Oncogene Cooperation and B-Lymphoid Tumorigenesis in Eμ-*myc* Transgenic Mice*

W. S. Alexander, O. Bernard, W. Y. Langdon, A. W. Harris, J. M. Adams, and S. Cory

A. Introduction

Alteration of the cellular *myc* oncogene has been strongly implicated in several types of lymphoid tumors. Although its normal role remains largely unknown, c-*myc* seems likely to play a crucial role in the control of cellular proliferation and appears to become oncogenic when genetic alterations deregulate its expression. The gene is activated by retroviral insertion in most avian bursal lymphomas and several mammalian T lymphomas and by chromosomal translocation to the active immunoglobulin heavy-chain (IgH) locus in human Burkitt's lymphomas, mouse plasmacytomas, and rat immunocytomas (reviewed by [3]). Direct evidence that deregulated *myc* expression causes malignancy has been provided by studies with transgenic mice. Mice bearing *myc* coupled to immunoglobulin enhancers [1, 12] or a retroviral long terminal repeat [8, 11] succumb to tumors. Particularly striking are the Eμ-*myc* transgenic mice, which bear c-*myc* driven by the IgH enhancer and invariably develop B-lymphoid tumors. Despite this absolute predisposition to lymphoma, the expression of Eμ-*myc* appears insufficient to tumorigenesis. Although lymphoid tissues in young Eμ-*myc* mice express the transgene at similar levels to the tumor cells [2], unlike the latter, they do not elicit tumors upon transplantation [5]. Furthermore, the time of onset for tumors in individual mice varies widely [5], and the lymphomas are monoclonal, even though many B-lineage cells express Eμ-*myc*. Thus, additional somatic change(s) are presumed necessary to confer a fully tumorigenic potential to Eμ-*myc* cells.

B. Involvement of Additional Oncogenes in Eμ-*myc* Tumorigenesis

An appealing hypothesis is that the somatic change(s) which renders Eμ-*myc* cells malignant involves the activation of other cellular oncogenes. To test this idea directly, we have assayed DNA from several Eμ-*myc* lymphomas for the presence of genes capable of inducing NIH3T3 cells to produce foci in culture and/or fibrosarcomas in nude mice. DNA from one tumor induced focus formation and particularly rapid fibrosarcoma development, and these transfected cells displayed amplified and/or rearranged N-*ras* genes. An N-*ras* cDNA was subsequently cloned from the original Eμ-*myc* lymphoma and shown to contain a GLN→HIS mutation at amino acid 61, a residue commonly implicated in *ras* gene activation. Gene transfer experiments using this clone in a retroviral vector have confirmed that this mutated N-*ras* gene can transform fibroblasts and also cause the tumorigenic conversion of preneoplastic Eμ-*myc* pre-B cells. Thus we have established that at least two oncogenes were involved in the development of an Eμ-*myc* lymphoma (Alexan-

Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Victoria 3050, Australia

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der, Bernard, and Cory, in preparation). Preliminary results suggest that activated c-*raf* and *ras* genes may also be involved in several other Eμ-*myc* tumors.

C. Eμ-*myc* B-Lymphoid Cells Progress Toward Autonomy in Culture

Although Eμ-*myc* appears insufficient for tumorigenesis, its constitutive expression in the B-lineage cells of young transgenic mice causes a dramatic perturbation in B-lymphoid development. Preneoplastic Eμ-*myc* mice display a marked polyclonal expansion of large pre-B cells at the expense or more mature B-lymphocytes and most of these cells appear to be in cycle [5]. To investigate how the B-lineage cells have been disturbed in these mice, we have investigated their growth properties in vitro [6]. Eμ-*myc* B-lineage cells were not autonomous, since they died rapidly when cultured in the absence of feeder cells. Nevertheless, long-term cultures could be established on a layer of bone marrow stromal cells, and these initially behaved similarly to parallel cultures from normal mice. However, after 3 weeks, the Eμ-*myc* cultures consistently grew at slightly higher densities than normal cells. As in vivo, the Eμ-*myc* cells were notably larger and showed considerably greater cell cycle activity.

After about 15 weeks in culture, the Eμ-*myc* populations dramatically shifted to growth at tenfold higher density. Nevertheless, these cells still remained strictly feeder layer-dependent and did not elicit tumors in histocompatible mice. Analysis of immunoglobulin gene rearrangements showed that these changes reflected the emergence of dominant pre-B clones. By 25 weeks, the Eμ-*myc* cultures no longer required stromal layers and now produced tumors upon transplantation. These long-term cultures thus permitted the evolution of Eμ-*myc* cells toward a fully transformed phenotype. Their sequential changes in growth characteristics may indicate that more than one alteration is involved in the development of growth autonomy and tumorigenicity.

D. The v-H-*ras* and v-*raf* Oncogenes Cause Autonomous Growth and Malignant Conversion of Eμ-*myc* Pre-B Cells

Preneoplastic Eμ-*myc* mice provide an ideal system to test directly which oncogenes can synergize with a deregulated *myc* gene for B-lymphoid transformation. Using retroviruses, we have introduced a second activated oncogene into preneoplastic Eμ-*myc* lymphoid cells and compared their growth properties with those of similarly infected normal cells. Uninfected cells from normal or Eμ-*myc* marrow did not proliferate in soft agar or in liquid culture and were not tumorigenic. In contrast, Eμ-*myc* marrow cells infected with helper virus-free stocks of Harvey murine sarcoma virus (H-MSV), which carries the v-H-*ras* gene, or with 3611-MSV, which harbors v-*raf*, produced 10–100 times more lymphoid colonies in soft agar than infected normal marrow cells. Moreover, in liquid culture, nonadherent cell lines emerged from H-MSV or 3611-MSV infected Eμ-*myc* marrow cultures at a higher frequency and with shorter latency than from infected normal marrows. Flow-cytometric analyses for surface markers on cells from the agar clones and the liquid culture populations indicated that they were all pre-B, although some contained subpopulations which had matured to express sIg.

All the Eμ-*myc* cultures established in liquid medium following viral infection were highly polyclonal, and a high proportion of the cells plated as colonies in agar. Furthermore, all 18 clones picked from primary agar cultures and injected into nude mice proved tumorigenic. Thus the activation of an H-*ras* or *raf* gene may be sufficient to convert preneoplastic Eμ-*myc* pre-B cells to malignancy.

Significantly, neither v-*raf* nor v-H-*ras* expression was sufficient to transform normal lymphoid cells. The cells derived by 3611-MSV infection of normal marrow were clearly only partially transformed, since they required feeder cells for growth in vitro, did not clone in soft agar, and were not tumorigenic upon transplantation. Although the H-MSV infected normal marrow cultures did contain tumorigenic cells, these appeared to be rare transformants, since early liquid cultures were only oligoclonal and the cells were density dependent for growth in vitro and cloned with poor efficiency in agar. It seems that somatic changes are required in addition to v-H-*ras* or v-*raf* expression to fully transform lymphoid cells.

E. Lymphoid Transformation by A-MLV Is Reduced in Eμ-*myc* Mice

Abelson murine leukemia virus (A-MLV), which harbors the v-*abl* oncogene, causes the preferential proliferation of pre-B cells from infected bone marrow [10, 14]. Although preneoplastic Eμ-*myc* marrow shows a three- to fourfold elevation in pre-B cells [5], it consistently yielded two- to threefold fewer lymphoid colonies than normal marrow following A-MLV infection. Similarly, while almost every normal marrow culture initiated in liquid medium by A-MLV infection yielded continuously proliferating pre-B cells, five of seven A-MLV infected Eμ-*myc* marrows failed to proliferate.

These results may indicate that a high proportion of cells which coexpress v-*abl* and a deregulated *myc* gene die. Alternatively, the primary target cell for A-MLV transformation may be a primitive B-lymphoid precursor rather than a pre-B cell and merely acquires the pre-B phenotype subsequent to infection. This putative target cell would presumably lack the capacity to activate the IgH enhancer and hence would actually be underrepresented in the Eμ-*myc* marrow in the face of the massive pre-B-cell expansion.

Previous studies have established that not all newly derived A-MLV clones from normal marrow are tumorigenic [13, 15]. Our results further indicate that deregulated *myc* expression makes no difference to the emergence of tumorigenic cells, since the proportion of A-MLV infected Eμ-*myc* clones which produced tumors was identical to that of infected normal marrow. Nevertheless, the liquid culture lines derived from Eμ-*myc* marrow with A-MLV plated in soft agar at significantly higher efficiency than those from infected normal marrow. Thus although the deregulated *myc* gene in A-MLV infected transgenic cells may confer some growth advantage in vitro, the combination of *myc* and v-*abl* is clearly insufficient to fully transform early B-lymphoid cells.

F. C-*myc* Gene Regulation in Infected Cells

As expected, the infected normal and Eμ-*myc* pre-B lines all abundantly expressed the appropriate viral transcripts, as shown for some liquid culture lines in Fig. 1. Furthermore, the Eμ-*myc* lines derived with H-MSV or 3611-MSV exhibited the 3.0-kb transgenic *myc* mRNA but not the smaller transcripts characteristic of the endogenous c-*myc* alleles (Fig. 1). This result confirms the data obtained from spontaneous Eμ-*myc* tumors [1, 2] and is consistent with the proposal that c-*myc* expression is controlled by a negative feedback loop: constitutive production of *myc* protein from a deregulated allele prevents expression of the normal c-*myc* gene [7, 9]. In contrast, the A-MLV infected Eμ-*myc* lines all expressed both the transgenic and endogenous *myc* genes (Fig. 1). It is unlikely that the Eμ-*myc* gene is being deleted (thereby releasing expression of the endogenous c-*myc* alleles), since in all of 16 lines subcloned from these populations, the transgene and the endogenous *myc* genes were still coexpressed in a similar ratio to the parental line. Perhaps v-*abl*

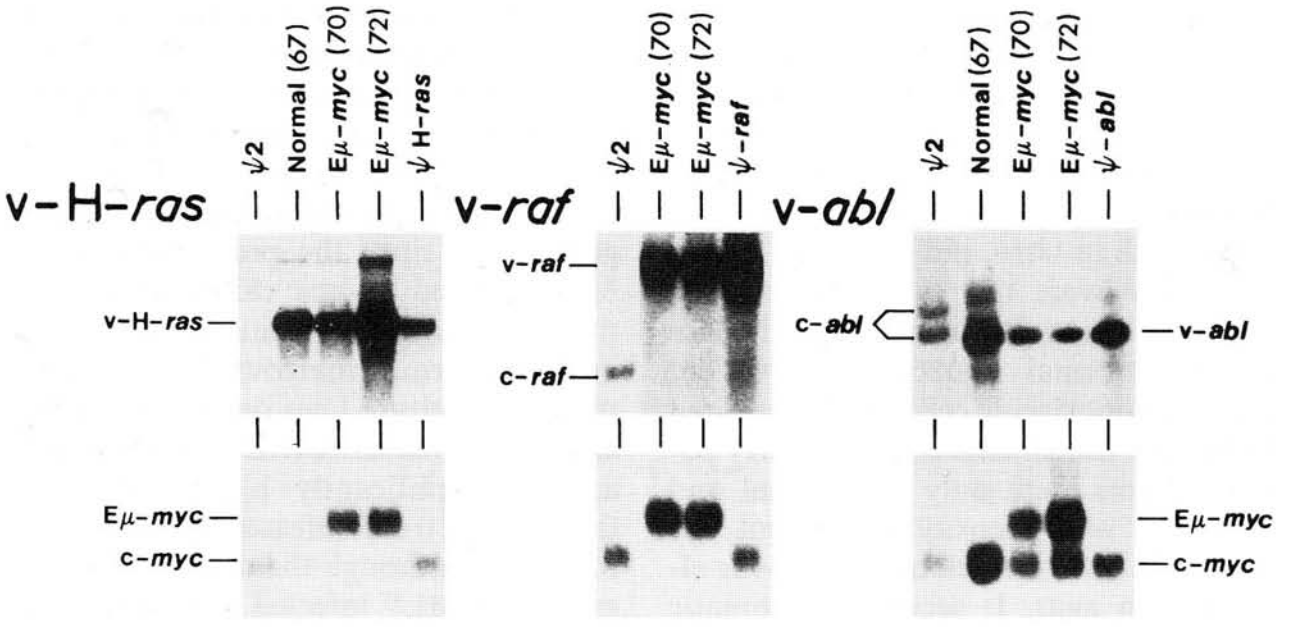

Fig. 1. Oncogene expression in lymphoid cell lines from virus-infected normal and Eμ-*myc* bone marrows. Ψ-H-*ras*, Ψ-*taf*,, and Ψ-*abl* denote the virus-producing fibroblasts and indicate the positions of the 5.4-kb v-H-*ras*, 7.2-kb v-*raf*, and 5.5-kb v-*abl* transcripts, respectively. *Below*, *myc* transcripts are shown; Eμ-*myc* as a 3.0-kb mRNA and the endogenous c-*myc* as the 2.3-kb species

can specifically induce expression of the normal c-*myc* alleles.

In summary, the constitutive expression of *myc* in young Eμ-*myc* mice profoundly disturbs B-lymphoid differentiation, but the onset of B lymphomas requires further somatic change. In vitro studies of the evolution of transgenic pre-B cells toward malignancy suggest that transformation correlates with the loss of growth factor requirements. Analysis of Eμ-*myc* tumor DNA has implicated mutation of the N-*ras* oncogene as a crucial somatic change in at least one tumor. The direct introduction of a second oncogene into preneoplastic lymphoid cells in vitro demonstrated that v-H-*ras* and v-*raf* transformed most (perhaps all) Eμ-*myc* pre-B cells, while v-*abl* appeared unable to cooperate with Eμ-*myc* for B-lymphoid transformation. This model system should help delineate which oncogenes can cooperate with c-*myc* to promote B-lymphoid neoplasia.

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