NOTES

v-myb Does Not Prevent the Expression of *c-myb* in Avian Erythroblasts

JOSEPH S. LIPSICK

Research Division, Veteran's Administration Medical Center, San Diego, California 92161, and Department of Pathology, University of California, San Diego, La Jolla, California 92093

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The *v-myb* oncogene of avian myeloblastosis virus transforms myeloid cells exclusively, both in vivo and in vitro. The *c-myb* proto-oncogene from which *v-myb* arose is expressed at relatively high levels in immature hematopoietic cells of the lymphoid, erythroid, and myeloid lineages but not in myeloblasts transformed by *v-myb*. This finding suggested that the nuclear *v-myb* gene product $p48^{v-myb}$ might act directly to inhibit the normal expression of the *c-myb* gene. I have therefore used a selectable avian retroviral vector to express $p48^{v-myb}$ in avian erythroblasts which normally express high levels of the *c-myb* gene product $p75^{c-myb}$. The results demonstrate that $p48^{v-myb}$ and $p75^{c-myb}$ can be coexpressed in the nuclei of cloned cells. Therefore, $p48^{v-myb}$ does not invariably prevent the expression of $p75^{c-myb}$.

Avian myeloblastosis virus (AMV) causes acute myelomonocytic leukemia in chickens and transforms only cells of the myeloid lineage in vitro (10, 22). Transformation by AMV is caused by the *v-myb* oncogene which has been transduced from the evolutionarily conserved *c-myb* proto-oncogene (18). The product of the *v-myb* gene, $p48^{v-myb}$, is a short-lived nuclear protein which binds to DNA in vitro (2, 3, 15-17, 21). The mechanism of transformation by $p48^{v-myb}$ is currently unknown.

The *c-myb* gene is expressed at high levels in immature hematopoietic cells of the lymphoid, erythroid, and myeloid lineages in a variety of species (1, 4-6, 9, 29). The *c-myb* gene product $p75^{c-myb}$ is also a short-lived nuclear protein which binds to DNA in vitro (1, 15-17). The expression of *c-myb* appears to be tightly regulated during the cell cycle (27, 28); however, its function remains to be determined.

Although normal immature avian myeloid cells express high levels of *c-myb* (6), myeloblasts which have been transformed by *v-myb* do not express detectable levels of *c-myb* mRNA or of $p75^{c-myb}$ (1, 5, 15). This lack of expression suggests that one essential transforming function of nuclear $p48^{v-myb}$ may be to inhibit the normal expression of the *c-myb* gene. Precedents exist for such a mechanism in that the nuclear protein products of the *fos*, *myc*, and adenovirus E1A oncogenes have all been shown to trans-regulate the transcription of various cellular genes (7, 11, 13, 26). Selective inhibition of *c-myb* but not *v-myb* expression by $p48^{v-myb}$ might be possible because *c-myb* is regulated by its normal cellular promoter complex, whereas *v-myb* is regulated by a retroviral long terminal repeat. Because no avian myeloid cell lines expressing *c-myb* have yet been described, I have tested this hypothesis by expressing $p48^{v-myb}$ in avian erythroblastosis virus (AEV)-transformed erythroblasts. Such erythroblasts seem appropriate for these experiments because (i) they express high levels of *c-myb* mRNA and protein (5, 9, 15), (ii) erythroblasts and myeloblasts arise from a common progenitor cell with

bipotential capability, and (iii) erythroblasts as well as myeloblasts are transformed by the *myb*-containing E26 leukemia virus (10, 22).

Expression of $p48^{v-myb}$ in avian erythroblasts. The 6C2 AEV-transformed chicken erythroblast cell line (12) was used to study the effect of $p48^{v-myb}$ on *c-myb* expression. These cells were infected with NEO-AMV, an avian retroviral vector which coexpresses both *v-myb* and the dominantly selectable *neo* gene (20). Efficient infection of the nonadherent 6C2 cell line by this virus was achieved by cocultivation with adherent mitomycin-treated QT6 cells which were productively infected with NEO-AMV(MAV-1) virus. After 24 h of cocultivation with either NEO-AMV(MAV-1)-infected QT6 cells or with uninfected control QT6 cells, the nonadherent 6C2 cells were removed into fresh culture dishes. After an additional 24 h of cultivation, 6C2 cells were selected for *neo* expression by the addition of G418 (1 mg/ml; GIBCO Laboratories). After 2 weeks of selection, no 6C2 cells which had been cocultivated with the control QT6 cells had survived, whereas those cocultivated with QT6-NEO-AMV cells grew rapidly.

6C2-NEO-AMV cells which were resistant to G418 were examined for $p48^{v-myb}$ expression by metabolic labeling with [³⁵S]methionine and immunoprecipitation (Fig. 1). Control 6C2 cells expressed $p75^{c-myb}$ which could be immunoprecipitated by anti-*myb* amino-terminal serum (1) but not by preimmune or anti-*v-myb* carboxyl-terminal serum (2). This was as expected because the carboxyl terminus of $p48^{v-myb}$ is of viral origin and is therefore not shared by $p75^{c-myb}$ (14, 23, 24). Primary chicken embryonic yolk sac myeloid cells transformed in vitro by NEO-AMV(MAV-1) expressed $p48^{v-myb}$ which could be immunoprecipitated by anti-*myb* amino- and carboxyl-terminal sera but not by preimmune serum. In addition, smaller proteins presumed to be the degradation products of $p48^{v-myb}$ were also detected by the amino-terminal antiserum in transformed myeloblasts as previously reported (19). No $p75^{c-myb}$ was detectable in these

FIG. 1. Expression of $p48^{v-myb}$ in avian erythroblasts. Control 6C2 erythroblasts, 6C2 erythroblasts infected with NEO-AMV(MAV-1) and G418-selected, or NEO-AMV(MAV-1)-transformed yolk sac (YS) myeloid cells were metabolically labeled with [35 S]methionine. Detergent lysates of these cells were then analyzed by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography (2). Lanes: P, preimmune serum; M, anti- $v-myb$ amino-terminal serum; E, anti- $v-myb$ carboxyl-terminal/ env serum. The positions of $p48^{v-myb}$, $p75^{c-myb}$, and $gp37^{env}$ are indicated on the left.

myeloblasts, further confirming the absence of $c-myb$ gene products in $v-myb$ -transformed cells. The uncloned population of G418^r 6C2-NEO-AMV cells expressed both $p75^{c-myb}$ and $p48^{v-myb}$ which could be immunoprecipitated by the appropriate antisera. This finding demonstrated that $p48^{v-myb}$ could be successfully expressed in AEV-transformed erythroblasts.

The anti- $v-myb$ carboxyl-terminal serum also precipitated $gp37^{env}$ from NEO-AMV(MAV-1)-infected myeloblasts and 6C2 cells but not from control 6C2 cells. This precipitation occurred because the carboxyl terminus of $p48^{v-myb}$ is shared by the $gp37^{env}$ of the MAV-1 helper virus (23) but apparently not by the RAV-2 helper virus present in 6C2 cells.

Coexpression of $p48^{v-myb}$ and $p75^{c-myb}$ in cloned avian erythroblasts. The presence of $p48^{v-myb}$ and $p75^{c-myb}$ in uncloned G418^r 6C2-NEO-AMV cells demonstrated that $p48^{v-myb}$ could be expressed in 6C2 cells but did not rule out the possibility that $p48^{v-myb}$ and $p75^{c-myb}$ were being exclusively expressed in different subpopulations. The approximately one-to-one ratio of $p48^{v-myb}$ to $p75^{c-myb}$ in these cells was somewhat surprising because the long terminal repeat of the NEO-AMV vector was expected to be a much stronger promoter than that of the endogenous $c-myb$ gene. To further investigate these questions, I isolated 11 independent clones of 6C2-NEO-AMV cells by limiting dilution, by plating a mean of one-third of a cell per well of a 96-well microtiter plate. Then these 11 clones were each examined for the expression of $p48^{v-myb}$ and $p75^{c-myb}$ by metabolic labeling with [35 S]methionine and immunoprecipitation. All of the clones expressed $p75^{c-myb}$, but only one clone, 6C2-NEO-AMV-3, also expressed detectable $p48^{v-myb}$ (Fig. 2). The ratio of $p48^{v-myb}$ to $p75^{c-myb}$ in these cells was five to one

as determined by densitometry of autoradiographs, which was consistent with the expected relative strengths of the viral and cellular promoters. These data show that only a small subpopulation of the uncloned 6C2-NEO-AMV G418^r cells expresses $p48^{v-myb}$.

Since all of the 6C2-NEO-AMV clones were G418^r, they must all contain proviruses which express the neo gene. The failure of 10 of these clones to coexpress $p48^{v-myb}$ might be due to a variety of reasons, such as proviral deletions in $v-myb$, low levels of splicing of proviral subgenomic $v-myb$ mRNA, or proviral integration in a site permitting only low-level expression which is sufficient for G418^r but not for $p48^{v-myb}$ detection. However, selection against a biologically active $v-myb$ appears unlikely because nontransforming deletion mutants of $v-myb$ are expressed at similar levels by the same vector in the same cells (data not shown).

The frequency of distribution of cells plated by limiting dilution conforms to the Poisson distribution, which predicts a probability of 1.7×10^{-1} that the 6C2-NEO-AMV-3 clone isolated by limiting dilution contained more than one founder

FIG. 2. Coexpression of $p48^{v-myb}$ and $p75^{c-myb}$ in a single clone (6C2-NEO-AMV-3) of avian erythroblasts. (A) Six independent clones of G418-resistant NEO-AMV(MAV-1)-infected 6C2 erythroblasts were metabolically labeled with [35 S]methionine. Detergent lysates of these cells were then analyzed as described in the legend to Fig. 1. (B) Cells of eight independent subclones of clone 3 shown above were metabolically labeled with [35 S]methionine. Detergent lysates of these cells were then analyzed as described in the legend to Fig. 1. Lanes: P, preimmune serum; M, anti- $v-myb$ amino terminal serum. The positions of $p48^{v-myb}$ and $p75^{c-myb}$ are indicated on the left.

FIG. 3. Both $p48^{v-myb}$ and $p75^{c-myb}$ are located in the nuclei of subcloned erythroblasts. Cells of subclone 6C2-NEO-AMV-3-1 (shown in Fig. 2) were metabolically labeled with [35 S]methionine and fractionated into nuclei (N), nuclear wash (W), and postnuclear supernatant (P) as previously described (3). Detergent lysates of each of these fractions were then analyzed as described in the legend to Fig. 1. Lanes: M, anti- $v-myb$ amino-terminal serum; G, anti- $p27^{gag}$ serum. The positions of $p48^{v-myb}$ and $p75^{c-myb}$ are indicated on the left. The positions of $Pr180^{gag-pol}$, $Pr76^{gag}$, $p75^{gag-erbA}$, and $p27^{gag}$ are indicated on the right.

cell. To ensure that $p48^{v-myb}$ and $p75^{c-myb}$ could be coexpressed in the same cells, 6C2-NEO-AMV-3 cells were recloned by limiting dilution. Eight independent subclones of 6C2-NEO-AMV-3 cells were isolated and analyzed by metabolic labeling with [35 S]methionine and immunoprecipitation which revealed that all of the subclones expressed both $p48^{v-myb}$ and $p75^{c-myb}$ (Fig. 2). The probability that each of these eight clones arose from more than one founder cell is 5.8×10^{-7} , which implies that $p48^{v-myb}$ and $p75^{c-myb}$ can be expressed in the same cell. Therefore, the expression of $v-myb$ in avian erythroblasts does not prevent the expression of $c-myb$ in these cells.

Colocalization of $p48^{v-myb}$ and $p75^{c-myb}$ in the nuclei of avian erythroblasts. Because $p48^{v-myb}$ is localized in the nuclei of transformed myeloblasts, its potential functions in transformation, including the inhibition of $p75^{c-myb}$ expression, were expected to occur within the cell nucleus. One explanation for the failure of $p48^{v-myb}$ to prevent $p75^{c-myb}$ expression in avian erythroblasts is that $p48^{v-myb}$ is nonnuclear in these cells. To determine whether $p48^{v-myb}$ and $p75^{c-myb}$ were coexpressed in the nuclei of avian erythroblasts, cells of the 6C2-NEO-AMV-3-1 subclone were analyzed by metabolic labeling with [35 S]methionine, subcellular fractionation, and immunoprecipitation (Fig. 3). Both $p48^{v-myb}$ and $p75^{c-myb}$ were evident predominantly in the nuclei of these cells and were not extracted after being washed with low-salt buffer. Most of the *gag*-encoded proteins in these cells, including $Pr180^{gag-pol}$ and $p27^{gag}$, were present in the postnuclear fraction (cytoplasm plus nonnuclear membranes), as expected. However, a *gag*-related protein of 75,000 molecular weight was unexpectedly present in both the nuclear fraction and the nuclear wash. This protein was presumed to be $p75^{gag-erbA}$, which is a product of the AEV present in 6C2 cells and has recently been shown to be a nuclear protein, in contrast to the $Pr76^{gag}$ precursor, which is a cytoplasmic protein (25). These data demonstrate that avian erythroblasts expressing both $p48^{v-myb}$ and $p75^{c-myb}$ coexpress these two proteins predominantly within the cell nucleus. Thus,

the expression of $p75^{c-myb}$ in these cells is not due to a failure of $p48^{v-myb}$ to localize in the nucleus.

It remains possible that additional factors present in myeloid but not erythroid cells are required for the inhibition of $c-myb$ expression by $v-myb$. An alternative explanation for the absence of $c-myb$ expression in $v-myb$ -transformed myeloblasts is related to the normal down regulation of $c-myb$ expression during myeloid differentiation (6, 8, 29). It is possible that myeloid cells transformed by $v-myb$ are frozen at a particular stage of differentiation subsequent to the normal cessation of $c-myb$ gene expression. The expression of $c-myb$ during earlier stages of myeloid differentiation may in fact be incompatible with transformation by $v-myb$.

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LITERATURE CITED

- Boyle, W. J., J. S. Lipsick, and M. A. Baluda. 1986. Antibodies to the evolutionarily conserved amino-terminal region of the $v-myb$ -encoded protein detect the $c-myb$ protein in widely divergent metazoan species. *Proc. Natl. Acad. Sci. USA* **83**: 4685-4689.
- Boyle, W. J., J. S. Lipsick, E. P. Reddy, and M. A. Baluda. 1983. Identification of the leukemogenic protein of avian myeloblastosis virus and of its normal cellular homologue. *Proc. Natl. Acad. Sci. USA* **80**:2834-2838.
- Boyle, W. J., M. A. Lampert, J. S. Lipsick, and M. A. Baluda. 1984. Avian myeloblastosis virus and E26 virus oncogene products are nuclear proteins. *Proc. Natl. Acad. Sci. USA* **81**:4265-4269.
- Chen, J. H. 1980. Expression of endogenous avian myeloblastosis virus information in different chicken cells. *J. Virol.* **36**:162-170.
- Coll, J., S. Saule, P. Martin, M. B. Raes, C. Lagrou, T. Graf, H. Beug, I. E. Simon, and D. Stehelin. 1983. The cellular oncogenes $c-myc$, $c-myb$ and $c-erb$ are transcribed in defined types of avian hematopoietic cells. *Exp. Cell. Res.* **149**:151-162.
- Duprey, S. P., and D. Boettiger. 1985. Developmental regulation of $c-myb$ in normal myeloid progenitor cells. *Proc. Natl. Acad. Sci. USA* **82**:6937-6941.
- Gaynor, R. B., D. Hillman, and A. J. Berk. 1984. Adenovirus early region 1A protein activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. *Proc. Natl. Acad. Sci. USA* **81**:1193-1197.
- Gonda, T. J., and D. Metcalf. 1984. Expression of myb , myc and fos proto-oncogenes during the differentiation of a murine myeloid leukemia. *Nature (London)* **310**:249-251.
- Gonda, T. J., D. K. Sheiness, and J. M. Bishop. 1982. Transcripts from the cellular homologs of retroviral oncogenes: distribution among chicken tissues. *Mol. Cell. Biol.* **2**:617-624.
- Graf, T., and H. Beug. 1978. Avian leukemia viruses: interaction with their target cells in vivo and in vitro. *Biochim. Biophys. Acta* **516**:269-299.
- Green, M. R., R. Treisman, and T. Maniatis. 1983. Transcriptional activation of cloned human β -globin genes by viral immediate early gene products. *Cell* **35**:137-148.
- Hayman, M. J., B. Royer-Pokora, and T. Graf. 1979. Defectiveness of avian erythroblastosis virus: synthesis of a 75K *gag*-related protein. *Virology* **92**:31-45.
- Kingston, R. E., A. S. Baldwin, and P. A. Sharp. 1984. Regulation of heat shock protein 70 gene expression by $c-myc$. *Nature (London)* **312**:280-283.
- Klempnauer, K.-H., T. J. Gonda, and J. M. Bishop. 1982. Nucleotide sequence of the retroviral leukemia gene $v-myb$ and its cellular progenitor $c-myb$: the architecture of a transduced

- oncogene. *Cell* 31:453–463.
15. Klemmner, K.-H., G. Ramsay, J. M. Bishop, M. G. Moscovici, C. Moscovici, J. P. McGrath, and A. D. Levinson. 1983. The product of the retroviral transforming gene *v-myb* is a truncated version of the protein encoded by the cellular oncogene *c-myb*. *Cell* 33:345–355.
 16. Klemmner, K.-H., and A. E. Sippel. 1986. Subnuclear localization of proteins encoded by the oncogene *v-myb* and its cellular homolog *c-myb*. *Mol. Cell. Biol.* 6:62–69.
 17. Klemmner, K.-H., G. Symonds, G. I. Evan, and J. M. Bishop. 1984. Subcellular localization of proteins encoded by oncogenes of avian myeloblastosis virus and avian leukemia virus E26 and by the cellular *c-myb* gene. *Cell* 37:537–547.
 18. Lipsick, J. S., and M. A. Baluda. 1986. The *myb* oncogene, p. 73–98. In T. S. Papas and G. F. Vande Woude (ed.), *Gene amplification and analysis*. Oncogenes, vol. 4. Elsevier Science Publishing, Inc., New York.
 19. Lipsick, J. S., and C. E. Ibanez. 1987. *env*-encoded residues are not required for transformation by *p48^{v-myb}*. *J. Virol.* 61:933–936.
 20. Lipsick, J. S., C. E. Ibanez, and M. A. Baluda. 1986. Expression of molecular clones of *v-myb* in avian and mammalian cells independently of transformation. *J. Virol.* 59:267–275.
 21. Moelling, K., E. Pfaff, H. Beug, P. Beimling, T. Bunte, H. E. Schaller, and T. Graf. 1985. DNA-binding activity is associated with purified *myb* proteins from AMV and E26 viruses and is temperature-sensitive for E26 ts mutants. *Cell* 40:983–990.
 22. Moscovici, C., and L. Gazzolo. 1982. Transformation of hemopoietic cells with avian leukemia viruses. *Adv. Viral Oncol.* 1:83–106.
 23. Perbal, B., J. S. Lipsick, J. Svoboda, R. F. Silva, and M. A. Baluda. 1985. Biologically active proviral clone of myeloblastosis-associated virus type 1: implications for the genesis of avian myeloblastosis virus. *J. Virol.* 56:240–244.
 24. Rushlow, K. E., J. A. Lautenberger, T. S. Papas, M. A. Baluda, B. Perbal, J. G. Chirikjian, and E. P. Reddy. 1982. Nucleotide sequence of the transforming gene of avian myeloblastosis virus. *Science* 216:1421–1423.
 25. Sap, J., A. Munoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Beug, and B. Vennstrom. 1986. The *c-erb-A* protein is a high-affinity receptor for thyroid hormone. *Nature (London)* 324:635–640.
 26. Setoyama, C., R. Frunzio, G. Liau, M. Mudryj, and B. de Crombrughe. 1986. Transcriptional activation encoded by the *v-fos* gene. *Proc. Natl. Acad. Sci. USA* 83:3213–3217.
 27. Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine. 1986. Expression of the *c-myb* proto-oncogene during cellular proliferation. *Nature (London)* 319:374–380.
 28. Torelli, G., L. Sella, A. Donelli, S. Ferrari, G. Emilia, D. Venturelli, L. Moretti, and U. Torelli. 1985. Activation of *c-myb* expression by phytohemagglutinin stimulation in normal human T lymphocytes. *Mol. Cell. Biol.* 5:2874–2877.
 29. Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal. 1982. Differential expression of the *amv* gene in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA* 79:2194–2198.