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Early Clonality and High-Frequency Proviral Integration into the c-*myc* Locus in AKR Leukemias

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Blot hybridization of thymocyte DNA from AKR/J mice was used to detect new proviral junction fragments as markers of clonality at different stages of viral leukemogenesis and to detect DNA rearrangements at the c-*myc* locus due to proviral insertion. Clonal populations of thymocytes were observed in mink cell focus-forming virus-injected mice as early as 35 days postinjection, at a stage distinguishable from frank leukemia by flow cytometric analysis and transplantation bioassay. Specific proviral integrations in the c-*myc* locus were detected in 15% of these early clones and in up to 65% of late-developing thymomas and frank leukemias. Thus, in this system c-*myc* activation appears to be a common mechanism in T-cell leukemogenesis.

Leukemogenesis in AKR mice appears to be initiated by infection of thymocytes with retroviruses which are genetically recombinant in the viral *env* gene and long terminal repeat sequences termed mink cell focus (MCF)-inducing viruses (for a review, see reference 5). The resulting thymomas are clonal or oligoclonal in origin (2, 7, 11). However, it is not known when clonal populations of proliferating cells first emerge during the preleukemic period in AKR mice. We explored this question in a system in which leukemia was induced synchronously by intrathymic inoculation of *env* recombinant virus in young AKR mice. This system permits flow-cytometric detection of early phenotypic alterations in thymocytes before overt thymoma or disseminated leukemia occurs (10). By analysis of new proviral-host junction fragments in thymus DNA blots, we found that the earliest phenotypically altered population represents outgrowth of one or a very few clones of cells. Specific proviral integrations into the c-*myc* locus were detected in some of these early clones. In later-developing thymomas and frank leukemias, up to 65% of the neoplasms exhibited clonal proviral alterations of c-*myc*. Thus it appears that, as with retrovirus-induced B-cell lymphomas of chickens, virus-induced T-cell leukemia in mice is associated with a high frequency of proviral insertion into the c-*myc* locus.

Recently, we described several stages of leukemogenesis that follow intrathymic injection of MCF 69L1 virus in young adult AKR/J mice (10). Stage I represented uniform virus infection of thymocytes without apparent changes in light-scatter properties of the cells or in the expression of several differentiation alloantigens on the major thymocyte subpopulations. Stage II was observed as early as 35 days postinjection and was distinguished by the presence of a subpopulation of cells with restricted transplantation properties which could be resolved from normal thymocytes by flow cytometry. Stage III was observed when considerable enlargement of the thymus had occured and represented the outgrowth of fully transformed cells that replaced the normal thymocyte subpopulations. Mice at this stage did not yet display signs of frank leukemia, i.e., ruffled fur, hunched appearance with chest enlargement, labored breathing, or lymph node enlargement.

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Figure 1 shows Southern blots (15) of thymus DNA from virus-injected AKR/J mice at the different stages of leukemogenesis hybridized with the pAKV5 probe, representing a sequence present in the p15(E) region of the *env* gene in MCF 69L1 virus, as well as in endogenous ecotropic murine leukemia virus (7). Staging was determined for each mouse by flow cytometry as described previously (10). In Fig. 1A and B the restriction endonucleases *Bam*HI and *Eco*RI, respectively, were used to cleave MCF 69L1 proviral DNA once in the *env* gene. Thus, a single junction fragment was observed for each new proviral integration. In each blot the germ line proviral junction fragments are indicated by arrows. It is apparent in Fig. 1A that new proviral junction fragments were seen first at stage II of leukemogenesis. The intensity of the new proviral fragments was lower than those of the germ line ecotropic proviruses of AKR/J mice, consistent with their being derived from the DNA of a minority population of cells which was emerging in thymus at this time. Mixing experiments have shown that such Southern blots can detect a clonal population present at a frequency of at least 5% under the conditions used in these experiments (data not shown). The intensity of new proviral fragments increased with thymus weight from mice at stage II (Fig. 1A), indicative of an increasing relative proportion of a new clonal population of cells. Densitometer tracings of stage II DNA blots suggested some degree of oligoclonality at this stage, as assessed by the differing intensities of new proviral fragments. For example, in lane 2 of Fig. 1A there are seven new junction fragments of equal intensity, consistent with a single clone, while in lane 3 there are six new junction fragments (three major and three minor) with relative intensities of 2.5:1, consistent with two emerging clones. We also observed such apparent oligoclonality frequently at stage III and in frankly leukemic mice (Fig. 1B), so it is not characteristic only of stage II. To verify that the DNAs of cells at stage I which appeared to be phenotypically normal contained newly integrated MCF 69L1 proviruses, we digested the DNAs with *Sma*I, which released an internal viral fragment of 2.2 kilobases (kb) from MCF 69L1 proviral DNA that was detected by the pAKV5 probe. DNAs from cells at both stage I and stage II contained the 2.2-kb internal fragment in the same relative intensity, confirming similar total proviral copy numbers in the populations of infected cells (Fig. 1C). A minor band was observed at 1.8 kb, which

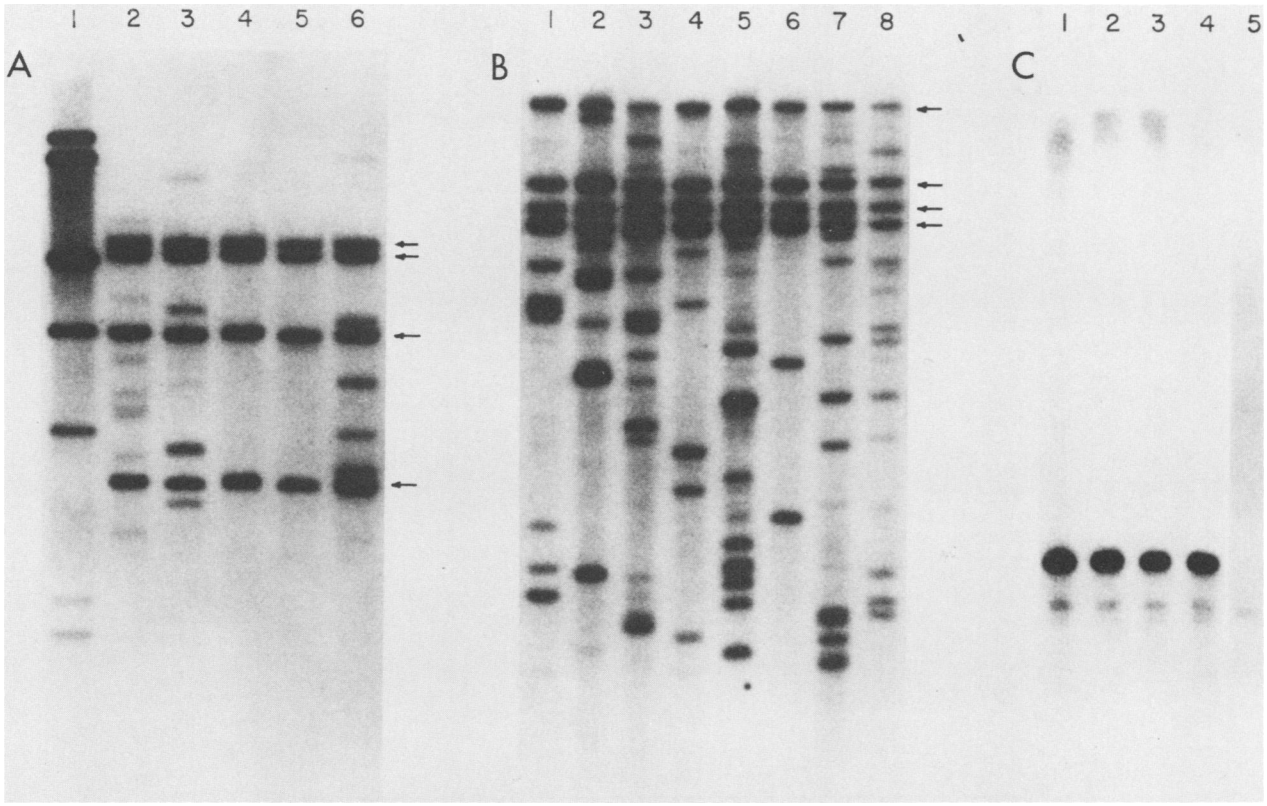

FIG. 1. Southen blotting of total thymus DNA from control and MCF 69L1 virus-injected AKR/J mice. Purified cellular DNA (15 μg) (6) was digested with the appropriate restriction endonuclease, electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized to a nick-translated (12) pAKV5 probe. (A) Digestion with *Bam*HI. Lane 1, DNA marker undigested (48 kb) and digested with *Hin*dIII which generated fragments of 23, 9.4, 6.7, 2.3, and 2.0 kb; lanes 2, 3, and 6, DNAs from mice at stage II with thymus weights of 118, 150, and 190 mg, respectively; lanes 4 and 5, DNAs from mice at stage I with thymus weights of 77 and 41 mg, respectively. Thymus weights of control AKR/J mice were 100 ± 32 mg. (B) Digestion with *Eco*RI. Lanes 1 and 8, DNAs from frankly leukemic mice with thymoma weights of 748 and 922 mg, respectively; lane 4, DNA from a mouse at stage II with a thymus weight of 225 mg; lanes 2, 3, 5, 6, and 7, DNAs from mice at stage III with thymus weights of 349, 234, 411, 376, and 398 mg, respectively. Flow cytometric analysis of thymocytes whose DNA is represented in lanes 4 and 5 was published previously (10). (C) Digestion with *Sma*I. Lane 1, DNA from a mouse at stage II also represented in panel A, lane 2; lanes 2 to 4, DNAs from mice at stage I (lanes 3 and 4 correspond to mice represented in lanes 4 and 5, respectively, in panel A); lane 5, DNA from a control AKR/J mouse injected with tissue culture medium.

is seen also in DNA of control thymus and is derived from replicating germ line ecotropic proviruses in AKR/J mice (1, 2).

Having shown the presence of clonal populations of cells at an early stage of leukemogenesis, we wanted to determine whether cells at this stage, as well as from frank thymomas, show evidence of DNA rearrangements at the c-*myc* locus which have been associated with some virus-induced leukemias in rodents (3, 8, 13, 17). DNAs from thymuses at various stages were digested with *Kpn*I and *Eco*RI, which cut at sites bracketing the c-*myc* coding region, and probed with pα25BH3.4, which recognizes one half of intron 1, exon 2, intron 2, and exon 3 (9). *Kpn*I blots of unselected DNA samples are shown in Fig. 2A, and the overall results are summarized in Table 1. No rearrangements of the germ line *Kpn*I fragment of 10.5 kb were observed at stage I, as expected because no clonality is evident at this stage. At stage II c-*myc* rearrangements were observed at a frequency of 15%; in two of three of these cases there were two different rearranged c-*myc* fragments which differed in intensity, confirming the frequent occurrence of oligoclonality. When DNAs were examined from mice at stage III or from frankly leukemic mice, a high frequency (up to 65%) of c-*myc* rearrangements was observed. Again, evidence for oligoclonality of c-*myc* rearrangements was observed in many cases (Fig. 2A, lanes 3, 5, and 8). Sites and orientation of proviral insertion were determined to a first approximation from the sizes of the rearranged *Kpn*I and *Eco*RI fragments and a knowledge of the restriction maps of the c-*myc* locus (13, 16) and of MCF 69L1 virus (S. Chattopadhyay and P. V. O'Donnell, unpublished data). Results

TABLE 1. DNA rearrangements at the c-*myc* locus[a]

Stage of leukemogenesis[b]	No. of rearrangements[c]		Frequency[d] (%)
	1	2	
I	0/17		0/17 (0)
II	1/20	2/20	3/20 (15)
III	7/12		7/12 (53)
Frank leukemia	28/48	3/48	31/48 (65)

[a] Novel *Kpn*I and *Eco*RI restriction fragments recognized in Southern blots of thymus DNAs by the pα25BH3.4 probe of the c-*myc* gene (9).

[b] Determined by flow cytometric analysis of thymocytes and by gross pathology (10).

[c] Number of new c-*myc*-containing restriction fragments per thymus DNA in addition to the germ line fragment.

[d] Number of DNAs showing rearrangements/total DNAs analyzed.

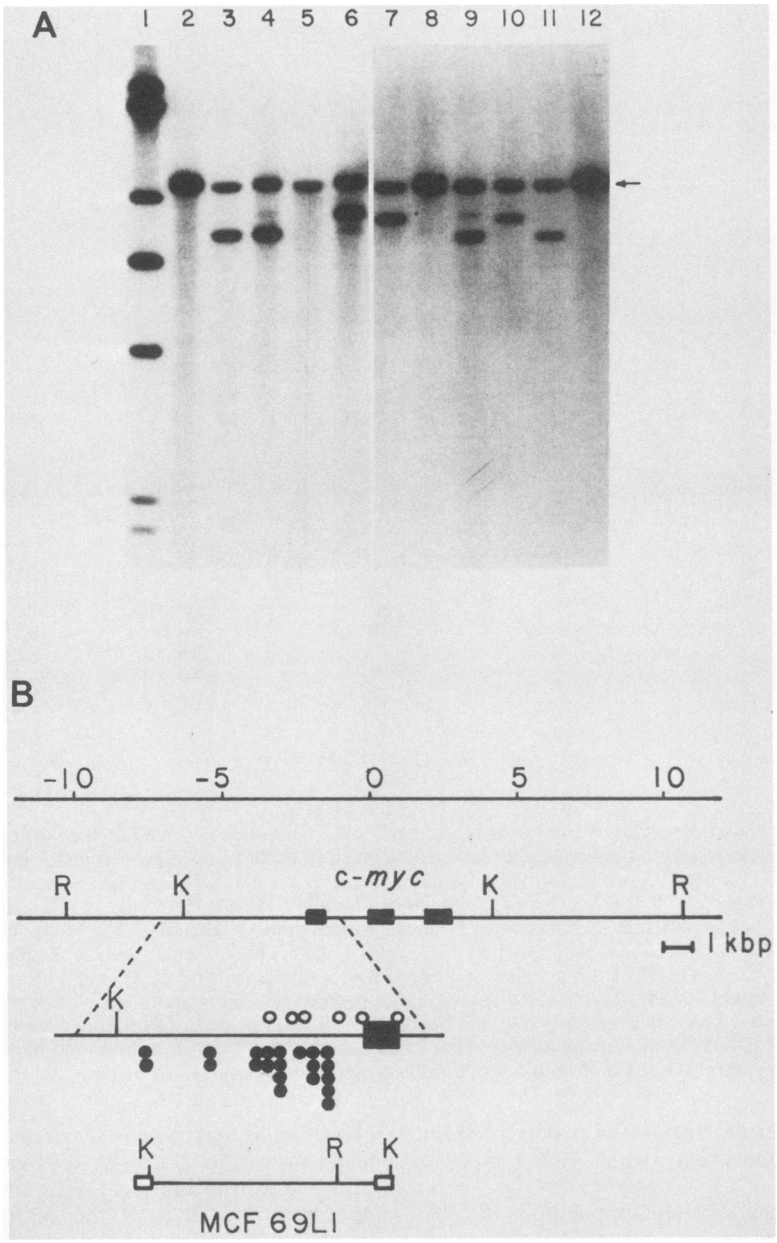

FIG. 2. DNA rearrangements at the c-*myc* locus in AKR/J mice injected with MCF 69L1 virus. (A) Southern blotting of *Kpn*I-digested thymus DNAs from frankly leukemic mice. Digested thymoma DNA (15 μg) was electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized to a nick-translated pα25BH3.4 probe (9). The germ line *Kpn*I fragment of 10.5 kb is indicated by the arrow. Lane 1, λ*Hin*dIII marker; lanes 2 to 12, unselected thymoma DNAs. (B) Restriction map of the c-*myc* locus and of MCF 69L1 proviral DNA showing approximate locations and transcriptional orientation of integrated MCF 69L1 proviruses which gave rise to the c-*myc* DNA rearrangements analyzed in this study. For purposes of illustration the region from the 5′-*Kpn*I site through exon 1 of the c-*myc* gene has been expanded twofold. Open symbols represent a transcriptional orientation of the provirus colinear with the c-myc gene, and closed symbols denote the reverse transcriptional orientation.

of this analysis are shown in Fig. 2B. Of 27 c-*myc* rearrangements analyzed, 25 appeared to result from proviral insertions upstream of exon 1 at a median distance of 1.2 kb, and 2 insertions occurred in or very close to exon 1. With respect to proviral orientation, 6 of the 27 appeared to be integrated in the same transcriptional orientation as the c-*myc* gene, and 21 were in the opposite orientation. The clustering of integration sites seen in our study and also observed by other workers (3, 8, 13) may reflect structural features of the c-*myc* transcriptional promoter including, possibly, the effects of a more distal negative regulatory site in the region upstream of the gene (14).

How can we account for the discrepancy in the frequency of c-*myc* rearrangements between stages II and III of leukemogenesis? We have already presented evidence that the transition from stage II to stage III represents progression to a more fully transformed phenotype (10). The finding of a higher frequency of c-*myc* rearrangements in fully transformed cells suggests that c-*myc* activation occurs both early and late in leukemogenesis by the same mechanism of

proviral insertion. Clones of proliferating cells at stage II not showing c-*myc* rearrangements most likely express other as yet uncharacterized genes which are activated to produce the observed phenotype. Some of these clones may require additional genetic changes, e.g., c-*myc* alteration, to become fully leukemic, thus accounting for the higher frequency of c-*myc* rearrangements found at late stages of the disease. Conversely, stage II clones which show c-*myc* rearrangements could require activation of other oncogenes for tumor progression to occur.

Other laboratories (3, 8, 13, 17) have reported a wide range of frequencies for murine leukemia virus integration into the c-*myc* region, from 15% in rat leukemias (induced by Moloney leukemia virus) to 45% in mice (representing mainly Moloney virus-induced leukemias in BALB/c mice). In AKR mice three laboratories have reported 15 to 25% frequencies of c-*myc* alteration in MCF 247 virus-accelerated leukemias and in spontaneous leukemias (3, 8, 10), similar to the stage II frequency observed in our study. We interpret these quantitative differences as reflecting a delicate balance between the rate of viral proliferation in the thymus and the selective potential of activating particular oncogenes. Important factors may be route of virus inoculation or effects which are specific to virus strain or mouse strain. It is interesting to note in this regard that Cuypers et al. (4) found a high frequency (65%) of proviral integrations at the *pim-1* locus in Moloney virus-induced disease in BALB/c mice, whereas we observed only five *pim-1* rearrangements in DNAs from 42 frank leukemias induced in AKR/J mice by MCF 69L1 virus.

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