Transformation of Myelomonocytic Cells by the Avian Myeloblastosis Virus Is Determined by the *v-myb* Oncogene, Not by the Unique Long Terminal Repeats of the Virus

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The avian myeloblastosis virus (AMV) induces acute monoblastic leukemia in chickens and transforms only myelomonocytic cells in vitro. The long terminal repeat (LTR) regulatory region of AMV is unique among the known classes of avian retrovirus LTRs. We demonstrate that the substitution of the AMV LTRs by Rous sarcoma virus LTRs did not alter the cell type specificity or the transforming ability of the virus.

The avian myeloblastosis virus (AMV) causes acute monoblastic leukemia in chickens and transforms exclusively myelomonocytic cells in vitro. In contrast, the Rous sarcoma virus (RSV) has been shown to transform only fibroblasts in vitro, whereas the MC29 virus transforms both fibroblasts and cells of the myelomonocytic lineage in culture (22). These viruses carry different oncogenes of cellular origin. In addition, the long terminal repeats (LTRs) of AMV, RSV, and MC29 share nearly identical R and U5 regions, but the sequence of U3 from AMV is very divergent from the U3 regions of RSV and MC29. Strong tissue-specific enhancer elements encoded in the U3 region of long-latency retroviruses have been shown to determine pathogenicity and tissue tropism (2–4, 7, 21). In acutely transforming retroviruses that carry a transduced oncogene in their genome, pathogenicity can be determined

solely by the transforming protein. However, there are also examples in which a unique transformation property is determined not by the oncogene but primarily by specific sequences in the viral enhancer (19, 20).

The transforming component of AMV has been genetically identified as the *v-myb* oncogene (12, 16). However, Press et al. (18) recently used a replication-competent virus in which *v-myb* was expressed from an RSV LTR; surprisingly, this construct was unable to transform myelomonocytic cells. The authors concluded that regulatory sequences within the LTR constitute a primary determinant of the transforming capability of *v-myb*-containing avian retroviruses. The nontransforming replication-competent virus from Press et al. (18) contained not only the RSV LTRs but also the *gag* and *pol* genes from RSV. Similar constructs also failed to transform cells in

Test Virus:

Helper Virus:

FIG. 1. Schematic representation of replication-deficient retroviral expression vectors (left) and their corresponding helper viruses (right) which were cotransfected into CEFs to generate producer cell lines. The relative positions of relevant restriction enzyme sites are indicated (C, *Clal*; B, *BglII*; E, *EcoRI*; H, *HindIII*; K, *KpnI*). SD, splice donor site; SA, splice acceptor site.

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FIG. 2. Expression of v-Myb protein in transformed cells. Levels of v-Myb protein in transformed cells were compared by immunoblot analysis of equal numbers of cells infected by PR-NEO-MYB (PR), SR-NEO-MYB (SR), or MAV-NEO-MYB (MAV). Lines at the right indicate molecular mass markers (116, 85, 58, 48, 36, and 26 kDa, in descending order).

our transformation assay. However, in this report, we demonstrate that replication-deficient *v-myb* viruses transformed myelomonocytic cells regardless of the LTRs from which the oncogene was expressed.

Construction of RSV LTR-MYB viruses. We tested two different RSV-derived, replication-deficient viruses which expressed a neomycin resistance gene (NEO) in addition to the *v-myb* oncogene (Fig. 1). The provirus SR-NEO-MYB was constructed by first subcloning the *Sst*I fragment from pUC-myb (14) into the *Sst*I site from CLA12 (11), generating

CLA12-MYB. The *Cla*I-restricted fragment from CLA12-MYB was then inserted into the *Cla*I site of RCAN (11). The resulting construct was digested with *Bgl*II and *Kpn*I to replace *gag*, *pol*, and *env* sequences with the neomycin phosphotransferase type II coding sequence of the *Bgl*II-*Kpn*I-resistant fragment from NEO-AMV (14). The resulting provirus contains the LTRs from the Schmidt-Ruppin A strain of RSV, which was used also by Press and coworkers in the publication mentioned above (6, 18).

The second virus that we tested for transformation ability, PR-NEO-MYB, contained the LTRs from the Prague A isolate of RSV. The Prague A LTR has a small insertion of 5 bp in U3 and a total of 16 substitutions in U3, R, and U5 relative to the Schmidt-Ruppin isolate (9). PR-NEO-MYB was constructed by the same strategy as the control virus MAV-NEO-MYB (formerly called NEO-AMV), which has been described elsewhere (14).

Transformation of avian myeloid cells by MAV-NEO-MYB and RSV-NEO-MYB retroviral vectors. Because AMV does not transform fibroblasts and because hematopoietic cells are difficult to transfect directly with DNA, the proviral constructs were stably introduced into chicken embryo fibroblasts (CEFs) to be used as a virus source in a cocultivation assay. Each proviral DNA was cotransfected with its corresponding helper virus by a modified calcium phosphate procedure as described previously (5, 13). CEFs were grown in Dulbecco's modified Eagle medium (high glucose) supplemented with 5% fetal calf serum, 2% heat-inactivated chicken serum, 4.5 g of glucose per liter, $1 \times$ nonessential amino acids, 2 mM L-glutamine, 1 mM

FIG. 3. Morphology of yolk sac cells transformed by MAV-NEO-MYB and RSV-NEO-MYB viruses. (A) Stained cytocentrifuge preparations of nonadherent cells from cultures infected by PR-NEO-MYB, MAV-NEO-MYB, or SR-NEO-MYB; (B) growth of the same transformed monoblasts in methylcellulose. Photographs of representative colonies were taken with a phase-contrast microscope.

sodium pyruvate, 100 μ g of streptomycin per ml, and 100 U of penicillin per ml in a humidified 10% CO₂-90% air incubator at 37°C. Transfections included 5 μ g of provirus (SR-NEO-MYB, PR-NEO-MYB, or MAV-NEO-MYB), 0.5 μ g of the appropriate helper virus DNA (RCAN [11], PRA- Δ MST [10], or MAV-1 [17], respectively; Fig. 1) to permit replication of the defective viruses, and 5 μ g of tRNA to optimize transfection efficiency. CEFs were selected for the expression of provirus by resistance to G418 (200 μ g/ml; GIBCO Laboratories, Grand Island, N.Y.), pooled, and grown in mass culture. Western blot (immunoblot) analysis from these three cell lines performed with monoclonal anti-Myb-2.2 antibodies (8) showed very similar v-Myb protein levels (data not shown). CEFs which had either SR-NEO-MYB or PR-NEO-MYB integrated did not show any significant phenotypic differences from CEFs which contained MAV-NEO-MYB or no virus (data not shown). We therefore conclude that v-myb does not transform fibroblasts even when expressed from an RSV LTR.

Myelomonocytic cells from 12-day-old chicken embryonic yolk sacs were infected by cocultivation for 24 h with virus-producing, mitomycin C-treated CEFs as described previously (12). Suspension cells were transferred to fresh dishes three times in the first week after cocultivation, and 3 ml of medium was exchanged every 3 to 5 days for 2 to 3 months. On day 5 after cocultivation, 500 μ l of a single-cell suspension was seeded into 4.5 ml of methylcellulose (0.8%, final concentration), and after 2 weeks, the number and size of colonies were examined.

Infection of yolk sac cells by any of the three viruses led to the proliferation of small round, highly refractile cells in liquid culture, as described for the natural isolate of AMV (1). Control cells cocultivated with untransfected CEFs differentiated and stopped dividing as expected. In addition, all three viruses gave rise to colonies in methylcellulose, whereas control cells did not. Transformation in liquid culture was first detectable at 9 days in the culture infected with MAV-NEO-MYB, whereas dividing cells in PR-NEO-MYB and SR-NEO-MYB were seen about 1 week later. The number of colonies in methylcellulose formed by yolk sac cells infected by MAV-NEO-MYB was 10 times higher than the number of colonies formed by either SR-NEO-MYB or PR-NEO-MYB. To determine whether levels of the v-Myb protein varied among cells transformed by the different viruses, we performed an immunoblot analysis with Myb-2.2 monoclonal antibody from 1.5×10^6 MAV-NEO-MYB-, SR-NEO-MYB-, or PR-NEO-MYB-transformed yolk sac cells (Fig. 2). The levels of v-Myb protein were very similar in all three cultures. These results demonstrate that all three LTRs can drive production of v-Myb to transform myelomonocytic cells.

Morphology of MAV-NEO-MYB- and RSV-NEO-MYB-transformed cells. Nonadherent cells from the cultures described above were spread with a cytocentrifuge, air dried, and then stained with Diff Quick (Baxter). By light microscopy, yolk sac cells transformed by PR-NEO-MYB, MAV-NEO-MYB, or SR-NEO-MYB virus were morphologically indistinguishable (Fig. 3A). They all showed the eccentric asymmetric nuclei, frequent mitoses, and vacuolated cytoplasm typical of immature monoblasts transformed by AMV. Colonies of all three cell types in methylcellulose were round and compact as reported for the natural isolate of AMV (15) (Fig. 3B). Some of the colonies from SR-NEO-MYB cultures were significantly smaller than colonies from MAV-NEO-MYB or PR-NEO-MYB cultures, suggesting a reduced growth rate.

Viral genomes in transformed myelomonocytic cells. A total of 5×10^5 transformed yolk sac cells from PR-NEO-MYB, MAV-NEO-MYB, and SR-NEO-MYB cultures were washed

FIG. 4. Genome structures of MAV-NEO-MYB and RSV-NEO-MYB viruses in transformed cells. PCR was performed on transformed cells and analyzed on a 2% agarose gel. (A) A schematic representation of the 3' terminus of each provirus is shown at the left, with restriction sites for *EcoRI* and *HindIII* as indicated. Arrows above each provirus depict the annealing positions of the 5' and 3' PCR primers. Sizes of the expected PCR products before and after digestion with either *EcoRI* or *HindIII* is given at the right. (B) Lanes: 1, 4, and 7, unrestricted PCR products; 2, 5, and 8, *EcoRI*-restricted PCR products; 3, 6, and 8, *HindIII*-restricted PCR products; 10, molecular mass markers. Lines at the right indicate relevant molecular mass markers (1,018, 516/506, 394, 344, 298, 220, 200, 154, 142, and 75 bp, in descending order). NS, nonspecifically amplified DNA products.

in phosphate-buffered saline, resuspended in 100 μ l of K buffer ($1 \times$ PCR buffer with 0.5% Tween 20, 0.5% Nonidet P-40, and 60 μ g of proteinase K per ml), incubated for 1 h at 56°C, and then boiled for 10 min. A standard PCR (35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min [last cycle, 10 min] at 72°C) was performed on 10 μ l of each lysate. The first primer, 5'-CCATTCCACAAGGACCAGAC-3', was complementary to a 3' sequence of v-myb, and the second primer, 5'-TCAACCAGGTGCACACC-3', was complementary to a region in U5 where the three different LTRs share 100% sequence identity.

The RSV LTRs contain an *EcoRI* restriction site in U3, whereas *HindIII* is a site unique for the MAV LTR (Fig. 4A). After PCR, three-fourths of the products were digested with either *HindIII* or *EcoRI* and then subjected to gel electrophoresis on a 2% agarose gel (Fig. 4B). The undigested PCR products from each cell line presented bands of the expected mobility (PR-NEO-MYB, lane 1, 1,096 bp; MAV-NEO-MYB, lane 4, 546 bp; SR-NEO-MYB, lane 7, 512 bp). Restriction

with *EcoRI* gave rise to a 94-bp fragment from cells infected with either one of the RSV constructs (lanes 2 and 8). Restriction of PCR products with *HindIII* showed a band at 68 bp (lane 6) for cells infected by MAV-NEO-MYB but no band of the same size from cells infected by PR-NEO-MYB (lane 3) or SR-NEO-MYB (lane 9). The 99-bp fragment in SR-NEO-MYB (lane 9) was the result from a *HindIII* site which was contained in the polylinker of CLA12. These results demonstrate that no rearrangement of the viruses had occurred and that transformation of yolk sac cells by SR-NEO-MYB and PR-NEO-MYB was due to the input virus and not the result of a cross-contamination by MAV-NEO-MYB.

We conclude that the unique LTRs from AMV can be replaced with LTRs from RSV without eliminating transforming ability or altering the phenotype of transformed cells. Therefore, the *v-myb* oncogene of AMV and not its unique LTRs determines cell type specificity of the virus.

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REFERENCES

1. Baluda, M. A., and I. E. Goetz. 1961. Morphological conversion of cell cultures by avian myeloblastosis virus. *Virology* **15**:185–199.
2. Brown, D. W., and H. L. Robinson. 1988. Influence of *env* and long terminal repeat sequences on the tissue tropism of avian leukosis virus. *J. Virol.* **62**:4828–4831.
3. Celander, D., and W. A. Haseltine. 1984. Tissue-specific transcription preferences as a determinant of cell tropism and leukaemogenic potential of murine retroviruses. *Nature (London)* **312**:159–162.
4. Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins. 1983. Role for the 3' end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. *Proc. Natl. Acad. Sci. USA* **80**:4408–4411.
5. Chen, C. H., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752.
6. Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, and J. M. Bishop. 1983. Corrections to the nucleotide sequence of the *src* gene of Rous sarcoma virus. *Nature (London)* **301**:737–738.
7. Des Groseillers, L. E., E. L. Rassert, and P. Jolicoeur. 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. *Proc. Natl. Acad. Sci. USA* **80**:4203–4207.
8. Evan, G. I., G. K. Lewis, and J. M. Bishop. 1984. Isolation of monoclonal antibodies specific for the products of the avian oncogene *myb*. *Mol. Cell. Biol.* **4**:2843–2850.
9. Gilmartin, G. M. 1978. Identification of transcriptional regulatory elements within the long terminal repeat of the Rous sarcoma virus. Ph.D. thesis. University of Virginia, Charlottesville.
10. Heaney, M. L., J. H. Pierce, and J. T. Parson. 1986. Site-directed mutagenesis of the *gag-myc* gene of avian myelocytomatosis virus 29: biological activity and intracellular localization of structurally altered proteins. *J. Virol.* **60**:167–176.
11. Hughes, S. H., J. J. Greenhouse, C. J. Petropoulos, and P. Suttrave. 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**:3004–3012.
12. Ibanez, C. E., and J. S. Lipsick. 1988. Structural and functional domains of the *myb* oncogene: requirements for nuclear transport, myeloid transformation, and colony formation. *J. Virol.* **62**:1981–1988.
13. Ibanez, C. E., and J. S. Lipsick. 1990. *trans* activation of gene expression by *v-myb*. *Mol. Cell. Biol.* **10**:2285–2293.
14. 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.
15. Moscovici, C., L. Gazzolo, and M. G. Moscovici. 1975. Focus assay and defectiveness of avian myeloblastosis virus. *Virology* **68**:173–181.
16. Moscovici, M. G., and C. Moscovici. 1983. Isolation and characterization of a temperature-sensitive mutant of avian myeloblastosis virus. *Proc. Natl. Acad. Sci. USA* **80**:1421–1425.
17. 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.
18. Press, R. D., A. Kim, D. L. Ewert, and E. P. Reddy. 1992. Transformation of chicken myelomonocytic cells by a retrovirus expressing the *v-myb* oncogene from the long terminal repeats of avian myeloblastosis virus but not Rous sarcoma virus. *J. Virol.* **66**:5373–5383.
19. Stocking, C., R. Kollek, U. Bergholz, and W. Ostertag. 1982. Long terminal repeat sequences impart hematopoietic transformation properties to the myeloproliferative sarcoma virus. *Proc. Natl. Acad. Sci. USA* **82**:5746–5750.
20. Stocking, C., R. Kollek, U. Bergholz, and W. Ostertag. 1986. Point mutations in the U3 region of the long terminal repeat of Moloney murine leukemia virus determine disease specificity of the myeloproliferative sarcoma virus. *Virology* **153**:145–149.
21. Weber, F., and W. Schaffner. 1985. Enhancer activity correlates with the oncogenic potential of avian retroviruses. *EMBO J.* **4**:949–956.
22. Weiss, R., N. Teich, H. E. Varmus, and J. M. Coffin. 1982. RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.