MOLECULAR AND CELLULAR BIOLOGY, Apr. 1996, p. 1316–1325 Vol. 16, No. 4
0270-7306/96/$04.00+0

Interaction of C/EBPβ and v-Myb Is Required for Synergistic Activation of the *mim*-1 Gene

SIGRUN MINK, ULRIKE KERBER, AND KARL-HEINZ KLEMPNAUER*

Hans Spemann Laboratory, Max Planck Institute for Immunobiology, D-79108 Freiburg, Germany

Received 4 August 1995/Returned for modification 27 September 1995/Accepted 4 January 1996

The retroviral oncogene v-*myb* encodes a transcription factor (v-Myb) which activates the myelomonocyte-specific *mim*-1 gene, a natural *myb* target gene, by cooperating with members of the C/EBP transcription factor family. The finding that v-Myb, together with C/EBP, is sufficient to activate the *mim*-1 gene in heterologous cell types has implicated Myb and C/EBP as a bipartite molecular switch, which regulates the expression of myelomonocyte-specific genes. To understand the relationship between v-Myb and C/EBP in more detail, we have examined the molecular basis of the activation of the *mim*-1 promoter by v-Myb and C/EBPβ, a member of the C/EBP transcription factor family highly expressed in myelomonocytic cells. We have identified a composite Myb and C/EBP response element which mediates synergistic activation of the *mim*-1 promoter by both factors and consists of closely spaced Myb- and C/EBP-binding sites. In vitro and in vivo protein-binding studies indicate that v-Myb and C/EBPβ interact with each other via their DNA-binding domains. We show that this interaction is essential for the synergistic activation of the *mim*-1 promoter by v-Myb and C/EBPβ. Our work therefore identifies C/EBPβ as an interaction partner of v-Myb involved in myelomonocyte gene expression.

Complex regulatory elements, containing binding sites for multiple transcription factors, are associated with many eucaryotic genes and provide a basis for the combinatorial action of different transcription factors in the control of gene expression. These elements play key roles in establishing a regulatory transcription factor network; they mediate the integration of different regulatory signals and allow different transcription factors to communicate with each other. The identification of such elements and of interacting transcription factors is therefore important in the elucidation of this regulatory network.

The oncogene v-*myb* of avian myeloblastosis virus (AMV) encodes a transcription factor which is capable of transforming specifically cells of the myelomonocytic lineage (for a review, see reference 11). v-*myb* is a structurally altered form of the chicken c-*myb* gene (19, 20), which itself plays a fundamental role in the development of the hematopoietic system. c-*myb* is highly expressed in immature cells of all hematopoietic lineages and is turned off during terminal differentiation of these cells. A block of c-*myb* expression reduces the proliferation potential of hematopoietic precursor cells (10), whereas sustained expression of c-*myb* arrests cells at an immature state of differentiation (6, 43). Finally, mice lacking a functional c-*myb* gene die during embryonic development from severe defects in fetal hepatic hematopoiesis (29). Together, these and other studies suggest an essential role for c-*myb* in the proliferation of hematopoietic progenitor cells.

The proteins encoded by v-*myb* and c-*myb* (referred to as v-Myb and c-Myb, respectively) specifically recognize the sequence motif PyAAC(G/T)G (3) and activate promoters containing this binding sequence (14, 17, 34, 45). One of the known natural Myb target genes, the chicken *mim*-1 gene, is highly expressed in myelomonocytic cells transformed by v-Myb (15, 33). The *mim*-1 gene is directly activated by v-Myb, and its promoter contains several Myb consensus binding sites (33); however, v-Myb alone is not sufficient to induce *mim*-1 expression. It has been shown recently that v-Myb activates the *mim*-1 gene by cooperating with members of the C/EBP transcription factor family (5, 32).

We have investigated the molecular basis of the activation of *mim*-1 by v-Myb and chicken C/EBPβ, a member of the C/EBP family highly expressed in myelomonocytic cells. We have identified a composite response element that mediates synergistic activation of the *mim*-1 promoter by v-Myb and C/EBPβ and consists of closely spaced Myb- and C/EBP-binding sites. We show that v-Myb and C/EBPβ are interaction partners and that their interaction is essential for the activation of the *mim*-1 promoter.

MATERIALS AND METHODS

***mim*-1 reporter genes, DNA transfections, and reporter gene assays.** The following *mim*-1 reporter genes were used in this study. p-240Luc has been described previously (33). p-180Luc, p-155Luc, and pS33/9, containing partially deleted versions of the *mim*-1 promoter, were generated by PCR with appropriate primers and cloned between the *Xho*I and *Hin*dIII sites of plasmid pXP2 (36). pDel130/33Luc is a derivative of p−180Luc in which sequences between −130 and −33 relative to the transcriptional start site are missing. pDel130/33 was constructed by fusing two PCR fragments containing sequences from −180 to −130 and −33 to +150 relative to the start site. pMut14, pMut19, and pMutT160 are point-mutated derivatives of p-180Luc. In pMut14, the distal C/EBP-binding site was destroyed by changing its sequence from GTCTTTC CCAACCA to GTCCCTACCGGCCA. In pMut19, the proximal C/EBP-binding site was destroyed by changing its sequence from TGATTGGCCAACAC to TGACCGACCGGCAC. In pMutT160, the *myb*-binding site was destroyed by changing its sequence from ACCGTTATAA to ACCGAAATAA. All mutants were generated by PCR with appropriate primers. All mutants showed a drastically reduced binding to bacterially expressed C/EBPβ or v-Myb protein, as determined by electrophoretic mobility shift assays. pDel-E-MutLuc and pDel-M-MutLuc are point-mutated versions of pDel130/33Luc containing the same mutations as pMut14Luc and pMutT160Luc, respectively. pDel130/33TRELuc is a point-mutated version of pDel130/33Luc in which the C/EBP-binding site was converted to an AP-1-binding site by changing the sequence from GTCTTTC CCAACCA to GTCTGAGTCAACCA. Plasmid p3×MimTKLuc contains three copies of the *mim*-1 promoter sequence from positions −175 to −135 placed upstream of the herpes simplex virus thymidine kinase promoter (positions −46 to +52 relative to the start site) and the luciferase gene. p3×MimTKLuc was generated by cloning a synthetic oligonucleotide containing the indicated region

* Corresponding author. Mailing address: Hans Spemann Laboratory, Max Planck Institute for Immunobiology, Stübeweg 51, D-79108 Freiburg, Germany. Phone: 0761/5108 590. Fax: 0761/5108 221.

of the *mim*-1 promoter flanked by *Bam*HI and *Bgl*II sites into the *Bam*HI site of plasmid pTATA (a kind gift of A. Hecht and A. E. Sippel).

In addition to the *mim*-1 reporter genes described above, plasmid pCH110 (Pharmacia) was used to monitor transfection efficiencies. DNA transfection was performed as described previously (5), with the quail fibroblast cell line QT6 (28). The amount of DNA used for transfection is indicated in the figure legends and refers to one 10-cm tissue culture dish. Preparations of cell extracts, luciferase, and β-galactosidase assays were performed as described previously (5).

Eucaryotic expression vectors. pVM134 (17) encodes full-length v-Myb; pVM111 (17) is a frameshift derivative of pVM134 encoding no functional v-Myb; pDIN1 (4) encodes a partially deleted v-Myb lacking its transactivation domain; pChB-*myb*/VP16 (8) encodes a chicken B-Myb/VP16 fusion protein; and pCRNC-A*myb* (9) encodes full-length chicken A-Myb. The chicken C/EBPβ expression vectors pCRNC-CCR, pCRNC-mutCCR, and pCRNC-fsCCR have been described previously (5). pCRNC-CCRΔ260–275 encodes a mutant version of chicken C/EBPβ with a deletion of the basic region (amino acids 260 to 275). In this vector, the sequence between an *Alu*I site and an *Hha*I site, located at positions 791 and 849, respectively, in the chicken C/EBPβ coding region (5), was replaced by a synthetic *Alu*I-*Hha*I oligonucleotide adapter. pCRNC-CCRΔN21, pCRNC-CCRΔN49, and pCRNC-CCRΔN110 encode deletion mutants of chicken C/EBPβ lacking 21, 49, or 110 amino acids, respectively, from the N terminus. These vectors were generated by PCR with appropriate primers. All PCR-derived sequences were verified by nucleotide sequencing. The human c-Jun expression vector pRSVc-jun and the control vector pRSV-0 have been described previously (2). pcDNA3-Bst-Jun-CCR encodes a c-Jun-C/EBPβ chimera consisting of the amino-terminal sequences (amino acids 1 to 248) from human c-Jun fused to the DNA-binding domain (amino acids 250 to 328) of chicken C/EBPβ. To construct this vector, a *Bst*XI site was introduced by PCR immediately upstream of position 763 of the chicken C/EBPβ coding sequence (5). A *Bst*XI-*Xba*I fragment encoding the carboxy-terminal end of chicken C/EBPβ and a *Hin*dIII-*Bst*XI fragment from pRSVc-jun (containing the coding region for the amino terminus of c-Jun) was then cloned between the *Hin*dIII and *Xba*I sites of pCDNA3 (Invitrogen). pCDNA3-CJ encodes a C/EBPb–c-Jun chimaera consisting of the amino-terminal sequences (amino acids 1 to 245) from chicken C/EBPβ fused to the DNA-binding domain (amino acids 244 to 331) of human c-Jun. To construct this vector, a *Bst*XI site was introduced by PCR immediately downstream of position 750 of the C/EBPβ coding sequence (5). A *Hin*dIII-*Bst*XI fragment encoding the amino-terminal end of C/EBPβ and a *Bst*XI-*Bst*XI fragment (encoding the DNA-binding domain of human c-Jun) were cloned into pCDNA3 (Invitrogen). All PCR-derived DNA fragments were sequenced.

Bacterial expression vectors. Bacterial expression vectors used are derivatives of the ptrp9 expression vector (21). ptrp9CCR encodes full-length chicken C/EBPβ. To construct this vector, a *Bgl*II restriction site was created upstream of the chicken C/EBPβ start codon by PCR, followed by insertion of the complete C/EBPβ coding region as a *Bgl*II-*Xba*I restriction fragment into the ptrp9 expression vector. The sequence immediately upstream of the C/EBPβ start codon is A G A T C T C A C C A T G (the *Bgl*II site and the ATG start codon are underlined). The *Xba*I site is located downstream of the C/EBPβ stop codon and is derived from polylinker sequences (5). The resulting expression vector encodes full-length chicken C/EBPβ fused at the N terminus to 16 amino acids (MLAIFVLKGSLDRDLT) encoded by the *trp* operon and by linker sequences. ptrp9CCR-mut and ptrp9CCRΔ260–275 are derivatives of ptrp9CCR that encode C/EBPβ variants carrying point mutations or a deletion in the basic region, respectively. To obtain ptrp9CCR-mut and ptrp9CCRΔ260–275, the *Nco*I-*Xba*I fragment of ptrp9CCR, containing most of the C/EBPβ coding region, was exchanged by the corresponding fragment from the eucaryotic expression vectors pCRNC-mutCCR and pCRNC-CCRΔ260–275, respectively. ptrp9CCRΔN21, ptrp9CCRΔN49, ptrp9CCRΔN84, and ptrp9CCRΔN110 encode chicken C/EBPβ deletion mutants missing the indicated number of amino acids from the N terminus and were derived from the corresponding eucaryotic expression vectors described above by subcloning the truncated C/EBPβ coding regions into the ptrp9 vector.

Bacterial expression vectors for full-length or partially deleted v-Myb have been described (37). Recombinant C/EBPβ, v-Myb, or *Escherichia coli* TrpE protein (encoded by the ptrp9 expression vector) was isolated in insoluble form (21). For some experiments, the insoluble purified bacterial protein was solubilized as described previously (21, 37).

In vitro translation. pSPmyb5 contains the complete v-*myb* coding region downstream of the SP6 promoter. pSPmyb5 was constructed by cloning a 1.3-kb *Hpa*I-*Xba*I fragment from plasmid pVM2028 (21) between the *Sal*I and *Xba*I sites of pSP64 (Promega). pBl-CCR(orf) contains the complete chicken C/EBPβ coding region placed downstream of the T3 promoter and was generated by cloning an *Eco*RI-*Xba*I fragment from plasmid pCRNC-CCR(orf) (5) between the *Eco*RI and *Xba*I sites of pBluescriptKSM13− (Stratagene). pPU.1 (22) contains the coding region for transcription factor PU.1 downstream of the T3 promoter. In vitro translation was carried out by standard protocols with the TNT reticulocyte lysate system (Promega).

In vitro protein-binding assays. Far-Western blotting was performed at room temperature as follows. Total protein from bacteria harboring the appropriate expression vectors or purified human c-Jun (Promega) was fractionated in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and blotted onto nitrocellulose. Filters were incubated first for 2 h in incubation buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, 0.2% Nonidet P-40) containing 5% milk powder and then for 2 h in incubation buffer containing 1% milk powder and [^{35}S]methionine-labeled, in vitro-translated v-Myb. As control, the incubation buffer was supplemented with radiolabeled in vitro translation reaction products to which no DNA had been added. Alternatively, bacterially expressed proteins, purified and solubilized as described previously (21), were used instead of radiolabeled proteins. To remove unbound protein, filters were washed three times for 10 min in incubation buffer. Filters incubated with radiolabeled proteins were exposed to autoradiographic film. Filters incubated with bacterially expressed proteins were immunostained with the appropriate antibodies. Blots were incubated with radiolabeled DNA as described above, except that ^{32}P-labeled nick-translated plasmid DNA (pBluescript; Stratagene) was used instead of in vitro-translated protein.

In vitro coimmunoprecipitation was performed as follows. In vitro translation reaction mixtures (10 to 20 μl) containing [^{35}S]methionine-labeled proteins were mixed with 500 ng of bacterially expressed proteins and incubated for 30 min at 30°C. The reaction mixtures were then diluted 50-fold with incubation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1 mM EDTA, 10% glycerol, 50 mM NaCl, 0.1% Triton X-100) and incubated with protein A-Sepharose (50-μl bed volume per ml of reaction mixture) for 30 min at 4°C. After centrifugation at 14,000 × *g* for 10 min, the supernatant was removed and supplemented with 5 μl of the appropriate rabbit serum. After addition of protein A-Sepharose, the samples were incubated at 4°C for 2 h with gentle agitation. The Sepharose beads were then collected by centrifugation and washed four times in incubation buffer and once in incubation buffer lacking Triton X-100. Prior to the last washing step, the samples were transferred to fresh tubes. Finally, the proteins were eluted from the Sepharose beads with SDS-sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

In vivo coimmunoprecipitation. To detect complexes of v-Myb and C/EBPβ, we used the AMV-transformed chicken myeloblast cell line BM2 (obtained from C. Moscovici), the MC29-transformed chicken macrophage cell line HD11 (obtained from T. Graf and H. Beug), or the quail fibroblast cell line QT6 (28). Cells were radiolabeled for 2 h in methionine-free medium containing 0.5 mCi of [^{35}S]methionine per ml. The cells were then washed twice with ice-cold phosphate-buffered saline and collected by centrifugation. The cell pellet was resuspended in 0.6 ml of hypotonic buffer (25 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$, 5 mM KCl) and left on ice for 5 min. An equal volume of hypotonic buffer containing 1% Nonidet P-40 was added, and the mixture was incubated on ice for 5 min. Nuclei were pelleted at 5,000 × *g* for 5 min and resuspended in 0.3 ml of resuspension buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl, 0.2% Nonidet P-40, 1% aprotinin, 0.1% leupeptin, 1 mM phenylmethylsulfonyl fluoride) with a syringe. The suspension was kept at 4°C for 2 h and clarified by centrifugation at 14,000 × *g* for 10 min. The extracts were then divided in three aliquots, diluted with resuspension buffer to a total volume of 0.6 ml each, and supplemented with the desired antiserum (5 μl) and protein A-Sepharose, which had been preincubated for 2 h at 4°C with unlabeled cell extract. After gentle agitation for 2 h at 4°C, the protein A-Sepharose was washed five times with resuspension buffer. The Sepharose was then mixed with 0.4 ml of 20 mM Tris-HCl (pH 7.4)–50 mM NaCl–1% SDS–5 mM dithiothreitol and boiled for 5 min. After the Sepharose was pelleted, the supernatant was divided into two aliquots. To both aliquots were added 0.2 ml of 20 mM Tris-HCl (pH 7.4)–50 mM NaCl–1% Nonidet P-40–1% sodium deoxycholate, a fresh aliquot of protein A-Sepharose, and 5 μl of the desired antiserum. After gentle agitation for 2 h at 4°C, the agarose was pelleted, washed five times with RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), and finally suspended in SDS-sample buffer. Samples were fractionated by SDS-PAGE followed by autoradiography.

Antibodies. Monoclonal Myb-specific antibodies CB100 and myb2-37 and polyclonal rabbit serum raised against bacterially expressed v-Myb have been described previously (7, 18, 20). Polyclonal C/EBPβ-specific rabbit serum was raised against bacterially expressed full-length chicken C/EBPβ. Immunostaining of proteins transferred to nitrocellulose was performed as described previously (18), except that protein-antibody complexes were visualized by using the enhanced chemiluminescence detection system (Amersham).

RESULTS

Nucleotide sequences involved in the activation of the *mim*-1 promoter by v-*myb* and C/EBPβ. To identify sequences which mediate the responsiveness of the *mim*-1 promoter to v-Myb and C/EBPβ, we analyzed the activation of *mim*-1 promoter mutants by v-Myb and C/EBPβ (Fig. 1). As shown previously (5), a reporter gene encompassing *mim*-1 promoter sequences from −240 to +150 bp (relative to the transcriptional start site) and containing three binding sites each for Myb and C/EBP was activated synergistically by v-Myb and C/EBPβ. The reporter gene −180Luc was also activated synergistically by both transcription factors, indicating that the Myb-binding

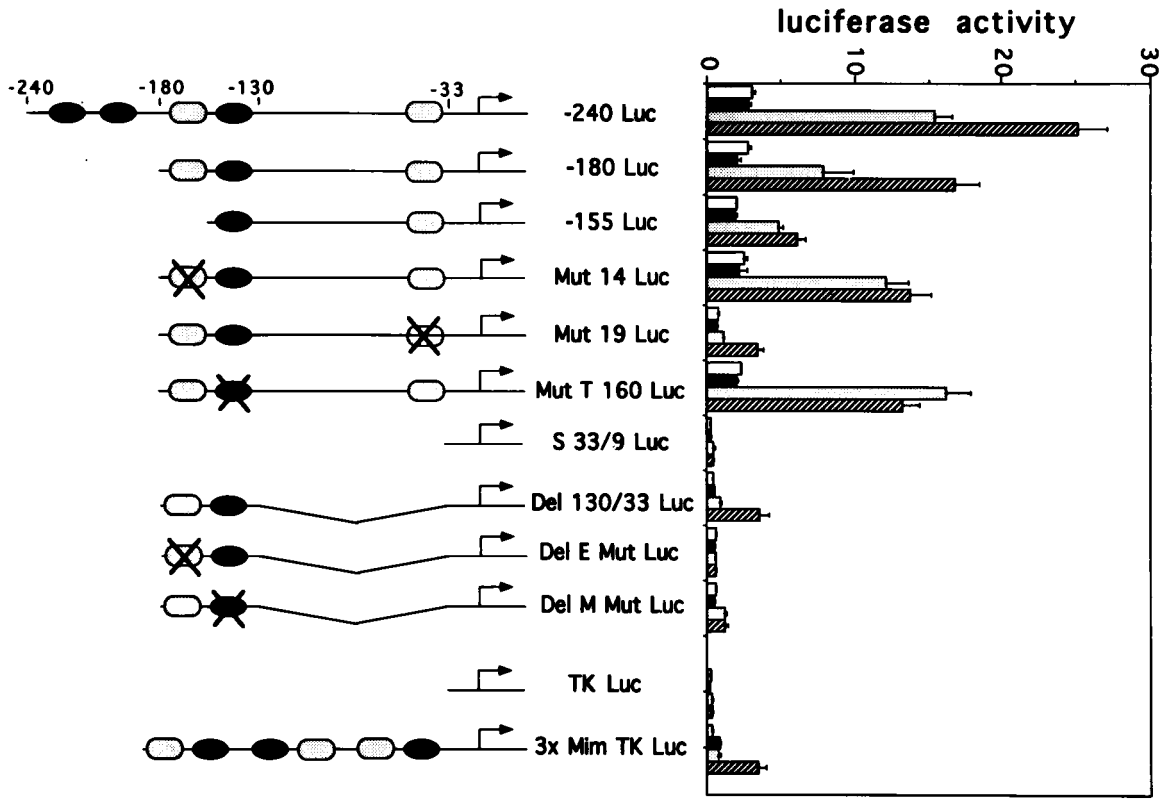


FIG. 1. Mapping of v-Myb- and C/EBP-responsive sites in the *mim*-1 promoter. The *mim*-1 reporter genes used are illustrated on the left. Myb- and C/EBP-binding sites are shown as black and stippled boxes, respectively. QT6 cells were cotransfected with 3 μg of the different luciferase reporter genes, 1 μg of pCH110, and different combinations of expression vectors for v-Myb (pVM134), chicken C/EBPβ (pCRNC-CCR), or the appropriate frameshift vectors (pVM111 and pCRNC-fsCCR). White bars, 1 μg of v-Myb frameshift vector and 0.3 μg of C/EBPβ frameshift vector. Black bars, 1 μg of v-Myb expression vector and 0.3 μg of C/EBPβ frameshift vector. Stippled bars, 1 μg of v-Myb frameshift vector and 0.3 μg of C/EBPβ expression vector. Hatched bars, 1 μg of v-Myb expression vector and 0.3 μg of C/EBPβ expression vector. Bars show the average activity of the luciferase plasmids, normalized with respect to the activity of the β-galactosidase plasmid pCH110. Thin lines show standard deviations.

site A (33) and one or both of the remaining C/EBP-binding sites are sufficient for synergistic activation. Deletion (−155Luc) or mutation (Mut14Luc) of the distal C/EBP-binding site or of the Myb-binding site (MutT160Luc) resulted in a reporter gene that was no longer activated synergistically by v-Myb and C/EBPβ. By contrast, mutation (Mut19Luc) of the proximal C/EBP-binding site did not abolish synergistic activation of the promoter. The overall promoter activity of this mutant was strongly reduced, however. These observations suggested that Myb-binding site A and the C/EBP-binding site located immediately upstream are necessary for synergistic activation. That these two binding sites were also sufficient for the synergistic effect was demonstrated in two ways. First, addition of Myb-binding site A and the adjoining C/EBP-binding site to a minimal *mim*-1 promoter (Del130/33Luc) was sufficient to confer synergistic Myb and C/EBP responsiveness onto the minimal promoter. Point mutation of either the Myb-binding site (DelMMutLuc) or the C/EBP-binding site (DelEMutLuc) abolished the synergistic effect, confirming that both binding sites were required. Second, fusion of three copies of a cassette containing Myb-binding site A and the adjoining C/EBP-binding site to the herpes simplex virus thymidine kinase promoter also yielded a reporter gene that was activated synergistically by v-Myb and C/EBPβ. We concluded from these results that the distal C/EBP-binding site and Myb-binding site A are necessary and sufficient for the synergistic activation of the promoter by v-Myb and C/EBPβ.

v-Myb and C/EBPβ interact with each other in vitro. The observation that closely spaced Myb- and C/EBP-binding sites are involved in the cooperation of v-Myb and C/EBPβ raised the possibility that the two factors interact with each other. To examine this possibility, we performed far-Western blotting experiments. In one experiment (Fig. 2A), protein extracts from bacteria expressing chicken C/EBPβ were fractionated by SDS-PAGE, blotted onto nitrocellulose, and incubated with in vitro-translated [^{35}S]methionine-labeled v-Myb. As a control, protein extracts from bacteria lacking C/EBPβ were analyzed in parallel. We used total bacterial protein rather than purified C/EBPβ to demonstrate the specificity of the binding reaction. The C/EBPβ was not the most abundant protein present. As an additional control, we performed far-Western blotting experiments with radiolabeled in vitro translation reactions to which no template DNA had been added. As shown in Fig. 2A, v-Myb specifically bound to C/EBPβ. Most of the bacterial proteins, some of which were more abundant than C/EBPβ, did not interact with v-Myb.

In a second experiment (Fig. 2B), protein extracts of bacteria expressing v-Myb were size fractionated, blotted onto nitrocellulose, and incubated with soluble, bacterially expressed C/EBPβ, which was then visualized by C/EBPβ-specific antiserum. As control, parallel blots were incubated with the unrelated *E. coli* TrpE protein expressed by the ptrp9 expression vector. As shown in Fig. 2B, bacterially expressed C/EBPβ

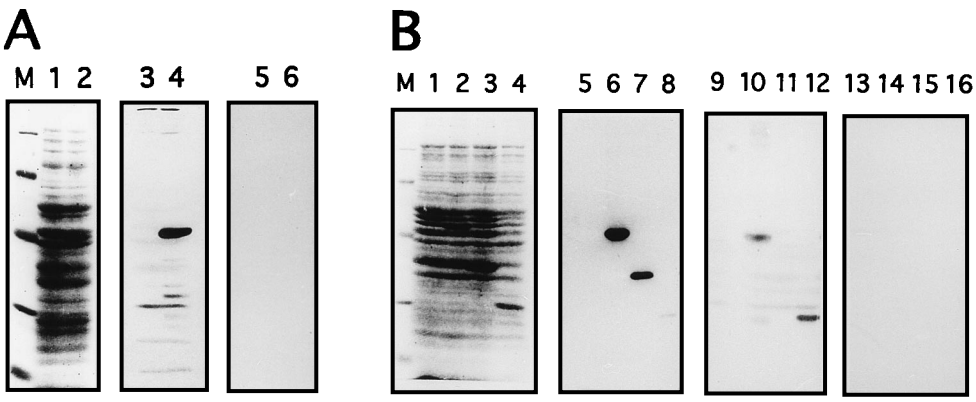


FIG. 2. Interaction of v-Myb and C/EBPβ in a far-Western blot. (A) Total protein from bacteria expressing chicken C/EBPβ (lanes 2, 4, and 6) or from control bacteria not expressing C/EBPβ (lanes 1, 3, and 5) was fractionated by SDS-PAGE and stained with Coomassie brilliant blue (lanes 1 and 2) or blotted onto nitrocellulose. Blots were incubated with in vitro-translated radiolabeled v-Myb (lanes 3 and 4) or with a control in vitro translation reaction mixture lacking v-Myb (lanes 5 and 6). The blots were then analyzed by autoradiography. Lane M shows molecular weight markers. (B) Total bacterial protein extracts lacking v-Myb (lanes 1, 5, 9, and 13) or containing full-length (lanes 2, 6, 10, and 14), amino-terminally truncated (lanes 3, 7, 11, and 15), or carboxy-terminally truncated (lanes 4, 8, 12, and 16) v-Myb (encoded by expression vectors pVM2028, pVM2008, and pVM2062, respectively) were fractionated by SDS-PAGE and stained with Coomassie brilliant blue (lanes 1 to 4) or blotted onto nitrocellulose. Blots were immunostained with a mixture of monoclonal Myb-specific antibodies myb2–37 and CB100 (lanes 5 to 8) or incubated with soluble, bacterially expressed chicken C/EBPβ (lanes 9 to 12) or *E. coli* TrpE protein (lanes 13 to 16), followed by immunostaining with antiserum against C/EBPβ. The poor visibility of carboxy-terminally truncated v-Myb (lane 8) is due to the low affinity of the CB100 antibody (relative to the myb2-37 antibody).

interacted specifically with v-Myb, confirming the result of the experiment shown in Fig. 2A.

Identification of domains of C/EBPβ and v-Myb involved in binding to each other. To determine which part of v-Myb interacts with C/EBPβ, we expressed partially deleted versions of v-Myb in bacteria and assessed their ability to bind to C/EBPβ by far-Western blotting. As shown in Fig. 2B, C/EBPβ did not bind to an amino-terminal v-Myb deletion mutant completely lacking the DNA-binding domain. A carboxy-terminal deletion mutant, however, bound to C/EBPβ with similar efficiency to that of full-length v-Myb. Thus, binding of C/EBPβ to v-Myb was dependent on the v-Myb DNA-binding domain.

The v-Myb DNA-binding domain consists of two related direct repeats, both of which are required for DNA binding (12, 21, 37). To map more precisely which part of this domain interacts with C/EBPβ, we used a set of v-Myb mutants carrying small deletions in the DNA-binding domain. These mutants are illustrated schematically in Fig. 3A. As shown in Fig. 3A, panel 1, deletion of a small region located in the center of the DNA-binding domain resulted in substantially reduced C/EBPβ-binding activity, suggesting that the central portion of the Myb DNA-binding domain is essential for the interaction of v-Myb and C/EBPβ. We also assessed the DNA-binding activity of the deletion mutants. As shown in Fig. 3A, panel 2, deletions within the second of the two repeats that make up the v-Myb DNA-binding domain completely destroyed the DNA-binding activity. Interestingly, some of these mutants still bound to C/EBPβ, indicating that DNA-binding and C/EBPβ-binding activities of v-Myb are independent of each other.

To identify which part of C/EBPβ is involved in interacting with v-Myb, we tested the ability of C/EBPβ mutants to bind to v-Myb. In the mutant CCRmut, 3 amino acids of the basic region of CEBPβ are replaced by proline residues (5). In the mutant CCRΔ260–275, the basic region of C/EBPβ has been deleted. As shown in Fig. 3B, both mutants no longer bound to v-Myb, suggesting that the basic region of C/EBPβ is required for the interaction. By contrast, deletion of N-terminal sequences of C/EBPβ did not interfere with binding to v-Myb. Taken together, the results illustrated in Fig. 3 show that the interaction of v-Myb and C/EBPβ is mediated by the DNA-binding domains of both proteins.

C/EBPβ and v-Myb interact in solution. To demonstrate the interaction between v-Myb and CEBPβ by a different assay, we incubated radiolabeled C/EBPβ with bacterially expressed v-Myb in solution and then determined the amount of bound C/EBPβ by immunoprecipitation with Myb-specific antiserum. To control the specificity of the interaction, we performed similar incubations with in vitro-translated transcription factor PU.1. In addition, we used the bacterially expressed v-Myb deletion mutant ID9, which shows greatly reduced binding to C/EBPβ (Fig. 3A). As illustrated in Fig. 4, in vitro-translated C/EBPβ interacted with bacterially expressed full-length v-Myb but not with the ID9 mutant of v-Myb. In complementary experiments, we incubated in vitro-translated, radiolabeled v-Myb with bacterially expressed C/EBPβ and immunoprecipitated it with antiserum raised against C/EBPβ. As control, we again used radiolabeled PU.1, as well as bacterial C/EBPβ carrying a deletion of the basic region (CCRΔ260–275). As shown in Fig. 3B, this deletion mutant does not interact with v-Myb. Figure 4 shows that in vitro-translated v-Myb interacts specifically with full-length but not partially deleted bacterial C/EBPβ. These experiments confirm that v-Myb and C/EBPβ interact in vitro and that the DNA-binding domains of both proteins are responsible for the interaction.

In vivo interaction of v-Myb and C/EBPβ. To investigate whether v-Myb and C/EBPβ interact in vivo, AMV-transformed myeloblasts expressing v-Myb, C/EBPβ, and the *mim*-1 gene were labeled with [^{35}S]methionine. An extract from these cells was then analyzed by immunoprecipitation, using preimmune serum and Myb- or C/EBPβ-specific antiserum. The immunoprecipitates were dissolved, divided into two aliquots, and reprecipitated with Myb- or C/EBPβ-specific antiserum. By performing sequential precipitations, the background of nonspecifically precipitated proteins is eliminated, thus permitting the detection of small amounts of coprecipitated protein. As shown in Fig. 5A, a small amount of v-Myb was coprecipitated with C/EBPβ. Likewise, some C/EBPβ was coprecipitated with v-Myb. Coprecipitation of the two proteins was not observed when control serum was used for the first immuno-

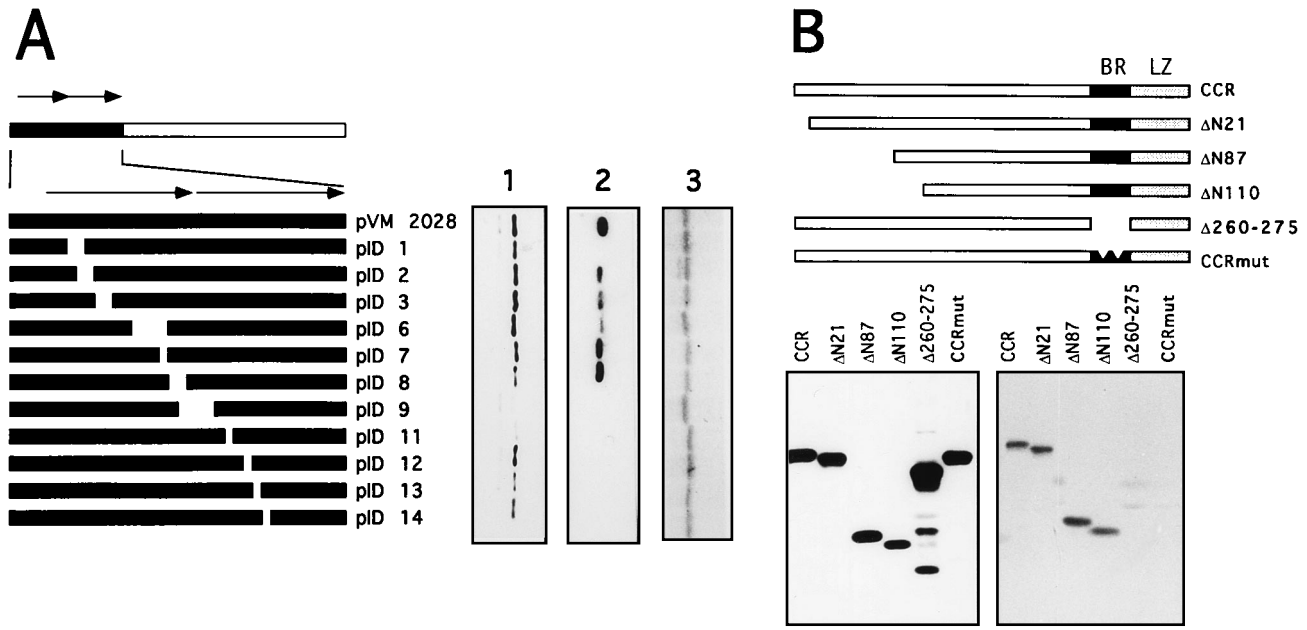


FIG. 3. Far-Western blot analysis of domains of v-Myb and C/EBPβ involved in direct interaction. (A) A schematic illustration of the v-Myb deletion mutants used and the two repeats making up the Myb DNA-binding domain are shown on the left. Plasmids encoding these mutants (pID1 to pID14) have been described previously (37). Equal amounts of purified, bacterially expressed full-length or partially deleted v-Myb proteins were fractionated by SDS-PAGE and blotted onto nitrocellulose. The blots were incubated with bacterially expressed chicken C/EBPβ and subsequently immunostained with C/EBPβ-specific antiserum (lane 1) or with ^{32}P-labeled pbluescript plasmid DNA (lane 2). Lane 3 shows the proteins stained with Coomassie brilliant blue. (B) Equal amounts of purified, bacterially expressed full-length, partially deleted, or mutated chicken C/EBPβ were fractionated by SDS-PAGE, blotted onto nitrocellulose, and immunostained with chicken C/EBPβ-specific antiserum (left) or incubated with in vitro-translated radiolabeled v-Myb, followed by autoradiography (right). The C/EBPβ mutants are illustrated schematically at the top. BR and LZ denote basic region and leucine zipper, respectively. The faint bands seen in the Δ260–275 and CCRmut lanes are derived from contaminating bacterial proteins.

precipitation step, suggesting that coprecipitation was not an artifact caused by insufficient washing or nonspecific binding of the proteins to the protein A-Sepharose. We also confirmed by Western blotting that neither the Myb-specific antiserum nor the C/EBPβ-specific antiserum cross-reacted with C/EBPβ and v-Myb (data not shown). As an additional control, we performed an identical experiment with the chicken macrophage cell line HD11; these cells express similar levels of C/EBPβ to AMV-transformed myeloblasts but lack v-Myb. The Myb-specific antiserum should therefore fail to coprecipitate C/EBPβ from HD11 cell extracts. As shown in Fig. 5B, C/EBPβ was indeed not coprecipitated by the Myb-specific antiserum.

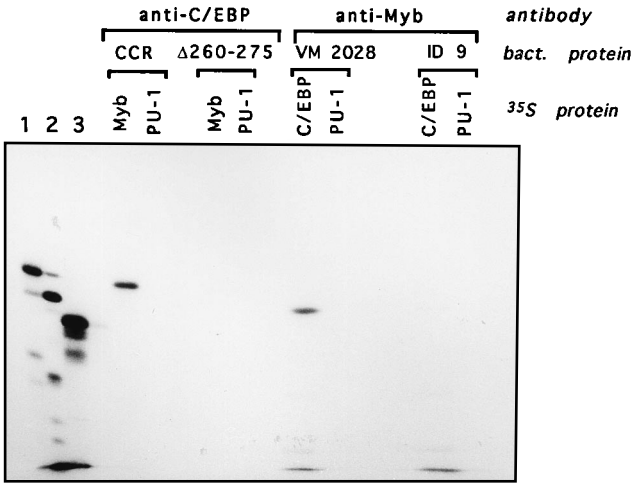


FIG. 4. Interaction of v-Myb and C/EBPβ in solution. Equal amounts of in vitro-translated [^{35}S]methionine-labeled v-Myb, PU.1, or C/EBPβ were incubated with unlabeled, bacterially expressed C/EBPβ (derived from ptrp9CCR or ptrp9CCRΔ260–275) or v-Myb (derived from expression vectors pVM2028 or pID9), followed by immunoprecipitation with C/EBPβ- or Myb-specific antiserum (as indicated at the top of the figure). The immunoprecipitated proteins were then analyzed by SDS-PAGE and autoradiography. Samples of the in vitro-translated proteins used for the experiment are shown in lanes 1 (v-Myb), 2 (C/EBPβ), and 3 (PU.1). The amounts used for coimmunoprecipitation were 50-fold larger that the amounts shown in lanes 1 to 3. Cross-contamination of proteins in lanes 1 and 2 was due to a loading artifact.

To demonstrate that the in vivo coprecipitation is dependent on the protein domains mapped in vitro, we cotransfected expression vectors for v-Myb and C/EBPβ into the quail fibroblast cell line QT6, which contains only low levels of endogenous C/EBPβ and no v-Myb, and used the transfected cells for coimmunoprecipitation studies. We used expression vectors for wild-type v-Myb and C/EBPβ (Fig. 5C) or for v-Myb lacking the DNA-binding domain and C/EBPβ lacking the basic region (Fig. 5D). As shown in Fig. 5C and D, the wild-type but not the mutant proteins could be coimmunoprecipitated. This experiment therefore confirms that the two proteins interact in vivo and shows that this interaction is dependent on the protein domains identified before.

Direct interaction of v-Myb and C/EBPβ is required for synergistic activation of the *mim*-1 promoter. The experiments described so far did not prove that the interaction of C/EBPβ and v-Myb is necessary for synergistic activation of the *mim*-1 promoter. Although the interaction of v-Myb and C/EBPβ was specific, as demonstrated by the use of mutant protein, the interaction appeared to be relatively weak, which raised some doubt about its relevance to the synergistic behavior of v-Myb and C/EBPβ. To demonstrate that the interaction is functionally relevant, we investigated whether we could replace C/EBPβ by another transcription factor not interacting with v-Myb and still observe synergistic activation. We selected hu-

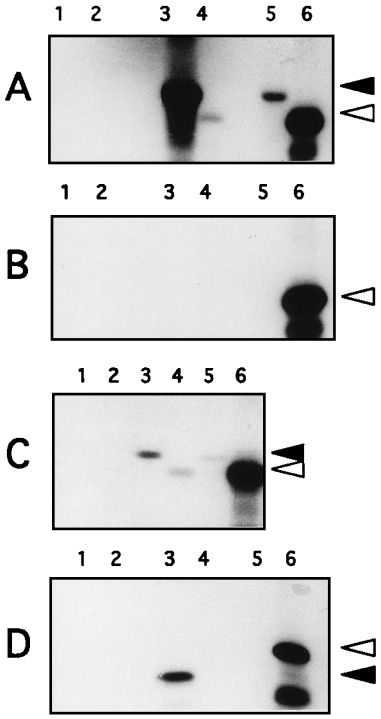


FIG. 5. C/EBPβ and v-Myb interact in vivo. (A and B) Extracts from [^{35}S]methionine-labeled BM2 cells (A) or HD11 cells (B) were immunoprecipitated with normal rabbit serum (lanes 1 and 2) or with rabbit serum against v-Myb (lanes 3 and 4) or C/EBPβ (lanes 5 and 6). Immunoprecipitates were dissolved, and aliquots were reprecipitated with rabbit serum against v-Myb (lanes 1, 3, and 5) or C/EBPβ (lanes 2, 4, and 6), followed by SDS-PAGE and autoradiography. Equivalent amounts of all fractions were loaded onto the gel. (C and D) QT6 cells were cotransfected with expression vectors pVM134 and pCRNC-CCR for full-length v-Myb and C/EBPβ (C) or with pVM130 (encoding v-Myb without the DNA-binding domain) and pCRNC-CCRΔ260–275 (encoding C/EBPβ without the basic region) (D). Transfected cells were radiolabeled and subjected to immunoprecipitation as described for panel A. Black and white arrowheads mark v-Myb and C/EBPβ, respectively.

man c-Jun for this experiment because we found that c-Jun does not bind efficiently to v-Myb (Fig. 6A and E). To replace C/EBPβ by c-Jun, we mutated the C/EBP-binding site of plasmid pDel130/33Luc such that it was recognized by c-Jun. It is important to note that the Jun-binding site (TRE) in the mutant reporter gene occupies the same position as the C/EBP-binding site in the original reporter gene. We then assessed the ability of the mutant promoter (referred to as pDel130/33TRELuc) to be activated by v-Myb, c-Jun, and C/EBPβ. As illustrated in Fig. 6B, C/EBPβ still activated the mutant promoter and synergized with v-Myb, indicating that the mutation per se had not destroyed the ability of the promoter to support synergistic activation. Binding of C/EBPβ to the mutant promoter was confirmed by in vitro binding experiments (data not shown). c-Jun bound to the mutant promoter in vitro (data not shown). c-Jun bound to the mutant promoter in vitro (data not shown) and *trans*-activated it in vivo but, surprisingly, did not synergize with v-Myb (Fig. 6B). Thus, C/EBPβ cannot be replaced by c-Jun without destroying the synergy with v-Myb. Failure to synergize was not due to saturation of the promoter with c-Jun, because increasing amounts of c-Jun expression vector increased the activity of the promoter proportionally. The lack of synergy could also not be explained by better binding of C/EBPβ (compared with c-Jun) to the TRE site in the mutated reporter gene pDel130/33TRELuc. In vitro DNA-binding experiments showed that C/EBPβ does not bind more efficiently to this site; mutation of the C/EBP-site to a TRE site in fact weakened the binding of C/EBPβ and increased the binding of c-Jun (data not shown). By performing gel retardation experiments with bacterially expressed c-Jun and v-Myb and an oligonucleotide containing the binding sites for both proteins, we also excluded the possibility that v-Myb and c-Jun sterically interfere with each other's binding. These experiments showed that both proteins can bind simultaneously to the mutant promoter (data not shown). To demonstrate a link between the interaction of v-Myb and C/EBPβ and their ability to synergize, we generated recombinants between c-Jun and C/EBPβ and investigated their properties. Interestingly, a recombinant (JC) whose DNA-binding domain is derived from C/EBPβ synergized with v-Myb as efficiently as did C/EBPβ itself. By contrast, the reverse recombinant (CJ) whose DNA-binding domain is derived from c-Jun failed to synergize with v-Myb (Fig. 6C and D). Thus, the DNA-binding domain of C/EBPβ is necessary as well as sufficient to confer the ability to synergize with v-Myb onto c-Jun. A recombinant containing only the basic region of C/EBPβ also synergized with v-Myb, whereas a recombinant containing only the basic region of c-Jun embedded in C/EBP failed to synergize (data not shown), suggesting that the basic region of C/EBP and not the leucine zipper is responsible for conferring the ability to synergize with v-Myb.

To investigate whether replacement of the Jun DNA-binding domain by that of C/EBPβ had also conferred the ability to interact with v-Myb on the hybrid protein, we performed the in vivo coprecipitation experiments in Fig. 6E. Expression vectors for v-Myb and c-Jun or the hybrid protein JC were cotransfected into quail fibroblasts, followed by coimmunoprecipitation analysis. As shown in Fig. 6E, lanes 7 to 12, v-Myb and c-Jun did not coprecipitate with, thus confirming that v-Myb does not interact with c-Jun. By contrast, the hybrid protein (JC) was coprecipitated with v-Myb (lanes 1 to 6). Taken together, the experiments in Fig. 6 clearly demonstrate a linkage between the ability of the proteins to interact and their ability to synergize with v-Myb. This strongly argues for the physiological relevance of the interaction between Myb and C/EBP and supports the notion that the synergistic properties of v-Myb and C/EBP are dependent on the interaction of the two proteins.

Synergistic activation of the *mim*-1 promoter by C/EBPβ and the Myb-related proteins A-Myb and B-Myb. We have shown that the DNA-binding domain of v-Myb is responsible for direct protein-protein interactions with C/EBPβ. Since the proteins encoded by the *myb*-related genes A-*myb* and B-*myb* have similar DNA-binding domains, it was of interest to determine whether the *myb*-related proteins A-Myb and B-Myb could also synergize with C/EBPβ. Since B-Myb lacks a transactivation domain (8), we used an expression vector for a B-Myb/VP16 fusion protein for cotransfection. As shown in Fig. 7, A-Myb as well as B-Myb/VP16 synergized with C/EBPβ. Thus, the ability to synergize with C/EBPβ is shared by all known members of the Myb family.

DISCUSSION

Regulatory sequences of eucaryotic genes commonly contain binding sites for multiple transcription factors and provide a basis for combinatorial interactions between different factors. These complex regulatory regions, referred to as composite response elements (26, 27, 38), are important junction points in the transcription factor network; they mediate the integration of multiple regulatory signals and allow different transcription

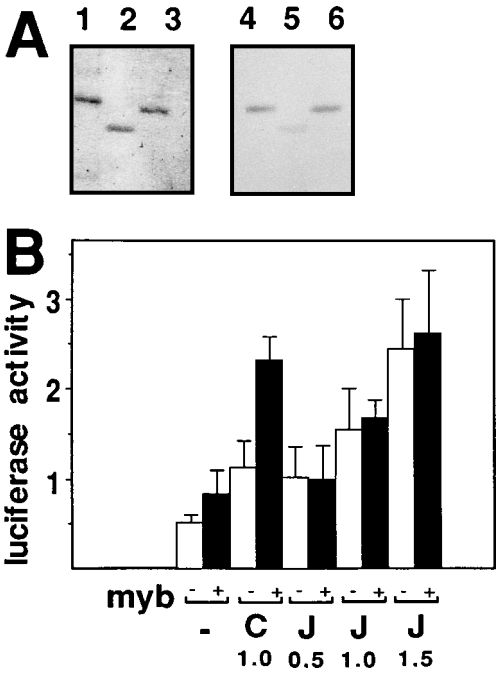
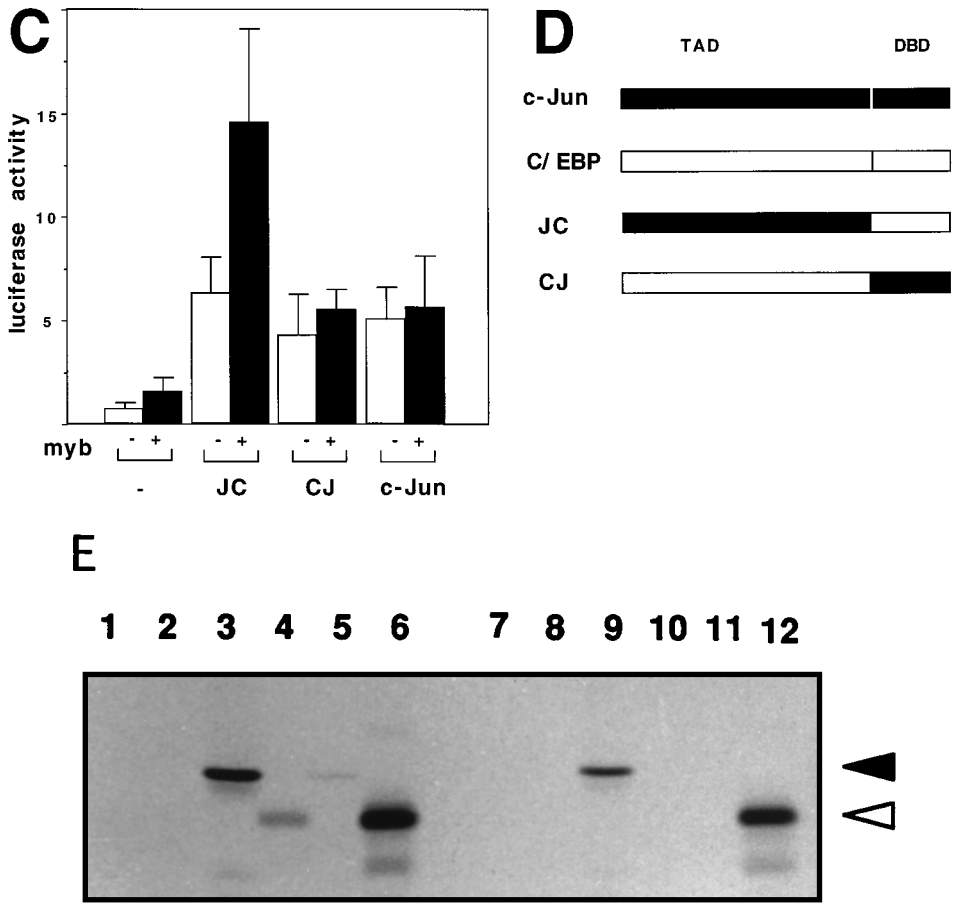


FIG. 6. Replacement of C/EBPβ by c-Jun: the DNA-binding domain of C/EBPβ mediates synergy with v-Myb. (A) Chicken C/EBPβ (lanes 1 and 4), human c-Jun (lanes 2 and 5), and chicken C/EBPα (lanes 3 and 6) were fractionated by SDS-PAGE and stained with Coomassie brilliant blue (lanes 1 to 3) or blotted and incubated with radiolabeled v-Myb (lanes 4 to 6). (B) QT6 cells were cotransfected with 3 μg of pDel130/33TRELuc, 1 μg of pCH110, and expression vectors for v-Myb, chicken C/EBPβ, and human c-Jun, as shown at the bottom. The following amounts of expression vectors were used: Myb expression vector pVM134 (+) and the frameshift control vector pVM111 (−), 0.2 μg; C/EBPβ expression vector pCRNC-CCR (C), 1 μg; c-Jun expression vector pRSVc-jun (J), 0.5, 1, or 1.5 μg. The bars show the average luciferase activity, normalized with respect to the activity of the β-galactosidase plasmid pCH110. Standard deviations are indicated by thin lines. (C) QT6 cells were transfected as in panel B, except that expression vectors for the Jun-C/EBPβ chimeras JC and CJ were used in this experiment. c-Jun, JC, and CJ expression vectors were used at 1 μg (c-Jun and CJ) or 0.5 μg (JC), respectively. As shown by Western blotting, similar amounts of the Jun proteins were produced under these conditions (data not shown). Transfections were analyzed as in panel B. (D) Schematic illustration of the Jun-C/EBPβ chimeras JC and CJ. TA and DBD refer to transactivation region and DNA-binding domain, respectively. (E) QT6 cells were cotransfected with expression vectors pVM134 and pcDNA3-Bst-Jun-CCR (lanes 1 to 6) or pVM134 and pRSVc-Jun (lanes 7 to 12) encoding full-length v-Myb, the Jun-C/EBP hybrid, or c-Jun. (E) The cells were radiolabeled, and extracts were immunoprecipitated with normal rabbit serum (lanes 1, 2, 7, and 8) or with rabbit serum against v-Myb (lanes 3, 4, 9, and 10) or c-Jun (lanes 5, 6, 11, and 12). Immunoprecipitates were dissolved, and aliquots were reprecipitated with rabbit serum against v-Myb (lanes 1, 3, 5, 7, 9, and 11) or c-Jun (lanes 2, 4, 6, 8, 10, and 12), followed by SDS-PAGE and autoradiography. Equivalent amounts of all fractions were loaded onto the gel. Black and white arrowheads mark v-Myb and JC or c-Jun, respectively.



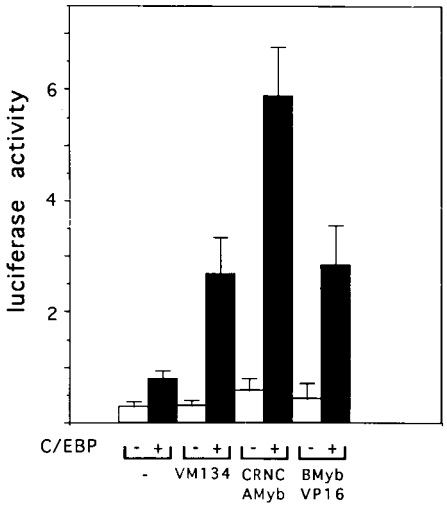


FIG. 7. Synergy between C/EBPβ and Myb-related proteins A-Myb and B-Myb. QT6 cells were transfected with 3 μg of pDel130/33Luc, 1 μg of pCH110, and the expression vectors indicated below the columns. The following amounts of expression vectors were used: C/EBPβ expression vector pCRNC-CCR (+) and frameshift control vector pCRNC-fsCCR (−), 0.3 μg; Expression vectors for v-Myb (pVM134), A-Myb (pCRNC-A*myb*), and B-Myb/VP16 (pChB-*myb*VP16), 1 μg. Bars show the average luciferase activity, normalized with respect to the activity of the β-galactosidase plasmid pCH110. Standard deviations are indicated by thin lines.

factors to communicate with each other. The analysis of the chicken *mim*-1 gene described here provides compelling evidence for the existence of a composite response element for Myb and C/EBP.

Identification of a composite Myb-C/EBP response element involved in myelomonocyte-specific gene expression. Previous work has shown that the activation of the myelomonocyte-specific *mim*-1 gene, a physiological target gene for v-Myb, requires synergistic cooperation of v-Myb with members of the C/EBP transcription factor family, such as C/EBPα, C/EBPβ, and C/EBPδ, all of which are highly expressed in myelomonocytic cells (5, 31, 32, 41, 42). Our work identifies a composite Myb-C/EBP response element, which consists of closely spaced Myb- and C/EBP-binding sites and is both necessary for synergistic activation of the *mim*-1 promoter by v-Myb and C/EBPβ and sufficient to confer synergistic Myb and C/EBP responsiveness onto a heterologous promoter. It is intriguing that *mim*-1 is not the only myelomonocyte-specific gene whose expression is dependent on Myb and C/EBP; the chicken lysozyme gene, another myelomonocyte-specific Myb target gene (4, 15), is also activated by v-Myb in combination with C/EBP family members (5, 32). Interestingly, the promoter of the chicken lysozyme gene contains C/EBP-binding sites (31; our unpublished observations) juxtaposed to potential Myb-binding sites; in addition, reporter genes containing the lysozyme promoter are activated synergistically by v-Myb and C/EBP (our unpublished observations). These observations raise the interesting possibility that composite elements such as the one identified here perhaps play a more general role in specifying myelomonocyte-specific gene expression. It will therefore be interesting to investigate whether Myb-C/EBP response elements are frequently found in myelomonocyte-specific genes.

Protein-protein interaction between v-Myb and C/EBPβ. A second major conclusion of our work is that v-Myb and C/EBPβ are interacting with each other. In vitro binding experiments with bacterially expressed v-Myb and C/EBPβ suggests that the interaction of the two proteins does not require other eucaryotic proteins and, hence, that it may be direct. We have shown that v-Myb and C/EBPβ interact via their DNA-binding domains. Thus, the potential leucine zipper region of v-Myb, which is located at the carboxy terminus of the protein and has been postulated to be involved in negative regulation of c-Myb function (40), is not involved in binding to C/EBPβ. Our deletion analysis of v-Myb has shown that some deletion mutants of v-Myb no longer bind to DNA but still interact with C/EBPβ. This indicates that DNA binding and C/EBP binding are distinct activities of v-Myb and that the interaction between v-Myb and C/EBPβ is DNA-binding independent.

C/EBPβ is the first interaction partner identified for v-Myb. We do not know whether the interaction with C/EBPβ is important only for the activation of myelomonocytic-specific genes or whether it is also relevant for the transforming activity of v-Myb. It is therefore possible that interaction with C/EBP is required for some but not all functions of v-Myb. Precise mapping of the interaction site will permit the construction of v-Myb mutants defective in C/EBP binding to address this issue.

The DNA-binding domain of v-Myb has previously been implicated in the interaction with another, as yet unidentified protein. The v-Myb proteins of AMV and avian leukemia virus E26, another *myb*-transducing chicken retrovirus, differ by several amino acids in their DNA-binding domains. It has been postulated that the differences in biological activity of the two viruses are due to differential interaction of another protein with the DNA-binding domains of the v-Myb proteins (15). It appears unlikely that C/EBPβ is identical to this hypothetical interaction partner, because we have found no difference in the ability of v-Myb from AMV or E26 to interact with C/EBPβ (unpublished results).

Several interacting proteins, such as NF-κB (24), c-Jun (13), and the glucocorticoid receptor (35), have already been described for C/EBPβ; we can now add v-Myb to this growing list. Interestingly, all of these proteins interact with the DNA-binding domain of C/EBPβ, either with the basic region or with the leucine zipper. It appears that C/EBPβ, in particular its DNA-binding domain, mediates crosstalk among several unrelated transcription factors and serves as a multifunctional integration point in the transcription factor network. This view is dramatized by the fact that C/EBPβ is the target of several signal transduction pathways (1, 16, 25, 30, 39, 44).

It is interesting that all of the interaction partners for C/EBPβ and v-Myb discussed above interact with the DNA-binding domains of these proteins. Clearly, the DNA-binding domains have multiple functions. They not only recognize specific nucleotide sequences but also function as contact points for intermolecular interactions with other transcription factors. Why the DNA-binding domains are hot spots for combinatorial interactions between different transcription factors can perhaps be rationalized from an evolutionary point of view. In general, the DNA-binding domains are the most highly conserved parts of most transcription factors. It seems quite possible that the strong evolutionary pressure to conserve the sequence recognition properties of these domains has generated highly conserved protein surfaces that have also been exploited for purposes other than sequence recognition, such as the establishment of combinatorial interactions and regulatory networks between different transcription factors.

Mechanistic and functional considerations of the C/EBP-Myb interaction. Because of the apparent weakness of the interaction of v-Myb and C/EBPβ, we were initially uncertain about its functional relevance. However, we have found that replacement of C/EBPβ by c-Jun in the Myb-C/EBP response

element leads to a complete loss of synergy with v-Myb and that the DNA-binding domain of C/EBPβ is sufficient to confer the ability to synergize and to interact with v-Myb on c-Jun. These surprising observations provide a strong argument for the idea that interaction between v-Myb and C/EBPβ is necessary for their cooperation. We have not yet addressed the mechanism by which the interaction of the two proteins leads to transcriptional synergy. Possibly, the interaction between Myb and C/EBP leads to cooperative DNA binding of the proteins to the adjacent binding sites in the composite response element. Another possibility is suggested by the recent finding that the activity of C/EBPβ is regulated by intramolecular interactions involving the basic region and adjacent sequences (23, 46). Interaction of C/EBPβ with Myb could lead to a conformational change of the protein and increase its transactivation potential. Clearly, further work is required to address these possibilities.

Previous work has shown that v-Myb in conjunction with C/EBP transcription factors is sufficient to activate the endogenous *mim*-1 gene even in cells in which the gene normally is not expressed (5, 32). The emerging view of Myb and C/EBP as a bipartite molecular switch specifying myelomonocyte-specific gene expression is dramatized by our identification of a composite response element for Myb and C/EBP and by the demonstration of an interaction between Myb and C/EBPβ. This close relationship of v-Myb with a transcription factor expressed mainly in myelomonocytic cells (at least within the hematopoietic system) (16) and the direct activation by Myb of differentiation-specific, myelomonocytic-specific genes (such as *mim*-1 and the lysozyme gene) is difficult to reconcile with the view of Myb as a transcription factor involved mainly in the self-renewal program of most hematopoietic progenitor cells. It seems that Myb, at least in the myelomonocytic lineage, performs a dual role. On one hand, Myb fulfills an essential role in the self-renewal program of immature hematopoietic cells, presumably by activating genes whose expression is required for proliferation. On the other hand, Myb also participates in the myelomonocytic differentiation program, by directly activating together with C/EBPβ or other C/EBP family member genes (such as *mim*-1 and the lysozyme gene), whose expression is induced as part of the differentiation program. Thus, Myb may be part of a switch mechanism that links and controls proliferation and differentiation of hematopoietic cells. The identification of a composite Myb-C/EBP response element and of C/EBPβ as an interaction partner for v-Myb provides a useful basis to further explore on the molecular level the role of v-Myb in this switch.

ACKNOWLEDGMENTS

We thank O. Burk and B. Mutschler for constructing some of the C/EBPβ mutants; P. Angel, C. F. Calkhoven, A. Hecht, P. Shaw, and A. E. Sippel for providing plasmids; and L. Lay for preparing the photographs.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB364/A4) and by the Max Planck Society.

REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression. NF-IL6 is a member of a C/EBP family. EMBO J. **9:**1897–1906.
2. **Angel, P., K. Hattori, T. Smeal, and M. Karin.** 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell **55:**875–885.
3. **Biedenkapp, H., U. Borgmeyer, A. E. Sippel, and K.-H. Klempnauer.** 1988. Viral *myb* oncogene encodes a sequence-specific DNA-binding activity. Nature (London) **355:**835–837.
4. **Burk, O., and K.-H. Klempnauer.** 1991. Estrogen-dependent alterations in differentiation state of myeloid cells caused by a v-*myb*/estrogen receptor fusion protein. EMBO J. **10:**3713–3719.
5. **Burk, O., S. Mink, M. Ringwald, and K.-H. Klempnauer.** 1993. Synergistic activation of the chicken *mim*-1 gene by v-*myb* and C/EBP transcription factors. EMBO J. **12:**2027–2038.
6. **Clarke, M. F., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. V. Prochownik.** 1988. Constitutive expression of a c-*myb* cDNA blocks friend murine erythroleukemia cell differentiation. Mol. Cell. Biol. **8:**884–892.
7. **Evan, G. I., G. K. Lewis, and J. M. Bishop.** 1984. Isolation of monoclonal antibodies specific for products of avian oncogene *myb*. Mol. Cell. Biol. **4:**2843–2850.
8. **Foos, G., S. Grimm, and K.-H. Klempnauer.** 1992. Functional antagonism between members of the *myb* family: B-*myb* inhibits v-*myb*-induced gene activation. EMBO J. **11:**4619–4629.
9. **Foos, G., S. Grimm, and K.-H. Klempnauer.** 1994. The chicken A-*myb* protein is a transcriptional activator. Oncogene **9:**2481–2488.
10. **Gewirtz, A. M., and B. Calabretta.** 1988. A c-*myb* antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. Science **242:**1303–1306.
11. **Graf, T.** 1992. Myb: a transcriptional activator linking proliferation and differentiation in hematopoietic cells. Curr. Opin. Genet. Dev. **2:**249–255.
12. **Howe, K. M., C. F. L. Reakes, and R. J. Watson.** 1990. Characterization of the sequence-specific interaction of mouse c-*myb* protein with DNA. EMBO J. **9:**161–169.
13. **Hsu, W., T. K. Kerppola, P.-L. Chen, T. Curran, and S. Chen-Kiang.** 1994. Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. Mol. Cell. Biol. **14:**268–276.
14. **Ibanez, C. E., and J. S. Lipsick.** 1990. *trans* activation of gene expression by v-*myb*. Mol. Cell. Biol. **10:**2285–2293.
15. **Introna, M., J. Golay, J. Frampton, T. Nakano, S. A. Ness, and T. Graf.** 1990. Mutations in v-myb alter the differentiation of myelomonocytic cells transformed by the oncogene. Cell **63:**1287–1297.
16. **Katz, S., E. Kowentz-Leutz, C. Müller, K. Meese, S. A. Ness, and A. Leutz.** 1993. The NF-M transcription factor is related to C/EBPβ and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. EMBO J. **12:**1321–1332.
17. **Klempnauer, K.-H., H. Arnold, and H. Biedenkapp.** 1989. Activation of transcription by v-myb: evidence for two different mechanisms. Genes Dev. **3:**1582–1589.
18. **Klempnauer, K.-H., C. Bonifer, and A. E. Sippel.** 1986. Identification and characterization of the protein encoded by the human c-myb proto-oncogene. EMBO J. **5:**1903–1911.
19. **Klempnauer, K.-H., T. J. Gonda, and J. M. Bishop.** 1982. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell **31:**453–463.
20. **Klempnauer, K.-H., G. Ramsay, J. M. Bishop, M. G. Moscovici, C. Moscovici, J. P. McGrath, and A. D. Levinson.** 1983. The product of the retroviral transforming gene v-myb is a truncated version of the protein encoded by the cellular oncogene c-myb. Cell **33:**345–355.
21. **Klempnauer, K.-H., and A. E. Sippel.** 1987. The highly conserved amino-terminal region of the protein encoded by the v-myb oncogene functions as a DNA-binding domain. EMBO J. **6:**2719–2725.
22. **Klemsz, M. J., S. R. McKercher, A. Celada, C. van Beveren, and R. A. Maki.** 1990. The macrophage and B cell-specific transcription factor Pu.1 is related to the ets oncogene. Cell **61:**113–124.
23. **Kowenz-Leutz, E., G. Twamley, S. Ansieau, and A. Leutz.** 1994. Novel mechanism of C/EBPβ (NF-M) transcriptional control: activation through derepression. Genes Dev. **8:**2781–2791.
24. **LeClair, K. P., M. A. Blanar, and P. A. Sharp.** 1992. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA **89:**8145–8149.
25. **Metz, R., and E. Ziff.** 1991. cAMP stimulates the C/EBP-related transcription factor rNF-IL6 to trans-locate to the nucleus and induce c-fos transcription. Genes Dev. **5:**1754–1766.
26. **Miner, J. N., and K. R. Yamamoto.** 1991. Regulatory crosstalk at composite response elements. Trends Biochem. Sci. **16:**423–426.
27. **Miner, J. N., and K. R. Yamamoto.** 1992. The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. Genes Dev. **6:**2491–2501.
28. **Moscovici, C., M. G. Moscovici, H. Jiminez, M. M. C. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of japanese quail. Cell **11:**95–103.
29. **Mucenski, M. L., K. McLain, A. B. Kier, S. H. Swerdlow, C. M. Schreiner, T. A. Miller, D. W. Pietryga, W. J. Scott, Jr., and S. S. Potter.** 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell **65:**677–689.
30. **Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira.** 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA **90:**2207–2211.
31. **Natsuka, S., S. Akira, Y. Nishio, S. Hashimoto, T. Sugita, H. Isshiki, and T. Kishimoto.** 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood **79:**460–466.
32. **Ness, S. A., E. Kowentz-Leutz, T. Casini, T. Graf, and A. Leutz.** 1993. Myb

and NF-M: combinatorial activators of myeloid genes in heterologous cell types. Genes Dev. **7:**749–759.
33. **Ness, S. A., A. Marknell, and T. Graf.** 1989. The v-myb oncogene product binds to and activates the promyelocyte-specific min-1 gene. Cell **59:**1115–1125.
34. **Nishina, Y., H. Nakagoshi, F. Imamoto, T. J. Gonda, and S. Ishii.** 1989. Trans-activation by the c-myb proto-oncogene. Nucleic Acids Res. **17:**107–117.
35. **Nishio, Y., H. Isshiki, T. Kishimoto, and S. Akira.** 1993. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat α1-acid glycoprotein gene via direct protein-protein interaction. Mol. Cell. Biol. **13:**1854–1862.
36. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–457.
37. **Oehler, T., H. Arnold, H. Biedenkapp, and K.-H. Klempnauer.** 1990. Characterization of the v-myb DNA binding domain. Nucleic Acids Res. **18:**1703–1710.
38. **Pearce, D., and K. R. Yamamoto.** 1993. Mineralcorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science **259:**1161–1165.
39. **Poli, V., F. P. Mancine, and R. Cortese.** 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell **63:**643–653.
40. **Sakura, H., C. Kanei-Ishii, T. Nagase, H. Nakagoshi, T. J. Gonda, and S. Ishii.** 1989. Delineation of three functional domains of the transcriptional activator encoded by the c-myb proto-oncogene. Proc. Natl. Acad. Sci. USA **86:**5758–5762.
41. **Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman.** 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood **80:**1725–1735.
42. **Sterneck, E., C. Müller, S. Katz, and A. Leutz.** 1992. Autocrine growth induced by kinase-type oncogenes in myeloid cells requires AP-1 and NF-M, a myeloid-specific, C/EBP-like factor. EMBO J. **11:**115–126.
43. **Todokoro, K., R. J. Watson, H. Higo, H. Amanuma, S. Kuramochi, H. Yanagisawa, and Y. Ikawa.** 1988. Down-regulation of c-myb gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. Proc. Natl. Acad. Sci. USA **79:**8900–8904.
44. **Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier.** 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature (London) **364:**544–547.
45. **Weston, K., and J. M. Bishop.** 1989. Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb. Cell **58:**85–93.
46. **Williams, S. C., M. Baer, A. J. Dillner, and P. F. Johnson.** 1995. CRP2 (C/EBPβ) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. EMBO J. **14:**3170–3183.