



# Transformation by v-Myb

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The *v-myb* oncogene of the avian myeloblastosis virus (AMV) is unique among known oncogenes in that it causes only acute leukemia in animals and transforms only hematopoietic cells in culture. AMV was discovered in the 1930s as a virus that caused a disease in chickens that is similar to acute myelogenous leukemia in humans (Hall *et al.*, 1941). This avian retrovirus played an important role in the history of cancer research for two reasons. First, AMV was used to demonstrate that all oncogenic viruses did not contain a single cancer-causing principle. In particular, although both Rous sarcoma virus (RSV) and AMV could replicate in cultures of either embryonic fibroblasts or hematopoietic cells, RSV could transform only fibroblasts whereas AMV could transform only hematopoietic cells (Baluda, 1963; Durban and Boettiger, 1981a). Second, chickens infected with AMV develop remarkably high white counts and therefore their peripheral blood contains remarkably large quantities of viral particles (Beard, 1963). For this reason AMV was often used as a prototypic retrovirus in order to study viral assembly and later to produce large amounts of reverse transcriptase for both research and commercial purposes.

Following the discovery of the *v-src* oncogene of RSV and the demonstration that it arose from the normal *c-src* proto-oncogene, a number of acute leukemia viruses were analysed by similar techniques and found to also contain viral oncogenes of cellular origin (Roussel *et al.*, 1979). In the case of AMV, it was shown that almost the entire retroviral *env* gene had been replaced by a sequence of cellular origin (initially called *mab* or *amv*, but later renamed *v-myb*) (Duesberg *et al.*, 1980; Souza *et al.*, 1980). Remarkably, sequences contained in this *myb* oncogene were shared between AMV and the avian E26 leukemia virus, but were not contained in any other acutely transforming retroviruses. In addition, the E26 virus contained a second sequence of cellular origin (*ets*) that was unique. The E26 leukemia virus was first described in the 1960s and causes an acute erythroblastosis in chickens, more reminiscent of the disease caused by avian erythroblastosis virus (AEV) than by AMV (Ivanov *et al.*, 1962).

**Keywords:** myb; oncogene; transcription

## The viruses

Molecular cloning and DNA sequencing revealed that AMV contains an internal subset of the exons that

encode c-Myb, as well as a portion of a *c-myb* intron that lies 5' of the first exon contained in *v-myb* (Klempnauer *et al.*, 1982; Rushlow *et al.*, 1982; Souza *et al.*, 1980). These results imply that the 5' viral/cellular recombination event that generated AMV occurred at the DNA rather than the RNA level (Klempnauer and Bishop, 1983). In addition, an in-frame termination codon occurs just 5' of the splice acceptor site for the first captured exon. As a result, the 48 kDa-v-Myb protein is produced by a sub-genomic spliced viral mRNA that fuses the first six codons of the *gag* gene in-frame to 371 codons of *v-myb* (Boyle *et al.*, 1983; Klempnauer *et al.*, 1983). The 3' cellular/viral recombination event occurred within an exon of *c-myb*, resulting in a fusion of the last eleven codons of the *env* gene in-frame with *v-myb*. A comparison of *v-myb* with *c-myb* cDNA sequences revealed that the v-Myb protein lacks 71 amino acids at its amino terminus and 199 amino acids at its carboxyl terminus relative to c-Myb (Gerondakis and Bishop, 1986; Rosson and Reddy, 1986). In addition, v-Myb contains ten amino acid substitutions in the region shared with c-Myb (note that a putative 3'-most, eleventh substitution found by comparison with a *c-myb* genomic clone was also found in the two published *c-myb* cDNAs). These substitutions can strongly affect the phenotype of the transformed cells (Dini *et al.*, 1995; Introna *et al.*, 1990).

Because AMV lacks almost the entire *env* gene and also the 3' portion of the *pol* open reading frame that overlaps the *env* open reading frame, this virus is defective for replication (reviewed in Baluda and Reddy, 1994). Stocks of AMV that were passaged in chickens for many years and selected for leukemogenicity contained two helper viruses, MAV-1 (subgroup A) and MAV-2 (subgroup B). Molecular cloning of these helper viruses demonstrated that AMV almost certainly arose by recombination with MAV-1 (Perbal *et al.*, 1985). However, cell culture studies have shown that subgroup B rather than subgroup A viruses are most efficient at infecting the hematopoietic cells that are transformed by AMV (Gazzolo *et al.*, 1975).

Nevertheless, subgroup A viruses have generally been used in transformation assays with molecular clones of AMV because of their ability to replicate in quail cells, which can be infected by subgroup A but not B viruses and which lack endogenous retroviruses related to AMV and RSV (Lipsick and Ibanez, 1987; Moscovici *et al.*, 1977).

A remarkable feature of the AMV/MAV viral complex is that the U3 region of the long terminal repeats (LTRs) of these viruses is unrelated to that of all other known avian retroviruses (Bizub *et al.*, 1984; Rushlow *et al.*, 1982). Although AMV-like viruses containing RSV LTRs can transform the same type of hematopoietic cells in culture, they are considerably less efficient (Engelke and Lipsick, 1994; Press *et al.*,

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1992). Interestingly, the MAV helper virus alone causes nephroblastomas, a cancer rarely caused by the oncogene-deficient Rous-associated viruses (RAVs) that contain an LTR similar to those of all other chicken retroviruses including the known endogenous viruses. Presumably, the reason for these biological differences is that the viral U3 region contains the enhancer and promoter that regulate viral transcription. The unusual MAV LTR appears to have arisen very recently during evolution, possibly by a recombination event within a single diseased animal. In this regard, it is interesting to note that AMV was initially isolated from a chicken with Marek's disease, a T cell lymphoma caused by an avian herpes virus. Intriguingly, at least one T cell line isolated from a Marek's virus-infected chicken also contains a retroviral insertion in the *c-myb* locus (Le Rouzic and Perbal, 1996). These results raise the possibility that the unusual AMV/MAV LTR may have arisen by recombination with Marek's disease virus rather than cellular DNA.

The E26 leukemia virus lacks much of the *gag* gene and all of the *pol* and *env* sequences. These viral sequences have been replaced by fragments of two different cellular genes, *c-myb* and *c-ets* (LePrince *et al.*, 1983; Nunn *et al.*, 1984). As a result, the virus encodes a single 135 kDa Gag-Myb-Ets fusion protein. The *c-myb* and *c-ets* proto-oncogenes are present on two different chicken chromosomes, implying that either two successive recombinations with cellular DNA occurred or that the virus captured a pre-existing chromosomal translocation (Symonds *et al.*, 1984b). The portion of c-Myb encoded by the E26 virus is a subset of that encoded by AMV (Nunn *et al.*, 1984). In addition, v-Myb of E26 contains only a single amino acid substitution relative to c-Myb that differs from all of those present in v-Myb of AMV. Although the *v-ets* gene alone is weakly oncogenic at best when encoded by a retrovirus (Metz and Graf, 1991b), retroviral insertions into *ets*-related genes have been observed in murine leukemias and chromosomal translocations involving *ets*-related genes have been observed in human cancers (reviewed in Graves and Petersen, 1998). Remarkably, avian retroviruses that separately encode the v-Myb and v-Ets proteins of E26 undergo a strong selection *in vivo* for rearrangements that encode a Gag-Myb-Ets fusion protein (Metz and Graf, 1991a).

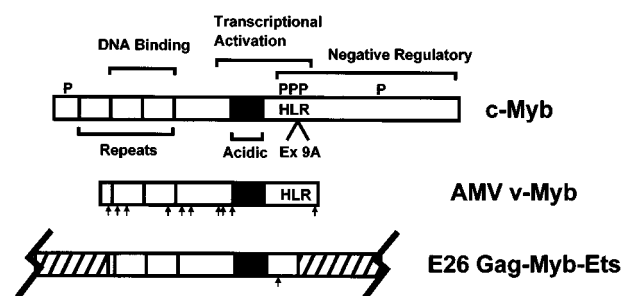
### The proteins (Figure 1)

The 48 kDa v-Myb protein encoded by AMV, the 135 kDa Gag-Myb-Ets protein encoded by the E26 virus, and the 75 kDa c-Myb protein are all nuclear and bind to the same DNA sequence (AACNG) (Biedenkapp *et al.*, 1988; Boyle *et al.*, 1983, 1984; Klempnauer *et al.*, 1983, 1984; Klempnauer and Sippel, 1987). The sequence that defines the Myb family of proteins is an approximately 50 amino acid motif with a series of conserved residues including three tryptophans separated by a characteristic spacing (reviewed in Lipsick, 1996). This motif is present in three tandem repeats in c-Myb, the first of which is drastically truncated in the AMV and E26 oncoproteins. Each repeat assumes a helix-turn-helix structure similar to that present in the bacterial

repressor proteins and the eukaryotic homeodomains (Ogata *et al.*, 1992, 1994). The second and third Myb repeats of c-Myb are together required for sequence-specific DNA-binding. The third helix of each of these repeats fits into the major groove of the DNA double-helix to allow base-specific recognition. The Myb proteins themselves bind to DNA as monomers (Garcia *et al.*, 1991; Howe *et al.*, 1990). Myb repeats have also been found in telomere-binding proteins and in non-DNA-binding transcriptional regulators (reviewed in Ganter and Lipsick, 1999).

Two regions have been implicated in the nuclear localization of the v-Myb protein (Ibanez and Lipsick, 1988). First, a series of deletion mutants established that a sequence motif contained in the first full Myb repeat (homologous to the second repeat of c-Myb) is required for nuclear localization. Second, a more carboxyl terminal sequence motif was required for efficient retention within the nucleus during biochemical fractionation. Presumably this second motif is required for protein-protein interactions that stabilize v-Myb within the nucleus. It has been reported that when v-Myb-transformed cells are induced to differentiate by treatment with phorbol ester (TPA), the v-Myb protein is no longer nuclear (Klempnauer *et al.*, 1984). However, these results are difficult to reconcile with the observation that transcriptional activation by v-Myb is actually increased following such differentiation (Ganter *et al.*, 1998).

A v-Myb-like deletion of the amino terminus of c-Myb including most of the first Myb repeat converts a very weakly transforming c-Myb protein into an efficient oncoprotein (Grasser *et al.*, 1991). This deletion causes a decreased affinity for DNA binding and also permits cyclin D to inhibit transcriptional activation in a CDK-independent fashion (Dini and Lipsick, 1993; Ganter *et al.*, 1998). The importance of this deletion in oncogenesis is underscored by the high frequency with which a similar deletion occurs in murine leukemia virus-induced myeloid malignancies in mice (reviewed in Shen-Ong, 1990). In addition to the absence of much of the first Myb repeat, v-Myb also lacks an amino terminal casein kinase II (CKII) phosphorylation site at residues 11 and 12 of c-Myb (Luscher *et al.*, 1989) and an adjacent stretch of acidic residues. However, mutagenesis of this CKII site in c-Myb does not cause efficient oncogenic transformation in cell culture or cancer in animals (Dini and Lipsick,



**Figure 1** v-Myb and c-Myb proteins. Lightly shaded boxes, Myb repeats. Darkly shaded boxes, acidic region. Small arrows, amino acid substitutions in v-Myb. Striped boxes, Gag- and Ets-encoded residues. P, phosphorylation site. HLR, heptad leucine repeat. Ex 9A, insertion site of c-Myb exon 9A-encoded residues

1993; Jiang *et al.*, 1997). Retroviral insertional mutagenesis that results in a truncation of amino acids 1–20 of c-Myb has been found in non-bursal B cell lymphomas caused by ALV in chickens (Kanter *et al.*, 1988). A recombinant retrovirus that encodes such a truncated protein causes lymphomas, sarcomas, and epithelial carcinomas in animals (Jiang *et al.*, 1997). These results suggest that the residues amino terminal to the first Myb repeat are also critical for proper regulation of c-Myb. Furthermore, these residues may regulate a cell type-specific function of c-Myb. Four amino acid substitutions are present within the DNA-binding domain of AMV v-Myb, but not c-Myb or E26 Gag-Myb-Ets. These substitutions do not appear to alter the sequence specificity of DNA-binding (Weston, 1992), but do affect the ability of the protein to transform different cell types, to regulate specific genes, and to be regulated by other cellular proteins (Dini *et al.*, 1995; Introna *et al.*, 1990; Levenson and Ness, 1998). Consistent with these observations, the biologically interesting substitutions occur on the surface of the protein that faces away from the DNA (Ogata *et al.*, 1994).

The central region of both v-Myb and c-Myb contain a transcriptional activation domain (Ibanez and Lipsick, 1990; Sakura *et al.*, 1989; Weston and Bishop, 1989). This activation domain can function with heterologous DNA-binding domains such as that of the budding yeast GAL4 protein and the *E. coli* LexA protein (Dubendorff *et al.*, 1992; Kalkbrenner *et al.*, 1990). The sequences necessary for transcriptional activation by v-Myb were initially mapped to a small acidic region using GAL4-fusion proteins (Weston and Bishop, 1989). However, in the context of v-Myb itself this region is not sufficient for transcriptional activation (Bortner and Ostrowski, 1991; Ibanez and Lipsick, 1990). Rather, a series of adjacent subdomains are required for transcriptional activation by v-Myb, including a sequence at its carboxyl terminus that includes a heptad leucine repeat and a conserved FAETL motif (Chen *et al.*, 1995; Fu and Lipsick, 1996). Interestingly, the amino acid substitutions accumulated during the passage and selection of AMV significantly reduce transcriptional activation (Dini *et al.*, 1995). Furthermore, the v-Myb protein can activate the expression of some genes even in the absence of Myb-binding sites within the target gene (Ibanez and Lipsick, 1990; Klempnauer *et al.*, 1989).

Carboxy-terminal to the minimal central activation domain of v-Myb lies a heptad leucine repeat that has also been referred to as the 'leucine zipper'. In the context of full-length c-Myb, mutations in this region can activate the protein for both transcriptional regulation and for oncogenic transformation (Kaneishi *et al.*, 1992). These results suggest that this region of the protein serves a negative regulatory function. A cellular protein, p160, that binds to this region has recently been isolated (Favier and Gonda, 1994; Tavner *et al.*, 1998). However, the same region appears to serve a positive regulatory function in v-Myb because it is required for both transcriptional activation and oncogenic transformation (Bartunek *et al.*, 1997; Fu and Lipsick, 1996). In the latter case, it is not the leucine residues that are essential, but rather a 'FAETL' domain that is more highly conserved among

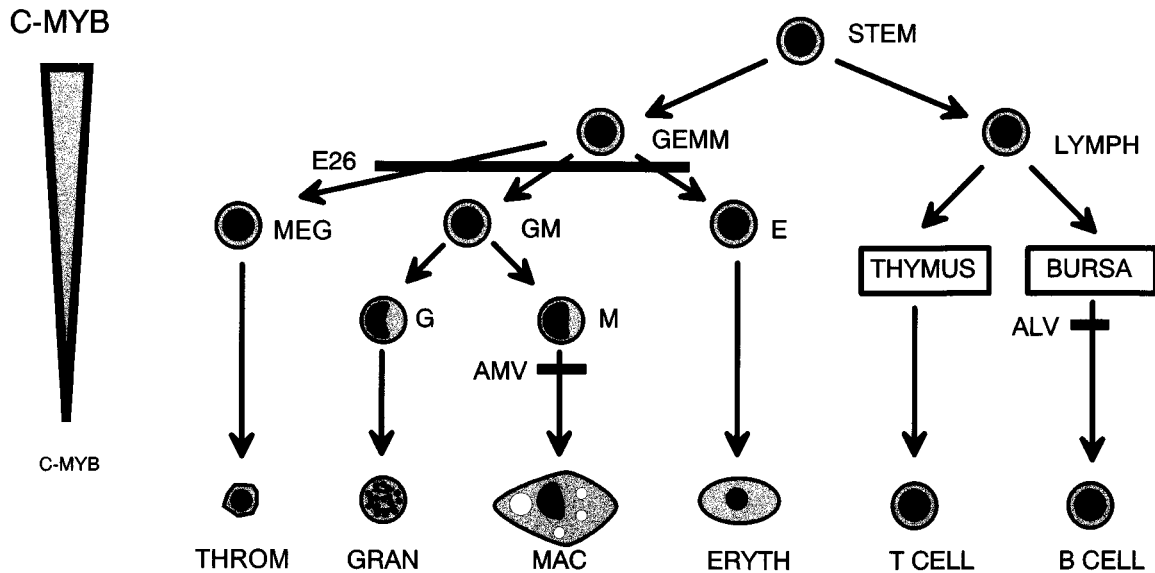
all c-Myb and A-Myb proteins than the leucines. This region of c-Myb is absent in the E26 Gag-Myb-Ets proteins and v-Myb-like proteins with a similar deletion are only very weakly transforming (Ibanez and Lipsick, 1988). However, the addition of Ets sequences to such a protein restores transformation, arguing that Ets can substitute for the function of the heptad leucine repeat/FAETL domain (Fu and Lipsick, 1996). Other oncogenes can also complement E26 Myb in *trans* in the absence of Ets (Metz *et al.*, 1991).

The carboxyl terminus of c-Myb that is absent in AMV v-Myb can negatively regulate transcriptional activation in animal cells (Sakura *et al.*, 1989; Kalkbrenner *et al.*, 1990), but not in yeast (Chen and Lipsick, 1993; Seneca *et al.*, 1993). This negative regulation can also operate in animal cells with heterologous DNA-binding domains and in *trans* to the Myb transcriptional activation domain (Dubendorff *et al.*, 1992). These results imply that an additional cellular protein absent in yeast is required for negative regulation of transcriptional activation by the carboxyl terminus of c-Myb. Several motifs in this region of the protein have been highly conserved in vertebrate c-Myb, A-Myb, B-Myb and in *Drosophila* Myb (Ganter and Lipsick, 1999). An adenovirus E1A-associated protein, BS69, has recently been shown to bind to this region of c-Myb and inhibit transcriptional activation (Hateboer *et al.*, 1995) (Ladendorff and Lipsick, unpublished).

The carboxyl terminus of c-Myb has also been reported to inhibit DNA binding by c-Myb (Ramsay *et al.*, 1991; Tanaka *et al.*, 1997). In support of this model, the amino and carboxyl termini of c-Myb have been shown to interact in yeast two-hybrid assays and in bacterial phage display (Dash *et al.*, 1996; Kiewitz and Wolfes, 1997). Furthermore, another cellular protein, p100, that contains an 'EVES' motif similar to that found in the carboxyl terminus of c-Myb has been proposed to regulate c-Myb function by competing with the carboxyl terminus for binding to the amino terminus (Dash *et al.*, 1996).

### Cells and transformation assays (Figure 2)

AMV causes rapidly fatal monoblastic leukemia when injected into newly hatched chicks. This property was developed into an end-point titration assay and hundreds of thousands of chickens were used to purify and study the virus (reviewed in Beard, 1963). Following the introduction of a cell culture assay for RSV by Rubin and Temin, similar assays were developed for AMV (Baluda and Goetz, 1961). Unlike RSV and most other acutely transforming retroviruses, AMV does not transform fibroblasts in culture. Therefore assays were developed using different chicken tissues that are rich in hematopoietic cells. In the simplest form of the assay, non-adherent cells from embryonic yolk sac (days 12–13), embryonic spleen (day 15), bone marrow, or peripheral blood are incubated with virus. During the course of a 2-week incubation, the immature progenitor cells will differentiate in culture into mature cell types. However, if AMV is present there will be a robust outgrowth of small round, highly refractile cells that are indistinguishable from the monoblasts present in the periph-



**Figure 2** v-Myb and c-Myb proteins in avian hematopoiesis. Arrows indicate the normal differentiation pathways of hematopoietic cells. Thick horizontal bars indicate the blocks in differentiation caused by three different avian retroviruses: AMV (v-Myb), E26 (Gag-Myb-Ets), and ALV (20 residue N-terminal truncation of c-Myb due to insertional mutagenesis). GEMM, common precursor of granulocytic (G), erythroid (E), monocytic (M), and megakaryocytic (MEG) cells. GM, common precursor of granulocytic and monocytic cells. THROM, thrombocyte (avian equivalent of platelets). GRAN, mature granulocyte. MAC, monocyte/macrophage. ERYTH, erythrocyte. Large triangle at left indicates that c-Myb expression declines as hematopoietic cells mature and is generally absent in the quiescent terminally differentiated cells

eral blood of leukemic animals. This 'liquid culture' assay remains the most sensitive method for the detection of weakly transforming viruses.

This assay can also be used as a quantitative tool by end-point dilution using multiple cultures, but two more useful quantitative assays have been developed for studying transformation by AMV. In the focus assay, embryonic yolk sac cells are permitted to differentiate into a monolayer of adherent macrophages. This monolayer is then inoculated with virus and an agar overlay is used to restrict further diffusion of the virus and cells. Foci of transformation are scored as bursts of round, rapidly growing cells arising from the monolayer (Moscovici *et al.*, 1975). In the colony-forming assay, non-adherent hematopoietic precursor cells are incubated with virus and then seeded directly into soft agar or methyl cellulose (Beug *et al.*, 1979). The formation of colonies within the semi-solid media can then be enumerated after 2–3 weeks. Colony formation in soft agar is a more stringent assay of transformation by mutants of AMV because normal colonies are never observed in agar, but do occur in methyl cellulose. The advantages of methyl cellulose are that colony morphology can be more informative and individual colonies can easily be recovered by plucking and chilling, which liquifies the methyl cellulose.

Once cell culture assays were developed for the acute leukemia viruses, a great deal of effort was directed at the question of whether different viruses transformed different 'target' cells (Gazzolo *et al.*, 1979; Graf *et al.*, 1981). It is now clear that AMV-transformed cells most closely resemble monoblasts, rapidly dividing progenitors that are committed to differentiate into monocytes and macrophages (McNagny and Graf, 1996; Moscovici, 1985). In this regard, AMV-transformed cells can

be induced to differentiate into macrophages by the addition of phorbol esters, activators of protein kinase C, despite the continued presence of the v-Myb protein (Pessano *et al.*, 1979; Symonds *et al.*, 1984a). Furthermore, although AMV-transformed cells are not very responsive to retinoic acid, the introduction of exogenous retinoic acid receptor causes these cells to differentiate into macrophage-like cells in response to retinoids (Smarda *et al.*, 1995; Zemanov and Smarda, 1998). These results stand in contrast to studies with bipotential human HL60 leukemia cells that differentiate into macrophages in response to phorbol esters and granulocytes in response to retinoids (Breitman *et al.*, 1980). Remarkably, cells at different stages of the monocytic lineage including what appear to be terminally differentiated macrophages can be infected with AMV and be transformed into monoblasts (Boettiger and Durban, 1984; Durban and Boettiger, 1981b). These results argue that AMV-v-Myb can reverse a differentiated phenotype.

Transformation assays of molecular clones of v-myb and c-myb were complicated by the failure of these genes to transform fibroblasts and the inability to transfect DNA efficiently into primary hematopoietic cells. These problems were circumvented using a two-step assay in which a defective provirus co-expressing a dominant selectable marker (*neo*) is transfected into avian fibroblasts together with a replication-competent 'helper' provirus (Lipsick and Ibanez, 1987; Lipsick *et al.*, 1986). Following marker selection, the virus-producing fibroblasts are treated with mitomycin C to prevent cell division but not viral replication. These cells are then used as a 'feeder layer' for the efficient transfer of the retrovirus into co-cultured non-adherent hematopoietic cells. One day later the non-adherent cells are then removed and used in either a

liquid culture transformation assay or a colony-forming assay. More recently a variation of this assay has been described using a replication-competent version of AMV which expresses a *gag*, *pol*, *env*, and a *myb* gene (Press *et al.*, 1992). A temperature-sensitive variant of AMV has been isolated and molecularly characterized, but has proven difficult to use in experiments directed at understanding the mechanism of transformation by v-Myb (Moscovici and Moscovici, 1983; Schirm *et al.*, 1990). In addition, viruses that produce estrogen-regulated forms of v-Myb have been described (Burk and Klempnauer, 1991; Engelke *et al.*, 1997). These viruses cause estrogen-inducible morphological changes in a v-Myc-transformed cell line, but are only weakly transforming in cultures of primary cells. Nevertheless, such viruses have proven useful for identifying genes that can be directly regulated by v-Myb (Burk *et al.*, 1997; Worpenberg *et al.*, 1997).

The transformation assays for molecular clones of v-*myb* have been used to ask which features of the v-Myb protein are required for biological activity. Nuclear transport, DNA binding, an essential cysteine within the DNA-binding domain, and another function provided by the carboxy terminal region of v-Myb appear to be required for oncogenic transformation (Grasser *et al.*, 1992; Ibanez *et al.*, 1988; Ibanez and Lipsick, 1988; Lane *et al.*, 1990). Similar assays were used to determine which alterations of c-*myb* induced by AMV are required for efficient oncogenic transformation. In brief, neither the *gag* nor *env*-encoded termini of v-Myb are required for transformation (Ibanez and Lipsick, 1988; Lipsick and Ibanez, 1987). The ten amino acid substitutions within v-Myb are also not required for transformation, but do affect the phenotype of the transformed cells (Stober-Grasser and Lipsick, 1988). In particular, reversion of individual substitutions within the DNA-binding domain cause the transformation of promyelocytes (granulocyte precursors) rather than monoblasts when assays are performed in the presence of cMGF, a chicken cytokine most similar to mammalian G-CSF (Introna *et al.*, 1990). However, substitutions in both the DNA-binding domain and the transcriptional activation domain are both required in order to cause a full AMV-like phenotype in which monoblasts are rapidly growing, have cell surface markers of the monocytic but not the granulocytic lineage, and are independent of exogenous growth factor by virtue of an autocrine loop (Dini *et al.*, 1995).

Deletion of the amino and/or carboxyl termini of c-Myb is required for efficient transformation of myelomonocytic cells in culture (Grasser *et al.*, 1991). Truncation of both termini of c-Myb similar to that of v-Myb causes the most robust transformation. Truncation of the amino terminus alone is strongly transforming, whereas truncation of the carboxyl terminus alone is weakly transforming. The single truncations were initially reported to transform only promyelocytes in the presence of exogenous cMGF. However, in the absence of this cytokine the transformed cells appear to be bipotential myelomonoblasts that continue to differentiate in culture (Wang and Lipsick, unpublished). Full-length c-Myb causes a much weaker outgrowth of similar bipotential cells (Ferraro *et al.*, 1995; Fu and Lipsick, 1997).

Similar transformation assays have been developed for the E26 virus (Graf *et al.*, 1979). Although the leukemia induced by this virus in chickens appears to consist primarily of immature erythroblasts, cell culture studies showed that both erythroid and myeloid cells could be transformed (Radke *et al.*, 1982). Further studies revealed the multipotential nature of these transformed progenitor cells (Graf *et al.*, 1992; Moscovici *et al.*, 1983a,b). Cell lines derived from these E26-transformed progenitors have proven very useful in dissecting the regulation of hematopoietic differentiation (Kulesa *et al.*, 1995; Rossi *et al.*, 1996). Temperature-sensitive variants of the E26 virus have been isolated and molecularly characterized (Beug *et al.*, 1984). Interestingly, a Gag-Myb-Ets protein with a substitution in the Myb DNA-binding domain is *ts* for transformation of myelomonocytic cells, whereas a mutant with a substitution in the Ets domain is *ts* for transformation of erythroid cells (Frykberg *et al.*, 1988; Golay *et al.*, 1988). Unlike AMV, the E26 virus has also been reported to stimulate mitogenesis of fibroblasts in culture (Ravel-Chapuis *et al.*, 1991).

A murine assay for transformation by mutants of c-Myb has been developed using fetal liver cultures in colony-forming assays (Gonda *et al.*, 1989a). In addition, a number of cell lines that are transformed by various Myb variants have been derived from such cultures (Gonda *et al.*, 1989b). In general, this fetal liver assay system has given results similar to those in the avian yolk sac and bone marrow assays described above with regard to activation by carboxy-terminal truncation of c-Myb (Hu *et al.*, 1991). Somewhat unexpectedly, the transformation of murine bone marrow cells with Myb viruses has not generally been successful. Although retroviral insertion into the c-*myb* locus is commonly found in murine leukemia virus-induced myeloid malignancies in mice (Shen-Ong *et al.*, 1984; Shen-Ong and Wolff, 1987), infection of mice with Myb viruses has not been reported to cause disease. In addition, the productive infection of bone marrow cells or whole mice carrying a transgene that encodes the quail receptor for subgroup A avian retroviruses with AMV-related viruses does not cause leukemic transformation (Woo and Lipsick, unpublished). These results suggest that mouse hematopoietic cells are more refractory to transformation by Myb proteins than are chicken cells, a finding that is similar to studies with v-Myc (Brightman *et al.*, 1986). However, v-Myb can cause long-latency T cell lymphomas in mice when produced by a T cell-specific transgene (Badiani *et al.*, 1996).

### The mechanisms of oncogenic transformation

As is the case for other nuclear oncoproteins, the precise mechanism of transformation by v-Myb remains unclear. Two major questions to be addressed for v-Myb transformation are cell type-specificity and the relationship between transcriptional regulation and oncogenic transformation.

The c-*myb* gene is normally expressed in all immature cells of the hemato-lymphoid system and this expression declines rapidly as the cells undergo terminal differentiation (Chen, 1980; Duprey and Boettiger, 1985; Westin *et al.*, 1982). Consistent with

this expression pattern, a homozygous disruption of *c-myb* in the laboratory mouse causes death *in utero* at day 15 with a dramatic failure of fetal liver erythro- and myelopoiesis (Mucenski *et al.*, 1991). Interestingly, the earlier extra-embryonic yolk sac hematopoiesis appears normal in these mice. The production of exogenous c-Myb can block the differentiation of cell lines that can otherwise be induced to differentiate into erythrocytes or myelomonocytic cells (Clarke *et al.*, 1988; Patel *et al.*, 1993; Selvakumaran *et al.*, 1992; Smarda and Lipsick, 1994; Todokoro *et al.*, 1988). Together these results had implied that the hematopoietic specificity of AMV and E26 may be due to a similar specificity of action of the *c-myb* proto-oncogene in regulating the proliferation and differentiation of blood and lymphoid cells. However, *c-myb* is also expressed in various other tissues including epithelia of the gut, lung, skin, mammary glands, and cancers thereof (Queva *et al.*, 1992; Ramsay *et al.*, 1992; Sitzmann *et al.*, 1995; Torelli *et al.*, 1987). Furthermore, a virus that produces a carboxy-terminal truncation of c-Myb has been reported to cause relatively long latency sarcomas in chickens (Press *et al.*, 1994). In addition, a 20 residue amino terminal deletion of c-Myb similar to that caused by retroviral insertional mutagenesis in chicken B cell lymphomas can cause epithelial carcinomas and sarcomas as well as lymphomas in chickens (Jiang *et al.*, 1997). Therefore, it seems more likely that the narrow cell-type specificity of v-*myb* observed with AMV and the E26 virus may be due to the specific mutations present in these viral oncogenes rather than representing a limited potential of the *c-myb* proto-oncogene itself.

It is clear that both v-Myb and c-Myb can activate the transcription of reporter genes in both animal cells and yeast (see above). Several studies of deletion, linker insertion, and substitution mutants of v-Myb pointed toward a strong correlation between transcriptional activation and oncogenic transformation (Fu and Lipsick, 1996; Grasser *et al.*, 1992; Ibanez and Lipsick, 1990; Lane *et al.*, 1990). However, more recent studies have called this conclusion into question. First, some mutants of v-Myb that are quite competent for transcriptional activation are incapable of oncogenic transformation (Chen *et al.*, 1995). Second, the highly leukemogenic v-Myb protein of AMV that contains *gag*- and *env*-encoded termini is an extremely weak transcriptional activator (Engelke *et al.*, 1995). Third, the amino acid substitutions in v-Myb accumulated during the repeated passage of AMV in animals increase the transforming efficiency of the protein but decrease its ability to activate transcription (Dini *et al.*, 1995). Indeed, the most intensely studied Myb-regulated gene, *mim-1*, is activated by c-Myb and by the E26 Gag-Myb-Ets fusion proteins but not by AMV v-Myb (Ness *et al.*, 1989; Queva *et al.*, 1992). Notably, the ability of AMV v-Myb to activate *mim-1* reporter genes in transient transfection assays does not accurately reflect the regulation of the endogenous gene.

Because v-Myb and c-Myb can act as transcriptional repressors as well as transcriptional activators (Ganter and Lipsick, 1997; Mizuguchi *et al.*, 1995; Nakagoshi *et al.*, 1989), these results raise the question of whether repression rather than activation of specific cellular genes might be critical for oncogenic transformation.

In this regard, it has been shown that transcriptional activation by the v-Myb protein increases when transformed cells are induced to terminally differentiate by treatment with phorbol esters (Ganter *et al.*, 1998). Interestingly, many of the putative 'target' genes positively regulated by c-Myb (such as *mim-1*, *CD4*, *CD13*, and the  $\delta$ - and  $\gamma$ -*TCR* genes; reviewed in Ness, (1996)) appear to be turned on during the differentiation of hematolymphoid cells, but the maintenance of their expression does not appear to require the continued presence of c-Myb (Queva *et al.*, 1992). These observations suggest a model in which c-Myb has two functions one to drive proliferation of progenitor cells and a second to permit the activation of genes required for terminal differentiation (Dini and Lipsick, 1993). v-Myb and other oncogenically activated forms of c-Myb would then cause transformation by carrying out the first but not the second function of normal c-Myb. v-Myb does not appear to function solely as a dominant inhibitor of c-Myb, because endogenous c-Myb is generally not expressed in cells transformed by v-Myb or activated forms of c-Myb (Lipsick, 1987). Conversely, the expression of exogenous c-Myb is compatible with the rapid growth of v-Myb-transformed monoblasts (Smarda and Lipsick, 1994). These results suggest that v-Myb is oncogenically dominant with respect to c-Myb when both proteins are present in the same cell.

One interesting exception to this model is the ability of v-Myb to activate an autocrine loop of cMGF production that specifically requires the amino acid substitutions found in AMV (Dini *et al.*, 1995). This occurs by a two-step pathway in which AMV v-Myb activates the transcription of the *GBX2* gene that encodes a homeodomain protein that in turn directly activates the *cMGF* gene (Kowenz-Leutz *et al.*, 1997). This activation of *GBX2* requires specific amino acid substitutions found in AMV v-Myb. c-Myb itself can activate the *GBX2* gene as well, but only in conjunction with other intracellular signaling pathways involving tyrosine kinases and/or Ras. These results suggest an additional wrinkle in which specific mutations in AMV v-Myb have been selected to activate specific downstream genes that are not normally activated by c-Myb alone. However, v-Myb-like proteins lacking these substitutions still transform myelomonocytic cells quite efficiently in cell culture even in the absence of exogenous growth factors (Dini *et al.*, 1995). The E26 Gag-Myb-Ets protein has been reported to directly regulate the *Bcl-2* anti-apoptotic gene, but the ability of AMV v-Myb to regulate this gene remains unclear (Frampton *et al.*, 1996).

To date much of the work on v-Myb and c-Myb function has focused on transcriptional regulation. However, an alternative mechanism of oncogenesis that remains to be considered is a more direct role of the Myb proteins in cell cycle progression. Three *myb*-related genes have been identified in vertebrates thus far, *c-myb*, *A-myb*, and *B-myb* (Nomura *et al.*, 1988). The first two are tissue-specific and are capable of strong transcriptional activation in various cell types. In contrast, *B-myb* is ubiquitously expressed and has generally been found to activate transcription weakly and only in specific cell types or following the introduction of exogenous cyclin A. *B-myb* itself appears to be a direct target of transcriptional

regulation by the cyclinD/Rb/E2F axis (reviewed in Saville and Watson, 1998). Similarly regulated genes encode DNA polymerase, PCNA, and many enzymes required to produce adequate pools of nucleotides for S phase (DeGregori *et al.*, 1995; Hurford *et al.*, 1997). The very short half-life of the Myb proteins suggests that they are likely to be produced at a time during the cell cycle shortly before their function is required. Therefore, one wonders whether the ancient function of Myb that is conserved among all of these proteins might be to advance cells through S phase. The transcriptional activation function appears to be a later development that was acquired during the duplication and evolution of *A-myb* and *c-myb* that serves a tissue-specific rather than a general purpose (Ganter and Lipsick, 1999). Oncogenic transformation

by v-Myb might therefore result from a loss of the more recently acquired strong transcriptional activation function of c-Myb that is essential for terminal differentiation of specific cell types, but a preservation of the more ancient cell cycle regulatory function present in all the Myb proteins.

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