MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 1176–1179
0270-7306/91/021176-04$02.00/0

Vol. 11, No. 2

Mice Bearing the Eμ-*myc* and Eμ-*pim*-1 Transgenes Develop Pre-B-Cell Leukemia Prenatally

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Received 13 August 1990/Accepted 6 November 1990

Previously, it has been shown that Eμ-*pim*-1 transgenic mice are predisposed to T-cell lymphomas, whereas Eμ-*myc* transgenic mice are predisposed to pre-B-cell lymphomas. Here we show that double-transgenic Eμ-*myc* Eμ-*pim*-1 mice exhibit pre-B-cell leukemia in utero. Upon transplantation into recipient mice, embryo-derived double-transgenic leukemic cells frequently progressed to highly malignant monoclonal tumors, indicating that additional (epi)genetic events had occurred during the progression of the disease.

The development of cancer is a multistep process of predominantly genetic and epigenetic events, the accumulation of which is directly correlated with the level of exposure to mutagens and with time (4, 8, 12, 17, 19). This fact predicts that certain genotypes may predispose to cancer. Such genetic linkage has indeed been demonstrated by classical genetics and in vitro transformation assays (7, 14, 20). Many of the genetic lesions have been shown to cause the activation of oncogenes or inactivation of tumor suppressor genes. Now that a number of these genes directly contributing to the tumorigenic process have been defined, we can predispose to cancer in a controlled and defined way by transgenesis (5, 6, 9). Previously, we have demonstrated that Eμ-*pim*-1 transgenic mice, which express the *pim*-1 oncogene at elevated levels in their lymphoid compartments, are predisposed to T-cell lymphomas (3, 21). In the preleukemic phase, lymphoid organs of Eμ-*pim*-1 transgenic mice do not show imbalances in the different subpopulations of cells, as analyzed by flow cytometric analysis, although a slight enlargement of the spleen is consistently observed. The occurrence of T-cell lymphomas, but not B-cell lymphomas, in Eμ-*pim*-1 transgenic mice does not preclude the possibility that *pim*-1 also promotes B-cell lymphomagenesis, as was suggested by the proviral activation of *pim*-1 in B-cell lymphomas of some mouse strains (18). T-cell lymphomagenesis in Eμ-*pim*-1 transgenic mice can be strongly accelerated by infection of newborn mice with murine leukemia virus (21). In over 95% of the tumors induced by murine leukemia virus in Eμ-*pim*-1 mice, overexpression of either c-*myc* or N-*myc* was found as the result of the nearby integration of a provirus (21). These results suggested, but did not prove, that *pim*-1 and *myc* directly synergize in lymphomagenesis, since one might argue that a high level of *pim*-1 expression only facilitates the activation of *myc*, perhaps by causing the expansion of a particular cell compartment. Previously, it has been demonstrated that Eμ-*myc* transgenic mice show a consistent enlargement of the pre-B-cell compartment with the concomitant predisposition to B-cell lymphomas (1, 2, 6, 10, 15). We have now crossed Eμ-*myc* and Eμ-*pim*-1 transgenic mice and show that *pim*-1 and c-*myc* directly synergize in lymphomagenesis.

Eμ-*pim*-1 transgenic mice have been described previously (21). For the generation of Eμ-*myc* mice, an Eμ-*myc* fusion gene, isolated from a plasmacytoma and generously provided by Suzanne Cory, was introduced into the germ line of mice by pronuclear injection of fertilized oocytes (11) recovered in cumulus from the oviducts of superovulated (CBA/BrA × C57BL/LiA)F1 females that had mated with F1 males. Transgenic founders were backcrossed with either (CBA/BrA × C57BL/LiA)F1 mice or with the C57BL/LiA parental strain.

The characteristics of the transgenic Eμ-*myc* mouse lines obtained (186 and 187) are essentially the same as those described by Adams et al. (1, 2, 6, 10, 15): the c-*myc* transgene is highly expressed in B cells but not in T cells or other somatic tissues (data not shown). Pre-B-cell lymphomas developed in 75% of the animals after a latent period of 2.5 to 12 months (Fig. 1). The prelymphomatous state was characterized by an enlarged pre-B-cell compartment in which the pre-B cells aberrantly expressed Ia major histocompatibility complex class II surface antigens, as detected by flow cytometry using antibodies directed against T- and B-cell-specific surface markers (for a summary of markers, see reference 21), as was previously noted by Cory et al. (6, 15). A third founder line, Eμ-*myc* 188, expressed the c-*myc* transgene at extremely low levels, with the concomitant absence of a high tumor incidence (Fig. 1).

Table 1 summarizes the numbers of offspring with the different genotypes born from crosses between heterozygous Eμ-*pim*-1 and Eμ-*myc* mice. With the exception of one animal that died at 5 weeks of age, no viable *pim*-1 c-*myc* double-transgenic mice were found in litters from crossbreedings of *pim*-1 line 64 or 66 with Eμ-*myc* line 186 or 187 (Fig. 1). Most of the dead newborns that could be collected immediately after birth carried both transgenes. In contrast, double-transgenic offspring from crosses between the Eμ-*pim*-1 lines and the low-expressor Eμ-*myc* line 188 were viable and showed a low tumor incidence. To gain insight into the cause of the perinatal lethality of transgenic mice expressing both *pim*-1 and c-*myc* at high levels, 17- to 19-day-old embryos from matings between heterozygous Eμ-*pim*-1 and Eμ-*myc* transgenic mice (lines 186 and 187) were collected, analyzed for the presence of the Eμ-*myc* and

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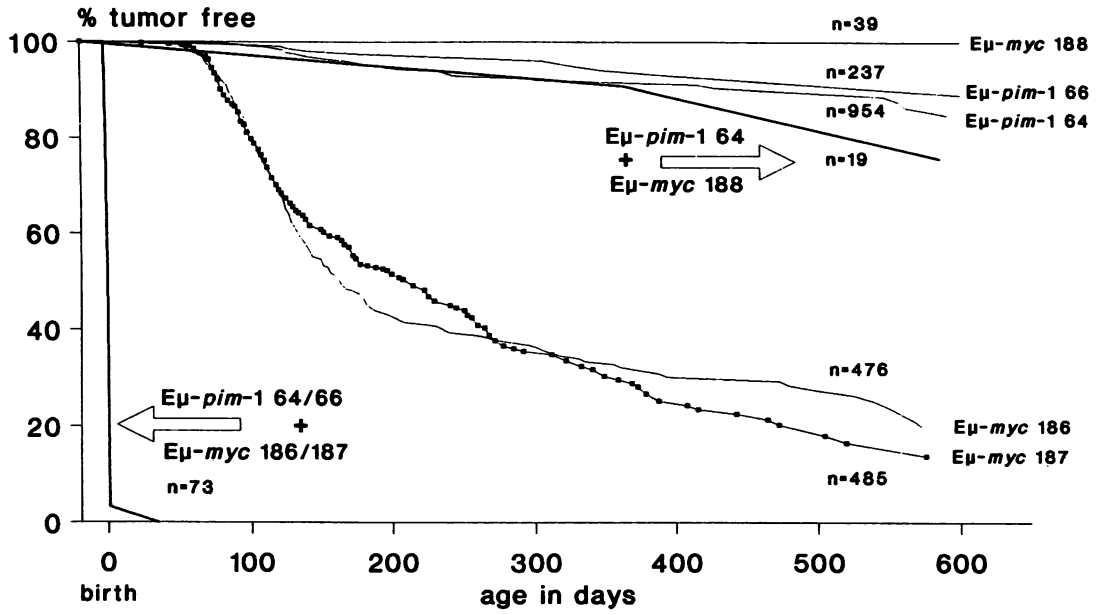

FIG. 1. Tumor incidence in the Eμ-*myc* and Eμ-*pim*-1 founder lines and double-transgenic progeny. The founder lines are indicated on the right, and the Eμ-*pim*-1 × Eμ-*myc* crosses are indicated by arrows. Numbers of mice on which these curves are based are indicated. Note that tumors in transgenic lines carrying only the Eμ-*pim*-1 transgene are of T-cell origin, whereas tumors in transgenic mice carrying the Eμ-*myc* construct are of B-cell origin.

Eμ-*pim*-1 transgenes, and examined histologically. Of these fetuses, 25% carried both transgenes (Table 1). The gross morphology of the double-transgenic fetuses was normal. However, peripheral blood of these fetuses contained large numbers of blastoid cells of variable size (Fig. 2). Histological examination of 17- to 19-day-old fetuses and placentas showed the involvement of predominantly liver and spleen but not of extraembryonic tissues. Whereas the liver exhibited a normal structure with an increased leukocyte cell density, the splenic architecture was hardly recognizable because of lymphomatous growth. Peripheral lymph nodes, Peyer's patches, and bone marrow did not show major alterations at this stage. However, accumulation of lymphoid cells was found at the base of the skull (between the hypophysis and the cochlear area), in the maxillary periosteal area, in the orbital-lining mesenchyme, in the venous plexus, and, incidentally, in cervical and cranial dermal mesenchyme. Immunohistochemistry revealed that the expanded cell population in the fetuses carried the B-cell-specific cell surface marker B220 (Fig. 3), whereas no staining was found for Thy-1, Mac-1, or surface immunoglobulin (Ig) (not shown), indicating that these cells represent pre-B cells.

TABLE 1. Transmission of Eμ-*myc* and Eμ-*pim*-1 transgenes to offspring from crossbreedings of heterozygous Eμ-*myc* and Eμ-*pim*-1 transgenic mice

Transgene(s)	No. with transgene(s)[a]			
	A	B	C	
			Lines 186 and 187	Line 188
None	27	4	37	14
Eμ-*pim*-1 —	33	7	22	13
— Eμ-*myc*	30	9	30	12
Eμ-*pim*-1 Eμ-*myc*	31	11[b]	1[c]	11

[a] Fetuses or newborn mice were analyzed for the presence of both transgenes at days 17 to 19 of gestation (A, lines 186 and 187), within 1 day after birth (B, lines 186 and 187), and at 4 to 8 weeks after birth (C).
[b] All were found dead.
[c] Died at 5 weeks of age from a pre-B-cell lymphoma.

To test the tumorigenic potential of the leukemic cells in these fetuses, peripheral blood was transplanted intravenously to histocompatible hosts. Six of twelve transplantations resulted in the outgrowth of lymphomas within 9 weeks. Transplantation into nude mice resulted in tumor outgrowth in four out of five cases within 8 weeks. In contrast, transplantation of peripheral blood from either single-transgenic or nontransgenic embryos did not result in the outgrowth of tumors (Table 2). Histological and flow cytometric analysis of the transplanted tumors conformed to the immunohistochemistry of the leukemic cells found in the fetuses. Southern blot analysis of the transplants showed distinct Ig heavy-chain gene rearrangements in tumors of both histocompatible recipients and nude mice (see below). No rearrangements of the Ig kappa light-chain locus were found (not shown). Apparently, overexpression of *pim*-1 and c-*myc* causes a dramatic expansion of early B cells in the developing embryo. The disease resembles that described for Eμ-*myc* transgenic mice except that the concomitant overexpression of *pim*-1 now causes disease during fetal life rather than several months after birth (Fig. 1).

Although these data show that *pim*-1 and c-*myc* synergize effectively in the induction of malignant pre-B-cell lymphomas in utero, the question remains as to whether overexpression of *pim*-1 and c-*myc* in the B-cell lineage early in embryonic development suffices for malignant transformation. If additional rare events are required for malignant growth, one expects the tumors to be of clonal origin. We first focused on the lymphoid cells present in double-transgenic fetuses. As early as days 15 to 16 of gestation, a significant increase in the number of lymphoid cells was

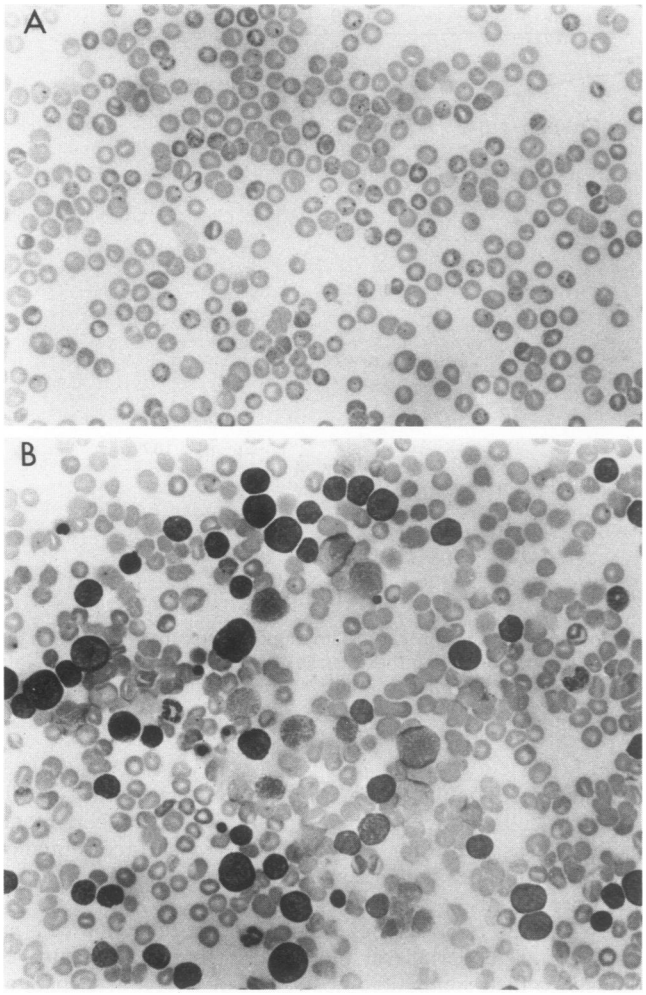

FIG. 2. Cytology of blood smears from the jugular veins of 17- to 19-day-old fetuses. (A) Normal nontransgenic or single-transgenic Eμ-*myc* or Eμ-*pim*-1 fetuses. All show the same cytology at this stage of development. (B) Eμ-*myc* Eμ-*pim*-1 double-transgenic fetus. Cells were stained with May-Grünwald-Giemsa.

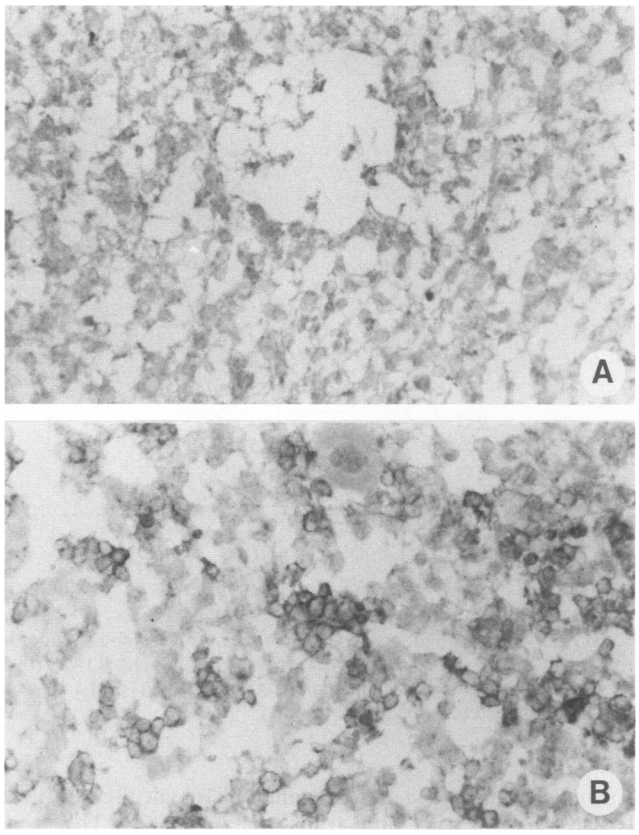

FIG. 3. Immunohistochemical detection of β-lineage cells in fetal liver. Sections are from a normal control (A) and a double-transgenic mouse (B). Note the abundance of B220-positive cells in the liver of the double-transgenic mouse. Immunohistochemistry was performed as described previously (13). Thin sections obtained from paraffin-embedded embryos were stained with hematoxylin and eosin and additionally with Giemsa.

observed. Their heterogeneous appearance suggested that they represent a polyclonal population of cells, although this heterogeneity might also reflect a limited capacity to differentiate. Furthermore, since the leukocyte count varied considerably among 17- to 19-day-old double-transgenic embryos, additional stochastic events may occur early in embryogenesis, thereby affecting the actual expansion of the embryonic lymphoid compartments. We were unable to sufficiently enrich the leukemic cells from these small blood samples for direct analysis. Moreover, it has been shown that normally certain DJ rearrangements predominate within the polyclonal B lineage (15), thereby limiting the usefulness of these markers to directly determine the clonality of the expanded pre-B cells in the fetuses. Therefore, we used an indirect approach to get insight into the clonality of the leukemic cells of the fetuses. A cell suspension was prepared from the spleen and liver of one fetus, and aliquots were transplanted into 15 histocompatible (CBA/BR × C57BL/LiA)F1 hosts (Table 2). Seven double-transgenic embryos were transplanted in this way. Four embryos proved to have transplantable tumor cells. DNA was isolated from the individual tumors, and the Ig heavy-chain rearrangement pattern was determined by Southern blot analysis, using the joining region probe pJ11 (16). Within a single recipient, Ig rearrangements, which probably are restricted to D-J joinings, were mostly identical in tumors from different anatomical sites. However, the rearrangement pattern differed among independent recipients inoculated with an aliquot of the same fetus (data not shown). This result strongly suggests that the transplanted tumors are derived from different ancestor cells, although we cannot formally exclude the possibility that cells from the same tumorigenic ancestor clone have undergone Ig rearrangements after transplantation. In either case transplantation has resulted in the selective outgrowth of only a limited number of (sub)clones.

Our data are most compatible with a model of tumorigenesis in which a polyclonal pre-B-cell proliferation, induced by the Eμ-*myc* and Eμ-*pim*-1 genes in the early embryo, is frequently followed or facilitated by additional (epi)genetic events leading to the formation of malignant cells. The stochastic nature of these events could explain the variation in the severity of the disease in 17- to 19-day-old fetuses and might also account for the lack of transplantability of some of the blood samples. Upon transplantation, additional selective forces then act on these cells, resulting in the

TABLE 2. Transplantability of leukemic cells from Eμ-*myc* Eμ-*pim*-1 double-transgenic fetuses

Inherited transgene(s)	Transplant	Ratio,[a] transplanted to:	
		Syngeneic host	Nude mice
None	Blood	0/2	ND
Eμ-*pim*-1 —	Blood	0/4	ND
— Eμ-*myc*	Blood	0/5	0/2
Eμ-*pim*-1 Eμ-*myc*	Blood	6/12	4/5
	Liver + spleen	4/7	ND

[a] Ratio of the number of transplantable leukemic cell samples to the total number of independently transplanted leukemic cell samples. Blood was collected in a equal volume of isotonic glucose-citrate from the jugular vein of fetuses and diluted with phosphate-buffered saline to 100 μl; 100 μl (equivalent with 5 to 20 μl of blood) was injected intravenously into two recipients. Cell suspensions prepared from fetal liver and spleen were injected intravenously into 15 syngeneic recipient mice (3×10^5 to 10^6 cells per mouse). Transplantability was scored positive if lymphomas developed in one of the recipients. ND, Not determined.

outgrowth of further adapted subclones of tumor cells that are marked by different D-J joinings. The massive expansion of pre-B cells seen already around 17 to 19 days of gestation indicates that the proliferative impulse, mediated by the high expression of c-*myc* and *pim*-1, acts on the hematopoietic system from early in embryogenesis. Finally, it is noteworthy that the search for synergizing oncogenes by an in vivo protocol using provirus tagging in transgenic mice has resulted in the identification of c-*myc* and *pim*-1, two oncogenes that appear to display the strongest synergism in in vivo tumorigenesis reported to date.

We thank Anneloes Beenders, Nel Bosnie, Patricia Kooiman, and Ellen Wientjens for excellent technical assistance, L. Kuyper-Pietersma, E. van Muylwijk, and J. Bulthuis for histological assistance, and Chris Saris and Peter Laird for discussion and critical reading of the manuscript.

This work was supported by the Netherlands Cancer Foundation (S.V.) and the Foundation for Medical and Health Research (MEDIGON) (M.V.L.).

REFERENCES

1. **Adams, J. M., A. W. Harris, C. A. Pinkert, L. M. Corcoran, W. S. Alexander, S. Cory, R. D. Palmiter, and R. L. Brinster.** 1985. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature (London) **318**:533–538.
2. **Alexander, W. S., J. W. Schrader, and J. M. Adams.** 1987. Expression of the c-*myc* oncogene under control of an immunoglobulin enhancer in Eμ-*myc* transgenic mice. Mol. Cell. Biol. **7**:1436–1444.
3. **Breuer, M., R. Slebos, S. Verbeek, M. van Lohuizen, E. Wientjens, and A. Berns.** 1989. Very high frequency of lymphoma induction by a chemical carcinogen in *pim*-1 transgenic mice. Nature (London) **340**:61–63.
4. **Cairns, J.** 1975. Mutation selection and the natural history of cancer. Nature (London) **255**:197–200.
5. **Compere, S. J., P. Baldacci, and R. Jaenisch.** 1988. Oncogenes in transgenic mice. Biochim. Biophys. Acta **948**:129–149.
6. **Cory, S., and J. M. Adams.** 1988. Transgenic mice and oncogenesis. Annu. Rev. Immunol. **6**:25–48.
7. **Der, C. J., T. G. Krontiris, and G. M. Cooper.** 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. USA **79**:3637–3640.
8. **Farber, E.** 1984. The multistep nature of cancer development. Cancer Res. **44**:4217–4223.
9. **Groner, B., C. A. Schoenenberger, and A. C. Andres.** 1987. Targeted expression of the ras and myc oncogenes in transgenic mice. Trends Genet. **3**:306–308.
10. **Harris, A. W., C. A. Pinkert, M. Crawford, W. Y. Langdon, R. L. Brinster, and J. M. Adams.** 1988. The Eμ-*myc* transgenic mouse: a model for high-incidence spontaneous lymphoma and leukemia of early B cells. J. Exp. Med. **167**:353–371.
11. **Hogan, B. L. M., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Klein, G., and E. Klein.** 1985. Evolution of tumors and the impact of molecular oncology. Nature (London) **315**:190–195.
13. **Kraal, G., M. Breel, M. Janse, and G. Bruin.** 1986. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. J. Exp. Med. **163**:981–997.
14. **Land, H., L. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
15. **Langdon, W. Y., A. W. Harris, S. Cory, and J. M. Adams.** 1986. The c-myc oncogene perturbs B lymphocyte development in Eμ-myc transgenic mice. Cell **47**:11–18.
16. **Marcu, K. B., J. Banerji, N. A. Penncavage, R. Long, and N. Arnheim.** 1980. 5′ Flanking region of immunoglobulin heavy chain constant region genes displays length heterogeneity in germ lines of inbred mouse strains. Cell **22**:187–196.
17. **Marx, J. L.** 1984. Oncogenes amplified in cancer cells. Science **223**:40–41.
18. **Mucenski, M. L., D. J. Gilbert, B. A. Taylor, N. A. Jenkins, and N. G. Copeland.** 1987. Common sites of viral integration in lymphomas arising in AKXD recombinant inbred mouse strains. Oncogene Res. **2**:33–48.
19. **Nusse, R., and A. Berns.** 1988. Cellular oncogene activation by insertion of retroviral DNA. Genes identified by provirus tagging, p. 95–119. *In* G. Klein (ed.), Cellular oncogene activation. Marcel Dekker, Inc., New York.
20. **Parada, L. F., C. J. Tabin, C. Shih, and R. A. Weinberg.** 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature (London) **297**:474–478.
21. **Van Lohuizen, M., S. Verbeek, P. Krimpenfort, J. Domen, C. Saris, T. Radaszkiewicz, and A. Berns.** 1989. Predisposition to lymphomagenesis in *pim*-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. Cell **56**:673–682.