Structural and Functional Domains of the *myb* Oncogene: Requirements for Nuclear Transport, Myeloid Transformation, and Colony Formation

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The *v-myb* oncogene of avian myeloblastosis virus causes acute myelomonocytic leukemia in vivo and transforms only myeloid cells in vitro. Its product, p48^{v-myb}, is a nuclear protein of unknown function. To determine structure-function relationships for this protein, we constructed a series of deletion mutants of *v-myb*, expressed them in retroviral vectors, and studied their biochemical and biological properties. We used these mutants to identify two separate domains of p48^{v-myb} which had distinct roles in its accumulation in the cell nucleus. We showed that the viral sequences which normally encode both termini of p48^{v-myb} were dispensable for transformation. In contrast, both copies of the highly conserved *v-myb* amino-terminal repeat were required for transformation. We also identified a carboxyl-terminal domain of p48^{v-myb} which was required for the growth of *v-myb*-transformed myeloblasts in soft agar but not for morphological transformation.

The acutely transforming retroviruses cause a broad spectrum of experimental malignancies due to a correspondingly wide variety of viral oncogenes which they have transduced from normal cellular DNA (reviewed in reference 6). The *v-myb* oncogene of avian myeloblastosis virus (AMV) is unique among these genes in that it causes only myelomonocytic leukemia in vivo and transforms myeloid cells but not fibroblasts in vitro (reviewed in reference 34). The *c-myb* proto-oncogene from which *v-myb* arose has been highly conserved during vertebrate evolution, and at least one domain is highly conserved in invertebrates and in green plants as well (2, 25, 43a, 47). The leukemogenic potential of the *c-myb* gene has been revealed by retroviral transduction with both AMV and avian E26 leukemia virus (7, 51). In addition, the *c-myb* gene has been activated in murine myeloid malignancies by retroviral insertional mutagenesis (49, 53). In all cases thus far examined, activation of *c-myb* has been correlated with predicted truncations of its normal protein-coding sequences.

The mechanism of myeloid transformation by p48^{v-myb} remains unknown. However, several properties of the protein products of *v-myb* and *c-myb* have been described. The product of the *v-myb* gene of AMV, p48^{v-myb}, is located almost entirely within the cell nucleus, as is its normal counterpart, p75^{c-myb} (11, 13, 28, 30). Both of these proteins also display an associated DNA-binding activity of as yet unknown specificity (29, 39). Although p75^{c-myb} is usually expressed in immature hematopoietic cells of all lineages, it is conspicuously absent in myeloblasts transformed by *v-myb*. However, recent studies have demonstrated that p48^{v-myb} does not itself prevent the expression of p75^{c-myb} (33).

As a first step toward studying the molecular genetics of transformation by *v-myb*, we recently developed a transformation assay for molecular clones of this oncogene (35). We have now constructed and analyzed a series of in-frame deletion and truncation mutants of *v-myb* in order to address

several basic questions about p48^{v-myb}. Are specific structural domains of p48^{v-myb} required for its nuclear accumulation? Are the viral *gag* and *env*-encoded amino and carboxyl termini of p48^{v-myb} required for transformation? Are one or both copies of the highly evolutionarily conserved amino-terminal repeat of p48^{v-myb} required for transformation? Are any relatively large domains of p48^{v-myb} dispensable for transformation?

MATERIALS AND METHODS

Cell lines. The methylcholanthrene-transformed QT6 quail cell line (41) was maintained in DME medium supplemented with 5% (vol/vol) fetal calf serum, 4.5 g of glucose per liter, 1× nonessential amino acids, 100 µg of streptomycin per ml, 100 U of penicillin per ml, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) in a humidified 10% CO₂-90% air 37°C incubator. The avian erythroblastosis virus-transformed HD4 chicken erythroblast cell line (3) was maintained in the same medium additionally supplemented with 5% (vol/vol) heat-inactivated (56°C for 1 h) chicken serum.

DNA constructions. All of the *v-myb* mutants except dGAG and dGE were constructed from pUCMYB (36) or a variant of this plasmid, pUCMYB-dE, lacking the *EcoRI* site in the pUC18 polylinker. To reconstruct these *v-myb* mutants into selectable proviruses, the *KpnI*- and *XbaI*-resistant *v-myb* DNA fragments were then used to replace the *KpnI*-*XbaI* *env* fragment of pNEO-MAV (36). All recombinant DNA manipulations were done by standard techniques as described previously (38). The structures of all mutants were verified by restriction enzyme mapping. Nucleotide numbering of the *v-myb* fragment begins at the *KpnI* restriction site (48).

The following deletion mutants (positions) were constructed: mutant d1, deleted from *SmaI* (650) to *HincII* (862); mutant d3, deleted from *HincII* (863) to *MstII* (1048); mutant d4, deleted from *MstII* (1052) to *BalI* (1231); mutant d5, deleted from *EcoRI* (511) to *PvuII* (777); mutant d6, deleted from *HinfI* (165) to *HinfI* (365) with recreation of the *v-myb*

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splice acceptor site (TTTTATATTTTCATTAG/AAT) at nucleotide 164 (26); mutant *d7*, deleted from *HinfI* (369) to *EcoRI* (506); mutant *d8*, deleted from *HhaI* (293) to *HhaI* (520); mutant *d9*, deleted from *HinfI* (168) to *EcoRI* (506) with recreation of the *v-myb* splice acceptor site; mutant *d10*, deleted from *EcoRI* (511) to *XbaI* (1309); mutant *d11*, deleted from *AvaII* (229) to *AvaII* (282); mutant *LX1*, deleted from *SmaI* (650) to *XbaI* (1309) following insertion of a synthetic *XbaI* linker (CTCTAGAG; New England BioLabs) at *SmaI*; mutant *LX2*, deleted from *HincII* (863) to *XbaI* (1309) following insertion of a synthetic *XbaI* linker at *HincII*; mutant *LX3*, deleted from *MstII* (1052) to *XbaI* (1309) following insertion of a synthetic *XbaI* linker at *MstII*; and mutant *LX4*, deleted from *BalI* (1232) to *XbaI* (1309) following insertion of a synthetic *XbaI* linker at *BalI*.

The *dGAG* and *dGE* mutants were first constructed in the SA CLA 12 NCO plasmid, which contains a synthetic splice acceptor site followed by the *v-src* in-frame termination codon and initiation codon, followed by the polylinker of pUC12N, all of which are flanked by *Clal* restriction enzyme sites (21). The *Clal*-resistant *myb*-specific fragments were then isolated and ligated to *Clal*-digested pNEO-MAV-CLA DNA. This latter plasmid is a variant of pNEO-MAV (36) in which the entire *KpnI* to *XbaI* *env* fragment has been replaced with a synthetic *Clal* linker (CATCGATG; Pharmacia). Mutant *dGAG*: The 1.3-kilobase (kb) *myb*-specific *SstI*-resistant fragment of pUCMYB165 (12), in which the TAG codon immediately upstream of the *v-myb* splice acceptor site was replaced by the 5' *lacZ'* open reading frame of pUC18, was subcloned into the *SstI* site of SA CLA 12 NCO. Mutant *dGE*: The *myb*-specific *BstEII*-*XbaI* fragment of pUCMYB-LX4 was used to replace the corresponding fragment of the *dGAG* mutant.

DNA transfection. QT6 cells were transfected with proviral plasmids by the DEAE-dextran method essentially as described previously (15), except that DEAE-dextran was used at 200 μ g/ml. Two days after transfection, cells were split 1:4 into selective medium containing G418 (GIBCO) at 200 μ g of active drug per ml. G418-resistant colonies were detected within 2 weeks after transfection.

Transformation assays. Single-cell suspensions were prepared from the yolk sacs of 12-day-old White Leghorn chicken embryos (McIntyre Poultry, Lakeside, Calif.) by first washing the sacs free of yolk with Hanks buffered salt solution (HBSS) and then mincing the sacs with sterile scalpels and finally dissociating the tissue with a 100-mesh tissue sieve (E-C, St. Petersburg, Fla.) (8). The cells were washed twice in HBSS and then resuspended to 4×10^6 /ml in yolk sac medium, which is identical to the QT6 medium described above except that the fetal calf serum was replaced by 5% (vol/vol) calf serum and 5% (vol/vol) heat-inactivated chicken serum. Ten milliliters of this cell suspension (4×10^7 cells) was then gently layered onto a 10-cm tissue culture dish containing a monolayer of adherent, virus-producing QT6 cells. These QT6 cells had been incubated with mitomycin C (10 μ g/ml in medium) for 2 h and then washed twice with phosphate-buffered saline (PBS). Twenty-four hours after applying the yolk sac cell suspension to this monolayer, the nonadherent cells and medium were removed into fresh dishes. These liquid cultures were then fed weekly with 3 to 5 ml of fresh medium and observed for at least 1 month for the appearance of transformed myeloblasts. To test for colony formation, single-cell suspensions of transformed myeloblasts were seeded into 0.3% agar (Difco) in the same medium and incubated for 2 weeks (40).

Infection of avian erythroblasts. Approximately 10^6 HD4 erythroblasts were cocultured with virus-producing QT6 cells as described above. Two days later, the cells were selected with G418 (1 mg/ml). Selection was continued for at least 2 weeks, during which all growth ceased in mock-infected control cultures.

Antisera. Rabbit antisera raised against a bacterial fusion protein containing the highly conserved amino-terminal domain of *v-myb* and against the carboxyl-terminal peptide of p48^{v-myb} have been described previously (12, 13). Three additional rabbit antisera were similarly raised against bacterial *trpE* fusion proteins partially encoded by the *NcoI*-*EcoRI* or the *SalI*-*MstII* fragment of *v-myb* or by the *BamHI*-*BglII* fragment of the *gag* gene of myeloblastosis-associated virus type 1 (MAV-1).

Cell labeling, immunoprecipitation, and SDS-PAGE. Cells were incubated at 10^7 per ml in methionine-free medium for 60 min at 37°C. [³⁵S]methionine (1,200 Ci/mmol; New England Nuclear) was then added (0.1 mCi/ml), and incubations were continued for 90 min. Cell lysis and immunoprecipitations were performed essentially as described with protein A-Sepharose CL4B as an immunoabsorbent (13). Samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 0.1% SDS–0.5 M urea gels and autoradiography.

Subcellular fractionation. Cells were fractionated as described previously (10, 11). Approximately 3×10^7 cells were pelleted at $500 \times g$ and then lysed by gentle pipetting for 3 min in 2 ml of ice-cold nuclear buffer (150 mM sucrose, 5 mM sodium phosphate, pH 7.4, 50 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 0.5 mM calcium chloride, 1 mM phenylmethylsulfonyl fluoride, 0.1% Kryo-EOB (Proctor and Gamble)). Nuclei were separated from the postnuclear supernatant by centrifugation at $500 \times g$ for 10 min at 4°C. The pelleted nuclei were suspended in 2.2 ml of ice-cold detergent lysis buffer as described above for immunoprecipitation. A 0.2-ml amount of $10\times$ concentrated detergent lysis buffer was added to the 2 ml of postnuclear supernatant. Samples of these fractions were then immunoprecipitated as described above.

RESULTS

Overall experimental strategy. Because *v-myb* does not transform fibroblasts and because hematopoietic targets for transformation by *v-myb* are difficult to transfect directly with DNA, our assay uses an infectious, selectable avian retroviral vector (Fig. 1) (36). The pNEO-AMV proviral plasmid contains a wild-type *v-myb* gene which is expressed via a spliced subgenomic mRNA in a fashion similar to the natural isolate of AMV. The amino terminus of p48^{v-myb} is encoded by the six initial *gag* codons which lie upstream of the normal viral splice donor site. The downstream *v-myb* segment of the pNEO-AMV plasmid, including the transduced splice acceptor site, is flanked by unique *KpnI* and *XbaI* restriction enzyme sites to facilitate the construction of mutant viruses. The NEO-AMV virus also contains the transposon Tn5 *neo* gene, which lies within the viral intron and allows dominant selection of virally infected cells with the drug G418 (14, 50).

The replication-defective NEO-AMV proviral DNA is first cotransfected into QT6 quail cells along with proviral DNA of the helper virus from which AMV arose, MAV-1 (44). These QT6 cells are then selected with G418, resulting in colonies which express the NEO-AMV virus as an MAV-1 pseudotype. The G418-resistant colonies are pooled, treated

FIG. 1. Strategy for the expression of wild-type and mutant *v-myb* gene products.

with mitomycin C to prevent cellular replication but not virus production, and then used to infect hematopoietic cells by cocultivation. To assay transformation by *v-myb*, the yolk sacs of 12-day-old chicken embryos are used as a convenient source of myeloid precursors (1). To also recover and study the protein products of nontransforming mutants of *v-myb*, the HD4 avian erythroblast cell line is used for viral infection and subsequent G418 selection.

Construction and biological assay of the *v-myb* mutants. A series of in-frame deletion and truncation mutants of *v-myb* were constructed (see Materials and Methods for details). The predicted protein products of these mutants are shown schematically in Fig. 2. Several of these constructions deserve additional comment. Mutants *d6*, *d7*, and *d9* represent in-frame deletions of the first, second, or both copies of the evolutionarily conserved 50-amino-acid repeats of *p48^{v-myb}*, respectively. Mutant *LX3* represents a carboxyl-terminal truncation of *p48^{v-myb}* very near the 3' recombination junction of the *myb* segment of the E26 leukemia virus (43). Mutant *LX4*, which has previously been shown to efficiently transform myeloid cells in vitro, represents a carboxyl-terminal deletion of 15 *myb*-specific residues and all 11 *env*-encoded residues of *p48^{v-myb}* (35).

Mutant *dGAG* has replaced the normal *gag*-encoded amino terminus of *p48^{v-myb}* with the following amino acids: wild type, Met-Asp-Ala-Val-Ile-Lys; *dGAG*, Met-Ala-Met-Ile-Thr-Asn-Ser-Ser-Ser-Val-Pro. This was accomplished by using a synthetic splice acceptor site followed by the *v-src* leader sequence and initiation codon to express all of the *v-myb* codons downstream of the normal AMV splice acceptor site. This segment of Rous sarcoma virus has previously been shown to contain an in-frame termination codon which results in translation of a presumptive short *gag* leader peptide followed by reinitiation at the authentic *v-src* AUG (21, 22). Mutant *dGE* is a recombinant between *dGAG* and *LX4* which lacks both the *gag*- and *env*-encoded termini of wild-type *p48^{v-myb}*. Each of the *v-myb* mutants was reconstructed into a selectable provirus to assay biochemical and biological properties.

Protein products of mutant *v-myb* genes. To assess the structure and stability of the protein products of the various

FIG. 2. Structures and functions of the protein products of wild-type and mutant *v-myb* genes. *myb*-encoded domains are shown as open boxes. Amino-terminal *gag*- and carboxyl-terminal *env*-encoded domains are shown as black boxes. Synthetic amino or carboxyl termini are shown as striped boxes. Deleted domains are indicated by gaps. The conserved repeats are shown above the wild type (WT); the solid bars indicate *c-myb* repeats which are completely retained in *v-myb*, and the dashed line indicates the *c-myb* repeat which is truncated in *v-myb*. Subcellular localization (LOCAL) of the proteins is indicated as nuclear (N), cytoplasmic (C), or both (N/C). Transformation in liquid culture (TF) and colony formation in soft agar (AGAR) are also indicated. n.t., Not tested; aa, amino acids.

v-myb mutants, HD4 avian erythroblasts were infected with the appropriate viruses. These cells were then selected for G418 resistance, metabolically radiolabeled, and analyzed for the presence of *myb*-related proteins by immunoprecipitation, SDS-PAGE, and autoradiography (Fig. 3). All of the *v-myb* mutant viruses tested except *d10* produced nearly wild-type levels of stable protein products following infection of avian erythroblasts. Immunoprecipitation of endogenous *p75^{c-myb}* from these cells served as an internal control for this quantitation.

The relative mobilities of the mutant *v-myb* proteins were reasonably consistent with their predicted molecular weights (Table 1). In addition, transient expression of each of these mutant *v-myb* genes by DNA transfection into simian COS cells produced proteins of similar relative mobilities (data not shown). This suggests that the coding capacities of the mutant viruses were not altered during proviral transfection and subsequent round(s) of viral replication in avian cells. The protein products of wild-type *p48^{v-myb}* and the mutants *d1*, *d3*, *d4*, *d5*, *d6*, *d7*, *d8*, *d9*, and *d11* were all immunoprecipitated by an anti-carboxyl terminus *myb*-specific antiserum, verifying that their respective deletions were in fact in frame (data not shown). As expected, the products of the carboxyl-terminal truncation mutants *LX1*, *LX2*, *LX3*, and

FIG. 3. Expression of wild-type and mutant *v-myb* gene products in avian erythroblasts. HD4 avian erythroblasts were infected with wild-type (WT) or various mutant *neo-myb* viruses, selected for G418 resistance, and then labeled with [35 S]methionine. Lysates of cells infected with the indicated viruses were immunoprecipitated twice and then analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography. Relative mobilities of wild-type p48^{v-myb} and p75^{c-myb} are indicated on the left. Relative mobilities of coelectrophoresed 14 C-labeled methylated protein molecular weight standards (Amersham) are also indicated (in kilodaltons). Left panel: Anti-amino terminus *myb*-specific antiserum (12). Right panel: Anti-*myb*-specific antiserum raised against the *Sall*-*MstII* domain of *v-myb*.

LX4 were not recognized by this anti-carboxyl terminus antiserum. The protein products of deletion mutants *d6*, *d9*, and *d11* were not immunoprecipitated by a previously described anti-amino terminus *myb*-specific antiserum (12), mapping the epitope recognized by this antiserum.

Nuclear accumulation of mutant *v-myb* proteins. Several nuclear proteins have recently been shown to have dominantly acting primary sequence domains which direct the import of these proteins into the cell nucleus (reviewed in reference 16). To determine whether p48^{v-myb} possesses any such karyophilic domains, we examined the nuclear accumulation of various mutant *v-myb* proteins in HD4 erythro-

TABLE 1. Characteristics of *v-myb* mutants^a

Mutant	Deletion	Predicted mol wt	<i>M_r</i>
<i>d6</i>	7-73	35,472	44,000
<i>d11</i>	30-47	41,444	47,000
<i>d8</i>	50-125	34,553	43,000
<i>d7</i>	75-120	38,204	44,000
<i>d9</i>	7-120	29,896	37,000
<i>d5</i>	123-211	34,209	38,000
<i>d1</i>	169-239	35,802	42,000
<i>d3</i>	240-301	37,063	37,000
<i>d4</i>	302-362	37,021	40,000
LX1	169-388 ^b	20,096	22,000
LX2	240-388 ^b	27,960	29,000
LX3	302-388 ^b	34,563	38,000
LX4	363-388 ^b	41,208	46,000
<i>dGAG</i>	1-6 ^c	44,131	48,000
<i>dGE</i>	1-6 ^c ;	41,655	46,000
	363-388 ^b		

^a Deletions are given in amino acid numbers (modified from the predicted protein sequence of Klempner et al. [27] by the addition of six amino-terminal *gag*-encoded residues). *M_r* is the relative mobility observed in SDS-PAGE (12.5% acrylamide).

^b Six additional amino acids (SRAQLZ) were inserted at the carboxyl terminus.

^c Eleven additional amino acids (MAMITNSSSPV) were inserted at the amino terminus.

FIG. 4. Subcellular localization of mutant *v-myb* gene products in avian erythroblasts. HD4 avian erythroblasts were infected with wild-type (WT) or various mutant *neo-myb* viruses, selected for G418 resistance, and then labeled with [35 S]methionine. These cells were then separated into nuclear (N) and cytoplasmic/membranous (C) fractions adjusted to represent equal cell concentrations. Each fraction was immunoprecipitated twice and then analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography. The relative mobilities of wild-type p48^{v-myb} and p75^{c-myb} are indicated on the left. However, the three panels represent separate experiments, and therefore relative mobilities are not comparable between panels. Left panel: Anti-*myb*-specific antiserum raised against the *NcoI*-*EcoRI* domain of *v-myb*. Middle panel, except mutant *d6*: anti-amino terminus *myb*-specific antiserum (12). Right panel and mutant *d6* in middle panel: anti-*myb*-specific antiserum raised against the *Sall*-*MstII* domain of *v-myb*.

blasts (Fig. 4). These cells were metabolically labeled with [35 S]methionine, then gently disrupted under isosmotic conditions, and separated into nuclear and nonnuclear fractions. No significant cross-contamination of these fractions was observed by two criteria. First, p75^{c-myb} served as an internal control for the normal nuclear distribution of *myb* proteins (Fig. 4). Second, samples of the same fractions were also immunoprecipitated with anti-*gag*-specific antiserum to verify the expected nonnuclear localization of the viral structural proteins (data not shown).

The protein products of mutants *d6*, *d8*, and *d9* were almost entirely absent from the nuclear fractions, whereas mutants *d7* and *d11* produced proteins which did enter the cell nucleus (Fig. 4). These results mapped a site that was absolutely required for the nuclear accumulation of p48^{v-myb} to a small domain of at most 29 amino acids (Fig. 5). This site was present in the first but not in the second amino-terminal repeat of the protein and was highly conserved during evolution. Mutants *d3*, LX1, LX2, and *d11* encoded proteins which were distributed in significant proportions in both the nuclear and nonnuclear fractions (Fig. 4). All other mutant *v-myb* proteins were localized almost entirely in the nuclear

FIG. 5. Conservation of the *myb* nuclear transport signal sequence. The domain absolutely required for nuclear transport of p48^{v-myb} as delineated by mutants *d6* (cytoplasmic), *d7* (nuclear), *d8* (cytoplasmic), and *d11* (predominantly nuclear) is given in the standard single-letter amino acid code. The homologous domains of the chicken (CHK), mouse (MOU), human (HUM), and *Drosophila* (DRO) *c-myb* protein products are compared with this sequence. Dashes indicate identity with the p48^{v-myb} sequence. Endpoints of the relevant deletions are indicated above the p48^{v-myb} sequence.

FIG. 6. Colony formation by myeloblasts transformed by wild-type and mutant *v-myb* genes. Primary embryonic chicken yolk sac cells were transformed in liquid culture by the indicated viruses. Transformed myeloblasts were then plated in 0.3% agar, and representative colonies were photographed 2 weeks later.

fractions with $p75^{c-myb}$, as was wild-type $p48^{v-myb}$. These results suggest that a second nonadjacent domain bounded by the deletion endpoints of mutant *d3* is required for wild-type levels of nuclear accumulation in addition to the absolute requirement for the domain present within the first amino-terminal repeat. The deletion in mutant *d11* also resulted in partial nuclear and cytoplasmic protein localization, which in this case may be due to the proximity of the deletion of the conserved 29-amino-acid site described above (Fig. 5).

Transformation by mutants of *v-myb*. All of the internal deletion mutants and several of the truncation mutants failed to transform myeloid cells, suggesting that AMV has transduced a nearly minimal transforming region of the *c-myb* proto-oncogene (Fig. 2). However, the truncation mutants LX3, LX4, *dGAG*, and *dGE* did transform myeloid cells in vitro to various degrees. The wild-type NEO-AMV virus and the LX4 truncation mutant transformed yolk sac myeloid cells equally well in vitro (35), and the resulting myeloblasts formed large colonies in soft agar as previously described for the natural isolate of AMV (40). Cells transformed by mutant *dGE* grew well in liquid culture and also formed robust colonies in soft agar (Fig. 6), demonstrating that neither *gag*-nor *env*-encoded sequences are required for transformation by *v-myb*. Cells transformed by mutant *dGAG* grew somewhat more slowly in liquid culture and formed characteristically smaller colonies in soft agar (Fig. 6).

Yolk sac cells infected with the mutant LX3 virus produced myeloblasts which grew very slowly in liquid culture

and formed only abortive colonies in soft agar, in which differentiation into macrophages could be observed (Fig. 6). In contrast to cells transformed by wild-type *v-myb* or mutants LX4, *dGE*, and *dGAG*, those transformed by mutant LX3 could not be subcultured in the absence of a yolk sac macrophage feeder layer. This suggests an absolute requirement for a macrophage-derived growth factor (MGF), presumably c-MGF (32), when a specific carboxyl-terminal domain of $p48^{v-myb}$ is deleted, as in mutant LX3, which lacks amino acids 302 to 388. Mutant *d4* contained a slightly smaller deletion (amino acids 302 to 362) than the truncation mutant LX3. Infection of yolk sac myeloid cells with the *d4* virus occasionally resulted in the persistence of small round myeloblastlike cells, but these cells proliferated slowly if at all and could not be further analyzed. This suggests that the extreme carboxyl terminus of $p48^{v-myb}$ (amino acids 363 to 388) may be inhibitory for cell proliferation. This notion was supported by the difference in growth characteristics of cells transformed by mutants *dGAG* and *dGE*, which differ only in this carboxyl-terminal domain (Fig. 2 and 6).

The differences in the morphologies of cells transformed by wild-type *v-myb* and the various transforming mutants were not due to differences in levels of protein expression. Immunoprecipitation and SDS-PAGE revealed that mutants *dGAG* and *dGE* expressed levels of *v-myb* proteins in transformed myeloblasts which were similar to that of the wild-type *v-myb* gene (Fig. 7). The absence of the carboxyl-terminal domain of $p48^{v-myb}$ in mutant *dGE* was documented by the failure of an anti-carboxyl terminus *myb*-specific

FIG 7. Expression of wild-type and mutant *v-myb* gene products in transformed myeloblasts. Myeloblasts transformed in liquid culture by the indicated viruses were labeled with [^{35}S]methionine, and lysates were immunoprecipitated with preimmune serum (lanes P), anti-amino terminus *myb*-specific antiserum (lanes N), or anti-carboxyl terminus *myb-env*-specific antiserum (lanes C). Samples were then analyzed by SDS-PAGE (10% polyacrylamide) and autoradiography.

antiserum to immunoprecipitate its protein product. Similar results had been reported for mutant LX4 (35). In addition, cells transformed in liquid culture by mutant LX3 also expressed nearly wild-type levels of mutant *v-myb* protein, which, as expected, also failed to be immunoprecipitated by anti-carboxyl terminus *myb*-specific antiserum (Fig. 7). This implies that the dissociation of morphological transformation and colony formation observed with mutant LX3 was not due to a protein dosage effect.

DISCUSSION

The *v-myb* leukemia gene has been difficult to study because, unlike other oncogenes, it transforms only myeloid cells and not fibroblasts. Hematopoietic cells themselves are not readily transfected with DNA, and therefore we had constructed a selectable avian retroviral vector containing both *v-myb* and the dominantly selectable *neo* gene. This vector is capable of transforming myeloid cells in vitro and also allows the recovery of the protein products of altered *v-myb* genes regardless of their transforming abilities (35, 36). We have now used this system to construct and express a series of adjacent and overlapping deletion mutations of the *v-myb* oncogene to delineate structure-function relationships in its protein product, *p48^{v-myb}*.

Although the function of *p48^{v-myb}* in transformation is unknown, this protein has been localized to the cell nucleus (11, 30). The transport of proteins to the cell nucleus has been shown in several cases to require a dominantly acting primary structural determinant (reviewed in reference 16). In one of the best-studied examples, a single amino acid change in the nuclear SV40 T antigen results in a cytoplasmic variant which, rather surprisingly, retains its transforming ability (24, 31). We have examined the nuclear transport of the protein products of our *v-myb* deletion and truncation mutants and identified two independent sites which function in nuclear accumulation of *p48^{v-myb}*. Mutants *d6*, *d7*, *d8*, *d9*, and *d11* delineate a common 29-amino-acid domain present in the first but not the second amino-terminal repeat which is absolutely required for transport of *p48^{v-myb}* into the nucleus. The conservation of this domain in the human, murine, avian, and *Drosophila myb* genes is consistent with the

nuclear location of the protein products of all of these genes (12). Mutant *d3* defined a second nonadjacent domain which functions only in the presence of the first domain and which is required for a completely nuclear localization of *p48^{v-myb}*. The reason for incomplete nuclear localization of mutants lacking this second domain is presently unknown but may be related to a lack of intranuclear binding to matrix structures or to nucleic acids (10, 29). The deletion mutants described in this report should be of use in defining domains of *p48^{v-myb}* which interact with other nuclear macromolecules.

We used endogenous *p75^{c-myb}* as an internal control of wild-type nuclear localization in these studies. Unlike the cytoplasmic SV40 T antigen mutant, which blocks the nuclear transport of wild-type T antigen, none of the mutant *v-myb* proteins acted in a dominant fashion to block the nuclear transport of *p75^{c-myb}*. We believe that we are in fact studying nuclear transport rather than passive nuclear accumulation for two reasons. First, we used a radiolabeling period (90 min) which is much shorter than the doubling time of the cells (greater than 12 h). Therefore, only a small percentage of the cell population can have undergone mitosis and dissolution of the nuclear membrane during the labeling period. Second, despite the relatively small size of *p48^{v-myb}* and its mutants, diffusion is unlikely to play a role in this process during the 90-min labeling period. Model studies have shown that only 5% of a protein of M_r 45,000 and 12% of a protein of M_r 13,000 can diffuse into the nucleus within 24 h (reviewed in reference 9).

The protein products of all of the known retroviral oncogenes differ structurally from their normal cellular counterparts (reviewed in reference 6). Many of these viral oncogenes produce fusion proteins which are encoded by sequences of both viral and cellular origin. In some cases the virally encoded residues have been shown to be required for transformation (17, 45). *p48^{v-myb}* is a hybrid protein composed of a short *gag*-encoded amino terminus, a *myb*-encoded central domain, and a short *env*-encoded carboxyl terminus. We have shown that variants of *p48^{v-myb}* which lack either or both of these virally encoded termini retain their transforming ability.

A number of viral oncogenes contain relatively large domains which are dispensible for transformation (e.g., *abl*, *myc*, *fos*) (20, 23, 45, 52). All of the internal deletion mutants of *v-myb* which we studied failed to transform myeloblasts in vitro, although they did produce wild-type levels of stable proteins in avian hematopoietic cells. Our results therefore suggest that *v-myb*, which itself is a truncated form of *c-myb*, represents a nearly minimal transforming domain of this proto-oncogene. In addition, these mutants provide formal genetic proof that *v-myb* is in fact responsible for AMV-induced leukemogenesis, because they contain the first mutations affecting transformation which have been definitively mapped to *v-myb*. Temperature-sensitive mutants of both AMV and the E26 virus have been described, but their genetic lesions remain unknown (5, 42).

A region of particular interest is present near the amino terminus of *p48^{v-myb}*. This region contains two copies of an imperfect 50-amino-acid tandem repeat which is present in three copies in *p75^{c-myb}*. This domain of *c-myb* is highly conserved in diverse species, including *Zea mays*, *Drosophila*, chicken, mouse, and humans (18, 19, 25, 37, 43a, 46). We have shown that deletion of the first (mutant *d6*), second (mutant *d7*), or both (mutant *d9*) of these repeats abolishes the transforming ability of *p48^{v-myb}*. Furthermore, deletion of only 18 amino acids within the first repeat (mutant *d11*)

completely abolishes transformation, although the resulting protein product is still present within the cell nucleus.

An additional domain of interest for transformation has been revealed by our deletional analysis of *v-myb*. The lack of a specific carboxyl-terminal domain of *v-myb* (mutant LX3) results in a dissociation of morphological transformation and the ability to form colonies in soft agar. Furthermore, unlike myeloblasts transformed by wild-type *v-myb*, these LX3-transformed myeloblasts cannot be cultured in the absence of macrophage feeder layers. Interestingly, the same domain lacking in mutant LX3 is also absent in the *v-myb* gene of the E26 leukemia virus, which transforms myeloblasts in vitro in a highly growth factor-dependent fashion (4). This suggests that this carboxyl-terminal domain of p48^{*v-myb*} may also be required for growth factor independence, which in turn implies that this region of the *myb* nuclear protein may interact directly with a signalling pathway which begins at the plasma membrane.

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