JOURNAL OF VIROLOGY, Dec. 1987, p. 3721–3725
0022-538X/87/123721-05$02.00/0

Vol. 61, No. 12

Moloney Murine Leukemia Virus-Induced Myeloid Tumors in Adult BALB/c Mice: Requirement of c-*myb* Activation but Lack of v-*abl* Involvement

GRACE L. C. SHEN-ONG* AND LINDA WOLFF

Laboratory of Genetics, National Cancer Institute, Bethesda, Maryland 20892

Received 24 July 1987/Accepted 3 September 1987

BALB/c mice treated with pristane and Abelson virus have been used as an animal model system for the rapid induction of plasmacytomas. Myelomonocytic tumors with helper Moloney murine leukemia virus clonally inserted into the c-*myb* locus were observed in about 10% of pristane-primed BALB/c mice infected with Abelson virus. However, v-*abl* was absent in almost all of those tumors. Since Moloney virus is thought to induce mostly T-cell lymphomas, we have carried out studies to investigate this alteration of disease specificity and to determine whether v-*abl* played an obligatory role in the development of these tumors. We found that, whereas lymphomas developed late (>3 months) in both pristane-primed and unprimed control mice, the myelomonocytic tumors arose at a high frequency, within 3 months, but only in pristane-treated mice. Clonal Moloney virus insertion was again found in each of the seven myelomonocytic tumors examined. Northern blot analyses and S1 mapping studies revealed the presence of virally promoted chimeric mRNAs that lack the three 5′-most *myb* coding exons. Hence it appears that the requirement for the v-*abl* gene product in tumor induction is not obligatory. Our results also indicate that tumor-specific alteration at the 5′ end of the *myb* gene plays an important role in the development of these tumors.

Insertional mutagenesis is thought to be a common initiating step in tumorigenesis by certain retroviruses, especially those that lack oncogenes derived from cellular genes (reviewed in reference 1). It has been shown that Moloney murine leukemia virus (M-MuLV) induces leukemogenesis in rodents, frequently T-cell lymphomas (20). Furthermore, a number of preferential M-MuLV integration sites, including the c-*myc* locus, have been identified (1).

A unique group of tumors, termed Abelson virus-induced plasmacytoid lymphosarcomas (ABPLs), have been shown to carry a disrupted allele of the c-*myb* locus (7) due to the insertion of the M-MuLV helper component of the Abelson virus complex (15). These tumors arose in less than 10% of BALB/c mice after the mice received intraperitoneal injections of pristane and the Abelson virus complex (10). The virus complex used in the tumor induction contains the replication-defective, oncogenic Abelson MuLV that carries the transforming v-*abl* sequence and the transmissible helper M-MuLV (18). Subsequent cellular and molecular studies have demonstrated that the tumor cells belong to the myelomonocytic lineage, and the cells have thus been renamed as myelogenous leukemia (ABML) (17). Only one of the six ABMLs examined contains and expresses the oncogenic v-*abl* sequences of A-MuLV (7). The loss of the v-*abl* sequences had been observed during experimental transplantation of certain lymphoid tumors originally induced by Abelson virus complex (4), indicating that the continued influence of the v-*abl* gene product was not required for the maintenance of those tumors. One postulated role of v-*abl* in tumorigenesis is to abrogate the growth factor(s) requirements of hematopoietic cells (2, 8, 9). It is therefore possible that v-*abl* is only involved in ABML induction, while the altered *myb* gene product or the deregulation of the *myb* gene expression as a result of M-MuLV insertion, or both, are required for the maintenance of these tumors. The present study was undertaken to determine whether v-*abl* is absolutely required in the tumorigenesis of ABML tumors and to gain insights into the mechanism(s) of disease specificity of M-MuLV. To this end, tumor induction experiments were performed by infecting pristane-primed adult BALB/c mice with M-MuLV alone.

* Corresponding author.

MATERIALS AND METHODS

Viruses. M-MuLV was prepared by transfecting *Hin*dIII-linearized cloned M-MuLV DNA (19) (a generous gift from E. Gilboa) onto NIH 3T3 cells by calcium phosphate precipitation (3). M-MuLV was prepared by collecting 24-h supernatant fluids from a confluent producer line that was cloned by limiting dilution. Infectivity was assayed by the XC plaque assay (13).

Mice and tumor induction. Young (5- to 7-week-old) female BALB/cAn mice were primed by intraperitoneal injection with 0.5 ml of pristane (Aldrich Chemical Co., Milwaukee, Wis.). After 4 weeks, the pristane-treated mice and a group of control mice were infected intravenously with 4×10^5 PFU of tissue culture-grown virus in a volume of 0.5 ml. Mice were monitored for disease by preparing smears from the peritoneal ascitic fluid and staining the cells with Wright-Giemsa. Animals were sacrificed when the disease was far advanced. Tumors were maintained via serial passage in the peritoneal cavities of pristane-primed mice.

Isolation of nucleic acids and blot hybridization. Extraction of total cellular RNA from frozen tissue samples by the guanidium-cesium chloride method has been described (6). Southern blot experiments were performed as described previously (14).

S1 nuclease mapping. Samples of 5 μg of poly(A) RNA were used in the S1 nuclease mapping as described (16).

RESULTS

Tumor induction by M-MuLV infection in pristane-primed and unprimed adult BALB/c mice. The tumor-inducing po-

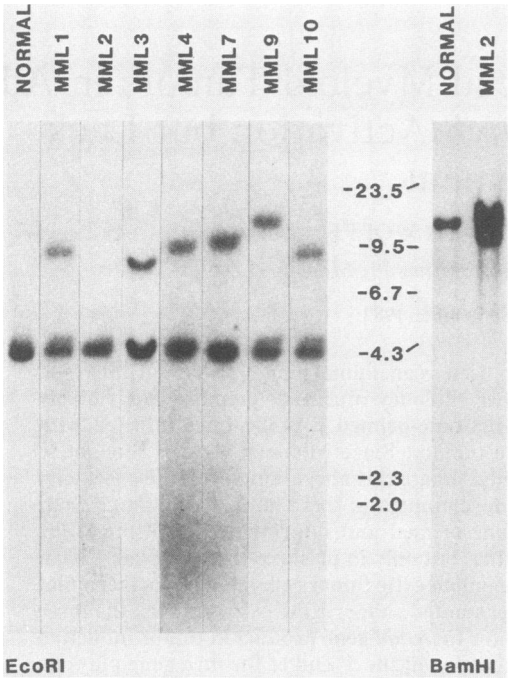

FIG. 1. *myb* hybridization of blots of *Eco*RI and *Bam*HI digests of genomic DNA (15 μg) from normal BALB/c liver and MMLs as indicated. The probe used for hybridization is a 1.4-kbp genomic m-*myb* *Bgl*II fragment that contains the vE2 exon (16). The sizes of standard markers (*Hin*dIII fragments of phage λ DNA) are in kilobase pairs.

tential of ecotropic M-MuLV was determined by intravenous inoculation of young adult pristane-primed and control unprimed BALB/c mice. Seven of the 13 pristane-primed mice that were infected with M-MuLV developed ABML-like myelomonocytic tumors. They were termed M-MuLV-induced myelogenous leukemias (MMLs). The mean latent period for MML development was 66 days; MMLs 1, 2, 3, and 4 developed 53 days post virus, MMLs 9 and 10 developed 75 days post virus, and MML 7 developed 102 days post virus. Similar to previous studies that used other animal model systems, lymphomas developed after a latent period of over 100 days post virus in the control unprimed BALB/c mice and the other pristane-primed mice that did not develop MMLs. Flow microflurometry analyses showed that cells from the seven MMLs express the Mac-1 and Ly-17 (Fc receptor) myeloid-associated surface antigens at levels comparable to that found on the ABML cells. The detailed classification of MMLs is described elsewhere (L. Wolff, J. F. Mushinski, G. L. C. Shen-Ong, and H. C. Morse III, manuscript in preparation). This suggests that the ecotropic M-MuLV alone can induce myelomonocytic tumors in the same animal model system used to derive the ABMLs.

Clonal rearrangement in the c-*myb* locus in MMLs. Previous studies that showed M-MuLV insertion in the c-*myb* locus in ABMLs (15, 16) suggest that insertional activation of this particular oncogene plays an important role in the tumorigenesis of these tumors. No c-*myb* rearrangement has been observed in M-MuLV-induced T or B lymphomas. In this study, six of seven MMLs were found to carry both a rearranged and normal 4.2-kilobase-pair (kbp) c-*myb* EcoRI fragments that contain the 5′-most exons (vE1 and vE2) with v-*myb* homology (Fig. 1A; see Fig. 4, top). Although no rearrangement was found in the 4.2-kbp c-*myb* *Eco*RI fragment of genomic DNA of the MML 2 tumor, subsequent restriction enzyme digestion of MML 2 DNA with *Bam*HI revealed the presence of a rearranged 9.7-kbp and the normal 10.8-kbp c-*myb*-hybridizing *Bam*HI fragment (Fig. 1B; see Fig. 4, top). It is therefore possible that the clonal rearrangement found in the c-*myb* locus in all seven MMLs may be due to the target-specific insertion of the M-MuLV.

Transcription of aberrant *myb* mRNAs in MMLs. Disruption of the c-*myb* locus in ABMLs has been shown to be associated with the expression of altered forms of *myb* RNA (7). RNA blot analysis of the poly(A) RNA obtained from the different MMLs also showed an altered pattern of *myb* RNA similar to that expressed by the ABMLs (data not shown). This indicates that similar abnormal *myb* expression occurs in both groups of myelomonocytic tumors irrespective of the presence or absence of v-*abl* in the virus stocks used in tumor induction. Previously, a novel viral *gag-myb* transcript was detected in five different ABMLs by means of S1 mapping studies using a tumor-specific *gag-myb* cDNA sequence as the hybridizing probe (16). The genetic organization of the virus-disrupted *myb* locus is diagrammed in Fig. 2, and the positions of the splice sequences that are utilized in the generation of the novel chimeric *gag-myb* transcript are indicated. As described in the previous S1 mapping studies of the ABML *myb* RNAs, the 433-nucleotide (nt) *gag-myb* fragment isolated from an ABML-2 cDNA clone was used to determine whether the same splice junction is present in the MML *myb* RNAs (16; Fig. 3, bottom). Hence, the protection of a 433-nt species with contiguous *gag-myb* sequences by the RNAs from the MMLs would indicate that the aberrant *myb* RNAs are promoted within the upstream 5′ long terminal repeat and that the cryptic splice donor sequence at nucleotide position 1594 in the *gag* sequence is used in splicing the M-MuLV

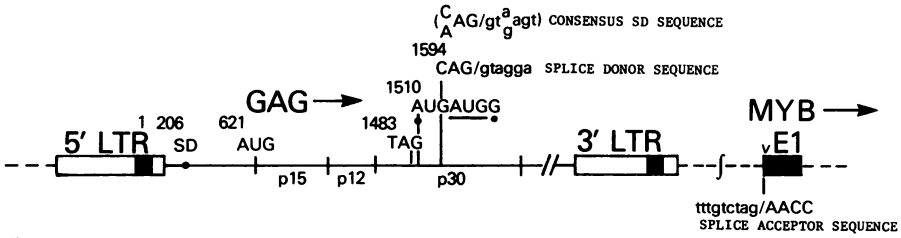

FIG. 2. Schematic diagram illustrating the mechanism of disruption of the c-*myb* locus in ABMLs. The numbers shown are positions of the following viral sequences: 1, the cap site of the M-MuLV; 206, the viral splice donor sequence (SD); 1483, upstream termination codon in the same reading frame as the *myb* sequence in the chimeric *gag-myb* transcripts; 1510, sequences around the putative tumor *myb* start codon; 1594, the novel splice donor sequence used to generate the chimeric RNA resulting in the sequence CAG/AACC at the splice junction.

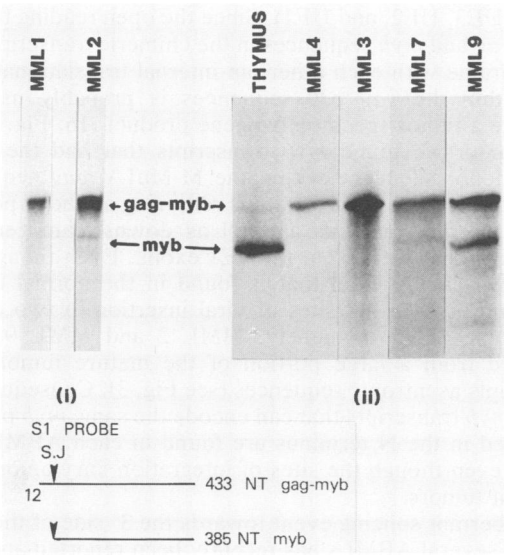

FIG. 3. Analyses of c-*myb* transcripts in MMLs. S1 nuclease mapping of protection of thymus and MML RNAs by a uniformly labeled *gag-myb* probe. The 455-nt (NT) S1 probe, which contains 48 residues of the *gag* sequences, 385 residues of the *myb* sequences, and 12 dC residues included in the *Pst*I fragment as a result of tailing reaction during cDNA cloning, is as shown (bottom). The 433-nt *gag-myb* species and 385-nt *myb* species that are protected by the probe are as indicated. Separate experiments are shown in panels i and ii.

sequence to the splice acceptor sequence in the vE1 exon. Alternatively, the 385-nt species containing only the *myb* sequences but not the 48 bases of *gag* sequences would be protected by c-*myb* transcripts either from the normal *myb* allele, from alternative splicing involving other splice donor sequences, or, in those MMLs with proviral insertion upstream of the UE1 exon, from splicing of the cryptic splice donor sequence in *gag* to the splice acceptor sequence in the UE1 exon. Figure 3B shows that a 433-nt species was protected by RNAs from all six MMLs, but not by thymus RNAs. Together with RNA blot and S1 analyses which showed the expression of altered forms of *myb* RNA in the seventh tumor, MML 10, we can conclude that the novel *gag-myb* transcript in MMLs is the same as that found in ABMLs.

Mapping the sites of M-MuLV insertion in the c-*myb* locus in MMLs. Previous studies had shown that in ABMLs, the M-MuLV helper components had inserted in the same transcriptional orientation within 2 kbp upstream of the 5′-most exon (vE1) with v-*myb* homology (16). In each case, the inserted provirus retained both the 5′ and 3′ long terminal repeats, most of the *gag* sequences, and variable portions of the *pol* and *env* sequences. Since M-MuLV does not contain any *Eco*RI sequences, the difference in the sizes of the rearranged c-*myb* *Eco*RI fragments in different MMLs as shown in Fig. 1 indicates the insertion of a variably deleted M-MuLV in the c-*myb* locus. We presume that the size increase in each rearranged MML c-*myb*-hybridizing *Eco*RI fragment represents the size of the M-MuLV insert in

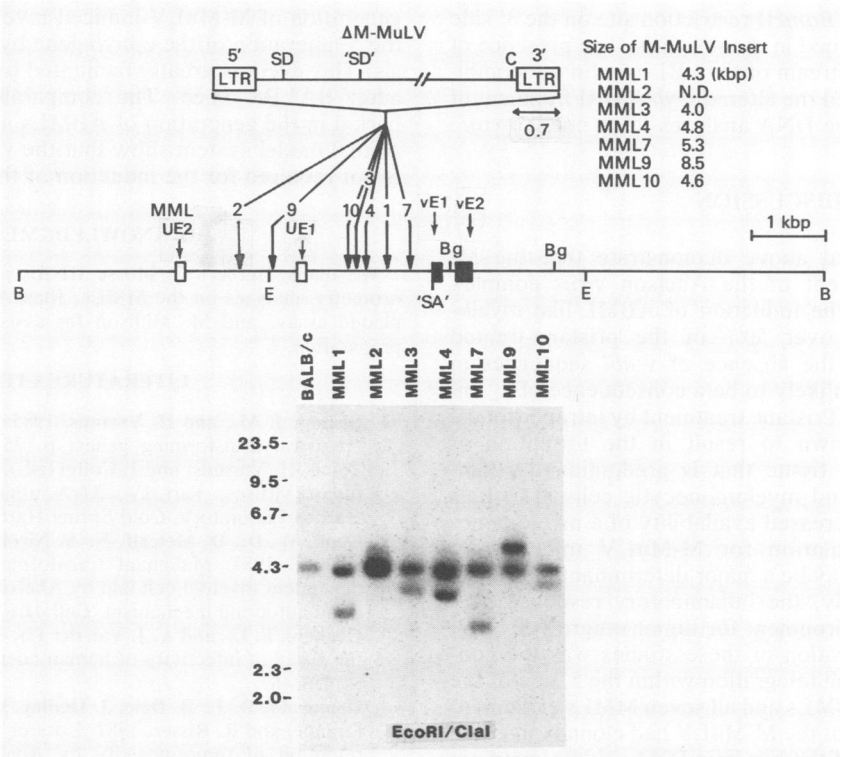

FIG. 4. Top, Map of the c-*myb* genetic region that is altered by the insertion of an M-MuLV component. Bottom, *myb* hybridization of blots of *Eco*RI + *Cla*I digests of genomic DNA (15 μg) from normal BALB/c liver and MMLs as described in the legend of Fig. 1. The size of the deleted M-MuLV insert in the rearranged fragment (top right) was estimated by determining the increase in size of the normal 4.2-kbp *Eco*RI fragment found in each MML and is equivalent to the size of the rearranged *Eco*RI band shown in Fig. 1 minus 4.2 kbp. The position of virus insertion in each MML was deduced as described in the text and is calculated using the following equation: (size of the rearranged *Cla*I/*Eco*RI fragment − 0.7 kbp). Open and solid boxes indicate the four *myb* coding exons from left to right as the upstream 5′ UE2 and UE1 exons, which are absent in the *gag-myb* mRNAs, and the first two vE1 and vE2 exons with v-*myb* homology. The positions of the exons are as previously mapped (5).

the c-*myb* locus. Our results suggest that an M-MuLV component ranging from 4.0 to 8.5 kbp has inserted into the 4.2-kbp *Eco*RI fragment of the different MMLs (Fig. 4; see Fig. 4 legend for the calculation employed in the determination of the size of each M-MuLV insert).

It is also possible to deduce the sites of M-MuLV insertion in the c-*myb* locus in MMLs by blot analyses of tumor DNAs. The presence of the chimeric *gag-myb* transcripts (Fig. 3) indicates that proviral insertion is in the same transcriptional orientation as the c-*myb* gene. Furthermore, we predict that the *Cla*I site located in sequences 0.7 kbp upstream of the 3′ long terminal repeat is retained in the inserted provirus in MMLs as has been found in the ABMLs (15). Since no *Cla*I sequences were found in the normal 4.2-kbp *Eco*RI *myb*-hybridizing fragment into which the M-MuLV had inserted, the novel *myb*-hybridizing bands for MMLs 1, 3, 4, 7, 9, and 10 observed in the blot of genomic DNAs doubly digested with the *Eco*RI and *Cla*I restriction enzymes would be due to the introduction of the viral *Cla*I site as shown (Fig. 4, top). Hence, the sizes of the *Cla*I-*Eco*RI *myb*-hybridizing fragments allow the positioning of the *Cla*I enzyme site within the provirus with respect to the 3′ *Eco*RI site of the *myb* fragment (see Fig. 4 legend for the calculation employed in the positioning of the *Cla*I site and the site of proviral insertion in each MML). The restriction map in Fig. 4 shows that M-MuLV has inserted in the same transcriptional orientation within a 3.0-kbp span of the genomic DNA upstream of the vE1 *myb* exons. The position of the M-MuLV insertion in MML 2 is predicted from the size of the rearranged *Bam*HI fragment (Fig. 1B), based on the assumption that the *Bam*HI restriction site on the 3′ side of the M-MuLV is retained in the provirus. The presence of a deleted M-MuLV upstream of the vE1 exon in a recombinant clone that contained the altered *myb Eco*RI fragment of MML7 also confirms the DNA analyses (data not shown).

DISCUSSION

The results described above demonstrate that the M-MuLV helper component of the Abelson virus complex alone is sufficient for the induction of ABML-like myelomonocytic tumors in over 50% of the pristane-treated BALB/c mice. Hence the absence of v-*abl* sequences in almost all ABMLs is unlikely to be a consequence of a "hit and run" phenomenon. Pristane treatment by intraperitoneal injection has been shown to result in the formation of chronic granulomatous tissue that is predominantly comprised of neutrophils and myelomonocytic cells (11). It is conceivable that the increased availability of a myelomonocytic target cell population for M-MuLV infection in pristane-treated mice may be a major determinant of disease specificity. Alternatively, the inflammatory response may provide the proper environment for tumor outgrowth.

An important observation of these studies was the consistent finding of proviral integration within the 5′ end of the c-*myb* gene in all six ABMLs and all seven MMLs examined. We have shown here that the M-MuLV had clonally inserted within a 3.0-kbp span of the genomic DNA, either upstream of the UE1 exon, as in MML 2 and MML 9, or upstream of the vE1 *myb* exons, as in the other five MMLs (Fig. 4). Interestingly, S1 mapping studies (Fig. 3) using a *gag-myb* fragment isolated from an ABML 2 cDNA clone as the hybridization probe indicated that, as for all ABMLs, the major form of *myb* transcripts in MMLs is the chimeric viral *gag-myb* species that lacks the three 5′-most c-*myb* coding exons (UE3, UE2, and UE1). Since the open reading frames of the *gag* and *myb* sequences in the chimeric transcripts are out of frame with each other, an internal translational start site within the *gag* p30 sequences is probably used to generate a tumor-specific *myb* gene product (16; Fig. 2). In each tumor we found *myb* transcripts that had the same splice donor sequence within the M-MuLV *gag* sequence joined to the vE1 *myb* exon, thus removing all the 3′ portion of the inserted provirus as well as downstream genomic sequences that were 5′ to the vE1 exons. Even though the UE1 *myb* coding exon that is found in the normal c-*myb* transcript is 3′ to the sites of viral insertion in two of the MMLs (see Fig. 4), namely, MML 2 and MML 9, it is removed from a large portion of the mature tumor *myb* transcripts as intronic sequences (see Fig. 3). Consequently, tumor *myb* transcripts that can encode the same *myb* protein truncated in the N terminus are found in each ABML and MML, even though the sites of integration vary among the different tumors.

An aberrant splicing event towards the 3′ side of the *myb* gene in several ABMLs has recently been reported and was suggested to be induced by M-MuLV insertion, thus playing a crucial role in the activation of the *myb* gene (12). We have also observed the same aberrant splicing event in one of the three clones obtained from an ABML 2 cDNA library (unpublished data). Further studies indicate that this splicing event is not only associated with ABMLs and MMLs (manuscript in preparation). The role of the 3′ splicing event in tumor development is therefore less clear. Our present studies show that a consistent and major determinant in the generation of M-MuLV-induced myelomonocytic tumors is the 5′ alteration of the c-*myb* gene by insertional mutagenesis. This event is greatly facilitated by pristane treatment in adult BALB/c mice. The comparable lengths of latency period in the generation of ABMLs and MMLs in the same animal model system show that the v-*abl* oncogene product is not required for the induction of these tumors.

ACKNOWLEDGMENTS

We thank Herbert C. Morse III for performing flow microfluorometry analyses on the MMLs, Joan Austin for help in the XC plaque assay, and M. Millison for assistance in the manuscript preparation.

LITERATURE CITED

1. **Bishop, J. M., and H. Varmus.** 1985. Functions and origins of retroviral transforming genes, p. 351–356. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), The molecular biology of tumor viruses. Part IV, RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Cook, W. D., D. Metcalf, N. A. Nicola, A. W. Burgess, and F. Walker.** 1985. Malignant transformation of a growth factor dependent myeloid cell line by Abelson virus without evidence of an autocrine mechanism. Cell **41**:677–683.
3. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 4 DNA. Virology **52**:456–467.
4. **Grunwald, D. J., B. Dale, J. Dudley, W. Lamph, B. Sugden, B. Ozanne, and R. Risser.** 1982. Loss of viral gene expression and retention of tumorigenicity by Abelson lymphoma cells. J. Virol. **43**:92–103.
5. **Lavu, S., and E. P. Reddy.** 1986. Structural organization and nucleotide sequence of mouse c-*myb* oncogene: activation in ABPL tumors is due to viral integration in an intron which results in the deletion of the 5′ coding sequences. Nucleic Acids Res. **14**:5309–5320.
6. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y.
7. **Mushinski, J. F., M. Potter, S. R. Bauer, and E. P. Reddy.** 1983. DNA rearrangement and altered RNA expression of the c-*myb* oncogene in mouse plasmacytoid lymphosarcomas. Science **220**:795–798.
8. **Oliff, A., O. Agranorsky, M. D. McKinney, V. V. V. S. Murty, and R. Bauchwitz.** 1985. Friend murine leukemia virus-immortalized myeloid cells are converted into tumorigenic cell lines by Abelson leukemia virus. Proc. Natl. Acad. Sci. USA **82**:3306–3310.
9. **Pierce, J. H., P. P. D. Fiore, S. A. Aaronson, M. Potter, J. Pumphrey, A. Scott, and J. N. Ihle.** 1985. Neoplasic transformation of mast cells by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. Cell **41**:685–693.
10. **Potter, M., M. D. Sklar, and W. P. Rowe.** 1973. Rapid viral induction of plasmacytomas in pristane primed BALB/c mice. Science **182**:592–594.
11. **Potter, M., J. S. Wax, A. O. Anderson, and R. P. Nordan.** 1985. Inhibition of plasmacytoma development in BALB/c mice by indomethacin. J. Exp. Med. **161**:996–1012.
12. **Rosson, D., D. Dugan, and E. P. Reddy.** 1987. Aberrant splicing events that are induced by proviral integration: implications for *myb* oncogene activation. Proc. Natl. Acad. Sci. USA **84**:3171–3175.
13. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
14. **Shen-Ong, G. L. C., E. J. Keath, S. P. Piccoli, and M. D. Cole.** 1982. Novel *myc* oncogene RNA from abortive immunoglobulin gene recombination in mouse plasmacytomas. Cell **31**:443–452.
15. **Shen-Ong, G. L. C., M. Potter, J. F. Mushinski, S. Lavu, and E. P. Reddy.** 1984. Activation of the c-*myb* locus by viral insertional mutagenesis in plasmacytoid lymphosarcomas. Science **226**:1077–1080.
16. **Shen-Ong, G. L. C., H. C. Morse III, M. Potter, and J. F. Mushinski.** 1986. Two modes of c-*myb* activation in virus-induced mouse myeloid tumors. Mol. Cell. Biol. **6**:380–392.
17. **Shen-Ong, G. L. C., K. Holmes, and H. C. Morse III.** 1987. Phorbol ester-induced growth arrest of murine myelomonocytic leukemic cells with virus-disrupted *myb* locus is not accompanied by decreased *myc* and *myb* expression. Proc. Natl. Acad. Sci. USA **84**:199–203.
18. **Shields, A., S. Goff, M. Paskind, G. Otto, and D. Baltimore.** 1979. Structure of the Abelson murine leukemia virus genome. Cell **18**:955–962.
19. **Shoemaker, C. S., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular M-MuLV DNA molecule containing an inverted segment: implication for retrovirus integration. Proc. Natl. Acad. Sci. USA **77**:3932–3936.
20. **Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy.** 1982. Pathogenesis of retrovirus-induced disease, p. 785–998. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.