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A Common Site for Immortalizing Proviral Integrations in Friend Erythroleukemia: Molecular Cloning and Characterization

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By using a tagged derivative of Friend spleen focus-forming virus, we previously obtained evidence that proviral integration(s) in the host genome can cause erythroblast immortality by abrogating the commitment of the cell to differentiate (C. Spiro, B. Gliniak, and D. Kabat, J. Virol. 62:4129–4135, 1988). Exploiting the fact that each leukemia was a single clone that contained one tagged provirus, we have now molecularly cloned and characterized one common genomic site for immortalizing proviral integrations.

Friend virus causes progressive erythroleukemia in susceptible mice (12, 14, 22, 25; D. Kabat, Curr. Top. Microbiol. Immunol., in press). Within 1 to 2 days, the spleens become engorged with numerous clones of proliferating erythroblasts that have only limited self-renewal capabilities (8, 21, 22, 24–26). After their proliferative potential is exhausted, these erythroblasts express their commitment to terminally differentiate (8, 24; Kabat, in press). Subsequently, between 3 to 8 weeks postinfection, immortal cells can be detected (12, 24–26). These leukemia cells have lost their commitment to differentiate, and they can be transplanted indefinitely in secondary recipients.

Several genetic factors have been implicated in leukemic progression. Although Friend virus is a mixture of a replication-defective spleen focus-forming virus (SFFV) and a replication-competent murine leukemia virus (MuLV), helper-free SFFV can occasionally induce transplantable erythroleukemias (24, 27, 28). In addition, advanced erythroleukemias caused by the viral complex usually have many and SFFV proviruses integrated into the cellular DNA (16–18). Often, these integrations occur in a common host genomic site, Spi-1 (18), and in the p53 proto-oncogene (4, 5, 9, 19). Additional important sites of integration might also occur in these leukemias. Advanced erythroleukemias also frequently have amplified copies of the c-*myc* proto-oncogene (B. Gliniak and D. Kabat, unpublished results) and various chromosomal rearrangements (15, 20). Because these changes in the genome of the cell can occur together, their individual roles in leukemogenesis have remained obscure. The clonal aspects of progression have also been difficult to analyze because SFFV contains only murine leukemia virus-related nucleic acid sequences that are present in many copies in the normal mouse genome (3, 16–18).

Recently, we described a method to address these issues (24). We modified a Lilly-Steeves polycythemia strain of SFFV by inserting a small tag of simian virus 40 DNA into its nonfunctional *pol* gene region. This SFFV clone was transfected into Psi-2 (13) retrovirus-packaging cells, which then released tagged helper-free SFFV virions. This tagged virus caused two types of disease in NIH Swiss mice. Most mice developed a transient mild polycythemia associated with polyclonal proliferation and subsequent differentiation of splenic erythroblasts. However, 20 to 30% of the mice developed severe anemias by 26 to 33 days postinfection and had large transplantable helper-free erythroleukemias in their spleens. Southern blot (hybridization) analyses indicated that these leukemias were all single clones with one tagged provirus per cell and that these proviruses were in a few tightly clustered sites in the host genome. Because these clonal erythroblast immortalization events were tightly associated with single proviral integrations at specific chromosomal sites, we proposed that these integrations were responsible for abrogating the commitment to differentiate and for the immortalization. We now describe one common site for SFFV proviral integrations in these immortalized erythroleukemias.

Genomic libraries were constructed in the EMBL-3 bacteriophage lambda vector by standard methods (7; Promega Biotec, Madison, Wis.) with DNAs prepared from leukemic spleen 33.1 (J) (24) and from erythroleukemia cell line IP/IR (24a). For the IP/IR library, two plaques that hybridized with simian virus 40 DNA were isolated from approximately 3×10^6 phage. For the J (33.1) library, one positive was isolated from approximately 4×10^6 phage. Restriction maps of the SFFV-host DNA junction fragment clones are presented in Fig. 1C.

Single-copy probes A, B, and C were prepared from these junction fragment clones (Fig. 1C). Probe B hybridized to both clones. These probes were then used to isolate additional host genomic clones from the integration site region. An extended map showing 30 kilobases of the region is shown in Fig. 1A; the maps of two overlapping genomic clones are shown in Fig. 1B.

By Southern blotting (23, 24), we then analyzed independently isolated Friend erythroleukemias to determine whether they also contained proviruses in this region. These leukemias included nine produced with our tagged helper-free SFFV, six (cell lines M-1, M-4, M-5, M-7, M-12, and M-13) that were produced with an untagged helper-free SFFV (28), and four other cell lines (FVT/A and FII [1], F745 [6], and F4N [20]) that contained both SFFV and MuLV proviruses. A typical Southern blot of *Kpn*I-digested genomic DNAs that was hybridized with probe B is shown in Fig. 2A; clearly, many of these samples contain rearranged loci, as indicated by extra DNA fragments (i.e., lanes 3 through 8, 12, and 13), in contrast to the single 12.5-kilobase fragment observed in the *Kpn*I digest of normal mouse DNA (lanes 2 and 14). By using DNAs digested with different restriction endonucleases (*Eco*RI, *Kpn*I, and *Bam*HI) and different single-copy probes, we were able to determine positions and orientations of proviruses in this gene region. Typical data are shown in Fig. 2. This information is

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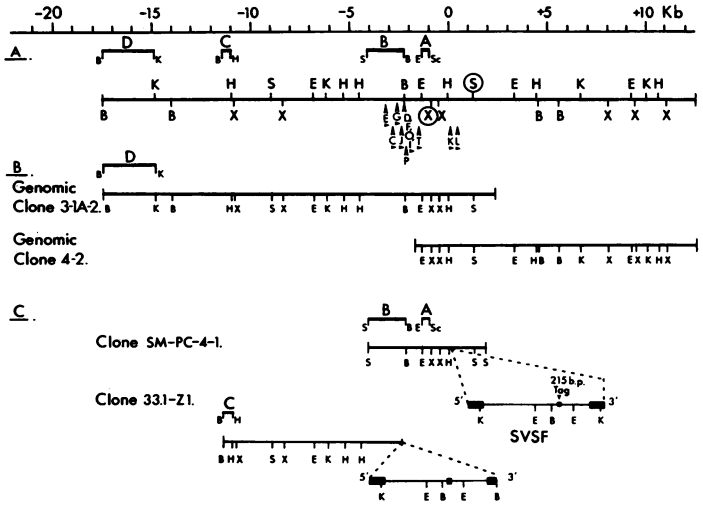

FIG. 1. (A) Map of the unrearranged imi-1 genomic site showing location and orientation of proviral integrations. Position zero is arbitrarily defined as the site of a tagged SFFV proviral integration in erythroleukemia cell line IP/IR (represented as integration K beneath the genomic map). The cleavage sites for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Sal*I (S), *Xba*I (X), and *Sac*I (Sc) are indicated. Circled restriction sites identify some of the differences between the imi-1 and Spi-1 (21) maps. ▲, Sites of proviral integration in erythroleukemias examined; ►, orientation of provirus 5′ to 3′. Erythroleukemias C, D, E, F, G, I, and J, which have tagged SFFV proviral integrations, are labeled as previously described (27). Proviral integrations in additional erythroleukemias are labeled as follows: L, FVT/A cells and FII cells; O, MI cells; P, M4 cells; T, M13 cells. Orientation of provirus P is not assigned because this DNA did not cut in one *Eco*RI digestion. Fragments A through D (indicated by thick underline) were subcloned into the vector pGEM-4 (Promega Biotec) and used as probes for our DNA and RNA blots. Kb, Kilobases. (B) Maps of overlapping genomic clones 3-1A-2 and 4-2. Abbreviations are described in the legend for map A. (C) Maps of clones SM-PC-4-1 and 33.1-Z1, including tagged SFFV (called here SVSF) proviral sequences present in each. Clones SM-PC-4-1 and 33.1-Z1 were obtained by hybridizing the 215-base-pair simian virus 40 probe to genomic libraries made with DNA from leukemias K and J, respectively. Single-copy probes A, B, and C were derived from fragments of these clones as indicated. Abbreviations are described in the legend for map A.

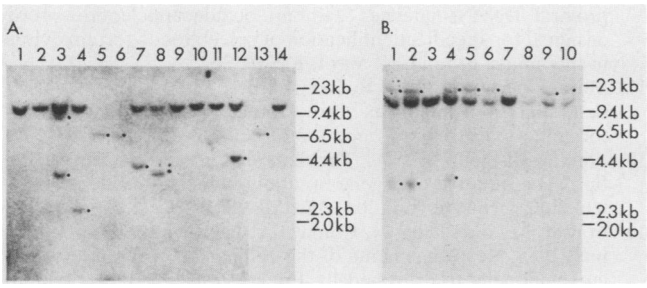

FIG. 2. Southern blot analyses of proviral integrations in the imi-1 genomic site. High-molecular-weight DNAs from SFFV-infected erythroleukemic spleens and from normal spleens were digested with *Kpn*I (A) or *Bam*HI (B). Blots were probed with the 1,900-base-pair probe B described in the legend to Fig. 1A. Leukemia DNAs described in the legend to Fig. 1A and by Spiro et al. (27) are identified here with the same nomenclature. (A) DNAs were as follows. Lanes: 1, F745 cells; 2, uninfected spleens; 3, tagged helper-free spleen C; 4, F-4N cells; 5, FVT/A cells; 6, FII cells; 7, MI cells; 8, M4 cells; 9, M5 cells; 10, M7 cells; 11, M12 cells; 12, M13 cells; 13, IP/IR cells; 14, uninfected spleens. Lanes 13 and 14 were from a reprobing of the same blot. This was done because these lanes were obscured by radioactive contamination in the original autoradiogram. (B) DNAs were as follows. Lanes: 1, tagged helper-free spleen C; 2, tagged helper-free spleen G; 3, tagged helper-free spleen H; 4, tagged helper-free spleen E; 5, tagged helper-free spleen F; 6, tagged helper-free spleen J; 7, uninfected spleen; 8, IP/IR cells; 9, tagged helper-free spleen I; 10, tagged helper-free spleen D. To ensure identification of faint bands that were clearly seen in the original autoradiograms, black dots were placed to the right of any such band.

summarized in Fig. 1A. Of the 18 leukemias studied, 12 contained assignable proviral integrations in this host genomic region. One additional leukemia that contained both MuLV and SFFV proviruses (F4N) also had a rearrangement (Fig. 2A, lane 4). However, this rearranged locus had an abnormal restriction map, and we were therefore unable to unambiguously locate and orient its provirus.

Eight of nine helper-free leukemias with tagged SFFVs contained tightly clustered integrations. Previously, we obtained evidence that the SFFV proviruses in leukemias C, G, and E and in D, F, and J were in tightly clustered groups (24). This confirms our earlier interpretations and also establishes close linkage of these groups. The C, G, and E group splits the B probe region, whereas the D, F, and J group occurs closer to its edge (compare lanes 1, 2, and 4 with lanes 5, 6, and 10, respectively, of Fig. 2B).

A significant proportion (ca. 5/18) of independently derived immortalized leukemias lack proviruses in this region. Southern blot analysis with a 900-base-pair *Bgl*II-*Pst*I fragment from the 27.1a molecular clone of p53 (11) suggested that the proviruses in these leukemias were not in the p53 oncogene (results not shown). Erythroleukemia cell lines with or without integrations in this common region differentiated similarly in response to dimethyl sulfoxide.

We infer that SFFV proviral integrations into one allele at this common site can immortalize erythroblasts. Thus, many immortalized leukemias contain both rearranged and normal alleles (Fig. 2). Nevertheless, loss of the normal allele had occurred in the erythroleukemia cell lines IP/IR, FVT/A, and FII (Fig. 2A, lanes 5, 6, and 13). Since FVT/A is a derivative of FII (1), the normal allele must have been lost before their divergence. A similar loss could have occurred in the splenic

leukemias, but it would have been masked by the presence of uninfected spleen cells (24a). The substantial frequency of this loss suggests that it might contribute to leukemic progression. According to this idea, the normal allele would act as a tumor suppressor gene whereas the allele with an integrated provirus would act as a dominant oncogene. Precedence for conversion of a tumor suppressor gene into an oncogene occurs with the p53 proto-oncogene (2, 10, 19).

This common integration site appears to be identical to the recently described Spi-1 site (18). First, the restriction map in Fig. 1 is similar but not identical to that of Spi-1. The circled *Xba*I site in Fig. 1A is absent in the Spi-1 map. Also, we find no other *Sal*I sites at the right of the circled *Sal*I site in Fig. 1A, whereas Spi-1 has two additional *Sal*I sites in this region. Our genomic clones also contain numerous *Sac*I sites, in contrast to the single *Sac*I site reported in Spi-1, and we also detected several additional *Pst*I and *Pvu*II sites that were absent from the Spi-1 map (data not shown). Because our clones and Spi-1 were derived from NIH Swiss and ICFW mice, respectively, these discrepancies could be due to strain differences. Our map also extends the published Spi-1 map. Second, S. Ruscetti had sent DNA from six helper-free erythroleukemias (M-1, M-4, M-5, M-7, M-12, and M-13) to F. Moreau-Gachelin and co-workers and had been informed which three of these contained Spi-1 integrations. In a blind study, we determined that the same three DNAs contained integrations in the region we had cloned. Finally, after all other aspects of this work were completed, we received single-copy Spi-1 probes and then confirmed the identity of these loci.

Although our data therefore generally support the conclusions of Moreau-Gachelin et al. (18), our results also differ significantly. Important differences include the restriction maps, the use of different leukemias, and our evidence for loss of the normal allele during progression. We have also found that probe D (corresponding to their probe C [18]) is not single copy. Consequently, the reported 4.0-kilobase RNA (18) appears not to be the true transcript of this gene. We are currently analyzing an abundant 1.5-kilobase poly(A)-containing RNA that is transcribed from the region adjacent to this probe. Most importantly, our approach with helper-free SFFV suggests that single integrations in Spi-1 contribute to immortalization of erythroblasts and that additional proviruses are unnecessary for leukemic progression.

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