Analysis of the v-myb structural components important for transactivation of gene expression

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ABSTRACT

In order to define the domains of the v-myb protein that are important for transactivation of gene expression, we have studied transactivation by the v-myb gene and a set of v-myb deletion mutants using transient transfection assays in NIH 3T3 cells. Analysis of the set of v-myb deletion products demonstrated that a previously unidentified region in the carboxyl-terminal portion of the protein is required for transactivation. This region lies between amino acids 295 – 356 with respect to the 5' end of the v-myb gene. Switching the v-myb DNA binding domain with the DNA binding domain of the rat glucocorticoid receptor (rGR) switched the cis-element requirement for v-myb action: only reporters containing glucocorticoid response elements were activated by myb-rGR fusion proteins. The carboxyl terminal region essential for transactivation by the intact v-myb gene was also necessary for transactivation by the rGR-fusion gene. Carboxyl-terminal deletion mutations that encompassed the novel transactivation region were able to block wild-type v-myb transactivation when tested in transient co-expression assays. In an unexpected sidelight to our studies, we could demonstrate that the lacZ gene present in the prokaryotic vector sequences contained a DNA element that fortuitously can act as a v-myb-dependent enhancer element, and that v-myb protein can bind to this element in vitro. The lacZ enhancer contains the myb consensus DNA binding site YAAC(G/T)G.

INTRODUCTION

The v-myb oncogene is the genetic component of avian myeloblastosis virus (AMV) and avian leukemia virus E26 responsible for the transformation of myelomonocytic hematopoietic cells by these retroviruses in vivo and in vitro (1). The v-myb gene encodes a 48 kD nuclear phosphoprotein that is a truncated and mutated version of the chicken proto-oncogene, c-myb (2). The c-myb gene is expressed primarily in immature hematopoietic and lymphoid cells (3), but also in serum stimulated

fibroblasts (4). Studies that used anti-sense c-myb oligonucleotides suggest that the gene is necessary for the differentiation and growth of lymphoid cells (5, 6).

It has been known for some time that the v-myb gene product binds to DNA (7). It was also demonstrated that this DNA binding activity is essential for cellular transformation by the oncogene (8). Thus, it is likely that both the oncogenic and normal forms of myb function as regulators of gene expression in eukaryotic cells (7, 8). Experiments that used myb genes fused to the region encoding the DNA binding domain of the yeast transcriptional activator GAL4 have conclusively demonstrated that myb proteins are transcriptional activators (9). One transcriptional activator domain of myb, that is discrete from the DNA-binding domain, is highly charged; however, it is not the typical negatively-charged alpha-helical activator domain found in many other transcription factors (9).

Klemppner and colleagues have demonstrated that the sequence YAAC(T/G)G is a consensus DNA binding site for the myb oncogene product (10). It has been shown by several groups that this sequence can act as a myb-dependent enhancer of transcription (9, 11, 12, 13). In addition, a gene expressed in monocytes has been shown to contain the myb-enhancer sequence (13).

As indicated above, the v-myb gene contains deletions relative to the c-myb proto-oncogene. In addition, v-myb contains multiple point mutations relative to the wild-type gene. Some of these changes are probably important for the oncogenic potential of the v-myb gene. For example, the v-myb gene lacks an amino-terminal phosphorylation site that can modulate the ability of the c-myb gene to bind to DNA in a sequence specific manner (14).

We are interested in characterizing additional differences between v-myb and c-myb. To this end, we began studying the ability of v-myb to transactivate several common eukaryotic promoters in transient assays, including the Herpes simplex thymidine kinase (TK) promoter. We present evidence that v-myb activated the TK promoter constructions studied, irrespective of whether the plasmid contained exogenous copies of the myb consensus binding site. We show that a 180 bp fragment of the E. coli lacZ gene present in the vectors used acted as a v-myb dependent enhancer element. A 60 bp sub-fragment of this

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enhancer element was recognized by v-myb protein produced in *E. coli*. We have characterized the protein domains of v-myb important for transactivation. Transient transfection assays were used to monitor the ability of truncated v-myb proteins to transactivate transcription. The studies used v-myb proteins that contained the myb-DNA binding domain, or fusion proteins between myb sequences and the DNA binding domain of the rat glucocorticoid receptor (rGR). The data indicate that in addition to the DNA binding domain and the transcriptional activation domain previously defined (9), the carboxyl-terminal portion of v-myb was also required for optimal transactivation. Co-expression of the carboxyl-terminal deletion mutants with wild-type v-myb could partially block transactivation by v-myb.

MATERIALS AND METHODS

Cell Culture

NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 9.5 mM L-glutamine, and 100 μ g/ml mezlocillin. Myb hybridoma cells (15) were purchased from American Type Culture Collection and were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, 9.5 mM L-glutamine, and 100 μ g/ml mezlocillin.

Plasmid Constructions

Standard procedures were used for recombinant DNA manipulations (16). Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer, followed by HPLC purification. Sequencing was performed using the Sequenase kit (U.S. Biochemicals Corp.). Plasmid pSL46 contains the Herpes virus thymidine kinase (TK) promoter (residues -120 to +32 relative to the RNA cap site, 17) cloned into the HindIII site of the firefly luciferase plasmid pJD204 (18). Plasmid pSL90 is identical to pSL46 except the XbaI site 5' of the TK promoter is converted to BglII. Construct TKLUC-M13 was made by cloning the 2.0 kb BglII/BamHI fragment of pSL90, which contains the TK promoter and luciferase gene, into the BamHI site of M13mp19. Plasmid pDB29 was constructed by cloning 7 copies of complementary oligonucleotides containing the consensus binding site for v-myb into the BglII site of pSL90 (5' to 3': GATCCTAGGAATAACGGAAT and GATCATTCCGTTATTCCTAG). Construct pDB37 was made by cloning the 2.0 kb BglII/BamHI fragment of pSL90 into the BamHI site of pAT153 (19). Plasmids pDB41 and pDB42 were constructed by cloning the 180 bp SalI-BglII carboxyl-terminal fragment of the lacZ coding sequences in both orientations relative to TK promoter in pDB37. Polymerase chain reaction (PCR) was used to amplify the lacZ region of pUC18 using the following oligonucleotides: (5' to 3') AGGAAACAGCTATGACCATGA and CCGTCGACGCCATTCAGGCTGCG. The PCR product was digested with SalI, and the 180 bp fragment cloned into the SalI site of pDB37. Plasmid pSL89 contains eight copies of a glucocorticoid response element (GRE) 5' to the TK promoter and luciferase gene of pSL46.

The expression vector pRDO2 contains a single Rous sarcoma virus (RSV) LTR inserted into the EcoRI site of pSP64. PCR was utilized to modify the 5' and 3' ends of the v-myb gene in pVM2 (American Type Culture Collection), which contains the v-myb coding region flanked by viral sequences. The 5' boundary was created by an oligonucleotide (32mer) which introduced a BamHI site and translation start site upstream of the v-myb coding

region (5' to 3': CCGGATCCACCATGAATCGGACAG-ATGTTTCAG). The 3' boundary was created by an oligonucleotide (28mer) which introduced a polyadenylation addition signal and HindIII site at the terminus of the v-myb coding region (5' to 3': CCAAGCTTTATTATAATTGAGCTCTAGA). Plasmid pMYB was made by cloning the PCR product digested with BamHI and HindIII into pRDO2 digested at these sites. Deletions of 3' portions of the v-myb gene were made from the XbaI site to the appropriate internal site in pMYB, and the fragments treated with Klenow, followed by blunt-end ligation. pMYBdC26 contains an 80 bp deletion from BalI to XbaI; pMYBdC87 contains a 261 bp deletion from MstII to XbaI; and pMYBdC148 contains a 449 bp SalI to XbaI deletion. Plasmid pMYBdI60, which contains an internal deletion of 188 bp from SalI to MstII, was constructed by digesting pMYB at these sites, followed by ligation with the following complementary oligonucleotides, which maintain the correct reading frame, and insert a BamHI site for screening purposes: (5' to 3') TCG-ACGGATCC and TCAGGATCCG. pMYBdN138, which contains a 5' deletion of 416 bp from NcoI to SmaI, was made by digesting pMYB at these sites, followed by Klenow treatment and blunt-end ligation. pMYBdN138-TAG, which contains the nuclear localization signal of SV40 large T antigen (20) fused with a 5' deletion of v-myb, was constructed as follows: pMYB was digested with BamHI and SmaI, creating a 484 bp deletion at the 5' end, and ligated with the following complementary oligonucleotides which encode the nuclear localization signal, and maintain the reading frame (5' to 3') GATCCAACATGGCTCC-AAAAAAGAAGAGAAAGGT and ACCTTTCTCTTCTTTTTTGGAGCCCATGTTG). Plasmid pGR-MYB contains the DNA binding domain of the rat glucocorticoid receptor (rGR, 21) fused with a 5' deletion of v-myb. PCR was used to amplify the region of the rGR corresponding to amino acids 407-560, using the following oligonucleotides: (5' to 3') AGCCTGAC-TTCCTTGGGATCCACCATGGGCCGTCAGTGTTT and CCCCCGGGACCAAGGTAGGGGTGAG. The PCR product was digested with BamHI and SmaI, and ligated with pMYB containing a 484 bp deletion of v-myb from BamHI to SmaI. Deletions of 3' regions of the fusion gene were made in pGR-MYB as described above for v-myb. pGR-MYBdC87 contains a 261 bp MstII to XbaI deletion; pGR-MYBdC148 contains a 449 bp SalI to XbaI deletion; and pGR, which contains the rGR DNA binding domain only, contains a 662 bp SmaI to XbaI deletion. The fusion constructs and those containing internal or 5' deletions were sequenced to confirm maintenance of the correct reading frame.

Transient Transfections and Enzyme Assays

Transient transfections of NIH 3T3 cells were performed by the calcium-phosphate precipitate method as previously described (22). Cells were plated at 3×10^5 per 100 mm collagen-treated dish. Unless otherwise indicated, 4.5 μ g reporter plasmid, 4.5 μ g expression plasmid, and 1 μ g of an internal control plasmid containing the RSV LTR driving the human placental secreted alkaline phosphatase gene (RSV-SEAP, 23) was added to each dish. Cell extracts or culture media were prepared and luciferase and SEAP activity assayed as previously described (22). Luciferase activity was normalized for protein concentration of the extracts, determined by the Bio-Rad protein assay, and for relative transfection efficiency, determined by SEAP activity. Normalized luciferase activity was expressed as [(luminescence/ μ g protein)/(milliunits of SEAP/ml)]. The values

presented are the averages of at least 3 independent experiments, and the level of myb induction varied by less than 10%.

Immunoprecipitation Analysis

NIH 3T3 cells were transiently transfected with full length and truncated myb expression vectors (4.5 μ g of wild type and 4.5 μ g of mutant per 100 mm dish), followed 32 hr later by metabolic labeling with 35 S-methionine. Immunoprecipitations were performed with pooled Myb-specific monoclonal antibodies 2-7.77, 2-3.76, and 2-37.63 (15). Monoclonal antibody preparations were obtained from culture supernatant by addition of NH_4SO_4 to 40%, and the resuspended precipitate was dialyzed against PBS. Conditions used for metabolic labeling, extract preparation, and immunoprecipitation were as previously described (9).

Expression of myb Proteins in Bacteria and Gel Retardation Assays

Selective expression of myb proteins in *E. coli* was performed using the T7 RNA polymerase system (24). Extracts were prepared from IPTG-induced cells by freeze-thaw lysis in TEN buffer (10mM Tris-Cl, pH7.8, 1 mM EDTA, 100 mM NaCl, 0.1 mM DTT, 0.5 mM PMSF). Insoluble material was collected by centrifugation, resuspended in TEN buffer that contained 6 M urea, and dialyzed versus TEN buffer containing 1 M urea. Gel retardation assays were performed similarly to those previously described (22). The lacZ probe was prepared by digesting pUC18 with BglII and PvuII, end-labeling with 32 P-ATP and polynucleotide kinase, and purifying the 60 bp fragment. Approximately 0.5 ng of probe (50,000 dpm) was used in each binding reaction.

RESULTS

The lacZ gene contains a myb-dependent enhancer element

NIH 3T3 cells were co-transfected with a RSV LTR/v-myb expression vector (see Methods) and reporter plasmids containing the firefly luciferase gene. The reporter genes were transcribed from the well-defined Herpes virus thymidine kinase (TK) promoter (−120 to +32, 17). The basal level of expression of the TK reporter plasmid was determined by co-transfecting with the RSV LTR expression vector containing no gene. Figure 1A shows that v-myb expression resulted in approximately 10-fold higher stimulation of the reporter plasmid pSL46 than the basal level obtained with the control vector. The presence of multiple copies of the v-myb consensus binding site upstream of the TK promoter (Figure 1A, pDB29, pUC based vector), resulted in only a slight additional increase in v-myb stimulation.

In addition to the TK promoter cassette, we also tested several other well characterized eukaryotic promoters including the mouse β -globin promoter, the RSV promoter, and SV40 promoter in the same reporter vector. All of these promoters were induced about 10-fold by v-myb co-expression (data not shown). The reporter gene luciferase could be replaced by the chloramphenicol acetyltransferase gene with no diminution of v-myb responsiveness (data not shown). Taken together, these data indicated to us that the prokaryotic vector sequences must contain a fortuitous myb-enhancer element.

To determine what reporter plasmid sequences are necessary in cis for v-myb induction, we placed the TK-luciferase reporter gene into either a M13 backbone, or into plasmid pAT153 (19),

A

REPORTER PLASMIDS:			Fold Induction by MYB
pSL46			10.1
TKLUC-M13			10.8
pDB29			12.7
pDB37			2.5
pDB41			10.7
pDB42			12.6

Figure 1. A) Activation of TK promoter constructs by v-myb. Reporter plasmids are depicted, with the HSV TK promoter, luciferase reporter gene, and vector background of pUC, M13 (M13mp19), or pAT153 indicated. Plasmid pDB29 contains 7 copies of consensus MYB binding site oligonucleotides upstream of the TK promoter. Plasmids pDB41 and pDB42 contain, in either orientation, a 180 bp fragment of lacZ gene upstream of the TK promoter. NIH 3T3 cells were transiently cotransfected with the indicated reporter plasmid and either the v-myb expression plasmid, pMYB, or a control expression plasmid lacking v-myb sequences. An internal control plasmid, RSV SEAP, was also included in each transfection. The fold induction by myb is the ratio of reporter activity in cells transfected with the myb expression vector to that in cells transfected with the empty expression vector. Results are the averages of 3 independent experiments. B). v-myb proteins recognize the lacZ enhancer element. Gel retardation was performed with a 60 bp fragment within the lacZ gene of pUC18 and v-myb proteins produced in *E. coli*. Lanes: M, MspI-digested pBR322 marker DNA; 1, extract from cells transformed with expression vector pET3a; 2, pMYB-ET3a extract; 3, pMYBdC26-ET3a extract; 4, pMYBdC87-ET3a extract. See Figure 2 for the identity of v-myb genes used for DNA binding studies.

Figure 2. Effect of v-myb deletions on transcription activation activity. Schematics of v-myb constructs are shown. Specific regions of v-myb were deleted using restriction sites, designated as follows: B, BamHI; N, NcoI; R, EcoRI; P, PstI; S, SmaI; V, PvuII; L, SalI; M, MstII; BI, BglI; X, XbaI. Regions of the v-myb gene which encode the DNA binding domain (hatched box), and a previously defined activation region (black box) of the protein are indicated. Dotted lines indicate deletions. Construct pMYBdN138-Tag contains sequences encoding the nuclear localization signal of SV40 large T antigen (stippled box) fused in frame with myb sequences. Each expression plasmid was cotransfected with 4.5 μ g pSL46 reporter plasmid, and 1 μ g RSV SEAP per 100 mm dish. The level of transactivation by the v-myb constructs was determined by the ratio of reporter activity in cells transfected with the indicated v-myb expression vector to that in cells transfected with the empty expression vector. Results are the averages of 3 independent experiments.

a vector that lacks the lacZ gene (Figure 1A, TK-LUC-M13 and pDB37, respectively). Transient assays with these vectors show that the reporter present in the M13 background still responds to v-myb, while that in the pAT153 vector is stimulated only 2.5-fold by v-myb (Figure 1A). When a 180 bp fragment of the *E. coli* lacZ gene (SalI-BglI fragment) is placed into the pAT153 based reporter in either orientation with respect to TK promoter element, full v-myb transactivation is recovered (pDB41 and pDB42, Figure 1A). Thus, the lacZ gene fragment contains a myb-responsive enhancer element.

Full length v-myb protein (48 kDa) was expressed in *E. coli* using the T7-derived system developed by Studier and co-workers (24). Two deleted v-myb genes, those present in pMYBdC26 and pMYBdC87 (46 kDa product or 38 kDa product, respectively, see below), were also cloned into the T7 vector. The v-myb proteins produced in *E. coli* were used for in vitro gel retention assays with a 60 bp DNA probe that represents a sub-fragments of 180 bp enhancer (see Figure 1B). This probe formed a complex with full length v-myb protein extract (Figure 1B, lane 2) and the truncated v-myb protein extracts (lanes 3 and 4), but not with control extract (lane 1). The C-terminal truncated 46 kD and 38 kD v-myb proteins formed complexes whose mobility increased as the size of the v-myb protein decreased (lanes 3 and 4, middle and lower arrow). The complexes were specific, as determined by competition experiments utilizing synthetic oligonucleotides that contained the myb consensus sequence, or the recognition sites for other nuclear transcription factors (data not shown). Inspection of the sequence of this 60 bp segment revealed a perfect match to the consensus v-myb binding site, YAAC(G/T)G (Figure 1B, bottom).

Figure 3. Analysis of rGR-myb fusion products. (A) Schematic of rGR-myb fusion constructs. The v-myb gene and restriction sites in pMYB are as described in the legend to Figure 2. Plasmid pGR-MYB contains the DNA binding domain of the rGR (large hatched box), amino acids 407–560, fused to an N-terminal deletion of v-myb, at the SmaI site. Restriction sites were used to make C-terminal deletions in the fusion gene, similar to those made in v-myb (see figure 2). Plasmid pGR contains the rGR DNA binding domain without v-myb sequences cloned into the expression plasmid. (B) Activity of rGR-MYB fusion constructs. Transient transfections were performed as described in the legend to Figure 2. The reporter plasmids, depicted below the graph, were pSL46 (black bars) and pSL89 (hatched bars), which contains 8 GRE copies upstream of the TK promoter. The data are the averages of 3 independent experiments.

Myb deletion mutants define a novel transactivation domain

To investigate what regions of the v-myb protein are required for the observed transcriptional activation effect, a series of C-terminal, N-terminal, and internal deletions were constructed. Figure 2 depicts the normal and deleted v-myb constructs, with the DNA binding domain (7), and a previously defined activation region (9) indicated. NIH 3T3 cells were co-transfected with a v-myb expression vector and pSL46 reporter plasmid. The control expression vector lacking a v-myb gene was also co-transfected with the reporter plasmids to determine the basal level of luciferase expression. The fold induction by each deletion

Figure 4. Immunoprecipitation of v-myb proteins from transiently transfected cells. Immunoprecipitation analysis was performed on lysates prepared from transiently transfected NIH 3T3 cells, radiolabeled 8 hr with 250 μ Ci 35 S-methionine per 100 mm dish. Lysates were either treated with myb-specific monoclonal antibodies, lanes 3 and 5, or with buffer, lanes 2 and 4, prior to addition of protein A agarose beads. Samples were subjected to electrophoresis through a 10% SDS-polyacrylamide gel, followed by fluorography. Sample were as follows: Lanes 2 and 3, pMYB and pMYBdC87 cotransfected cell extracts; lanes 4 and 5, pMYB and pMYBdC148 cell extracts. The large arrowheads show the position of wild type v-myb migration (48 kDa), while the small arrowheads indicate the position of the pMYBdC87 product (38 kDa, lane 3) or the pMYBdC148 product (28 kDa, lane 5). Lane 1 contained protein size standards.

construct, an average of three independent transfection experiments, is shown to the right of the corresponding construct in Figure 2. Deletion construct pMYBdC26, in which the C-terminal 26 amino acids are removed, retains transcriptional activation function, and actually causes a slight increase in reporter gene expression relative to wild type v-myb (pMYB). A deletion which removes 87 amino acids from the C-terminus results in a 80% reduction in transactivation (pMYBdC87, Figure 2). These two deletions indicate that amino acid residues 295–356 with respect to the 5' end of v-myb coding sequences is important for transactivation. An internal deletion of 60 amino acids (pMYBdI60), which encompasses the previously defined activation region, also results in a 80% loss of activity. Deletion of the C-terminal 148 amino acids (pMYBdC148) results in a total loss of transactivation function.

Deletion of the N-terminal 138 amino acids (pMYBdN138), which eliminates the DNA binding domain, also results in total loss of transactivation function. In addition to removing the DNA binding domain, this deletion encompasses a portion of v-myb important for nuclear localization (25). To address the possibility that loss of v-myb function caused by deletion of this region might be due to an inability of the truncated protein to locate to the nucleus, a construct was made which fuses the nuclear localization signal of SV40 large T antigen (20) with the N-terminal deletion

Figure 5. Interference with v-myb activity by C-terminal deletion constructs. Mixed cotransfections were performed in NIH 3T3 cells with RSV expression vectors and pSP70 carrier DNA totalling 6 μ g, 3 μ g pSL46 reporter plasmid, and 1 μ g RSV SEAP internal control per 100 mm dish. Plasmid pMYB was cotransfected with an equimolar amount of pMYBdC87 expression vector (pMYB + pMYBdC87) or pMYBdC148 expression vector (pMYB + pMYBdC148). Each of the v-myb constructs (pMYB, pMYBdC87, and pMYBdC148) was also cotransfected with an equimolar amount of the control expression vector for comparison. For each transfection sample, the total molar amount of RSV expression plasmids remained constant. Basal activity is the level of luciferase expression observed when pSL46 is cotransfected with the empty RSV expression vector. The relative luciferase activity of each transfection sample is shown as a percentage of the activity in extracts from cells transfected with pMYB expression plasmid and pSL46 reporter plasmid (100%). Values are indicated beside the bars. The data are the averages of 3 separate experiments.

of v-myb (pMYBdN138-TAG). This construct also failed to mediate transcriptional activation.

The activity of the deleted versions of v-myb was the same whether the reporter plasmid was pSL46 or pDB29 (data not shown). The latter contained v-myb consensus binding sites (see Figure 1).

rGR-myb fusion genes require the novel C-terminal region for transactivation

To confirm our findings concerning the importance of amino acid residues 295–356 in transcriptional activation by v-myb observed in our system, fusion constructs were created in which the v-myb DNA binding domain is replaced by that of the rat glucocorticoid receptor (rGR, see Figure 3A, 21). The fusion genes were tested in transient transfection assays for their ability to transactivate the TK-luciferase reporter plasmid, pSL46, or a similar reporter plasmid, pSL89, which has 8 copies of a glucocorticoid response element (GRE) 5' of the TK promoter. Figure 3B shows the average results of 3 independent experiments. The fusion protein encoded by pGR-MYB, which has the rGR DNA binding domain fused to a deletion of v-myb that lacks the N-terminal 138 amino acids, was only able to induce reporter gene expression if the GRE was present (Figure 3, pSL46 vs. pSL89). Deletions were made in the v-myb region of the fusion gene in order to assess the role of v-myb domains in transcriptional activation by the fusion protein. As was the case with the normal v-myb protein, removal of the C-terminal 87 amino acids of the fusion protein (pGR-MYBdC87) resulted in a 80% reduction in transactivation. A deletion which extends 61 amino acids further from the C-terminal end (pGR-MYBdC148) results in a total loss of function. A control construct encoding only the rGR DNA binding domain (pGR) had no transcriptional activation activity, indicating that v-myb regions were responsible for the transactivation activity of the fusion protein.

v-myb proteins encoded by the deletion constructs are synthesized during the transient assay

Immunoprecipitation of ³⁵S-labeled proteins with pooled myb monoclonal antibodies (15) following transfection indicated that wild-type and mutant proteins were expressed at the same levels (Figure 4). In these experiments, equal amounts of wild type and deleted myb expression vectors were transfected. Analysis of the antibody-reactive proteins synthesized showed that a 48 kDa protein corresponding to v-myb could be detected (lanes 3 and 5, large arrow point). Additionally, cells transfected with pMYBdC87 and pMYBdC148 synthesize proteins of 38 kDa and 28 kDa, respectively (small arrow points, lanes 3 and 5; the 38 kDa product encoded by the former is the bottom half of a poorly resolved doublet in this gel). Proteins of the same size were detected by western analysis when these myb genes were expressed in *E. coli* (data not shown).

Further evidence for stable expression of the truncated proteins was provided by mixed co-transfection experiments. C-terminal truncated v-myb proteins that retain the DNA binding domain, but have lost transactivation function, were assessed for their ability to interfere with normal v-myb function. Equimolar amounts of normal v-myb expression vector (pMYB) and a C-terminal truncated v-myb expression vector (either pMYBdC87 or pMYBdC148) were co-transfected with the TK-luciferase reporter plasmid. The effect of the deletion construct on transactivation by the wild-type v-myb gene was compared to co-transfection with the control expression vector. The average results of 3 mixed co-transfection experiments are presented in Figure 5. Co-transfection of equimolar amounts of pMYB with pMYBdC87 caused a 2.5-fold reduction in reporter gene expression relative to co-transfection of pMYB with the control expression vector. Co-transfection of pMYB with pMYBdC148 caused a 5-fold reduction of myb activity.

DISCUSSION

It is now well established that v-myb and its cellular counterpart c-myb encode proteins that can act as transcription factors (9, 11, 12, 13). What remains less well defined is the differences between the two genes that allow v-myb to transform hematopoietic cells. To begin to address this question, we decided to map the domains of the v-myb protein required in transactivation, and later to compare these domains to those important for c-myb action. As a preliminary step in our investigations, we tested the effect of a v-myb expression vector on luciferase reporter plasmids that contained well characterized mammalian or viral promoters.

Surprisingly, these constructs were strongly upregulated by v-myb (e.g., Figure 1). In the case of the Herpes TK promoter, adding 7 copies of an oligonucleotide that contains the v-myb consensus binding site had little effect on the magnitude of v-myb stimulation (Figure 1, pDB29). Inspection of the sequences of these promoters did not reveal good homologies to the published v-myb binding sequence (YAAC(T/G)G, 10). A simple hypothesis that accounts for this data is that v-myb binds to DNA sequences outside of the eukaryotic promoters used. Since v-myb activated either luciferase or CAT chimeric genes (data not shown), sequences in the prokaryotic plasmid would be predicted as the most likely candidates for v-myb-dependent enhancer activity.

The experiments presented in Figure 1 demonstrate that a 180 bp fragment of the lacZ gene could act as a myb-dependent

enhancer element. We also found that v-myb protein produced in *E. coli* could specifically recognize a portion of the lacZ enhancer fragment in vitro (Figure 1B). These results are similar to those recently reported by Lipsick and colleagues (12). However, this group did not map the location of putative vector enhancer elements. While the myb-dependent enhancer in the lacZ gene has no biological significance, our data raise a cautionary message concerning assays of transactivators. Vector sequences are not necessarily neutral, especially when relatively high levels of transactivator may be expressed.

What domains of the v-myb peptide are involved in transactivation? As expected (9, 12, 26), the N-terminal portion of v-myb, which contains the DNA binding domain and nuclear translocation signals, is necessary for transactivation (Figure 2). In agreement with published results (9, 26), we find that deletion of a highly charged region near the C-terminal end of the v-myb protein diminishes transactivation by 80% (Figure 2, pMYBdl60, 2-fold induction over basal levels of activity). However, we find that deletions of amino acids more proximal to the C-terminal end of the protein also have significant effects on transactivation.

Deletion of the last 26 amino acids of v-myb actually increases transactivation (Figure 2, pMYBdC26). This deletion removes primarily amino acids derived from the envelope region of the transducing avian retrovirus (1). A similar activation of c-myb by deletion of C-terminal residues has been reported, and interpreted to be due to removal of a negative regulatory domain from the c-myb protein (26). Deletion of the next 61 amino acids results in loss of 80% of transactivation (Figure 2, pMYBdC87, 2-fold induction over basal). Deletion of an additional 61 amino acids, including the previously defined 'activation' region (9, 26), results in a loss of the final 20% of activity (Figure 2, pMYBdC148). The same results are obtained when the myb DNA-binding domain is replaced by the glucocorticoid receptor DNA-binding domain (Figure 3, pGR-MYBdC87 and pGR-MYBdC148). In this case, the reporter plasmid must contain multiple copies of the GRE in order to be transactivated (Figure 3, see above).

A trivial explanation of this data is that the different deletion constructs have different stabilities or different abilities to translocate to the nucleus. Immunoprecipitation experiments with anti-myb monoclonal antibodies (15) indicate that deletions are expressed at roughly the same level as the wild type v-myb protein (Figure 4). As a separate means of addressing this problem, we asked whether the deletions, when co-expressed with the wild-type v-myb gene could block transactivation by full length v-myb. These experiments were conducted so that any inhibition seen could not be due to competition for transcription factors between the expression vector sequences (see legend to Figure 5). As demonstrated in Figure 5, both inactivating C-terminal deletions, pMYBdC87 and pMYBdC148, were able to block transactivation of the TK-luciferase chimera by full length v-myb. The former inhibits transactivation 2.5 fold, and the latter inhibits 5-fold. This data provides further evidence that the mutant proteins are expressed and are probably localized in the cell nucleus.

The distal C-terminal region required for transactivation by the v-myb protein contains a motif of repeated leucine residues spaced at intervals of 7–8 amino acids (1, 10). Such a structure is reminiscent of the 'leucine zipper' dimerization motif of the fos/jun family of transcription factors (27). The fos/jun leucine zippers are adjacent to the basic DNA binding domain (27), while the leucine repeat is far removed from the myb DNA binding

domain. However, the published work of two groups has demonstrated that the myb repeated leucine motif is not required for DNA binding in vitro (28, 29). The data presented in Figure 1B (lane 4) confirms these results.

Therefore, our data defines a second C-terminal region that is important for v-myb transactivation. Mutations that have either only this region or the previously identified 'activation' region (9, 26), have weak 2-fold activation activity (Figure 2, pMYBdC87 and pMYBdI60). However, together, the two domains activated transcription by 10-fold. We suggest that the distal C-terminal domain important for transactivation by v-myb may be important for interaction with a 'co-activator' protein (30, 31, 32). Recent evidence suggests that 'co-activators' are proteins that bridge transactivators to the RNA polymerase II transcriptional complex (30, 31, 32). For example, the well-characterized transcription factor Sp1 requires such a co-activator in order to stimulate transcription in vitro (30). We hypothesize that the distal C-terminal portion of myb is involved in linking the 'activation' domain of v-myb (9) to the RNA polymerase II transcriptional machinery through interaction with a 'co-activator', just as the glutamine rich activation domain of Sp1 is linked by its 'co-activator' to the RNA polymerase II complex (30). The interaction of a transactivator with co-activator is likely to be an important factor in tissue-specific gene expression during growth and differentiation of eukaryotic organisms (30). Thus, our hypothesis may have important implications in understanding how c-myb activates transcription during the differentiation of various hematopoietic lineages. Further experiments will be required to test this hypothesis, and to address the role of the two v-myb domains in transcriptional activation by c-myb proteins.

A v-myb protein that lacked the distal C-terminal activation domain had a partial dominant negative phenotype, that is, expression of this protein partially blocks the full activity of the wild-type v-myb protein (Figure 5, pMYBdC87). A myb protein lacking both C-terminal domains required for transactivation exhibited a stronger dominant negative phenotype (Figure 5, pMYBdC148). The mechanism for this phenotype is unknown. One likely possibility is that the truncated proteins can bind to DNA and act as repressors. Whatever the mechanism, such dominant-negative mutations of v-myb may be useful tools in exploring the role of c-myb in hematopoietic cell growth and differentiation.

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