAL  147
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B

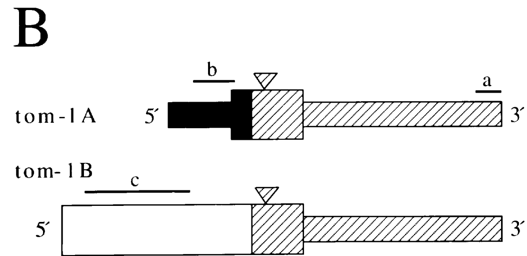


Fig. 2. Structure of *tom-1* RNAs and putative translation products. (**A**) Deduced amino acid sequences of the putative *tom-1*A and *tom-1*B proteins. (**B**) Schematic illustration of the structure of the *tom-1*A and *tom-1*B RNAs. Large boxes: ORFs; small boxes: presumed untranslated sequences; hatched regions: sequences common to *tom-1*A and *tom-1*B; black and white regions: sequences specific for *tom-1*A (black) and *tom-1*B (white). The triangles denote a sequence present only in a subfraction of the RNAs. The lines marked by small letters identify the probes used for Nothern blotting. a, differential display fragment; b and c, *tom-1*A and *tom-1*B-specific probes.

the cells, the putative *tom-1*A protein lacks a hydrophobic signal sequence and thus appears not to be exported through the endoplasmatic reticulum. Homology searches have identified a human nucleotide sequence (EST08420; Adams *et al.*, 1993) which is highly related to the specific part of the *tom-1*B transcript and presumably is derived from the human homolog of *tom-1*. Thus, *tom-1* seems to be conserved between chickens and humans.

tom-1 is a direct v-Myb target gene activated in AMV- and E26-transformed myeloblasts

To examine whether *tom-1* is a direct target gene for v-Myb we studied its activation in the absence of *de novo* protein synthesis. 10.4 cells were treated with estrogen in the presence or the absence of the protein synthesis inhibitor cycloheximide, followed by Northern blotting. As illustrated in Figure 3, activation of *tom-1* expression by v-Myb did not require ongoing protein synthesis, strongly suggesting that v-Myb activates the *tom-1* gene directly.

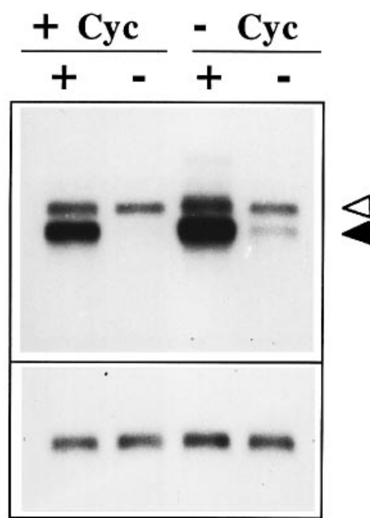


Fig. 3. Induction of *tom-1* expression in the presence of cycloheximide. 10.4 cells were grown in the presence (+Cyc) or absence (–Cyc) of 50 μg/ml cycloheximide either with (+) or without (–) 2 μM β-estradiol. The hormone was added to the cultures 15 min later than cycloheximide. Cells were harvested 5 h after addition of hormone. Polyadenylated RNA prepared from these cells was analyzed by Northern blotting using the cloned differential display fragment as probe. The bottom panel shows a control hybridization with a S17-specific probe. The *tom-1*A and *tom-1*B RNAs are marked by black and white arrowheads, respectively.

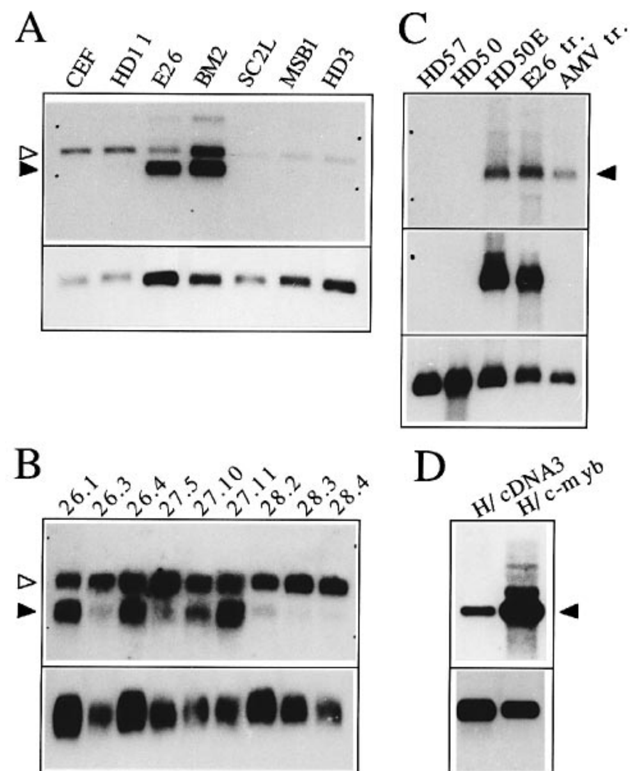


Fig. 4. Expression of *tom-1* RNA in chicken cells. Polyadenylated (A, B and D) or total (C) RNA of various chicken cells was analyzed by Northern blotting using the cloned differential display fragment (**A** and **B**) or a *tom-1*A-specific cDNA fragment (**C** and **D**) as probe. Control hybridizations of the same blots with a S17-specific probe are shown at the bottom. In (C) an additional hybridization using a *mim-1*-specific probe is shown in the middle. *tom-1*A and B RNAs are marked by black and white arrowheads, respectively. CEF, primary chicken embryo fibroblasts; HD11, v-*myc* transformed chicken macrophage cell line; E26 and BM2, E26- and AMV-transformed chicken myeloblast cell lines; SC2L and MSB1, chicken B- and T-lymphoid cell lines; HD3, AEV-transformed chicken erythroblast cell line; 26.1, 26.3 and 26.4, HD11 suclones expressing the AMV version of v-Myb; 27.5, 27.10 and 27.11, HD11 subclones expressing an E26 version of v-Myb; 28.2, 28.3 and 28.4, control HD11 subclones expressing no v-Myb; HD50 and HD57, E26-transformed MEP cell lines; HD50E, derivative of HD50 committed to the eosinophilic lineage; E26 tr and AMV tr, primary E26- and AMV-transformed chicken myeloblasts. H/c-myb, HD11 subclone expressing chicken c-Myb; H/cDNA3, HD11 subclone expressing no c-Myb.

So far only two myeloid-specific genes, *mim-1* and the lysozyme gene, are known to be regulated directly by v-Myb (Ness *et al.*, 1989; Introna *et al.*, 1990; Burk and Klempnauer, 1991). Since both genes are activated only in E26-transformed, but not in AMV-transformed myelomonocytic cells, these genes do not appear to play crucial roles in v-*myb* induced transformation. To investigate whether the activation of the *tom-1* gene is specific for a particular version of v-*myb*, we analyzed its expression in a panel of chicken cell lines, including E26- and AMV-transformed myeloblasts. *tom-1*A RNA was abundantly expressed in both v-*myb* transformed cell lines (Figure 4A) suggesting that both viruses activate the gene. The expression of *tom-1*A RNA in these cell lines also demonstrates that the activation of the gene is not an artifact of the v-Myb–ER system. We also generated stable transfectants of the HD11 cell line using expression vectors for the E26 or AMV versions of v-Myb or a frameshift control vector (Figure 4B). Nothern blot analysis of the resulting clones showed that expression of the *tom-1*A transcript was induced by both versions of v-*myb*.

To confirm that the E26 and AMV versions of v-*myb* both induce *tom-1*A expression we performed Northern blot analyses of primary E26- or AMV-transformed chicken myeloblasts, kindly provided by T.Graf. As illustrated in Figure 4C *tom-1*A expression was expressed at similar levels in cells transformed by either virus.

Figure 4A also shows that neither erythroid nor lymphoid cell-lines expressed detectable levels of the *tom-1*A transcript. E26-transformed multipotent progenitor cell-lines (Metz and Graf, 1991; Kulessa *et al.*, 1995) also did not express *tom-1*A mRNA; however, a variant of the MEP HD50 cell line committed to the eosinophilic lineage expressed *tom-1*A (Figure 4C). *tom-1*B RNA, whose expression is not affected by v-Myb, is present at similar levels in all cells analyzed so far, suggesting that its function is not specific for a particular set of hematopoietic cells. By contrast, the *myb*-inducible *tom-1*A RNA is expressed at detectable levels only in committed myelomonocytic or eosinophilic cells, suggesting that it has a specific role in these cells.

To investigate whether *tom-1*A is also activated by c-Myb, we stably expressed c-Myb in the HD11 cell line. We found that *tom-1*A mRNA is expressed in such cells at elevated levels similar to those seen in v-Myb transfectants (Figure 4D), indicating that *tom-1*A is also activated by c-Myb.

We analyzed further the level of *tom-1*A expression in various normal chicken tissues. In hematopoietic tissues, *tom-1*A expression was clearly visible only in a bone marrow fraction enriched for immature cells (Figure 5B).

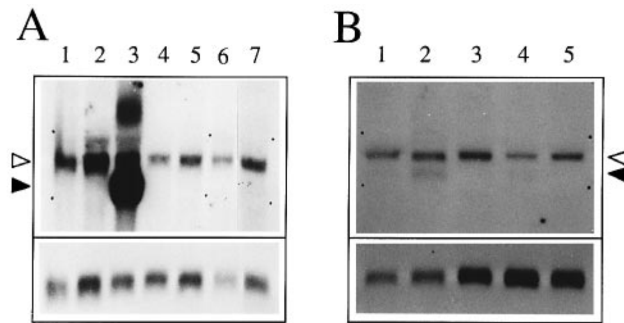


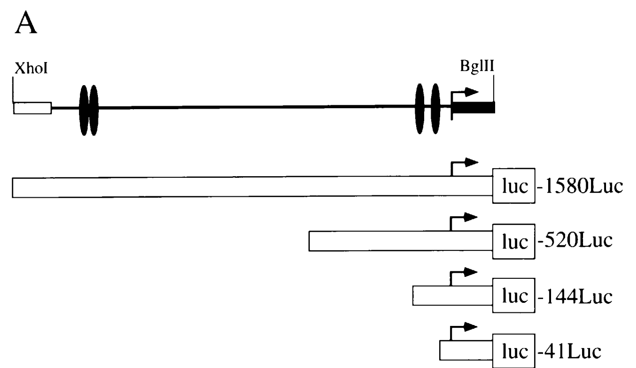
Fig. 5. Expression of *tom-1*A mRNA in normal chicken tissues. Total RNA from different non-hematopoietic (**A**) or hematopoietic (**B**) chicken tissues was analyzed by Northern blotting using the cloned differential display fragment as probe. Control hybridizations of the same blots with a S17-specific probe are shown at the bottom. RNA was prepared from the following tissues: (A), lung (1); heart (2); skeletal muscle (3); liver (4); kidney (5); brain (6); small intestine (7) and in (B), bone marrow fraction containing mostly mature red blood cells and granulocytes (1); bone marrow fraction enriched for immature myeloid and lymphoid cells (2); spleen (3); thymus (4); bursa (5). *tom-1*A and B RNAs are marked by black and white arrowheads, respectively.

Weak *tom-1*A expression could be detected in the thymus after longer exposure of this blot or analysis of polyadenylated RNA (data not shown). The expression of *tom-1*A in hematopoietic cells, particularly in immature bone marrow cells, suggests that *tom-1*A normally performs a role in these cells. The analysis of non-hematopoietic tissues showed, surprisingly, that skeletal muscle expressed *tom-1*A RNA very abundantly (Figure 5A). Thus, *tom-1*A expression is not restricted to hematopoietic cells.

Identification and analysis of the myb-inducible tom-1A promoter

To identify the *tom-1*A promoter we screened a phage library of genomic chicken DNA with a *tom-1* probe and identified several chicken *tom-1* clones. Further analysis showed that the *myb*-inducible *tom-1*A transcript is initiated within an intron of the *tom-1* gene (Figure 6A). The precise start of the *tom-1*A RNA was identifed by nuclease S1 mapping as well as by primer extension analysis (data not shown). A potential TATA box is located immediately upstream of the transcriptional start site. Interestingly, consistent with the idea that *tom-1* is a direct target gene for v-Myb several Myb binding sites are present upstream of the TATA box (Figure 6A).

To demonstrate that the *tom-1*A promoter is Myb-inducible and to delineate the sequences mediating its Myb-responsiveness we constructed a series of *tom-1*A luciferase reporter genes containing nested 5′ deletions of the *tom-1*A promoter (Figure 6A). As shown in Figure 6B the activity of most of the reporter genes was substantially increased by v-Myb or c-Myb, indicating that the activity of the *tom-1*A promoter is Myb-inducible. Deletion of promoter sequences upstream of –41 bp resulted in a complete loss of the *myb*-reponsiveness, suggesting that the sequences between –144 and –41 bp upstream of the start site are crucial for the activation of the promoter by v-Myb or c-Myb. As shown in Figure 6A, this region of the promoter contained two Myb binding sites (referred to as MBS-A and MBS-B).



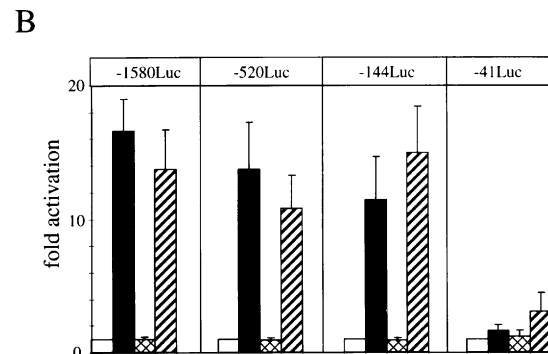


Fig. 6. Activation of the *tom-1*A promoter by Myb proteins. (**A**) A 1.7 kb *Xho*I–*Bgl*II genomic DNA fragment from the *tom-1* gene encompassing the *tom-1*A promoter is shown schematically at the top. The white box at the left corresponds to the end of the last *tom-1*B-specific exon, the black box at the right corresponds to the start of the first *tom-1*A-specific exon and the thin line corresponds to an intron of the *tom-1* gene. The *Xho*I and *Bgl*II sites are present at position 1065–1070 and position 161–166 of the *tom-1*B and *tom-1*A cDNA sequences, respectively. The transcriptional start site of *tom-1*A RNA is marked by an arrow and *myb* binding sites are marked by black ellipses. Reporter genes containing the entire *Xho*I–*Bgl*II fragment upstream of the luciferase gene or 5′ truncated parts of the fragment are shown schematically below. (**B**) Co-transfection experiments of the reporter genes described in (A). QT6 cells were co-transfected with 3 μg of the reporter gene indicated at the top, 0.1 μg of the β-galactosidase reference plasmid pCMVβ and 5 μg of expression vector for v-Myb (pVM134, black columns), v-Myb lacking the DNA-binding domain (pVM130, cross-hatched columns), c-Myb (pCM100, hatched columns) or a control vector not expressing Myb (pVM111, white columns). Cells were harvested 24 h after transfection and analyzed for luciferase and β-galactosidase activity. The columns show the average activation factors of the luciferase reporter genes. The activity of each reporter in the absence of exogenous Myb protein was designated as 1. Thin lines show standard deviations.

Figure 6B also shows the results of co-transfections of the *tom-1*A reporter genes with an expression vector encoding a truncated v-Myb protein lacking the DNA-binding domain. As expected, the activation of the *tom-1*A promoter requires the v-Myb DNA-binding domain.

To address the role of the two Myb binding sites located in the –144 to –41 bp region of the *tom-1*A promoter we destroyed each of them by point mutation. Gel retardation assays using bacterially expressed v-Myb confirmed that the protein recognizes both sites in the *tom-1*A promoter and that the mutations had destroyed both of them (Figure 7B). As shown by the co-transfection experiments illustrated in Figure 7A, mutation of MBS-A strongly diminished the Myb-inducibility of the promoter. By contrast, mutation of MBS-B had only a minor effect. We therefore concluded that the proximal Myb binding site

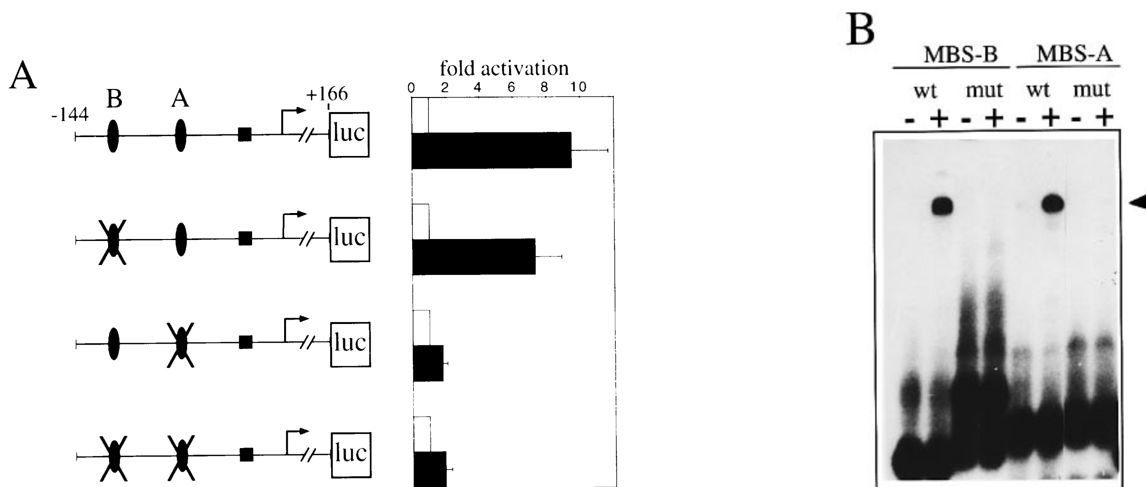


Fig. 7. Mutation analysis of the Myb binding sites of the *tom-1*A promoter. (**A**) *tom-1*A reporter genes (from top: p-144Luc; p-144Luc–mutMBS-B; p-144Luc–mutMBS-A, p-144Luc–mutMBS-AB) are shown schematically on the left. The TATA box (black box) and the Myb binding sites A and B are highlighted. Numbers indicate positions relative to the transcriptional start site (arrow). The results of co-transfection experiments are illustrated on the right. QT6 cells were co-transfected with 3 μg of the appropriate reporter gene, 0.1 μg of the β-galactosidase reference plasmid pCMVβ and 5 μg of v-Myb expression vector pVM134 (black bars) or of control vector pVM111 (white bars). Cells were harvested 24 h after transfection and analyzed for luciferase and β-galactosidase activity as described in Figure 6B. (**B**) Gel retardation experiments using bacterially expressed v-Myb bound to radiolabeled oligonucleotides corresponding to unmutated (wt) or mutated (mut) Myb binding sites A (MBS-A) and B (MBS-B) of the *tom-1*A promoter. Binding reactions contained (+) or lacked (–) v-Myb protein. Complex of v-Myb and the oligonucleotides are marked by an arrowhead. The intense bands at the bottom correspond to unbound oligonucleotides.

A is crucial for the activation of the *tom-1*A promoter by v-Myb.

Activation of the tom-1A promoter by v-Myb requires a C/EBP family member

Previous work has identified the C/EBP family members as crucial partners for v-Myb in the activation of the *mim-1* gene (Burk *et al.*, 1993; Ness *et al.*, 1993; Mink *et al.*, 1996). Dissection of the *mim-1* promoter has delineated a composite response element, which mediates the activation of the promoter by v-Myb and consists of a binding site each for v-Myb and C/EBP (Mink *et al.*, 1996). As shown in Figure 8A, a potential C/EBP binding site is located immediately upstream of the MBS-A site. It was therefore of interest to investigate whether C/EBP is also involved in the activation of the *tom-1*A promoter by v-Myb.

Figure 8C shows a gel retardation experiment confirming that different C/EBP isoforms indeed bind to the C/EBP site located upstream of MBS-A. To explore further the role of C/EBP we first determined the effect of a dominant-negative variant of C/EBPβ on the activation of the *tom-1*A promoter by v-Myb. We used an amino-terminally truncated C/EBPβ, which dimerizes and binds to DNA but which does not activate transcription due to deletion of its transactivation domain. As illustrated in Figure 8A, v-Myb activated the *tom-1*A promoter much less efficiently in the presence of the dominant-negative C/EBP than in its absence. As a control, we performed a similar experiment using the Myb-responsive but C/EBP-independent reporter gene 3xATkLuc (which contains three copies of the Myb binding site ‘A’ from the *mim-1* gene, fused to the HSV Tk promoter). The activation of this reporter gene was not affected by the dominant-negative C/EBPβ (Figure 8A). As an additional control, we showed that the amount of v-Myb was not decreased in the presence of the dominant-negative C/EBPβ (Figure 8B). These observations indicated that a C/EBP transcription factor is involved in the Myb-dependent activation of the *tom-1*A promoter. To substantiate this conclusion and to determine whether the C/EBP binding site located immediately upstream of MBS-A is necessary for the activation of the promoter, we mutated this C/EBP binding site. Gel retardation experiments confirmed that the mutation had effectively destroyed the C/EBP binding site (Figure 8C). We then investigated whether the reporter gene containing the point-mutated C/EBP binding site was still activated by v-Myb. As shown in Figure 8A, mutation of the C/EBP binding site indeed substantially diminished the Myb-responsiveness of the promoter. In control experiments (data not shown) we confirmed that mutation of the C/EBP binding site did not affect binding of v-Myb to the adjacent Myb binding site. Taken together, our results support the notion that v-Myb and a C/EBP family member cooperate in the activation of the *tom-1*A promoter. Furthermore, the data suggest that the C/EBP binding site located immediately upstream of the Myb binding site A is responsible for the effect of C/EBP on the activation of the promoter.

To show further that v-Myb and C/EBP transcription factors cooperate on the *tom-1*A promoter we studied the effect of co-expressing v-Myb and C/EBP on the activity of the promoter. Figure 9B shows that the *tom-1*A promoter was activated by different C/EBP family members in the absence of v-Myb. The strongest activation was observed for C/EBPα and C/EBPδ, whereas C/EBPβ was inactive. In the presence of v-Myb, synergistic activation was observed for all three C/EBP family members, most notably for C/EBPδ. As shown by the control experiment illustrated in Figure 8D, the amount of v-Myb present in the transfected cells was not affected by C/EBP, and vice versa. Mutation of the C/EBP binding site abolished C/EBP-dependent transactivation as well as synergy with v-Myb, supporting the crucial role of the C/EBP site located immediately upstream of MBS-A (Figure 9C).

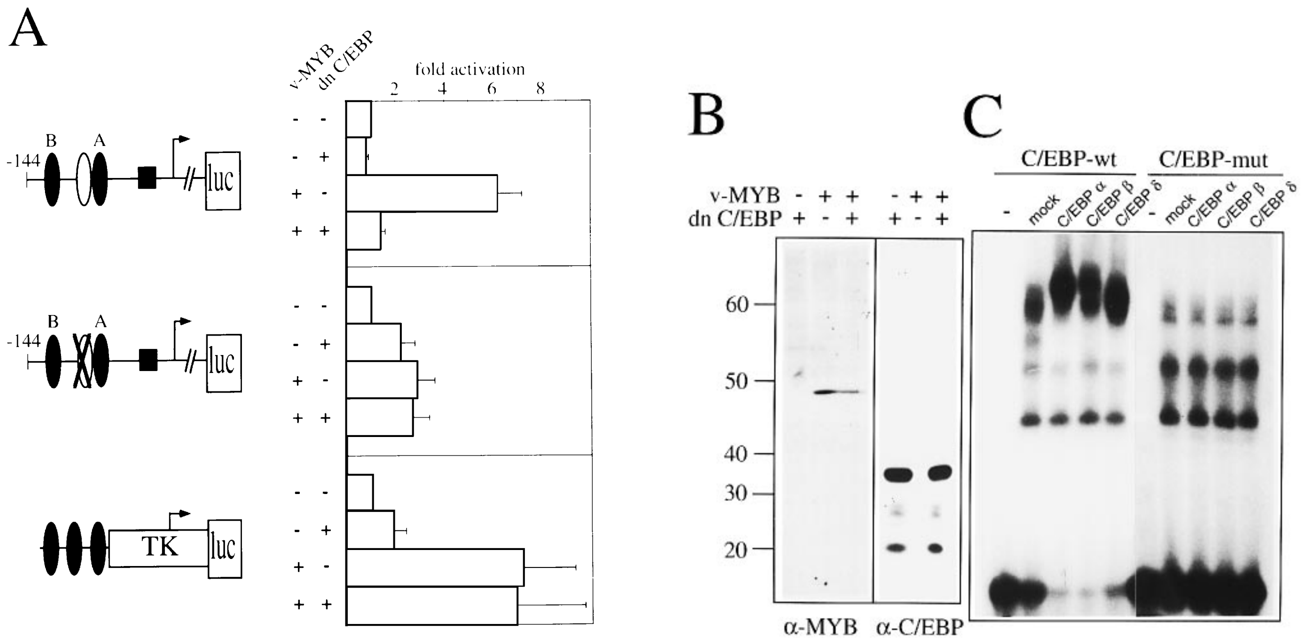


Fig. 8. Effect of dominant-negative C/EBPβ on activation of the *tom-1*A promoter by v-Myb. (**A**) Relevant reporter genes (from top: p-144Luc; p-144Luc–mutCEBP; p3xATkLuc) are shown schematically on the left. Myb and C/EBP binding sites are indicated by black and white ellipses, respectively, and the TATA box is shown as a black box. The reporter gene p3xATkLuc contains the Tk promoter and three Myb binding sites derived from the *mim-1* promoter. The results of co-transfection experiments are illustrated on the right. QT6 cells were co-transfected with 3 μg of the appropriate reporter gene, 0.1 μg of the β-galactosidase reference plasmid pCMVβ and different combinations of expression vectors for v-Myb (pVM134, 5 μg) and dominant-negative C/EBPβ (pCRNC-CCRΔN110, 0.5 μg) as indicated. Control transfections contained equivalent amounts of the appropriate empty expression vectors. Cells were harvested 24 h after transfection and analyzed for luciferase and β-galactosidase activity as described in Figure 6B. (**B**) QT6 cells were co-transfected with 0.1 μg pCMVβ and expression vectors for v-Myb (10 μg), dominant-negative C/EBPβ (1 μg) or the corresponding amount of the appropriate empty expression vector, as indicated at the top. After 24 h the cells were harvested and total cellular protein was analyzed by SDS–PAGE and Western blotting using Myb- or C/EBP-specific antibodies. Molecular weight markers (in kilodaltons) are shown on the left. The activity of the co-transfected pCMVβ plasmid was determined in aliquots of the transfected cells to ensure that the transfection efficiencies were similar. (**C**) Gel retardation experiments using nuclear extract of QT6 cells transfected with expression vectors for rat C/EBPα, mouse C/EBPβ or mouse C/EBPδ or of mock-transfected cells bound to a radiolabeled oligonucleotide corresponding to the unmutated (C/EBP-wt) or the mutated (C/EBP-mut) C/EBP binding site from the *tom-1*A promoter. Lanes marked (–) show the oligonucleotides in the absence of nuclear extract.

Comparison with the *mim-1* promoter revealed clear differences in the relative activities of the different C/EBP isoforms on the two promoters (Figure 9A). In particular, the relative activities of C/EBPα and C/EBPδ were reversed and C/EBPβ was virtually inactive on the *tom-1*A promoter. Thus, in addition to demonstrating cooperation between v-Myb and members of the C/EBP family our results also provide direct evidence for the selective action of different C/EBP isoforms.

We have shown previously that ectopic expression of v-Myb and C/EBP family members in fibroblasts results in the activation of the endogenous *mim-1* gene (Burk *et al.*, 1993). By contrast, the endogenous *tom-1* gene is not activated under these conditions (data not shown). Possibly, the *myb*-inducible *tom-1* promoter is not accessible to transcription factors in these cells.

Discussion

tom-1, a novel direct v-Myb target gene expressed in AMV- and E26-transformed myelomonocytic cells

Numerous studies have suggested that transformation of myelomonocytic cells by the oncogene v-*myb* of the chicken retroviruses AMV and E26 is due to the activation of specific genes whose deregulated expression interferes with the proliferation and differentiation of these cells. So far, only two genes expressed in v-*myb* transformed myeloblasts have been identified as direct targets for v-Myb, *mim-1* and the lysozyme gene (Ness *et al.*, 1989; Introna *et al.*, 1990; Burk and Klempnauer, 1991). Although both genes are directly activated by v-Myb they are expressed only in cells transformed by the E26 virus and not in AMV-transformed myeloblasts, suggesting that neither of them is essential for the transformation of myelomonocytic cells by v-*myb*. Genes acting as direct targets for both oncogenic versions of v-*myb* have not been identified so far.

The novel v-Myb target gene described here, *tom-1*, is expressed at elevated levels in AMV-transformed as well as in E26-transformed myeloid cells. The activation of *tom-1* expression by v-Myb does not require *de novo* protein synthesis and, finally, the *myb*-inducible *tom-1* promoter contains a Myb binding site the mutation of which abolished transactivation by v-Myb. We conclude from these data that *tom-1* is a direct target gene for both oncogenic forms of v-*myb*. Since *tom-1* is the first example of a v-Myb target gene activated in AMV- as well as in E26-transformed cells, it will be very interesting to address the molecular function of *tom-1* and its possible role in v-*myb*-induced cell transformation.

Our results show that *tom-1* is also a target for c-*myb* and that it is expressed —and presumably functions— in normal hematopoietic cells. Surprisingly, however, *tom-1*

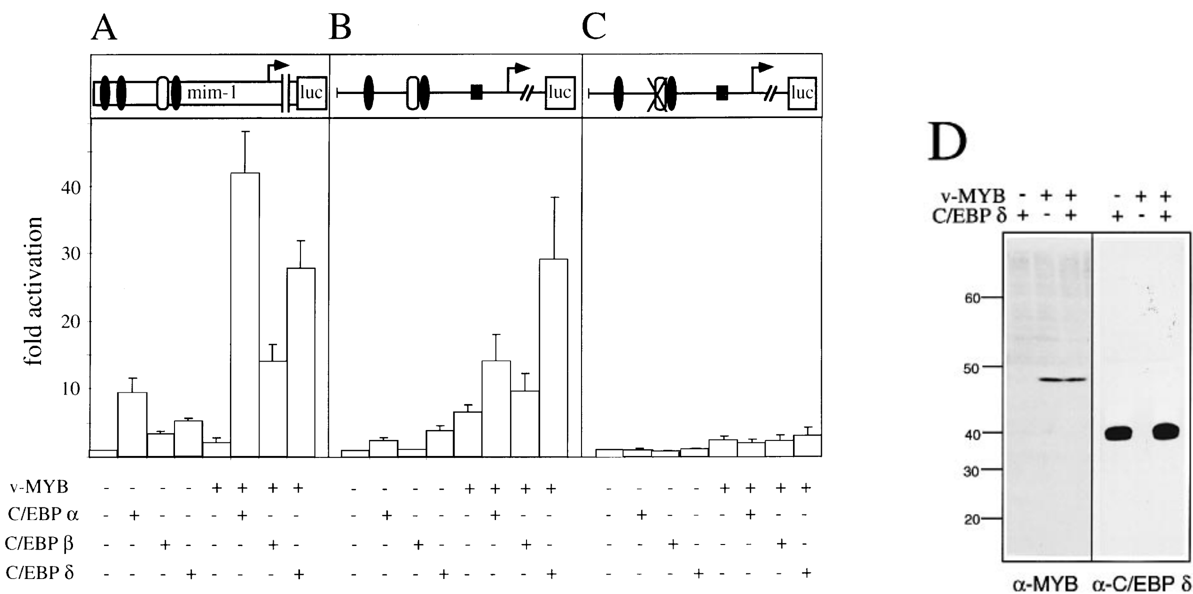


Fig. 9. Synergistic activation of the *tom-1*A and *mim-1* promoters by v-Myb and C/EBP transcription factors. The relevant *mim-1* (p-240Luc) (**A**) and *tom-1*A (p-144Luc and p-144Luc–mutCEBP) (**B** and **C**) reporter genes are shown schematically at the top of each panel. Myb and C/EBP binding sites are marked by black and white ellipses. QT6 cells were co-transfected with 3 μg of the reporter genes shown at the top, 0.1 μg of the β-galactosidase reference plasmid pCMVβ and different combinations of expression vectors for v-Myb (pVM134, 1 μg), rat C/EBPα (pMSV–C/EBP), mouse C/EBPβ (pMSV/EBPβ) and mouse C/EBPδ (pMSV/EBPδ) (0.3 μg each), as indicated below the columns. Control transfections lacking v-Myb or C/EBP contained equivalent amounts of the appropriate empty expression vectors. Cells were harvested 24 h after transfection and analyzed for luciferase and β-galactosidase activity as described in Figure 6B. (**D**) QT6 cells were co-transfected with 0.1 μg pCMVβ and expression vectors for v-Myb (5 μg), mouse C/EBPδ (1.7 μg) or the corresponding amount of the appropriate empty expression vectors, as indicated at the top. After 24 h the cells were harvested and total cellular protein was analyzed by SDS–PAGE and Western blotting using Myb- or C/EBPδ-specific antibodies. Molecular weight markers (in kilodaltons) are shown on the left. The activity of the co-transfected pCMVβ plasmid was determined in aliquots of the transfected cells to ensure that the transfection efficiencies were similar.

expression is very high in skeletal muscle. The significance of this finding will remain unclear, however, until we know more about the possible role of the gene. Due to a lack of homology to other proteins or known amino acid motifs the function of *tom-1* presently remains unknown.

Cooperative activation of the Myb-inducible tom-1 promoter by v-Myb and C/EBP

Physiological Myb target genes are ideal tools to study at the molecular level how Myb affects the expression of other genes. Previous work, based on the *mim-1* promoter, has identified the C/EBP transcription factors as cooperation partners for v-Myb (Burk *et al.*, 1993; Ness *et al.*, 1993). Recently, we have shown that a pair of closely spaced Myb and C/EBP binding sites in the *mim-1* promoter comprises the minimal Myb-responsive element and is responsible for cooperation between v-Myb and C/EBP (Mink *et al.*, 1996).

The data presented here demonstrate that C/EBP is also a partner for v-Myb at the *tom-1*A promoter. As in the *mim-1* promoter the most relevant Myb binding site of the promoter is juxtaposed to a C/EBP consensus binding site. Our data show that a dominant-negative variant of C/EBPβ inhibits activation of the promoter by v-Myb and, furthermore, that mutation of the C/EBP binding site abolishes both the inhibitory effect of dominant-negative C/EBP as well as the activation of the promoter by v-Myb. These results indicate that v-Myb activates the *tom-1*A promoter in conjunction with a C/EBP family member binding to the adjacent C/EBP binding site. Together with previous work (Burk *et al.*, 1993; Ness *et al.*, 1993), this leads to the interesting conclusion that in fact all direct v-Myb inducible genes whose activation by v-Myb has been studied in detail, *tom-1*, *mim-1* and the lysozyme gene, are activated by the synergistic interplay of v-Myb with C/EBP transcription factors. Thus, C/EBP family members may act as general cooperation partners for v-Myb in myelomonocytic cells.

The cooperation of v-Myb and C/EBP has been studied in detail at the *mim-1* promoter and involves a direct interaction between the two proteins (Mink *et al.*, 1996). In addition, v-Myb has been shown to recruit CREB-binding protein (CBP) as a coactivator (Dai *et al.*, 1996; Oelgeschläger *et al.*, 1996). Activation by v-Myb thus appears to involve at least a trimolecular complex of v-Myb, C/EBP and CBP. We have shown recently that CBP/p300 also interacts with C/EBP and thereby presumably provides additional stabilization of the complex (S.Mink and K.-H.Klempnauer, manuscript in preparation). However, it remains to be seen whether or not all of these interactions are equally important for the activation of different target genes.

It has been shown that myelomonocytic cells express high levels of several members of the C/EBP family, such as C/EBPα, β and δ and that individual C/EBP family members have distinct temporal patterns of expression during myelomonocytic differentiation (Scott *et al.*, 1992). A cascade of expression of different C/EBP isoforms has also been observed during fat cell differentiation (Cao *et al.*, 1991; Yeh *et al.*, 1995). It is presumed that the different isoforms activate specific and perhaps overlapping sets of genes and thereby orchestrate the activation of a large number of target genes. Our finding that the *tom-1*A and *mim-1* promoters have distinct patterns of

responsiveness to different C/EBP isoforms provides direct evidence for the preferential activation of physiological C/EBP target genes by specific C/EBP family members. The availability of these genes and their promoters should facilitate further analysis of the molecular basis for this specificity.

Materials and methods

Cell lines and chicken tissues

The chicken cell lines BM2 (AMV-transformed myeloblasts), HD11 (MC29-transformed macrophages) and HD3 (AEV-transformed erythroblasts) have been described (Klempnauer *et al.*, 1983; Burk and Klempnauer, 1993). 10.4 is a derivative of the HD11 cell line expressing a v-Myb–ER fusion protein (Burk and Klempnauer, 1991). QT6 is a line of quail fibroblasts derived from a methylcholanthrene-induced fibrosarcoma (Moscovici *et al.*, 1977). QT6 cells were grown in Iscove's modified DMEM (IMDM) supplemented with 8% fetal calf serum and 2% chicken serum. An E26-transformed myeloblast cell line was obtained from T.Graf and was grown in the same medium as QT6 cells. S2CL (Chen *et al.*, 1983) and MSB1 (Akiyama and Kato, 1974) are chicken B- and T-cell lines grown in RMPI 1640 medium supplemented with 8% fetal calf serum, 2% chicken serum and 32 μM β-mercaptoethanol. Chicken embryo fibroblasts were obtained from Flow laboratories and were grown in the same medium as QT6 cells. The MEP cell lines HD50 (Kulessa *et al.*, 1995), HD57 (Metz and Graf, 1991) and HD50E (Kulessa *et al.*, 1995) and primary E26- and AMV-transformed myeloblasts were obtained from T.Graf and were used directly for preparation of RNA. Subclones of the HD11 cell line expressing v-*myb* or c-*myb* were generated by G418 selection of stable transfectants of HD11 cells. Cells were transfected with expression vectors pVM116 (encoding the AMV version of v-Myb; clones 26.1, 26.3, 26.4), pVM134 (encoding an E26-like version of v-Myb; clones 27.5, 27.10, 27.11), pVM111 (control vector encoding no v-Myb protein; clones 28.2, 28.3, 28.4), pcDNA3/c-Myb (encoding chicken c-Myb, clone H/c-myb) or pcDNA3 (expressing no c-Myb protein, clone H/cDNA3). Cells were selected in the presence of 400 μg/ml G418 and cultivated further at 200 μg/ml G418. Normal tissues were prepared from 4-week-old chickens. Bone marrow was prepared from 3-week-old chickens. Immature myeloid and lymphoid cells were separated from mature red blood cells and granulocytes by centrifugation through a ficoll cushion (density 1.077 g/ml).

Differential display

Differential display was performed as described by Liang and Pardee (1992) with the following modifications: total RNA was prepared from 10.4 cells treated with or without 2 μM β-estradiol for 24 h and digested with DNase as described (Liang *et al.*, 1993) to remove contaminating genomic DNA. 2.5 μg of total RNA was reverse transcribed with 200 U MMLV–RT (Superscript, Gibco-BRL) in the presence of 2.5 μM oligo(dT)-based primer and 500 μM dNTP at 50°C in a volume of 20 μl. After heat inactivation of the enzyme at 95°C (5 min) the reaction was diluted with 10 mM Tris–HCl, pH 7.5, 1 mM EDTA to 250 μl. 2 μl of the diluted cDNA was then used for PCR [2 μM dNTP, 2 μCi [α-^{33}P]dATP, 1 U native *Taq* polymerase (Perkin-Elmer), 2 μM oligo(dT)-based primer, 1 μM random decamer primer in buffer containing 50 mM KCl, 10 mM Tris–HCl, pH 8.4, 1 mM $MgCl_2$, 0.01% gelatin]. Cycling parameters were as follows: 2 min at 94°C were followed by 36 cycles of 94°C (30 s), 40°C (1 min), 72°C (30 s), followed by final 5 min at 72°C. The amplified DNA was separated on a 6% sequencing gel. Recovery and reamplification of fragments of interest was performed as described (Liang *et al.*, 1993). The reamplified fragments were separated on agarose gels, the DNA extracted and cloned into pCR-Script vector (Stratagene). Sequencing was carried out using the T7 sequencing kit (Pharmacia). The *tom-1* differential display fragment was obtained with the oligo(dT)-based primer T_{12}CG and the random primer GATCCAGTAC.

Isolation of tom-1 cDNA and genomic clones

A λgt11 cDNA library of the BM2 cell line was screened under conditions of high stringency with the cloned *tom-1* differential display fragment. *Eco*RI insert fragments of positive phages were subcloned into pBluescript vector (Stratagene) and sequenced using the T7 sequencing kit (Pharmacia). The EMBL/GenBank/DDBJ accession numbers for *tom-1*A and B cDNA sequences are Y08740 and Y08741, respectively. A λEMBL-3 Sp6/T7 genomic library of chicken liver (Clontech) was screened under high stringency conditions with a 2100 bp *tom-1* probe (position 738–2821 of the *tom-1*B cDNA sequence). *Xho*I insert fragments of positive phages were subcloned into pBluescript vector. A Southern blot of these cloned fragments was hybridized with a *tom-1*A 5′-specific probe (position 61–166 of the *tom-1*A cDNA sequence) to identify the promoter region of the *tom-1*A transcript. An ~1.7 kb *Xho*I–*Bgl*II fragment hybridizing with the probe was sequenced (EMBL/GenBank/DDBJ accession number Y08742).

Western blotting

Monoclonal Myb-specific antibodies myb2-2 and polyclonal chicken C/EBPβ-specific antiserum have been described (Evan *et al.*, 1984; Mink *et al.*, 1996). Polyclonal mouse C/EBPδ-specific antiserum was obtained from Santa Cruz. Immunostaining was performed using the ECL detection system (Amersham).

Eukaryotic expression vectors

Expression vectors for v-Myb (pVM134 and pVM116), amino-terminally truncated v-Myb lacking its DNA-binding domain (pVM130), c-Myb (pCM100), the v-Myb frameshift vector (pVM111) and amino-terminally truncated chicken C/EBPβ (pCRNC-CCRΔN110) have been described (Klempnauer *et al.*, 1989; Burk and Klempnauer, 1991; Mink *et al.*, 1996). Expression vectors for rat C/EBPα (pMSV–C/EBP) and mouse C/EBPβ and δ (pMSV/EBPβ, pMSV/EBPδ) were obtained from S.McKnight (Friedman *et al.*, 1989; Cao *et al.*, 1991).

Reporter genes, transfections, luciferase and β-galactosidase assays

The reporter plasmids p-240Luc and p3xATkLuc have been described (Ness *et al.*, 1989). pCMVβ was obtained from Clontech. Reporter genes p-1580Luc, p-520Luc, p-144Luc and p-41Luc encompass *tom-1* promoter sequences from the indicated upstream positions to +166 bp (relative to the transcriptional start site). These promoter fragments were generated by digestion with restriction enzymes or by PCR, using appropriate primer combinations, and cloned into the pGL-2 basic vector (Promega). In addition, the following point-mutated derivatives of the reporter gene p-144Luc were constructed. p-144Luc–mutMBS-A contains a mutation of the Myb binding site A (the promoter sequence between positions –74 and –69 was changed from TAACGG to TCCAGG), p-144Luc–mutMBS-B contains a mutation of the Myb binding site B (the promoter sequence between positions –122 and –117 was changed from CAGTTG to CATAAG), p-144Luc–mutMBS-AB contains the combination of both mutations described above. p-144Luc–mutCEBP contains a mutation of a C/EBP binding site located adjacent to the Myb binding site A (the promoter sequence between positions –86 and –78 was changed from TGGCGCAAT to CCGCCCGCA). All mutants were generated by PCR using appropriate primers and the identity of the PCR products was confirmed by sequencing. DNA transfection using the quail fibroblast cell line QT6 (Moscovici *et al.*, 1977) was performed as described (Burk *et al.*, 1993). The amounts of DNA used for transfection of cells in a 10 cm tissue culture dish are indicated in the figure legends. Cells were harvested 24 h after transfection. Preparation of cell extract, luciferase and β-galactosidase assays were performed as described (Burk *et al.*, 1993).

Gel retardation assays

The following single-stranded oligonucleotides were annealed and used for gel retardation assays: MBS-A-wt (–92 to –61 bp of the *tom-1*A promoter), 5′-CCAATGTGGCGCAATCCTTAACGGA-3′ and 5′-GCCTCAGTCCGTTAAGGATTGCGCC-3′; MBS-A-mut, 5′-CCAATGTGGCGCAATCCTTCCAGGA-3′ and 5′-GCCTCAGTCCTGGAAGGATTGCGCC-3′; MBS-B-wt (–131 to –110 bp of the *tom-1*A promoter), 5′-GCTGAGGAACAGTTGGGCAGGG-3′ and 5′-CCCTGCCCAACTGTTCCTCAG-3′; MBS-B-mut, 5′-GCTGAGGAACATAAGGGCAGGG-3′ and 5′-CCCTGCCCTTATGTTCCTCAG-3′; C/EBP-wt (the same oligonucleotides as for MBS-A-wt); C/EBP-mut, 5′-CCAATGCCGCCCGCACCTTAACGGA-3′ and 5′-GCCTCAGTCCGTTAAGGTGCGGGCG-3′.

After annealing, oligonucleotides were radiolabeled by filling in the ends using [α-^{32}P]dCTP. Bacterial v-Myb to be used for gel retardation experiments was prepared as described (Oehler *et al.*, 1990). Nuclear extracts containing different C/EBP isoforms were prepared from QT6 cells transfected with the appropriate expression vectors as follows: first, nuclei were prepared from the transfected cells 24 h after transfection by washing the cells twice in hypotonic buffer (10 mM Tris–HCl, pH 7.8; 5 mM KCl; 2 mM $MgCl_2$) and then lysing them in hypotonic

buffer supplemented with 0.25% NP40 for 5 min on ice. Nuclei were pelleted, washed twice with hypotonic buffer, pelleted again and suspended in two volumes of hypotonic buffer containing 0.3 M NaCl. The suspension was kept on ice for 30 min and agitated occasionally. Finally, the nuclei were pelleted and the supernatant was used directly for electrophoretic mobility shift experiments as described (Barberis *et al.*, 1987).

Northern blotting

Preparation of polyadenylated RNA, Northern blotting and detection of the chicken *mim-1* RNAs was performed as described (Burk *et al.*, 1993). Total RNA was prepared according to Chomczynski and Sacchi (1987). To detect *tom-1*A and B transcripts, the cloned 150 bp differential display fragment was used. A *tom-1*A-specific probe was derived from a *tom-1*A cDNA fragment (position 161–404 of the *tom-1*A cDNA sequence). A *tom-1*B-specific probe was derived from a *tom-1*B cDNA fragment (position 119–743 of the *tom-1*B cDNA sequence). A probe specific for gene encoding ribosomal protein S17 was generated by PCR, based on the sequence of the chicken S17 gene (Trüeb *et al.*, 1988). S17 RNA is ubiquitously expressed and has been used previously for standardization of RNA expression data (Leonard *et al.*, 1993).

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