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CHARACTERIZATION AND CLONING OF THE KIRSTEN MURINE LEUKEMIA VIRUS GENOME

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We have characterized the intracellular and circular unintegrated proviral DNA species of Kirsten murine leukemia virus by restriction mapping using the Southern blotting technique. These studies show the 8.5 kilobase pair genome to possess long terminal repeats (0.5 kilobase pairs in length) which are indistinguishable from those of the derivative Kirsten murine sarcoma virus. In addition, we have identified a 3'-located region in Kirsten murine leukemia virus which is very similar to the putative leukemogenic region of Gross murine leukemia virus. We also report the cloning of the leukemia virus genome using DNA obtained from the endogenous reverse transcriptase reaction of detergent disrupted virions.

The initial intracellular event in the replication cycle of a retrovirus is the reverse transcription of the RNA genome of the virus into a linear DNA copy (the provirus). This DNA copy enters the nucleus and circularizes before becoming covalently integrated into the host cell genome. The integrated provirus subsequently serves as a template for expression of viral genes and production of progeny virus RNA [1].

Kirsten murine leukemia virus (KiMLV) is a weakly oncogenic retrovirus that causes erythroblastosis in mice [2]. The virus also induces lymphomas in rats and mice which are indistinguishable from those induced by Gross murine leukemia virus [3]. A highly oncogenic variant, Kirsten murine sarcoma virus (KiMSV) was derived from KiMLV during growth of the latter virus in a rat [3,4]. Extensive analysis of the KiMSV genome has shown that it resulted from recombination between the parental leukemia virus and two sets of rat-derived sequences; the 'K-ras' oncogene (conferring the increased oncogenicity of the virus) and a member of a class of retrovirus-like genetic elements (denoted virus-like 30S sequences) [5,6].

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We are investigating the molecular basis for the pathogenicity of the Kirsten virus isolates. In particular we wish to determine the mechanism by which rat-derived cellular sequences were transduced by the parental leukemia virus. Towards this end we report here the characterization and cloning of unintegrated KiMLV proviral DNA. Our analysis shows that the linear KiMLV genome possesses long terminal repeats indistinguishable from those of the derivative KiMSV, and contains a 3'-located region very similar to the putative leukemogenic region of Gross murine leukemia virus.

MATERIALS AND METHODS

Viral DNA. The source and growth conditions of mouse NIH/3T3 fibroblasts and KiMLV have been described previously [7]. For preparation of *in vivo* synthesized KiMLV DNA, sub-confluent NIH/3T3 cells were infected in roller bottles at a multiplicity of ten plaque forming units of KiMLV per cell. Eighteen hours later, low molecular weight DNA was extracted by the method of Hirt [8] and viral DNA further purified by either centrifugation through a sucrose gradient (for linear DNA) or by caesium chloride - ethidium bromide centrifugation (for circular DNA) essentially as described previously [9]. Virus DNA was also synthesized *in vitro* by using the endogenous reverse transcriptase reaction of detergent disrupted virions [10]. We have described the reaction conditions for synthesis and the methods for gel purification of virus DNA elsewhere [9].

Restriction enzyme analysis. Digestion of DNA with restriction enzymes, agarose gel electrophoresis, Southern blotting and filter hybridization were all carried out as described previously [9]. ^{32}P-labelled DNA probe (cDNA-specific activity 10^8 cpm per μg) was synthesized from a purified KiMLV RNA template using avian myeloblastosis reverse transcriptase essentially as described by Norton *et al.* [9].

Cloning of KiMLV DNA. For cloning of *in vitro* synthesized KiMLV DNA, gel purified viral DNA was cleaved with *Pst* I and ligated into *Pst* I digested, alkaline phosphatase treated, pAT153 plasmid DNA. The resulting recombinants were used to transform competent *E. coli* MC1060 cells essentially as described by Khan *et al.* [11] and transformants were screened by colony hybridization using a KiMLV cDNA probe [12]. Plasmid DNA was isolated from positive colonies by the cleared lysate method [13]. In a separate cloning procedure, a 1.7 kilobase pair 3' terminal *Bam* HI fragment was inserted into the *Bam* HI site of pAT153 DNA. To facilitate the cloning, molecular linkers containing the *Bam* HI recognition site (Bethesda Research Laboratories) were blunt-end ligated onto the viral DNA fragment. Following *Bam* HI digestion, unligated linkers were removed by sepharose 4B chromatography.

RESULTS AND DISCUSSION

Linear KiMLV DNA synthesized shortly after infection of NIH/3T3 cells was purified to remove low molecular weight cell DNA. The proviral DNA sequences obtained were analysed using a KiMLV cDNA probe and the Southern blotting technique. Fig. 1 lane (a) shows undigested linear DNA which can be seen to migrate as a single species with a size of about 8.5 kilobase pairs (kbp).

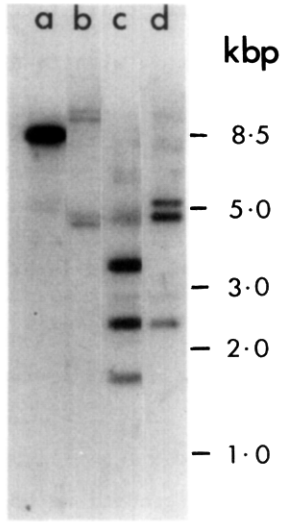

Fig. 1. Analysis of linear and circular KiMLV DNA. In vivo synthesized viral DNA was digested with restriction enzymes as appropriate, electrophoresed on a 1.0% agarose gel and the DNA transferred to a nitrocellulose filter. Viral DNA fragments were detected by annealing with a KiMLV cDNA probe followed by autoradiography. (a) undigested linear viral DNA, (b) undigested circular viral DNA, (c) Bam HI digested linear DNA, (d) Bam HI digested circular DNA.

When the supercoiled fraction of KiMLV infected cell DNA was examined by the same procedure (fig. 1 lane (b)) two doublet bands representing the closed circular (fastest migrating) and relaxed, open circular (slowest migrating) species of viral DNA were observed. The circular configuration of these DNAs was confirmed by analysis with restriction enzymes. For example, fig. 1 lanes (c) and (d) show the restriction fragments generated by Bam HI digestion of linear and circular virus DNA respectively. Two fragments released from linear DNA which are terminally located (see later) are absent in the digest of circular DNA and are replaced by a doublet of about 5.0 kbp. The size of the largest component of the doublet band (5.2 kbp) corresponds to the combined sizes of the missing terminal fragments (3.5 + 1.7 kbp) whereas the other component is 0.5 kbp smaller.

By digestion of KiMLV DNA with combinations of different enzymes we have ordered restriction fragments on the linear viral DNA. Fig. 2 shows the complete physical map of KiMLV DNA aligned with that of the derivative KiMSV [9]. It can be seen that the 0.5 kbp long terminal repeats which we previously mapped on the KiMSV genome are shared by KiMLV. Therefore as with

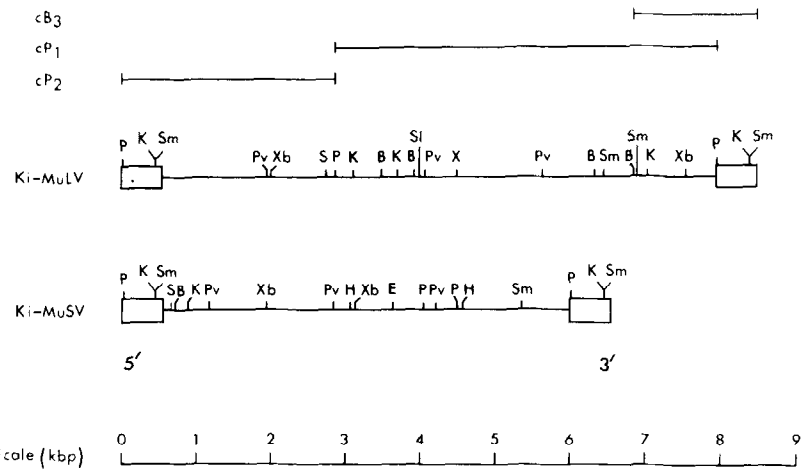

Fig. 2. Physical map of linear KiMLV and KiMSV DNAs showing the structure of cloned KiMLV DNA. Open boxes represent the long terminal repeats. The 5'-3' genome orientation (with respect to virion RNA) was determined by hybridization of a cDNA probe specific for the 3' end of the genome to restriction fragments of KiMLV DNA (data not shown). The KiMSV physical map is redrawn from reference 9. The bars indicate three restriction fragments which were selected for cloning. Restriction enzyme abbreviations: P = Pst I, K = Kpn I, Sm = Sma I, Pv = Pvu II, Xb = Xba I, S = Sac I, B = Bam HI, Sl = Sal I, X = Xho I, E = Eco RI, H = Hind III.

KiMSV and other retroviral DNAs [1,9] the larger size class of circular KiMLV DNA seen in fig. 1 most likely represents an exact circularization of linear DNA, whilst the smaller size class lacks one of the long terminal repeats.

We have previously localized leukemia virus derived sequences on the recombinant KiMSV genome by hybridization studies using a KiMLV cDNA probe [14]. Our data indicated that the long terminal repeats and an additional short sequence, contiguous with the 3' long terminal repeat of the sarcoma virus genome are derived from KiMLV. Recently however, it has been suggested that the KiMSV genome may have arisen by a complex series of recombinational events such that a region of its long terminal repeat is different from that of the parental KiMLV [15]. The data we present here suggest that the long terminal repeats of KiMLV and KiMSV are identical. In addition we can infer from fig. 2 that the additional 3'-located KiMLV derived sequences extend no further than 0.4 kbp from the long terminal repeat in KiMSV, since in KiMLV there is an Xba I site at this position that is absent from the sarcoma virus genome.

On surveying published restriction maps of different murine leukemia virus isolates, we were struck by the similarity between our KiMLV physical map and those of isolates originating from the AKR strain of mouse (e.g. see ref. 16). Recently Des Groseillers et al. [17] have localized sequences responsible for the leukemogenic potential of the Gross passage A, AKR-derived virus to within a 3'-located, 1.35 kbp Kpn I fragment. This fragment is shared by KiMLV (7.0-8.4 kbp in fig. 2). A comparison of the nucleotide sequence of this region in the Gross and Kirsten viruses may therefore identify conserved sequence elements responsible for the leukemogenic properties of these viruses.

In order to facilitate the detailed analysis of KiMLV DNA we have cloned three restriction fragments derived from DNA synthesized by the endogenous reverse transcriptase reaction of purified KiMLV particles. Fig. 2 shows two

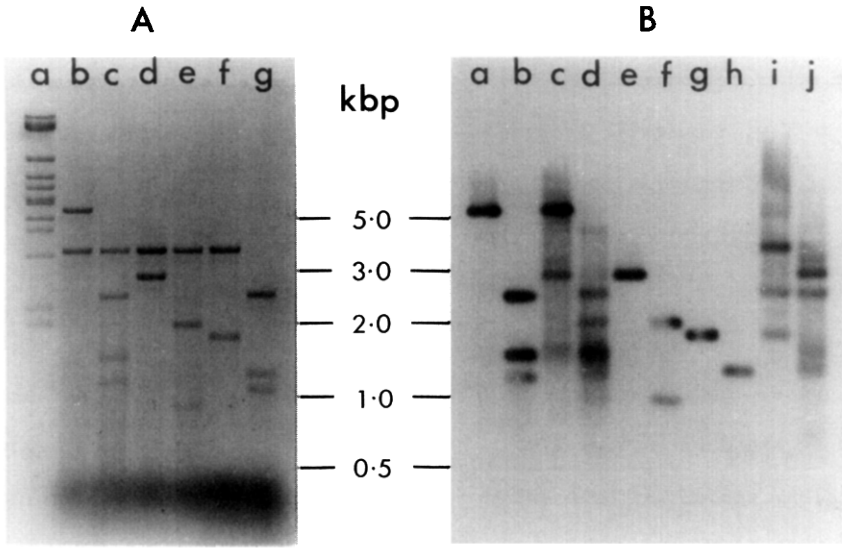

Fig. 3. Analysis of recombinant plasmids containing KiMLV DNA. A: 1.0% agarose gel stained with ethidium bromide and photographed under U.V. light showing (a) Eco RI + Hind III fragments of bacteriophage λ DNA as a marker, (b) clone P_1 digested with Pst I, (c) clone P_1 digested with Pst I + Pvu II, (d) clone P_2 digested with Pst I, (e) clone P_2 digested with Pst I + Pvu II, (f) clone B_3 digested with Bam HI, and (g) clone B_3 digested with Bam HI + Pst I. B: autoradiograph of viral DNA fragments detected with KiMLV cDNA probe (as in fig. 1). Showing (a) clone P_1 digested with Pst I, (b) clone P_1 digested with Pst I + Pvu II, (c) in vivo linear KiMLV DNA digested with Pst I, (d) in vivo viral DNA digested with Pst I + Pvu II, (e) clone P_2 digested with Pst I, (f) clone P_2 digested with Pst I + Pvu II, (g) clone B_3 digested with Bam HI, (h) clone B_3 digested with Bam HI + Pst I, (i) in vivo viral DNA digested with Bam HI and (j) in vivo viral DNA digested with Bam HI + Pst I.

adjacent Pst I fragments and a terminally located Bam HI fragment, together representing the complete genome, which were chosen for insertion into the plasmid vector pAT 153. The identities of the recombinant clones obtained were confirmed by the analysis shown in fig. 3. Panel A shows an ethidium bromide stained gel of restriction digests of the three plasmid DNAs. Adjacent tracks compare digestion with one enzyme to remove the insert and digestion with this and a second enzyme in concert. In panel B the same digests were analysed in parallel with in vivo KiMLV DNA by Southern Blotting using a KiMLV cDNA probe. In each case it can be seen that the cloned DNAs generate the predicted restriction fragments and are therefore homologous to the original viral DNA. Analysis of the cloned DNAs with several other enzymes also yielded results consistent with the restriction maps in fig. 2 (data not shown). We are presently performing sequence analysis of cloned KiMSV and KiMLV DNAs. This will elucidate the sequences at the points of recombination on the sarcoma virus genome, and identify possible sequence elements responsible for the leukemogenicity of KiMLV in the 3' region of the genome.

ACKNOWLEDGEMENTS

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