Protein Truncation Is Required for the Activation of the *c-myb* Proto-Oncogene

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The protein product of the *v-myb* oncogene of avian myeloblastosis virus, v-Myb, differs from its normal cellular counterpart, c-Myb, by (i) expression under the control of a strong viral long terminal repeat, (ii) truncation of both its amino and carboxyl termini, (iii) replacement of these termini by virally encoded residues, and (iv) substitution of 11 amino acid residues. We had previously shown that neither the virally encoded termini nor the amino acid substitutions are required for transformation by v-Myb. We have now constructed avian retroviruses that express full-length or singly truncated forms of c-Myb and have tested them for the transformation of chicken bone marrow cells. We conclude that truncation of either the amino or carboxyl terminus of c-Myb is sufficient for transformation. In contrast, the overexpression of full-length c-Myb does not result in transformation. We have also shown that the amino acid substitutions of v-Myb by themselves are not sufficient for the activation of c-Myb. Rather, the presence of either the normal amino or carboxyl terminus of c-Myb can suppress transformation when fused to v-Myb. Cells transformed by c-Myb proteins truncated at either their amino or carboxyl terminus appear to be granulated promyelocytes that express the Mim-1 protein. Cells transformed by a doubly truncated c-Myb protein are not granulated but do express the Mim-1 protein, in contrast to monoblasts transformed by v-Myb that neither contain granules nor express Mim-1. These results suggest that various alterations of c-Myb itself may determine the lineage of differentiating hematopoietic cells.

Retroviruses have become important tools in studying the molecular mechanisms of tumorigenesis. In particular, the acutely oncogenic retroviruses were found to carry transforming genes derived from normal cellular genes (for a review, see reference 3). Subsequent work has shown that many of these cellular proto-oncogenes are also activated in spontaneously occurring tumors, including those caused by non-acutely oncogenic retroviruses. In the majority of the cases studied, various changes in the structures of the protein products of these proto-oncogenes have been linked to the induction of the transformed phenotype. In some instances, however, no change in protein structure has been implicated in the activation of a potential oncogene. Instead, aberrant or excessive expression of an otherwise normal gene product appears to be the cause of malignant activation. Given the diversity of such mechanisms of proto-oncogene activation, the elucidation of the specific mode(s) of activation of a given proto-oncogene appears essential for understanding its function in the transformed cell.

Avian myeloblastosis virus (AMV) induces a rapidly fatal monocytic leukemia in infected chickens and transforms only myelomonocytic cells in vitro (18, 41). The *v-myb* oncogene of AMV, which encodes v-Myb, arose through viral transduction of sequences derived from a cellular gene, *c-myb*, which encodes a much larger protein, c-Myb (for a review, see reference 53). During this transduction, the c-Myb protein underwent three structural changes: (i) large domains at both the amino (N) and carboxyl (C) termini of c-Myb were deleted; (ii) the deleted N and C termini were replaced by short sequences encoded by the viral *gag* and

env genes, respectively; and (iii) v-Myb sustained 11 amino acid substitutions relative to the homologous region in c-Myb (13, 51). A segment of the *c-myb* gene has also been transduced independently by the avian E26 leukemia virus, which transforms both myeloid and erythroid cells. However, in addition to similar structural changes in *c-myb*, a second cell-derived oncogene, *v-ets*, has been linked in frame to the *gag*- and *myb*-related sequences of E26 (30, 44). The *v-ets* oncogene has recently been found to transform erythroid cells on its own (39).

As well as being transduced during the genesis of the two acutely oncogenic avian retroviruses, AMV and E26, *c-myb* has been activated by the insertion of retroviruses that do not carry their own oncogenes. Murine leukemia viruses have been found to insert within *c-myb* and to direct the transcription of hybrid viral-*myb* mRNAs that are associated with the induction of myeloid leukemias (for a review, see reference 53). These leukemic cells express Myb proteins that are truncated at either the N or C terminus (9, 49, 54). In addition, the integration of either the EU-8 or RAV-1 avian retroviruses within the *c-myb* gene is strongly associated with the induction of nonbursal B-cell lymphomas (25, 46).

To understand the mechanisms of activation of the *c-myb* proto-oncogene, we have chosen the *v-myb* oncogene of AMV as a model. Molecular clones of this oncogene can transform myeloid cells in vitro (33). In addition, a system has been described that permits the recovery and analysis of nontransforming as well as transforming alleles of *v-myb* (34). We have previously shown that neither the addition of virally encoded *gag* or *env* residues nor the presence of amino acid substitutions is required for transformation by v-Myb (21, 33, 56). This study was therefore undertaken to determine whether the expression of the full-length c-Myb

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protein by a retrovirus would result in the transformation of hematopoietic target cells or whether deletions at either one or both ends of the protein were required for oncogenic activation. Included were three mutants of c-Myb that affected a region of homology near its C terminus that is conserved between vertebrate and *Drosophila* Myb proteins in addition to the more widely conserved N-terminal DNA-binding domain (26, 45). Although the specific amino acid substitutions of v-Myb are not necessary for transformation, we also wished to determine whether they might be sufficient for the activation of c-Myb. Therefore, we tested a second set of mutants that contained a central v-Myb region linked to either one or both of the N- or C-terminal regions of c-Myb that are not present in v-Myb.

MATERIALS AND METHODS

Cell lines. Quail QT6 fibroblasts were grown in Dulbecco's modified essential medium supplemented with 5% fetal calf serum, 4.5 g of glucose per liter, nonessential amino acids, L-glutamine, sodium pyruvate, 100 µg of streptomycin per ml, and 100 U of penicillin per ml in a humidified 10% CO₂-90% air incubator at 37°C. All cells infected with Neo-Myb retroviruses were maintained under selection with G418 (Geneticin; GIBCO) at 200 µg of active drug per ml.

DNA constructions. During viral transduction, sequences encoding both the N- and C-terminal ends of c-Myb were deleted. In naming the various mutants, c-Myb was therefore considered to have three domains, and this protein and the virus encoding it are referred to as CCC. Deletions of either terminus are indicated by the letter d. If, for example, the N terminus of c-Myb was deleted, then the resulting viral construct and protein are denoted as dCC. Likewise, wild-type v-Myb is referred to as dVd.

Plasmids pMAV-1, pUCMYB-dE, pNEO-AMV, pUCMYB-LX4-NM, pNEO-AMV-LX4-NM, pNEO-MAV, and pNEO-MAV-CLA have been described elsewhere (21, 34, 56). For consistency, the virus pNEO-AMV-LX4-NM and the protein that it encodes are referred to in this report as dCd. Plasmids pSG3 (13) and pSA-CLA-12-NCO (19) were kindly provided by Steve Gerondakis and Mike Bishop and by Steve Hughes, respectively. Recombinant DNA manipulations were performed by using standard techniques (37).

The complete coding region of a chicken *c-myb* cDNA was initially excised from pSG3 as an *EagI*-*Bsp*MI resistant fragment and inserted into the *Bam*HI site of the adaptor plasmid pSA-CLA-12-NCO by using synthetic oligonucleotide linkers. To allow the efficient expression of the *c-myb* coding region within a retroviral context, the 5'-untranslated region immediately upstream of the *c-myb* initiation codon was replaced with the corresponding *v-src* 5' untranslated region contained in the adaptor plasmid by insertion of a double-stranded synthetic *NcoI*-*Bsu*36I linker that encoded amino acids 1 to 31 of c-Myb. This construction was verified by the presence of an *NcoI* site that contains the modified *c-myb* initiation codon and by DNA sequencing by the dideoxy-chain termination method (6). The resulting plasmid contained nucleotides 127 to 2074 of the chicken *c-myb* cDNA derived from pSG3. To express full-length c-Myb (CCC) or mutant proteins with intact N termini, *Clal*-resistant *c-myb*-derived fragments were inserted into the unique *Clal* site of pNEO-MAV-CLA (Fig. 1). Mutants containing N-terminal truncations similar to that of v-Myb were constructed from plasmid pUCMYB-LX4-NM or pUCMYB-dE. The *KpnI*-*XbaI*-resistant *myb*-derived fragments were then used to replace the *env* gene of pNEO-MAV.

FIG. 1. Structure of proviral expression vectors. The prototype retroviruses for expression of Myb proteins with intact N termini (NEO-CCC) or truncated N termini (NEO-dCd) are diagrammed. The black boxes represent MAV-1-derived long terminal repeats. The splice donor (SD) and splice acceptor (SA) sites are indicated, as are the initiation codons (↑) and termination codons (↓) relevant for Myb protein expression as described in the text. The relative positions of selected restriction enzyme sites are also indicated (*Clal*, *KpnI*, and *XbaI*).

The predicted structures of the various MYB proteins are indicated in Table 1 and Fig. 2. Details of plasmid constructions are available upon request and are omitted here by request of the editor. All of the N-terminally truncated proteins are predicted to contain six amino acids (MEAVIK) encoded by the spliced *gag* leader sequences as is the case for v-Myb of wild-type AMV (Fig. 1). The C terminus of dCd and CCd is identical to that of the v-Myb mutants LX4 and dGAG/ENV (21, 33, 56) and is compatible with myeloid transformation.

Transient and stable expression of Myb proteins in QT6 and COS cells. All *myb* proviral DNAs were transiently transfected as calcium phosphate coprecipitates into quail QT6 cells (5). The expression of Myb proteins was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, using monoclonal anti-Myb-2.2 antibody (11) and rabbit anti-mouse immunoglobulin.

TABLE 1. Proteins encoded by Myb viruses

Virus	Myb residues ^a	N terminus	C terminus	Predicted size (kDa)
CCC	C: 1-641	c-Myb	c-Myb	72
dCd	C: 72-427	Gag	SRAQL	41
dCC	C: 72-641	Gag	c-Myb	65
CCd	C: 1-427	c-Myb	SRAQL	49
SH	C: 1-462	c-Myb	LD	53
BH	C: 1-498	c-Myb	PRVL	57
SB	C: 1-462	c-Myb	c-Myb	69
	C: 498-641			
CVC	C: 1-71	c-Myb	c-Myb	72
	V: 72-442			
	C: 443-641			
dVd	V: 72-442	Gag	Env	44
dVC	V: 72-442	Gag	c-Myb	65
	C: 443-641			
CVd	C: 1-71	c-Myb	Env	51
	V: 72-442			

^a The residues derived from c-Myb (C) or v-Myb (V) are numbered according to their position within the c-Myb sequence (13).

FIG. 2. Structure of wild-type and mutant Myb proteins. The c-Myb protein (CCC) and various truncations, deletions, and recombinations with v-Myb (dVd) are diagrammed. The stippled boxes indicate the highly conserved Myb DNA-binding domain, which is composed of three imperfect direct repeats of approximately 50 amino acids each. The striped boxes indicate a second more C-terminal domain of approximately 30 amino acids that is conserved between vertebrate and *Drosophila* Myb proteins. The black boxes indicate a domain that is required for transcriptional transactivation by the Myb proteins. dVd and its derivatives (CVC, dVC, and CVd) all contain the 11 amino acid substitutions caused by mutations in *v-myb* of AMV. The number of transformed colonies obtained per 10^4 bone marrow cells seeded 5 days after infection as described in Table 2 is indicated: 0, no colonies; +, fewer than 100 colonies; ++, 100 to 500 colonies; +++, more than 500 colonies. The phenotype of the transformed cells is indicated as depicted in Fig. 8.

lin G coupled to alkaline phosphatase (Promega, Madison, Wis.) as previously described (22). Protein production was also assayed in simian COS cells by similar transient expression and radioimmunoprecipitation, using polyclonal rabbit anti-TrpE-Myb7 serum as previously described (12, 34).

Each *myb* proviral DNA (5 μ g) was stably cotransfected with pMAV-1 helper viral DNA (1 μ g) into quail QT6 cells by the same calcium phosphate method. G418-resistant colonies were selected at 200 μ g of active drug per ml, pooled, and grown in mass culture. Culture supernatants containing each virus were then used to infect fresh QT6 cells, followed by a second round of G418 selection. The presence of mutant proteins in the pooled G418-resistant colonies was detected by immunoblotting as described above.

Transformation assays. The various *myb* viruses were assayed by cocultivation of virus-infected QT6 cells with hematopoietic cells from chicken bone marrow cultures as

described previously (17, 29). Briefly, 2×10^7 chicken bone marrow cells were cocultivated for 2 days with 10^6 virus-producing, mitomycin-treated QT6 cells, and then the medium was changed or the cells were passaged at 2-day intervals. Myeloid culture conditions included crude chicken myelomonocytic growth factor (cMGF) (31); erythroid culture conditions included anemic chicken serum (1 to 3%) as a source of erythropoietin (48). After 5 days, 10^5 cells from myeloid conditions were seeded into 35-mm dishes in growth medium containing cMGF and Methocel (18). Each mutant was tested at least three different times with similar results. The presence of Myb proteins in the transformed cells from the mass cultures was assayed by metabolic labelling with [35 S]methionine, immunoprecipitation with rabbit anti-TrpE-Myb7 (12) or anti-Mim-1 serum (43), SDS-PAGE, and autoradiography. Transformed cells were characterized by various histochemical stains, including a modified Wright-Giemsa, astra blue, toluidine blue, peroxidase, and ATPase (14). The transformed cells were further characterized by immunofluorescence with monoclonal antibodies IC3, which is specific for granulocytes (36), and 47/83, which is a general marker of the myeloid lineage (28). For transmission electron microscopy, cells were collected on Millipore filters (0.45- μ m pore size) and fixed in situ with 3% glutaraldehyde prior to embedding and sectioning.

RESULTS

Construction of proviral vectors. The main goal of this study was to determine whether the expression of a full-length protein or truncated versions of the chicken c-Myb protein by an avian retrovirus would transform hematopoietic target cells. To express c-Myb in an AMV-like retrovirus, we used a strategy similar to that used for the *v-src* gene of the Rous sarcoma virus (20). In this case, *v-src* is transcribed as a spliced, subgenomic mRNA containing two open reading frames. The first is a short open reading frame initiating in *gag* and terminating downstream of the splice acceptor site. This termination codon is followed by the authentic *v-src* initiation codon. Thus, a single mRNA directs the synthesis of both a presumptive small *gag*-related peptide as well as the full-length *v-src* protein. To express c-Myb in a similar fashion, the complete coding region of a chicken *c-myb* cDNA was excised from plasmid pSG3 (13) and ligated into plasmid pSA-CLA-12-NCO (19) as described in Materials and Methods. pSA-CLA-12-NCO itself contains a functional splice acceptor followed by the *v-src* leader and initiation codon, including the upstream termination codon described above. We precisely replaced the *v-src* initiation codon with that of the *c-myb* cDNA in plasmid pNEO-CCC. The resulting virus should therefore express the authentic c-Myb protein without any viral components and also permit coexpression of the neomycin resistance gene when introduced into our previously described selectable avian retroviral vectors (Fig. 1) (21, 34).

A series of N- and C-terminal deletion mutants of c-Myb was also constructed (Table 1 and Fig. 2). Comparison of the predicted amino acid sequences of chicken c-Myb and the *Drosophila* Myb protein had revealed the existence of a small conserved domain in their C-terminal regions in addition to the more widely conserved domain located in the N-terminal regions of these proteins (Fig. 2) (26, 45). The chicken *c-myb* sequence encoding this C-terminal region of homology 3' of the *v-myb* transduction point is conveniently located between the unique *Sst*I and *Bgl*II sites of the *c-myb* cDNA. To address the functional significance of this do-

main, mutants CCC-SH and CCC-BH were constructed by deletion of all *c-myb* coding sequences 3' of the *Sst*I and *Bgl*II sites, respectively. Mutant CCC-SB contains an in-frame deletion of this conserved C-terminal domain of c-Myb.

Previous studies had demonstrated that the amino acid substitutions in v-Myb were not required for myeloid transformation (56). Although these point mutations were not required for the activation of this doubly truncated protein, they might nevertheless be sufficient for activation of a full-length or a singly truncated c-Myb protein. We therefore constructed a set of mutants that contained a v-*myb*-specific central domain flanked by the coding regions for either one or both ends of c-Myb that were deleted during viral transduction. Mutant CVC encodes a mutant form of c-Myb that contains the 11 amino acid substitutions of v-Myb. Mutants CVd and dVC encode proteins in which either the N or C terminus of c-Myb has been fused to v-Myb in a manner that preserves the 11 amino acid substitutions.

Proviral expression of Myb proteins. The various *myb* genes were transferred into the proviral vector pNEO-MAV or pNEO-MAV-CLA, both of which express the Tn5 neomycin resistance gene as well as a spliced, subgenomic *myb* mRNA under the control of the viral long terminal repeat (21, 34). The capacity of the viral constructs to express their *myb* gene products was assayed by immunoblotting following transient expression in QT6 cells by DNA-calcium phosphate coprecipitation as described previously (5, 22). As shown in Fig. 3, all of the *myb* proviruses expressed proteins of the predicted size. The presence of less abundant additional immunoreactive proteins was likely due to partial proteolysis of the Myb proteins during the experimental procedures. Proteins of essentially identical mobility were also expressed in transiently transfected simian COS cells (data not shown).

The proviral constructs were also stably introduced into quail QT6 fibroblast cells to be used in cocultivation assays to test for their transforming potential. First, the proviral DNAs were cotransfected with MAV-1 helper DNA into QT6 cells. After selection with G418, the resistant colonies were pooled and grown in mass culture. Culture supernatants containing each virus were then used to infect fresh QT6 cells, followed by selection with G418. The colonies obtained were pooled, grown in mass culture, and assayed for the presence of Myb-related proteins by immunoblotting. As can be seen in Fig. 3, proteins of the expected size could again be detected in all cases. This demonstrated that the viruses had not undergone significant rearrangements during DNA transfection and a single round of viral infection. The increased background in immunoblots of infected cells versus transfected cells is due to a lower level of protein expression in the infected cells, which generally harbor only a small number of integrated proviruses.

Activation of c-Myb by N- or C-terminal truncation. The QT6 cells obtained after viral infection and G418 selection were used in cocultivation assays to test for the ability of the viruses to transform primary chicken bone marrow cells (17, 29). These assays were conducted in the presence of either cMGF or anemic chicken serum containing erythropoietin in order to promote the outgrowth of either myeloid or erythroid (as well as myeloid) cells, respectively (31, 48). All constructs that were positive for transformation in myeloid conditions also transformed under erythroid conditions. However, in either growth condition, only myeloid and not erythroid transformation was observed. An aliquot of cells grown under myeloid conditions for 5 days was analyzed for

FIG. 3. Proteins produced by *myb* proviruses. (A) Proviral DNAs encoding the Myb proteins shown in Fig. 1 were transiently transfected into QT6 quail cells and analyzed for protein expression by SDS-PAGE and immunoblotting with monoclonal anti-Myb-2.2 antibody. The relative mobility of coelectrophoresed prestained protein molecular weight markers is indicated on the right by bars in descending order: 180, 116, 84, 58, 49, 37, and 27 kDa. (B) The same proviruses were also cotransfected into QT6 cells with DNA of the MAV-1 helper virus and selected for G418 resistance. Culture supernatants from the pooled transfected colonies were used to infect fresh QT6 cells that were again selected for G418 resistance. The pooled infected colonies were then analyzed for protein expression as described above. The Myb-specific proteins are highlighted with black dots. The relative mobility of coelectrophoresed prestained protein molecular weight markers is indicated on the right by bars in descending order: 116, 84, 58, 49, and 37 kDa.

the capacity to form colonies in semisolid medium (Methocel) in the presence of cMGF (Table 2 and Fig. 2). Most importantly, no transformed colonies were observed when the bone marrow cultures were infected with the viral construct expressing the full-length c-Myb protein (CCC).

The infection with dCC induced colonies of similar number and size in semisolid medium as did wild-type v-Myb (dVd) or a doubly truncated c-Myb protein (dCd). The Myb protein encoded by dCC in transformed cells grown in liquid culture was analyzed by metabolic radiolabelling and immunoprecipitation (Fig. 4). A single *myb*-specific 68,000-molecular-weight protein was detected in these cells with a relative mobility similar to that of the dCC proteins expressed in QT6 and in COS cells. This indicated that a second adventitious truncation was not required for transformation by dCC. Interestingly, cells transformed by dCC did not express detectable endogenous c-Myb (75 kDa), which is also not expressed in cells transformed by wild-type v-Myb or dCd (4, 32, 56).

The three truncations of the C terminus of c-Myb (CCd,

TABLE 2. Transforming capacity of Myb viruses

Virus	No. of colonies ^a	% Marker-positive cells			Mim-1 expression
		Granules	IC3 antigen	47/83 antigen	
CCC	0	NR ^b	NR	NR	NR
dCd	2,000	0	NT ^c	40	+
dCC	800	93	85	60	+
CCd	40	24	45	100	+
SH	200	30	90	95	+
BH	40	99	60	80	NT
SB	0	NR	NR	NR	NR
CVC	0	NR	NR	NR	NR
dVd	1,000	0	0	90	+
dVC	0	NR	NR	NR	NR
CVd	0	NR	NR	NR	NR

^a Number of transformed colonies obtained in semisolid medium per 10⁴ cells seeded 5 days after infection for a representative experiment. Mutant dCd was tested separately, and the number of colonies was normalized with respect to the number of colonies formed by dVd.

^b NR, not relevant.

^c NT, not tested.

SH, and BH) also transformed myeloid cells (Table 2 and Fig. 2). However, an in-frame internal deletion of the C-terminal region of homology to the *Drosophila* Myb protein was nontransforming (SB). The cells transformed by CCd, SH, and BH grew much more slowly in liquid culture and formed fewer colonies in semisolid medium than cells transformed by dVd, dCd, or dCC. In the case of SH, immunoprecipitation detected a single *myb*-specific 54,000-molecular-weight protein with a relative mobility similar to that of the SH proteins expressed in QT6 and COS cells (data not shown).

FIG. 4. Expression of Myb proteins in transformed cells. Cells transformed by dCC or dVd were metabolically labelled with [³⁵S]methionine, and detergent lysates were subjected to immunoprecipitation, SDS-PAGE, and autoradiography. The allele of v-Myb used in this experiment lacks the 11 C-terminal *env*-encoded residues. The relative mobility of coelectrophoresed ¹⁴C-labeled protein molecular weight markers is indicated on the right by bars in descending order: 200, 93, 68, 46, and 30 kDa.

This indicates that a concomitant N-terminal truncation is not required for transformation by this C-terminal deletion mutant. Once again, no expression of endogenous c-Myb was detected in cells transformed by SH. Several attempts at growing sufficient CCd- or BH-transformed cells for a similar characterization of Myb proteins were unsuccessful.

Phenotype of the transformed cells. In all cases, the transformed cells grew more rapidly in myeloid than in erythroid culture conditions. The phenotype of the transformed cells was further analyzed by cytocentrifugation and modified Wright-Giemsa staining (Fig. 5). The myelomonocytic nature of the transformed cells was consistent with their expression of the antigen detected by monoclonal antibody 47/83, which is characteristic for monocytic and granulocytic cells (Table 2) (28). They were also positive for ATPase, another marker characteristic of myelomonocytic cells (data not shown).

Although all of the transformed cells appeared to be of the myelomonocytic lineage, those cells transformed by dCC, CCd, SH, and BH exhibited a morphology that differed from that of cells transformed by dCd or dVd. A significant percentage of the cells transformed by dCC, CCd, SH, and BH contained large, membrane-bound cytoplasmic granules that were not present in cells transformed by dVd or dCd (Table 2; Fig. 5 and 6). These granules stained positively with astra blue (characteristic of promyelocytes) but not with toluidine blue (characteristic of basophils) or peroxidase (characteristic of eosinophils). This suggested that the cells transformed by dCC, CCd, SH, and BH were promyelocytes rather than the monoblasts typically transformed by v-Myb. Consistent with this notion, cells transformed by dCC, CCd, and SH expressed Mim-1, an abundant Myb-inducible component of the granules of promyelocytes (Fig. 7) (43). The granulocytic nature of these cells was further supported by immunohistochemical staining with monoclonal antibody IC3, which specifically stains chicken granulocytes (36) (Table 1). Interestingly, cells transformed by dCd but not dVd also expressed Mim-1 protein, although no granules were observed in either of these cell types. The nuclei of cells transformed by dCd also appeared to be more compact and contain denser chromatin than those transformed by dVd (Fig. 5).

Suppression of the transforming potential of v-Myb by addition of N- or C-terminal sequences of c-Myb. Previous experiments had shown that the amino acid substitutions of v-Myb were not required for transformation (56). However, the possibility remained that these specific substitutions might be sufficient for the oncogenic activation of c-Myb or for altering the phenotype of cells transformed by a singly truncated form of this protein. To test this hypothesis, mutants CVC, CVd, and dVC were constructed (Fig. 2). They did not transform chicken bone marrow cells in the presence of either cMGF or erythropoietin (Table 1). Therefore, the amino acid substitutions of v-Myb are neither necessary nor sufficient for the oncogenic activation of c-Myb. Furthermore, the addition of either a normal N or C terminus of c-Myb suppressed transformation when fused to wild-type v-Myb which contains these amino acid substitutions.

DISCUSSION

The aim of this study was to determine whether the constitutive expression of a full-length chicken c-Myb protein would transform hematopoietic cells or whether truncation of one or both ends of c-Myb was necessary for

FIG. 5. Morphology of cells transformed by dCC, CCd, dCd, and dVd. Transformed cells were cytocentrifuged and stained with Wright-Giemsa. Note the presence of dense cytoplasmic granules in many of the cells transformed by dCC and CCd but not in the cells transformed by dCd and dVd.

FIG. 6. Ultrastructure of cells transformed by CCd and dVd. Transformed cells were collected on filters and fixed in 3% glutaraldehyde prior to embedding and sectioning for transmission electron microscopy. Note the presence of abundant membrane-bound cytoplasmic granules in the cells transformed by CCd but not in those transformed by dVd.

activation. We found that an AMV-like virus expressing authentic *c-Myb* did not transform avian bone marrow cells. We therefore do not believe that an increased dosage of *c-Myb* is sufficient for transformation for two reasons: first, *c-Myb* was expressed from the nontransforming viral construct at levels comparable to those for *v-Myb*. Second, the Gag-Myb-Ets protein product of the E26 virus is expressed

at much lower levels than *v-Myb* but nevertheless transforms hematopoietic cells. We found instead that truncation of either the N or C terminus of *c-Myb* was sufficient to activate the transforming potential of this protein.

The cellular as well as the viral Myb proteins bind DNA and are activators of gene expression (2, 22, 27, 40, 43, 52, 57). Furthermore, transactivation of gene expression by a series of mutants of *v-Myb* correlates with their transforming ability (29). The N terminus of *c-Myb* that is deleted in dCC, dCd, and dVd contains three structural elements that could potentially regulate this transactivation function. First, it has recently been suggested that the deletion of an N-terminal site(s) (Ser-11 and/or Ser-12) phosphorylated by casein kinase II or a related protein kinase might be involved in the regulation of DNA binding by *c-Myb* (35). Second, there is an adjacent stretch of negatively charged amino acids (10 of 15) located between residues 13 and 27 that might also be involved in the regulation of the activity of *c-Myb*, because a variety of transcription factors appear to interact via stretches of charged amino acids (47). Finally, the activating N-terminal truncation of *c-Myb* results in the deletion of most of the first of the three direct repeats of *c-Myb* that comprise its DNA-binding domain. Although the first repeat, situated between amino acids 38 and 89, is not necessary for DNA binding, it has been highly conserved in evolution. It is therefore possible that this first repeat regulates the sequence specificity of DNA binding by *c-Myb* or determines its interaction with other cell-type-specific proteins. Further experiments will be required to determine which of these specific structural elements are relevant for the activation of *c-Myb* by amino terminal truncation.

Truncation of the C terminus of *c-Myb* also caused transformation. Interestingly, a negative regulatory domain for transcriptional transactivation has recently been mapped to this general region of the human, murine, and chicken *c-Myb* proteins (24, 52; our unpublished data). A comparison of the predicted vertebrate Myb protein sequences with that of the *Drosophila* Myb protein revealed the presence of a conserved stretch of approximately 30 amino acids located near

FIG. 7. Expression of the Mim-1 protein in transformed cells. Cells transformed by dCC, CCd, SH, dCd, and dVd were metabolically labelled with [35 S]methionine, and detergent lysates were subjected to immunoprecipitation, SDS-PAGE, and autoradiography. The relative mobility of Mim-1 protein is indicated by an arrowhead on the right, and that of coelectrophoresed 14 C-labeled protein molecular weight markers is indicated on the right by bars in descending order: 200, 93, 68, 46, 30, and 14 kDa.

the C terminus of c-Myb in addition to the N-terminal DNA-binding domain that is more widely conserved in evolution (26, 45). We found that the removal of this particular region from the protein was neither necessary (mutant dCC) nor sufficient (mutant SB) for transformation. It remains possible, however, that such a C-terminal deletion in conjunction with other alterations of c-Myb might be sufficient for transformation.

The portion of *c-myb* that was transduced into *v-myb* contains point mutations resulting in 11 amino acid substitutions. We had previously shown that these substitutions were not required for transformation by *v-Myb* (56). We have now shown that the introduction of these substitutions into c-Myb does not cause transformation. This implies that the amino acid substitutions in *v-Myb* are not sufficient for the activation of c-Myb and are likely to have arisen by mutation and selection during the serial passages of the virus in chickens that occurred between the genesis of AMV around 1940 and the eventual isolation of a molecular clone in 1980 (55). These passages *in vivo* are likely to have selected a panel of interacting mutations that in concert produce a more leukemogenic virus. A specific aspect of this phenotype is the failure of AMV to activate the expression of the endogenous *mim-1* gene, in contrast to E26, which lacks the mutations of AMV, and all c-Myb viruses tested thus far. Surprisingly, the addition of either the N or C terminus of c-Myb to *v-Myb* inactivated its transforming potential. This implies a functional incompatibility of these normal termini of c-Myb with the constellation of amino substitutions of *v-Myb* that appear to have evolved in the absence of these termini.

The oncogenic activation of c-Myb by truncation of either its N or C terminus is consistent with a number of previous observations. Both AMV and the E26 avian leukemia virus encode Myb proteins that are truncated at both their N and C termini relative to c-Myb. These viruses are able to induce acute leukemias in animals and transform cells *in vitro* that grow in semisolid media. In addition, insertional mutagenesis of *c-myb* by avian retroviruses lacking their own oncogene appears to be a rapid, one-step process resulting in short latency nonbursal B-cell lymphomas that presumably cause N-terminal truncations of c-Myb (25, 46). This is in contrast to insertional activation of *c-myc*, which seems to be a multistep process involving the activation of additional genes (8). In the murine system, the insertion of retroviruses into *c-myb* is associated with long-latency myeloid neoplasms (53). Most of these insertions cause N-terminal truncations of the c-Myb protein, but a few tumors with C-terminal truncations have also been identified (9, 49, 54). However, no fully tumorigenic murine *myb*-containing retroviruses have been observed. These observations suggest that the induction of tumors by an activated *c-myb* gene in the avian system may be more direct than in the murine system for reasons that remain unclear.

In this regard, two recent reports described experiments designed to address the question of whether the murine c-Myb protein, when encoded by a murine retrovirus, was able to transform (15, 16). These authors could detect the enhanced proliferation of hematopoietic colonies from fetal liver with a rearranged *c-myb* virus that produced a fusion protein containing *gag*-encoded sequences as well as a small N-terminal and a large C-terminal deletion of c-Myb. In contrast, a related protein with a full-length c-Myb C terminus was inactive in the same assay. These results are consistent with our finding that a C-terminal truncation of avian c-Myb by itself is sufficient for oncogenic activation.

FIG. 8. Transformation of different cell types by *v-Myb* and *c-Myb* proteins. An idealized pathway of differentiation from a bipotential myelomonocytic precursor is shown. The horizontal bars represent blocks of differentiation caused by the indicated Myb proteins. The SH and BH proteins (not shown) appear to act at the same stage as dCC and CCd. The large black dots represent azurophilic granules that contain the Mim-1 protein and are typical of promyelocytes (43). The small stippled dots represent the specific granules typical of neutrophils. The lightly outlined, irregular hollow shapes represent phagocytic vacuoles typical of cells of the monocyte/macrophage lineage.

Another recent report describing experiments that attempted to address the same question in the avian system suggested that deletions of both N and C termini were required (50). However, there was no detectable protein production by the nontransforming proviral constructs used in those studies.

Interestingly, cells transformed by a series of singly truncated c-Myb proteins (dCC, CCd, SH, and BH) had a promyelocytic phenotype (positive for Mim-1 and for granules) that differed from that of the myeloblastic cells transformed by dCd (positive for Mim-1; negative for granules) and the monoblastic cells transformed by *v-Myb* (negative for Mim-1 and for granules). One interpretation of these results is shown in Fig. 8, in which cells transformed by these viruses are blocked at different stages of differentiation from a bipotential myelomonocytic precursor that gives rise to either neutrophils or macrophages. However, it is also possible that the transformed cells represent aberrant phenotypes which have no true normal counterparts. The promyelocytic phenotype of cells transformed by singly truncated c-Myb proteins was similar to that previously observed with an *ets* mutant of the E26 leukemia virus that encodes a single Gag-Myb-Ets fusion protein and with DNA-binding domain mutants of AMV *v-Myb* (14, 23). A number of experiments have also suggested that *v-Myb* and c-Myb can block or reverse the differentiated phenotype of various hematopoietic cells (1, 7, 10, 38, 41, 42). Together, these results imply that alterations in the structure of Myb proteins may be an important factor in determining the lineage of differentiating hematopoietic cells. In the case of the normal c-Myb protein, such alterations could be regulated by posttranslational modifications or by alternate splicing of coding exons rather than by the truncations and amino acid substitutions found in various transforming alleles of Myb.

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