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Transformation by v-*myb* Correlates with *trans*-Activation of Gene Expression†

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The v-*myb* oncogene of avian myeloblastosis virus causes acute myelomonocytic leukemia in chickens and transforms avian myeloid cells in vitro. Its protein product p48$^{v\text{-}myb}$ is a nuclear, sequence-specific, DNA-binding protein which activates gene expression in transient DNA transfection studies. To investigate the relationship between transformation and *trans*-activation by v-*myb*, we constructed 15 in-frame linker insertion mutants. The 12 mutants which transformed myeloid cells also *trans*-activated gene expression, whereas the 3 mutants which did not transform also did not *trans*-activate. This implies that *trans*-activation is required for transformation by v-*myb*. One of the transformation-defective mutants localized to the cell nucleus but failed to bind DNA. The other two transformation-defective mutants localized to the cell nucleus and bound DNA but nevertheless failed to *trans*-activate. These latter mutants define two distinct domains of p48$^{v\text{-}myb}$ which control *trans*-activation by DNA-bound protein, one within the amino-terminal DNA-binding domain itself and one in a carboxyl-terminal domain which is not required for DNA binding.

The v-*myb* oncogene of the avian myeloblastosis virus (AMV) causes acute myelomonocytic leukemia in chickens and transforms avian myeloid cells but not fibroblasts in vitro (for a review, see reference 29). The related E26 avian leukemia virus causes acute erythroid leukemia in chickens and transforms avian erythroid and myeloid cells in vitro (36, 43). The c-*myb* proto-oncogene from which v-*myb* arose is expressed at high levels predominantly in immature hemopoietic cells including those of the erythroid, lymphoid, and myeloid lineages (8, 16). The expression of c-*myb* declines as these cells mature (11, 48). c-*myb* itself appears to control this differentiation process because the expression of exogenous c-*myb* or v-*myb* can block or reverse the differentiated phenotype of various hemopoietic cells (3, 10, 12, 33, 37, 47). *myb*-related genes have also been identified in mammals, *Drosophila melanogaster*, *Zea mays*, *Saccharomyces cerevisiae*, and *Dictyostelium discoideum* (23, 39,41, 44, 46; U. Stober-Grasser, R. Firtel, and J. Lipsick, unpublished data).

The protein products of v-*myb* and c-*myb* are present in the cell nucleus, bind DNA in a sequence-specific fashion, and *trans*-activate gene expression in transient DNA transfection assays (4–6, 22, 24a, 25, 27, 38, 49). Consistent with these findings, the *myb*-related *BAS1* gene of *S. cerevisiae* encodes a genetically defined transcription factor which binds directly to DNA (46). In addition, the *myb*-related product of the *C1* locus of *Z. mays* controls several unlinked, developmentally regulated genes involved in the synthesis of anthocyanin pigments (41).

A number of oncogenes which encode nuclear proteins have been shown to regulate gene expression (for a review, see reference 24). However, for the adenovirus E1A proteins, genetic analyses have shown that transcriptional regulation and cellular transformation are functionally and physically separable (35). Previous studies of v-*myb* had shown that nuclear localization and DNA binding by p48$^{v\text{-}myb}$ are together not sufficient for transformation, implying that an additional function is required (20, 21). To specifically investigate the relationship between *trans*-activation of gene expression and transformation by v-*myb*, we constructed and biologically tested a series of 15 additional mutants.

MATERIALS AND METHODS

Linker insertion mutagenesis. A selectable linker insertion cassette was constructed by first ligating a synthetic double-stranded *Apa*I linker (GGGCCC) to the end-filled *Hin*dIII-resistant *neo* fragment of the pUC-NEO plasmid (31). This DNA was digested with *Sma*I to trim excess linkers, purified by agarose gel electrophoresis, and then cloned into the *Sma*I site of pUC18 to create the plasmid pANA (Fig. 1). This plasmid was then digested with *Sma*I to release a 1.4-kilobase *neo* fragment flanked by single *Apa*I linkers. *Apa*I was chosen because it does not cut within v-*myb*; however, the method can easily be adapted for other restriction sites.

The plasmid pUC-MYB-dE was used as a target for mutagenesis (21). Plasmid DNA was digested with the desired restriction enzyme, and linear DNA was purified by agarose gel electrophoresis and then ligated to the 1.4-kilobase *neo* fragment described above. Digestion of the target plasmid with restriction enzymes with multiple recognition sites was limited by titration of ethidium bromide to maximize the yield of linear DNA (40). In rare cases, certain individual sites (e.g., *Ava*II at 960) were never mutagenized by this method, suggesting a strong restriction enzyme site preference during partial digestion in the presence of ethidium bromide. Target DNAs produced by enzymes which generated three-nucleotide 5′ extensions were end filled with the Klenow fragment of DNA polymerase I and deoxynu-

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† This paper is dedicated to the memory of Abraham Lipsick.

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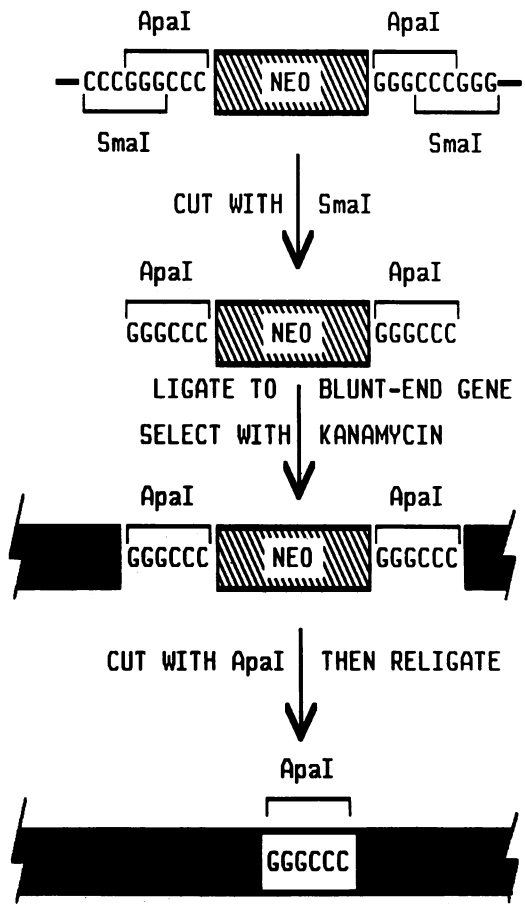

FIG. 1. Method of linker insertion mutagenesis. The blunt-ended *neo* fragment (striped box) bounded by *Apa*I sites was released from the pANA plasmid by digestion with *Sma*I and gel purified. This fragment was then ligated to the v-*myb* target plasmid (black box) which had been linearized and blunt ended as described in the text. After positive selection for insertions, the *neo* fragment was removed by digestion with *Apa*I and religation.

cleoside triphosphates before ligation of the 1.4-kilobase *neo* fragment. The ligation reactions were then used to transform *Escherichia coli* DH5, and colonies were selected with both kanamycin (30 μg/ml) and ampicillin (50 μg/ml) to select for the *neo* fragment but against insertions into the plasmid backbone.

Plasmid DNAs from these colonies were first screened with *Sst*I, the sites for which flank v-*myb*, to determine whether *neo* was inserted within v-*myb* itself or within the plasmid backbone. Plasmid DNAs from those colonies with insertions into v-*myb* were mapped by digestion with *Kpn*I and *Apa*I. The *neo* gene was then excised by digestion with *Apa*I and intramolecular ligation, leaving behind a single six-nucleotide *Apa*I linker. The mutated v-*myb* genes were then excised with *Kpn*I and *Xba*I and inserted into the pNEO-AMV retroviral expression vector. Protein expression in COS cells after DNA transfection was screened by immunoprecipitation as previously described (31).

Assays of gene expression. Quail QT6 cells were maintained as previously described (21) and transfected by a modified calcium phosphate precipitation method (9). Two days after transfection of 0.2 μg of the cytomegalovirus-β-galactosidase expression vector pON249 (constructed in the laboratory of Ed Mocarski, Stanford University, Stanford, Calif.), 1 μg of the TK-dAX reporter plasmid, 3 μg of the indicated effector plasmid, and 5.5 μg of yeast tRNA, cell lysates were prepared by freeze-thawing (17). The β-galactosidase activity was determined with a TK100 minifluorometer (Hoefer Scientific, San Francisco, Calif.) as recommended by the manufacturer. (No *myb* dependence of cytomegalovirus-β-galactosidase expression was observed.) Chloramphenicol acetyltransferase (CAT) activity was then assayed in normalized cell lysates by standard procedures with [^{14}C]chloramphenicol, thin-layer chromatography, and scintillation counting (17).

Immunoblotting and immunoprecipitation. For CAT assays, samples of total cell lysates prepared and normalized for β-galactosidase activity as described above were precipitated with 4 volumes of acetone at −70°C overnight. After centrifugation, the insoluble pellets were suspended in loading buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with prestained protein molecular weight markers (Sigma Chemical Co., St. Louis, Mo.). The proteins were then electrophoretically transferred to nitrocellulose membranes and incubated with the monoclonal antibody *myb*2.2 (13), kindly provided by Gary Ramsay and Mike Bishop (University of California at San Francisco). The blots were developed with alkaline phosphatase-coupled rabbit anti-mouse immunoglobulin G (Promega Biotec, Madison, Wis.) as recommended by the manufacturer. For HD4 cells, the nuclear and cytoplasmic cell fractions were prepared as previously described (21) and then analyzed as above. Radiolabeling and immunoprecipitation were performed as previously described (21).

Transformation assays. The mutant proviral plasmid DNAs were each cotransfected with MAV-1 helper virus DNA into QT6 cells by the calcium phosphate method and then selected for resistance to G418 (31). G418-resistant colonies for each mutant were pooled and treated with mitomycin C for use as sources of infectious retroviruses (30). The infection of HD4 cells by cocultivation, myeloid yolk sac transformation assays, and the detection of protein expression by immunoprecipitation have previously been described (21). Transformation of bone marrow cells was assayed after cocultivation as previously described (18).

DNA binding assays. Wild-type and mutant alleles of p48$^{v\text{-}myb}$ were expressed in *E. coli* from the pT7-MYB plasmid which contains in succession a T7 promoter, an MS2 ribosome-binding site, an *Nco*I cloning site at the initiation codon, a segment of pUC18 polylinker, and the entire v-*myb* coding sequence downstream of its normal splice acceptor site. This plasmid was derived from pCYCRBS (34), which was kindly provided by John Pines (Salk Institute, La Jolla, Calif.). Expression of v-*myb* protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in the BL21(DE3) LysS host strain which was kindly provided by F. W. Studier (F. W. Studier, A. H. Rosenberg, and J. J. Dunn, Methods Enzymol., in press). The *myb* protein produced in *E. coli* comigrates with authentic p48$^{v\text{-}myb}$ in SDS-PAGE and is predicted to be identical in sequence to the protein product of the v-*myb* mutant dGAG which transforms avian myeloid cells (21).

Bacterial proteins were prepared by three cycles of rapid freeze-thawing in buffer containing 10 mM Tris hydrochloride (pH 7.8), 50 mM NaCl, 1 mM EDTA, 6 M urea, 1 mM phenylmethylsulfonyl fluoride, 0.3 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin. These samples were then diluted 20-fold into the same buffer without urea but made 20% in glycerol. After centrifugation at 15,000 × *g* for 15 min, the supernatants were saved and stored at −70°C.

Protein concentrations were determined by dye binding with bovine serum albumin as a standard (7). Gel mobility shift assays were performed employing as a target the ^{32}P-end-labeled 115-base-pair *Hin*dIII-*Bam*HI fragment of KHK-TK-CAT which contains nine consensus binding sites for p48$^{v\text{-}myb}$ (22). Protein-DNA incubations were performed in reaction buffer containing 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 80 μg of poly(dI · dC) (Pharmacia, Inc., Piscataway, N.J.) per ml, and 100 μg of bovine serum albumin per ml for 30 min at 4°C. Free and bound DNA complexes were detected by electrophoresis through a 5% acrylamide gel (80:1 acrylamide bisacrylamide) in 0.25× Tris-borate-EDTA buffer and autoradiography as previously described (1).

RESULTS

Construction of v-*myb* linker insertion mutants. We had previously constructed and biologically tested a series of 16 deletion mutants of v-*myb* (21, 22). However, most of these mutants were transformation defective and therefore were not informative about the relationship between *trans*-activation and transformation by p48$^{v\text{-}myb}$. We therefore constructed a new series of v-*myb* mutants, each of which bears a unique two- or three-amino-acid insertion in p48$^{v\text{-}myb}$. Twenty such mutants were constructed by a selectable linker insertion technique which was based on a previously described method (32).

Each mutant contains a new GGGCCC sequence at a preexisting restriction enzyme site, which therefore introduces a novel *Apa*I restriction site into v-*myb*. The mutants are designated by the nucleotide at which the target restriction site occurs within the v-*myb* sequence (45). The amino acids encoded by these insertions vary depending on both the reading frame of the insertion and the adjacent nucleotides. Mutations created by linker insertions at blunt-ended restriction enzyme sites resulted in the insertion of two amino acids, while mutations created at restriction enzyme sites with three-nucleotide 5′ overhangs were filled in before linker insertion, resulting in the insertion of three amino acids. Proviruses containing each of these mutants were screened for transient protein expression after DNA transfection of simian COS cells. Of the 20 initial mutants, 15 produced immunoprecipitable proteins which were similar in abundance to and comigrated with wild-type p48$^{v\text{-}myb}$ in SDS-PAGE (data not shown). These 15 mutants were therefore selected for further analysis (Fig. 2).

***trans*-activation by mutants of v-*myb*.** The 15 linker insertion mutants which produced stable v-*myb* proteins were tested for *trans*-activation of CAT expression in quail QT6 cells by transient DNA transfection with the TK-dAX reporter plasmid. This plasmid contains the herpes simplex virus thymidine kinase promoter, the *cat* gene, and a simian virus 40 polyadenylation signal cloned into a pUC18 backbone. We have previously shown that p48$^{v\text{-}myb}$ *trans*-activates the expression of properly initiated thymidine kinase-CAT transcripts from this plasmid in such assays (22). The pNEO-MAV and pNEO-AMV plasmids which produce *env* and wild-type v-*myb*, respectively, were used as negative and positive controls for *trans*-activation. In addition, a cytomegalovirus-β-galactosidase reporter plasmid was used as an internal control in each assay to normalize for the efficiency of DNA transfection. Twelve of the linker insertion mutants *trans*-activated at levels equal to or greater than that of wild-type v-*myb* (Fig. 3; Table 1). However, three of the mutants (281, 364, 859) were essentially inactive in this assay. To determine whether the various mutant proteins were expressed equivalently, normalized samples of the same lysates used for CAT assays were immunoblotted with an anti-*myb* monoclonal antibody. All these mutant proteins were clearly expressed and comigrated with authentic p48$^{v\text{-}myb}$ (Fig. 3). Furthermore, those mutants which did not *trans*-activate (mutants 281, 364, and 859) were expressed at steady-state levels equal to or greater than that of the active wild-type protein, which itself is known to be present in excess in this assay (22). This demonstrates that the failure of mutants 281, 364, and 859 to *trans*-activate was not due to a lack of protein production or stability.

DNA binding by mutants of v-*myb* which do not *trans*-activate. The DNA-binding domain of p48$^{v\text{-}myb}$ has previously been mapped to its highly conserved amino terminus (20, 26). Two of the v-*myb* mutants which did not *trans*-activate mapped within this DNA-binding domain (mutants 281 and 364), whereas a third mutant which did not *trans*-activate mapped outside of this domain (mutant 859). To assay the intrinsic ability of these mutant proteins to bind to DNA, we expressed each of them in *E. coli* using the T7 RNA polymerase system (Studier et al., in press). Protein extracts from these bacteria were then used to assay sequence-specific DNA binding by the gel mobility shift assay (14, 15). The proteins expressed by wild-type v-*myb* and mutants 281 and 859 bound DNA in this assay, whereas the protein expressed by mutant 364 did not (Fig. 4A). The *myb* proteins were all present at similar levels in the *E. coli* extracts as shown by SDS-PAGE followed by Coomassie blue staining and by immunoblotting of portions of the same samples used for the gel mobility shift assays. Mutant 364 failed to bind DNA even at protein concentrations sufficient to allow the wild type and mutants 281 and 859 to shift essentially all the available DNA (Fig. 4A). The specificity of this protein-DNA interaction has been demonstrated both by specific competition with unlabeled oligonucleotide and by alteration of the mobility of the complexes with anti-*myb* antisera (data not shown). These results show that *trans*-activation by p48$^{v\text{-}myb}$ can be abolished by mutating either its amino-terminal DNA-binding domain or a second nonadjacent site in the carboxyl-terminal region which is not required for DNA binding.

At high protein concentrations in which DNA is limiting, mutant 281 reproducibly generated a protein-DNA complex of higher electrophoretic mobility than that of the wild type (Fig. 4A). However, at lower protein concentrations, the gel mobility shifts generated by the wild type and mutant 281 were identical (Fig. 4B). A change in complex mobility caused simply by an altered protein geometry or isoelectric point due to the three-amino-acid insertion (R-A-R) in mutant 281 should be seen at low as well as high protein concentrations. Therefore, these results suggest that the differences in the mobilities of wild-type and mutant 281 protein-DNA complexes at higher protein concentrations are due to altered protein-protein interactions or altered affinities for a specific subset of the available binding sites.

The failure of mutant 281 to *trans*-activate (and also to transform as described below) despite its ability to bind DNA might be due to a failure of its protein product to localize to the cell nucleus. This seemed plausible because the insertion in mutant 281 lies adjacent to the primary nuclear transport signal of p48$^{v\text{-}myb}$ (21). To test this hypothesis, we infected chicken HD4 erythroid cells with avian retroviruses expressing either wild-type v-*myb* or the mutant 281, 364, or 859. These cells were then fractionated to

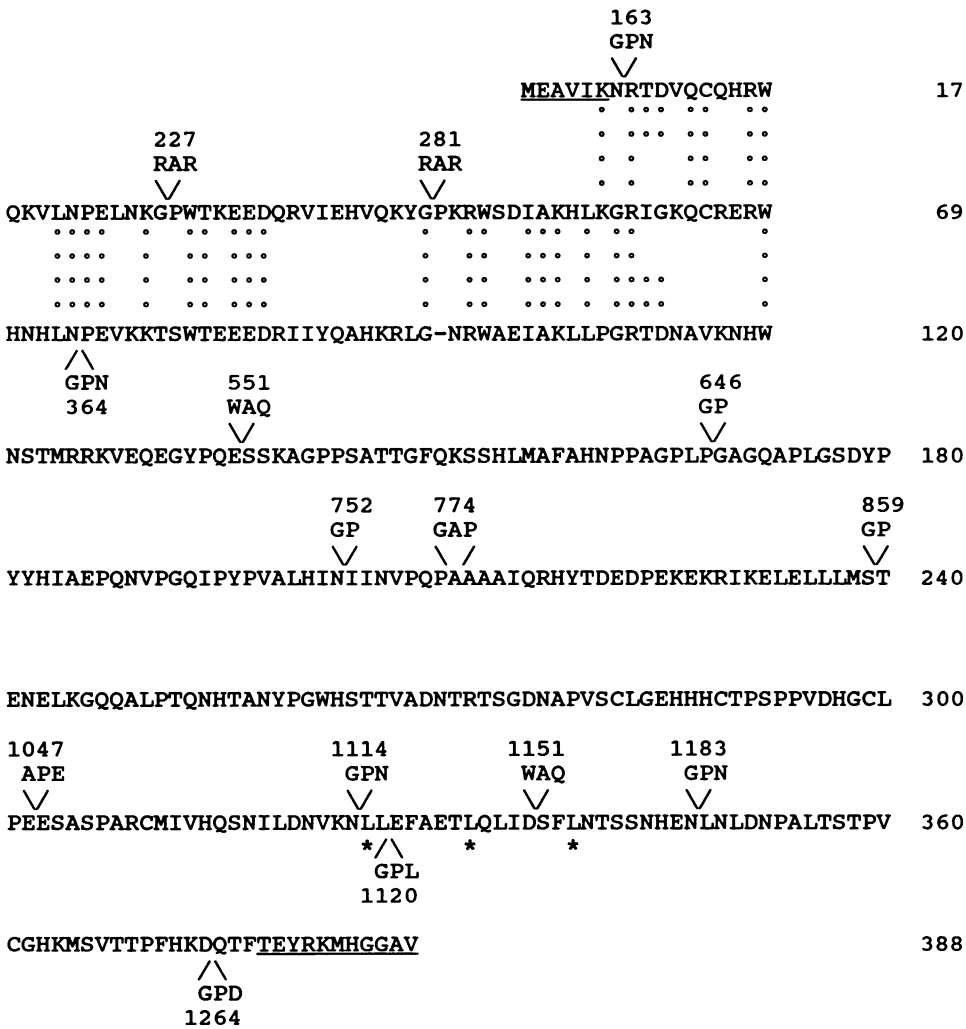

FIG. 2. Linker insertion mutants of p48$^{v\text{-}myb}$. The complete amino acid sequence of wild-type p48$^{v\text{-}myb}$ is shown, as are the alterations of each linker insertion mutant. Amino acid numbers are indicated on the right. Each arrowhead indicates the site of an insertion adjacent to which are indicated the designation of the mutant (number) and the amino acids inserted (letters). The first three lines are arranged to show the portion of the three amino-terminal repeats of p75$^{c\text{-}myb}$ which are retained in p48$^{v\text{-}myb}$. The dotted lines indicate residues which are identical in two or more repeats of p48$^{v\text{-}myb}$. The *gag*- and *env*-encoded amino and carboxyl termini are underlined. The heptad leucine repeats are indicated by asterisks.

separate the nuclei from the cytoplasm and plasma membrane. Immunoblotting demonstrated that the wild-type and all three mutant v-*myb* proteins were present within the cell nucleus (data not shown). Thus, mutants 281 and 859, which both bind DNA and localize to the cell nucleus, have lesions which specifically affect the *trans*-activation function of p48$^{v\text{-}myb}$.

Transformation correlates with *trans*-activation by mutants of v-*myb*. To assay the transforming ability of the 15 linker insertion mutants of v-*myb*, the wild-type or mutant proviral DNAs were each cotransfected into QT6 cells with proviral DNA of the MAV-1 helper virus (31). The transfected QT6 cells were selected for the expression of *neo* with G418, and then pooled colonies were used as sources of virus for several different cocultivation assays. In each assay, the QT6 cells were pretreated with mitomycin C to prevent cellular replication but not virus production. First, to test protein production by the viruses, HD4 avian erythroblasts were cocultivated overnight with mitomycin C-treated, G418-resistant QT6 cells. The HD4 cells were then replated and themselves selected with G418. The drug-resistant HD4 cells were metabolically labeled with [^{35}S]methionine, and lysates were analyzed by immunoprecipitation with anti-*myb* antiserum, SDS-PAGE, and autoradiography (Fig. 5). The wild-type v-*myb* virus and each of the 15 mutant viruses all produced comigrating, immunoreactive p48$^{v\text{-}myb}$-related proteins. As expected, this protein was not present in the original HD4 cells. However, all the HD4 cells did express p75$^{c\text{-}myb}$, which provides a useful internal control for judging the relative abundance of the mutant v-*myb* proteins.

Chicken yolk sac cells from 12-day-old embryos, which are rich in myeloid targets for transformation by v-*myb*, were assayed for transformation by cocultivation with mitomycin C-treated QT6 cells expressing the wild-type and mutant viruses (2, 30). Nine of the mutant viruses transformed yolk sac myeloid cells well, two mutants were weakly transforming (752 and 1114), and four mutants did not transform (227, 281, 364, and 859) (Table 1). p48$^{v\text{-}myb}$ related proteins were demonstrated by immunoprecipitation in yolk sac myeloid cells for all the transforming mutants

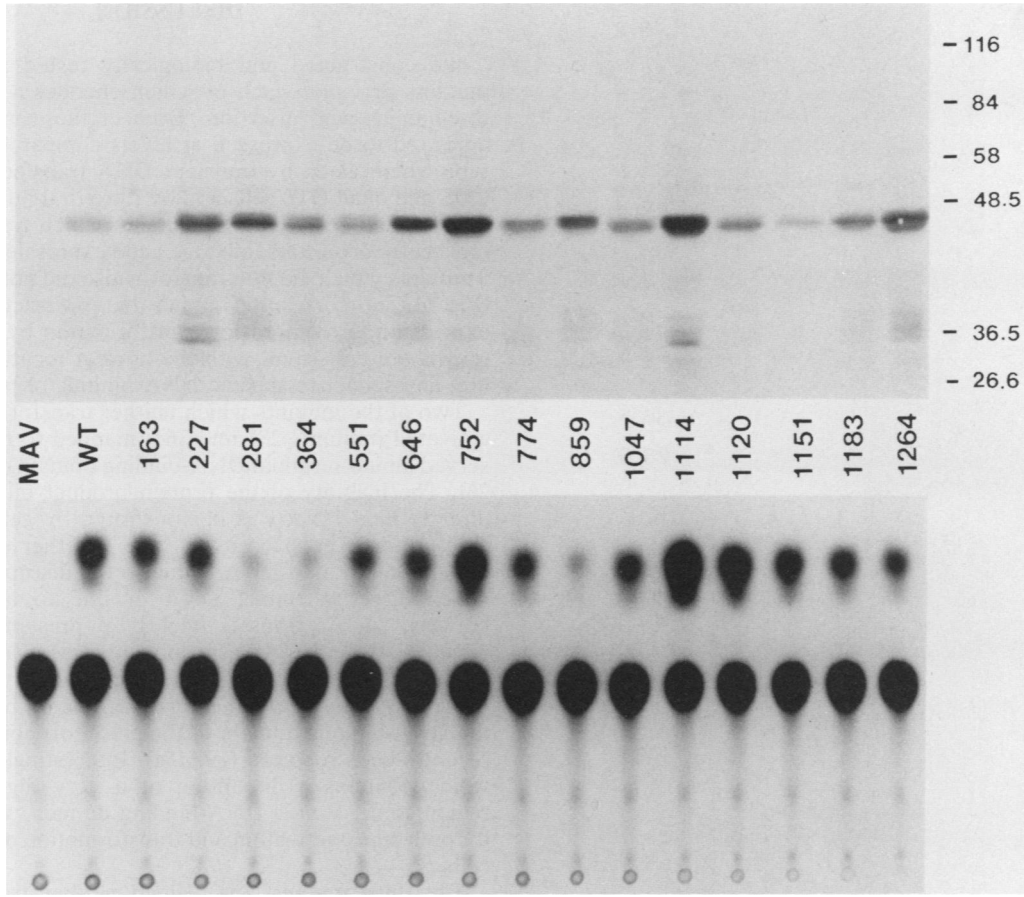

FIG. 3. *trans*-activation by mutants of p48$^{v\text{-}myb}$. Various proviral plasmid DNAs (3 μg) expressing *env* (MAV), wild-type v-*myb* (WT), or the indicated mutants of v-*myb* were cotransfected into quail QT6 cells with 0.2 μg of cytomegalovirus-β-galactosidase DNA and 1 μg of the pTK-dAX reporter plasmid DNA. Two days later, cells were harvested and lysates were assayed for β-galactosidase activity to normalize for transfection efficiency. Each normalized lysate was assayed for the expression of v-*myb* protein by immunoblotting (upper panel; numbers on right show molecular size in kilodaltons) and for CAT activity (lower panel).

except 752, which grew poorly in liquid culture (data not shown). The cells transformed by the various mutants were similar in phenotype to monoblasts transformed by wild-type v-*myb* when examined by cytocentrifugation, Wright-Giemsa staining, and light microscopy (data not shown).

Similar transformation assays were performed with bone marrow cells from 1- to 2-week-old chicks, which contain target cells of all hemopoietic lineages (18). Eleven of the mutant viruses transformed bone marrow cells well, one of the mutants was weakly transforming (774), and three mutants did not transform (281, 364, and 859) (Table 1). Further analysis demonstrated that all transformed bone marrow cells grew well at both 37 and 42°C and displayed a monoblast phenotype similar to that induced by wild-type v-*myb*, with the exception of cells transformed by mutant 752. The latter cells became slightly adherent at 42°C and displayed a macrophagelike morphology, with about 10% of the cells expressing promyelocytelike granules. The ability of mutant 227 to transform bone marrow but not yolk sac cells was observed in two independent experiments and may reflect differences in the target cell populations used or in the various nutrients and growth factors which differ somewhat in these assays. Most importantly, the three mutants which were transformation defective in both the yolk sac and bone marrow assays were the same three mutants which did not *trans*-activate gene expression in QT6 cells (Fig. 3; Table 1).

TABLE 1. *trans*-activation and transformation by mutants of v-*myb*[a]

Mutant	CAT assay (fold activation)	Yolk sac	Bone marrow
Wild type	12	+++	+++
163 (*Hin*fI)	8	+++	+++
227 (*Ava*II)	10	0	+++
281 (*Ava*II)	2	0	0
364 (*Hin*fI)	2	0	0
551 (*Hin*fI)	6	+++	+++
664 (*Sma*I)	8	+++	+++
752 (*Ssp*I)	21	+	+++ (ts)
774 (*Pvu*II)	8	+++	+
859 (*Hin*cII)	3	0	0
1047 (*Mst*II)	8	+++	+++
1114 (*Hin*fI)	38	+	+++
1120 (*Dde*I)	21	+++	+++
1151 (*Hin*fI)	13	+++	+++
1183 (*Hin*fI)	9	+++	+++
1264 (*Ava*II)	8	+++	+++

[a] The restriction enzyme site used to create each mutant is indicated in parentheses. CAT assay results are shown as fold activation over control (NEO-MAV) for a representative assay (Fig. 3). Transformation assays of yolk sac and bone marrow cells were scored as strongly transforming (+++), weakly transforming when outgrowth of transformed cells was very slow (+), or nontransforming (0). All transformed bone marrow cells were tested for temperature sensitivity (ts). Only the cells transformed by mutant 752 had altered morphology and growth properties at 42°C (see text for details).

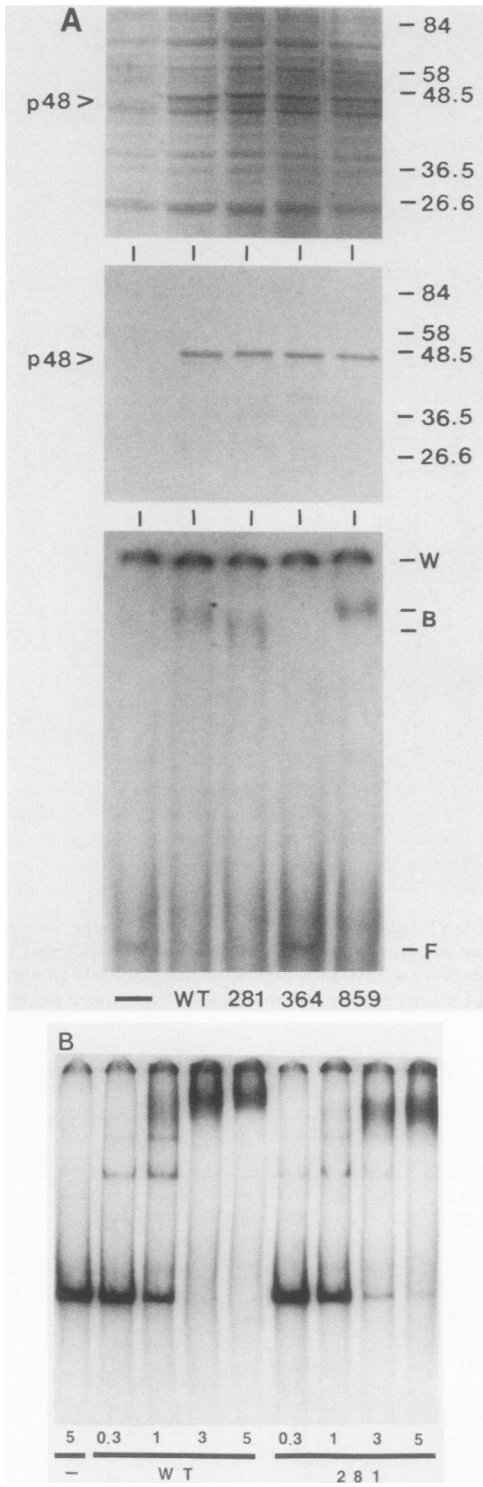

FIG. 4. DNA-binding by mutants of v-*myb* which do not *trans*-activate. *E. coli* harboring the plasmids pT7-MYB-less (−), pT7-MYB (WT), pT7-MYB-281, pT7-MYB-364, and pT7-MYB-859 were induced for protein expression with IPTG. (A) Lysates of bacterial cells were analyzed for total proteins by SDS-PAGE and staining with Coomassie blue (upper panel), for *myb*-related protein by SDS-PAGE and immunoblotting (middle panel), and for DNA binding by gel mobility shift of a ^{32}P-labeled DNA fragment containing nine copies of the consensus *myb* binding site with 5 μg of protein (lower panel). The mobility of p48$^{v\text{-}myb}$ is indicated by an arrowhead on the left, and the mobility of protein molecular size markers is indicated in kilodaltons on the right of the upper two panels. The position of the wells (W) and the mobility of free (F) and bound (B) DNA are indicated on the right of the lower panel. (B) Gel mobility shift assays were conducted with various amounts of protein (micrograms) from extracts of *E. coli* induced for the expression of pT7-MYB-less (−), pT7-MYB (WT), or pT7-MYB-281.

DISCUSSION

We constructed and biologically tested a series of 15 mutants of v-*myb* each of which encodes a single two- or three-amino-acid insertion. Each of these mutant proteins appeared to be expressed at levels comparable to the wild type when tested by transient DNA transfection of simian COS and quail QT6 cells and by retroviral infection of avian hemopoietic cells. The 12 mutants which transformed myeloid cells also *trans*-activated gene expression, whereas the 3 mutants which did not transform also did not *trans*-activate (Fig. 6). This correlation implies that *trans*-activation of gene expression is required for transformation by v-*myb*. These results are consistent with the nuclear location, short half-life, and sequence-specific DNA binding of p48$^{v\text{-}myb}$.

Two of the mutants which neither transformed nor *trans*-activated (mutants 281 and 364) mapped to the highly conserved amino-terminal DNA-binding domain of p48$^{v\text{-}myb}$ (21, 26). The inability of the protein product of mutant 364 to directly bind DNA was demonstrated by gel mobility shift assay after expression in *E. coli*. Whether the inability of mutant 364 to bind DNA is due to the disruption of regions which directly contact DNA or due to altered spacing between such regions is unclear at present. Mutant 281, which also neither transformed nor *trans*-activated, was, however, able to bind DNA and localize within the cell nucleus. A similar effect on transactivation by mutations in the DNA-binding domain of the glucocorticoid receptor has recently been reported (19, 45a). Interestingly, mutant 227, which contains a disruption of a very highly conserved region of the v-*myb* DNA-binding domain, was competent for both *trans*-activation and transformation of bone marrow cells.

The third mutant (859) which neither transformed nor *trans*-activated is located in a more carboxyl-terminal region of p48$^{v\text{-}myb}$ which is not required for nuclear transport or DNA binding (20, 21). The intrinsic ability of the protein product of this mutant to bind DNA was demonstrated by gel mobility shift assays of protein expressed in *E. coli*. It also localized to the cell nucleus in chicken hemopoietic cells. This mutant therefore maps an additional specific domain of p48$^{v\text{-}myb}$ which is required for *trans*-activation but not for DNA binding by p48$^{v\text{-}myb}$. This domain is conserved among vertebrate *myb* proteins, but unlike the DNA-binding domain, it is not conserved in the known *myb*-related proteins of invertebrates, green plants, or lower eucaryotes. The region of v-*myb* containing the insertion site of mutant 859 has recently been shown to be specifically required for *trans*-activation by *GAL4-myb* hybrids as well (49). Several transcriptional activation domains of other proteins resemble amphipathic helices bearing a net negative charge (for a review, see reference 42). In this regard, mutant 859, which differs from the wild type only by the insertion of a Gly-Pro dipeptide, has no change in net charge. However, this mutation would be expected to disrupt an alpha helix if it were present in this region. In summary, p48$^{v\text{-}myb}$ contains two distinct domains which control *trans*-activation by

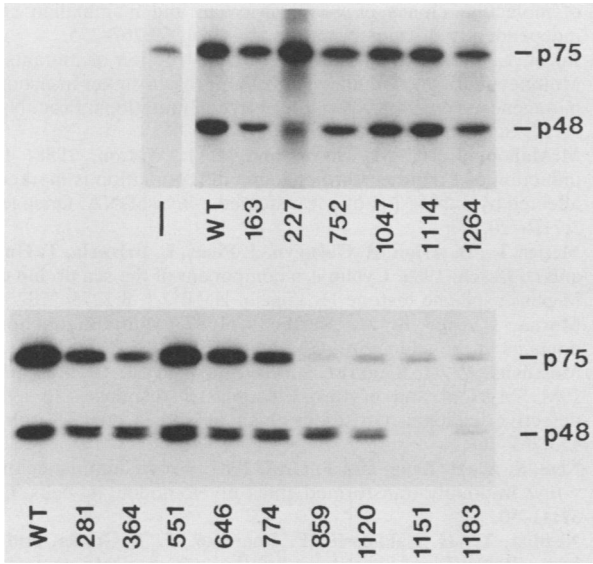

FIG. 5. Expression of wild-type and mutant v-*myb* proteins by infection of avian hemopoietic cells. Avian HD4 cells were infected with no virus (−), wild-type NEO-AMV (WT), or the indicated mutant NEO-AMV viruses and selected with G418. The cells were lysed and assayed for *myb*-related proteins by immunoprecipitation and SDS-PAGE. The mobilities of $p48^{v\text{-}myb}$ and $p75^{c\text{-}myb}$ are indicated on the right.

DNA-bound protein, one within the DNA-binding domain itself and one in the less conserved carboxyl terminus of the protein. This suggests a greater complexity for the action of $p48^{v\text{-}myb}$ than might be expected from studies of the GAL4 and GCN4 proteins, which appear to contain functionally and physically separable DNA-binding and transcriptional activation domains (for a review, see reference 42).

A family of proteins including C/EBP, GCN4, and the products of the *jun*, *fos*, and *myc* oncogenes contain a leucine zipper motif which appears to be required for dimerization and DNA binding by these proteins (28). A series of three leucines with the characteristic heptad spacing of the leucine zipper is also present in the carboxyl terminus of $p48^{v\text{-}myb}$ (Fig. 2). This region of $p48^{v\text{-}myb}$ is not required for the morphologic transformation of myeloid cells but is required for colony formation in soft agar and methocel (21; T. Graf and J. Lipsick, unpublished data). Three of the linker insertions (mutants 1114, 1120, and 1151) are predicted to disrupt this putative leucine zipper structure in $p48^{v\text{-}myb}$ by altering the spacing and local environments of the leucines. However, these mutations prevented neither *trans*-activation nor transformation (Table 1).

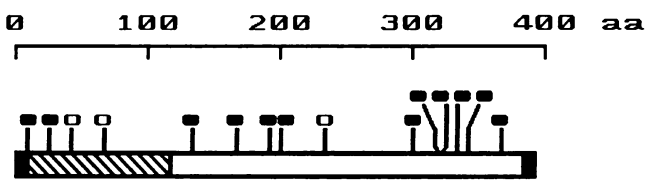

FIG. 6. Transformation and *trans*-activation by mutants of v-*myb*. $p48^{v\text{-}myb}$ is represented by the large rectangular bar, on which the DNA-binding domain (striped bar) and the *gag*- and *env*-encoded termini (black bars) are indicated. The site of each linker insertion mutant of v-*myb* is shown above by a flag. Black flags indicate mutants that are positive for transformation and *trans*-activation; white flags indicate those that are negative for transformation and *trans*-activation. aa, Amino acids.

The strong correlation between transformation and *trans*-activation by $p48^{v\text{-}myb}$ suggests that transformation by v-*myb* is likely to involve the regulation of specific target genes. Because $p48^{v\text{-}myb}$ can be expressed in the nuclei of fibroblasts without any changes characteristic of the transformed phenotype (27), the genes which are targets of v-*myb* are likely to be tissue specific in their accessibility to $p48^{v\text{-}myb}$, in their effects on cell growth and differentiation, or both. The identification and characterization of these target genes may therefore be of interest in understanding normal hemopoietic differentiation as well as its malignant aberrations.

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ADDENDUM IN PROOF

An endogenous gene which is induced by the *myb*-containing avian E26 leukemia virus but not by AMV has recently been identified (S. A. Ness, A. Marknell, and T. Graf, Cell **59**:1115–1125, 1989).

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