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Insertion and Truncation of c-*myb* by Murine Leukemia Virus in a Myeloid Cell Line Derived from Cultures of Normal Hematopoietic Cells

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A retroviral insertion into the c-*myb* gene, which resulted in a 3′ truncation, was found in an in vitro-derived myeloid cell line. The retroviral insertion occurred at precisely the same nucleotide at which another murine leukemia virus insertion occurred in an in vivo-induced myeloid leukemia. These findings suggest that comparable events may be required for the derivation of myeloid cell lines in vitro and for induction of myeloid leukemia in vivo.

Among the known oncogenes, the *myb* oncogene is unique in its predominant association with myeloid leukemia. The v-*myb* oncogene was initially identified as the transforming gene of avian myeloblastosis which induces only myeloblastic leukemias (3, 5, 14, 29). v-*myb* is also found in the avian E26 virus which induces myeloid and erythroid disease (17, 19). In mice, rearrangements of the c-*myb* locus have been associated with myeloid leukemia cell lines from a series of tumors induced in BALB/c mice given Abelson murine leukemia virus (MuLV) (16, 26). In addition, a rearranged c-*myb* gene was found in a myeloid leukemia cell line isolated from a primary tumor induced in NFS mice by a wild-mouse ecotropic virus (Cas-Br-M) (31).

Although retroviral insertion and activation of proto-oncogenes is a common mechanism for transformation in vivo, similar events have not been shown to be responsible for transformation of hematopoietic cells in vitro. In the course of experiments examining the effects of transforming retroviruses on the growth and differentiation of primary hematopoietic cells, clonal immature myeloid lines were obtained. One of the cell lines had a retroviral insertion in the c-*myb* gene which was identical to an insertion associated with transformation of myeloid cells in vivo.

Derivation of cell lines from primary cultures. To study the effects of transforming viruses containing the v-*raf* or v-*myc* oncogenes, or both, on the growth and differentiation of hematopoietic cells, we have cultured murine fetal liver cells with interleukin 3 (IL-3) as an obligatory growth factor. Under these conditions, hematopoietic stem cells undergo differentiation during the first several weeks, and the cultures consist of a variety of hematopoietic lineages (10). After this initial phase, the cultures progressively become populated by fully differentiated mast cells which continue to proliferate, although at a slow rate, for several months. In most of the cultures which were infected with the J2 retroviral construct containing the v-*myc* and v-*raf* oncogenes (22), the early phase of growth and differentiation was not noticeably altered, and after several weeks, the cultures contained predominately mast cells. Comparable effects have been observed with Abelson MuLV (18) and Harvey murine sarcoma virus (23). From 3 of approximately 20 experiments, however, three unusual cell lines were obtained. The culture flasks giving rise to the lines were initially identified by a significantly faster growth rate and by the observation, by Giemsa staining, that the cells were not mast cells but rather were lymphoid or immature myeloid cells in morphology. For these reasons, the lines were further characterized.

Initially, the lines were examined by Southern blot analysis with v-*myc* probes for the presence of the retroviral vector. Both the IFLJ2 and VFLJ2 lines contained the construct, whereas the URFL line did not. Restriction enzymes which do not cut in the provirus were used, and the IFLJ2 and VFLJ2 lines were found to contain a single integrated provirus, demonstrating that both lines arose as single clones from the infected cultures. In addition, the lines were examined for the presence of the helper virus, the Leuk-1 strain of Moloney leukemia virus (22), by Southern blot analysis with an *env*-specific probe. The VFLJ2 and URFL lines contained the helper virus, while the IFLJ2 line did not (data not shown).

The lines were subsequently typed for markers which are characteristic of various hematopoietic lineages. Both the VFLJ2 and URFL lines expressed Mac-1, which is characteristic of myeloid lineage cells (20) (Table 1). In addition, both cell lines had receptors for IL-3, although neither line required IL-3 for growth. Previous studies have shown that the expression of IL-3 receptors is restricted to early hematopoietic-lymphoid stem cells or committed myeloid progenitors (17). In addition, the VFLJ2 cell line expressed a myeloid-lineage-specific cell surface determinant detected by the monoclonal antibody RB6-8C5 (R. Coffman, personal communication). In contrast, the IFLJ2 line did not express myeloid-lineage-specific markers and had the morphology of lymphoid cells. Additional typing has shown that the IFLJ2 cell line is of B-cell lineage (data not shown).

For comparison, the properties of three tumor-derived myeloid cell lines are shown in Table 1. The ABPL-2 and ABPL-4 cell lines were isolated from tumors induced in pristane-primed mice inoculated with Abelson MuLV; however, neither cell line contains the transforming virus (26). Although the cells were initially thought to represent pre-B cells, careful examination of the phenotypes has shown that

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TABLE 1. Phenotypic characteristics of in vivo- and in vitro-derived myeloid cell lines

Line[a]	Reference	IL-3[b] dependence	% IL-3[c] receptors expressed	% Cells positive for:			Morphology[d]
				Mac-1[e]	Thy-1	RB6-8C5	
IFLJ2		−	<0.1	<1	<1	ND[f]	Lymphoblastic
VFLJ2		−	6.8	70	<1	70	Myelobastic
URFL		−	7.1	90	<1	ND	Myeloblastic
NFS-60	29	+	7.5	<4	95	10	Myeloblastic
ABPL-2	14	−	6.2	65	<1	69	Myeloblastic
ABPL-4	14	−	6.5	90	<1	87	Myeloblastic

[a] IFLJ2, VFLJ2, and URFL cell lines were derived in the studies described in the text. The origins of NFS-60 cells and ABPL-2 and ABPL-4 cell lines are given in the indicated references. ABPL-2 and ABPL-4 lines were kindly provided by E. Premkumar Reddy.

[b] IL-3 dependence was determined by comparing growth curves for the lines in the presence or absence of homogenous IL-3 and by [^{3}H]thymidine incorporation assays (7).

[c] The expression of receptors was determined by assessing the binding of iodinated IL-3 by previously described procedures (13, 15). The percentage of the input of iodinated IL-3 specifically bound is given.

[d] The cell surface phenotypes were assessed by fluorescence-activated cell sorting. Monoclonal antibodies against Thy-1 and Mac-1 were obtained commercially. The monoclonal antibody RB6-8C5 was kindly provided by Robert Coffman, DNAX, Palo Alto, Calif.

[e] Assessed with Wright-Giemsa-stained cytocentrifuge preparations of the cells.

[f] ND, Not determined.

the cells are of the myeloid lineage (27). The phenotypes of the ABPL-2 and ABPL-4 lines were very similar to that of the VFLJ2 line (Table 1). Also shown in Table 1 are the properties of the NFS-60 cell line which was isolated from a Cas-Br-M MuLV-induced myelogenous leukemia. This line expresses IL-3 receptors and requires IL-3 for growth (31). NFS-60 cells express high levels of Thy-1 and little Mac-1 or the marker detected by RB6-8C5. This phenotype is consistent with an early myeloid progenitor (7, 10, 11).

Rearrangement of the *myb* locus in VFLJ2 cells. Since rearrangements of the c-*myb* locus have been associated with transformation of myeloid cells in vivo and because the phenotypic properties of the VFLJ2 and URFL cells were very similar to those of the ABPL-2 and ABPL-4 lines which have rearrangements of the c-*myb* locus, we examined the lines by Southern blot analysis for possible *myb* gene rearrangements. For these studies we used a 4.2-kilobase (kb) *Eco*RI genomic fragment from the 5′ region of the gene which has been shown to be the site of retroviral integrations in the ABPL tumors. In addition, we used a 0.5-kb *Eco*RI-*Eco*RI (star reaction) genomic fragment containing sequences corresponding to the 3′ region of v-*myb* sequences. This probe detects a normal 1.5-kb genomic *Eco*RI fragment which contains a retroviral integration site in the NFS-60 cell line.

None of the cell lines contained rearrangements in the 5′ region of the c-*myb* gene (Fig. 1). When the 3′ probe was used, the URFL cell line had two *Bam*HI fragments. This pair of fragments is due to a genetic polymorphism in a *Bam*HI site found in the outbred NIH Swiss mice used for the experiments. However, the VFLJ2 cell line contained an apparent 3′ rearrangement which was detectable in both *Bam*HI- and *Eco*RI-digested DNAs. The patterns were consistent with a rearrangement of one of the c-*myb* alleles involving the insertion of sequences within the normal 1.5-kb genomic *Eco*RI fragment comparable to the rearrangement in the NFS-60 cell line.

Expression of a truncated c-*myb* RNA in VFLJ2 cells. In previous studies (31), we have shown that in NFS-60 cells, rearrangement of the *myb* locus causes the truncation of the c-*myb* RNA. We therefore initially compared the c-*myb* transcripts of the VFLJ2 cells with those found in the NFS-60 cell line. The major c-*myb* transcript in VFLJ2 cells was approximately 2.0 kb and comigrated with the truncated c-*myb* transcript found in NFS-60 cells (Fig. 2). For comparison, the RNA from the WEHI-3 myeloid cell line is also shown. This line expresses the normal 4.0-kb *myb* transcript. RNA from the VFLJ2 cells was also similar to that from NFS-60 cells in the absence of the normal 4.0-kb c-*myb* transcript, although both cell lines contain one normal, unrearranged c-*myb* allele. Because of the remarkable similarity of the rearrangements in the NFS-60 and VFLJ2 cells, we cloned the rearranged VFLJ2 c-*myb* *Eco*RI fragment.

Rearrangement due to retroviral integration. A genomic library of *Eco*RI-restricted and size-selected DNA was made in EMBL-3 and screened with the 0.5-kb 3′ *myb* probe by hybridization. Several identical clones were isolated, and the insert from one was subcloned into pUC12 for further characterization. The restriction map of the clone is shown in Fig. 3. By comparison of restriction sites and by hybridization with Moloney leukemia virus long terminal repeat (LTR) and *env*-specific probes, the rearrangement was found to be due to the insertion of a retrovirus into the 1.5-kb genomic *Eco*RI c-*myb* fragment in a manner similar to that previously found in NFS-60 cells. The integrated provirus

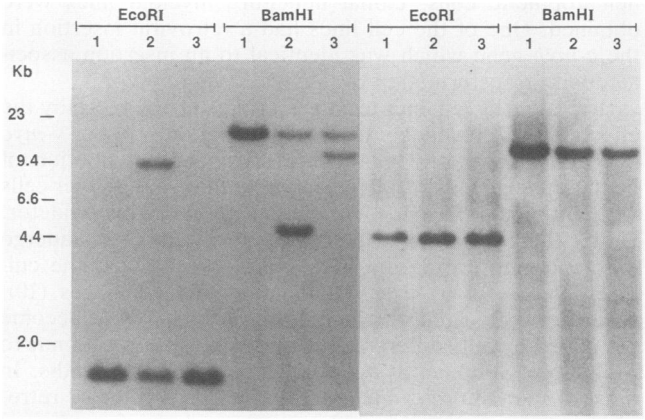

FIG. 1. Detection of rearrangements of the c-*myb* locus by Southern blots of DNA from myeloid cell lines. DNA was purified from the indicated cell lines and digested with *Eco*RI or *Bam*HI. The DNAs were subjected to electrophoresis in 0.8% agarose gels, transferred to nitrocellulose (28), and hybridized with the 0.5-kb 3′ *myb* fragment (left set of lanes) or the 4-kb 5′ *myb* fragment (right set of lanes) labeled with ^{32}P by nick translation. The migrations of *Hin*dIII-digested λ DNA are used as size markers, and the sizes are given in kilobases. Lanes 1, 2, and 3 contain DNA from IFLJ2, VFLJ2, and URFL cell lines, respectively.

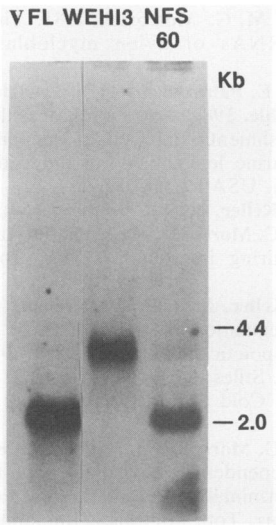

FIG. 2. Expression of a truncated c-*myb* RNA in VFLJ2 cell line. Poly(A)$^+$ RNA was purified from the indicated cell lines, and 10 μg of each sample of RNA was denatured with glyoxal, subjected to electrophoresis in 1% agarose gels, and transferred to nitrocellulose (28). The filters were hybridized with the 0.5-kb c-*myb* probe labeled with ^{32}P by nick translation. For comparison, the results obtained with the NFS-60 and WEHI-3 cell lines are shown. The sizes are given in kilobases.

contained the restriction sites characteristic of the Leuk-1 strain of Moloney leukemia virus which was used as the helper virus in the experiments. Unlike the integration of a complete Cas-Br-M MuLV in NFS-60 cells, the integrated provirus in the VFLJ2 cells contained a deletion of approximately 1.0 kb in the *pol* gene region.

Identical integration sites in VFLJ2 and NFS-60 cells. To further characterize the rearrangement, the proviral *myb* junction regions were subcloned and sequenced. The sequences are shown in Fig. 3 and compared with the sequences of the junctions in NFS-60 cells. As shown, the 5′ and 3′ junctions demonstrate that the provirus had integrated at precisely the same nucleotide position in the c-*myb* locus as in NFS-60 cells. Importantly, the integration also contained the same four-base direct repeat that was found in NFS-60 cells. Like the Cas-Br-M MuLV insertion, the Leuk-1 Moloney leukemia virus proviral LTR sequences begin with TGA, a sequence which is introduced in frame relative to the c-*myb* coding sequences and is predicted to cause termination of protein synthesis. Consistent with the restriction mapping, the sequence of approximately 300 bases of the LTR showed only 3 base differences relative to the sequences of the Leuk-1 MuLV LTR (U. R. Rapp, unpublished data) compared with 38 base differences relative to the Cas-Br-M MuLV LTR sequence.

Conclusions. The establishment of the URFL and VFLJ2 myeloid cell lines constituted rare, clonal events, although during the initial culture period, a high percentage of cells were infected and replicated the retroviral construct expressing both the v-*raf* and v-*myc* oncogenes (data not shown). Therefore, neither of these oncogenes alone nor both together are sufficient for the establishment of this type of myeloid cell line. Moreover, whether the J2 virus played any role in the establishment of these myeloid cell lines is not clear. In particular, the lack of the construct in the URFL line would suggest that the J2 virus was not necessary, at least in this case, in any direct manner. A possible indirect role for the J2 virus in the derivation of such lines can only be suggested by the observation that similar lines have not been obtained in comparable experiments with other transforming viruses.

The phenotypic properties of the VFLJ2 line and the URFL line are similar to those of cell lines derived from primary retrovirus-induced myeloid tumors and suggest that transformation of these cells has involved an alteration in their ability to terminally differentiate. In the NFS-60 and ABPL cell lines, this phenotype has been attributed to the rearrangement of the c-*myb* gene based on the properties of avian myeloblastosis-transformed myeloid cells, specifically the inability of the cells to terminally differentiate (1). The similarity of properties of the URFL and VFLJ2 lines would suggest that other proto-oncogenes may cause a comparable phenotype of transformation. This possibility is further indicated by the existence of a number of cell lines, derived from retrovirus-induced myeloid leukemias, which have comparable phenotypes (6, 9) and do not contain rearrangements of the c-*myb* locus.

In the VFLJ2 line, which expresses the v-*raf* and v-*myc* oncogenes, it can be speculated that one or both of the oncogenes contribute to the properties of the cell line. In particular, we have previously shown that v-*myc* but not v-*raf* abrogates the requirement of myeloid cell lines for IL-3 (21). Moreover, the continued requirement of the NFS-60 cells for IL-3 demonstrates that the rearrangement of the c-*myb* locus does not confer factor independence. Thus, it is conceivable that in the VFLJ2 line, the v-*myc* gene is responsible for the factor independence of the cells. In addition, we have shown that v-*raf*, like v-Ha-*ras*, immortalizes mast cells for growth in vitro (8). Thus, it is conceivable that v-*raf* performs a similar function in VFLJ2 cells.

The rearrangement in the VFLJ2 line represents a second

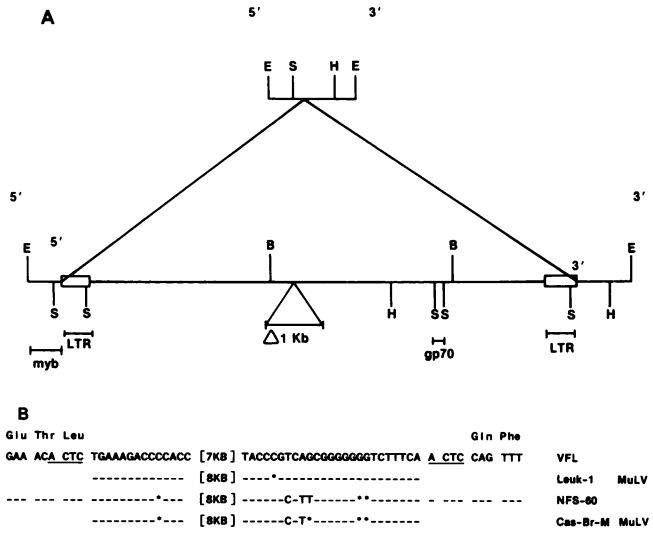

FIG. 3. (A) Restriction enzyme map of the normal and rearranged *Eco*RI fragments from the VFL cell line. The restriction map for the normal 1.4-kb fragment (upper) (4) is compared with that for the rearranged 10-kb fragment (lower) and was determined by standard double digests with the indicated enzyme and by hybridization of the fragments to specific LTR and gp70 probes. E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; B, *Bam*HI. (B) Comparison of sequences of rearranged *myb* in the VFLJ2 and NFS-60 (5) cell lines. The junction areas were subcloned into pUC12 and sequenced by the Maxam-Gilbert technique (12). Underscored letters indicate a four-base repeat at the site of integration. *, Deletion.

example of a 3′ rearrangement and truncation of the c-*myb* locus. These rearrangements contrast with the 5′ retroviral integrations that have been shown to occur in the ABPL tumors (16, 26). Although this finding initially suggested that two mechanisms exist for the activation of the c-*myb* locus, more recent studies suggest that the 3′ and 5′ integrations may share the deletion or alteration of the 3′ region of the c-*myb* locus. In particular, recent studies (D. Rosson and E. P. Reddy, personal communication) have demonstrated that the 5′ integrations affect splicing and alter the structure of the c-*myb* transcript by introducing intron sequences in the same region as the 3′ truncations.

Perhaps the most striking feature of the integration in the VFLJ2 line is that it occurred at precisely the same nucleotide in the *myb* gene as in the NFS-60 line. Whether this identity was fortuitous or whether it occurred because of the limited sites available for activation of the *myb* locus or a sequence specificity or both is not known. It should be noted, however, that in a recent study (25), it was shown that among seven integrations of Moloney leukemia virus in the *pim*-1 gene, two had apparently occurred at the same site. However, there was no apparent sequence homology between this site and the site of integration in the *myb* gene. Nor have we been able to find a similar site of integration in other published sequences of retroviral insertions. Examination of more rearrangements of the c-*myb* locus should help to better define the requirements and possible sequence specificities.

While integration and activation of cellular proto-oncogenes by retroviruses has been shown to be a common mechanism in the induction of leukemia in vivo, we are aware of only one other example of the in vitro activation of a proto-oncogene by a retroviral integration. In this case, a Friend MuLV provirus integrated 5′ of the first coding exon of the c-K-*ras* gene, resulting in a 25- to 30-fold increase in the expression of Ki-*ras* p21 (4, 24) in a cell line derived from Friend MuLV infection of long-term bone culture (2). In our experience, the establishment of myeloid cell lines from fetal liver cultures is an extremely rare event and possibly requires transformation. Our results demonstrate that this transformation may involve the activation of proto-oncogenes that are also associated with transformation of myeloid cells in vivo.

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