45. Demonstration of Xenotropic Murine Leukemia Virus-related Sequences in Various Rodent Species

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Introduction. All vertebrates appear to carry in their genomes endogenous retroviruses which are transmitted vertically.¹⁾ In mice, endogenous type C retroviruses are divided into three classes on the basis of their host range; ecotropic, xenotropic and amphotropic murine leukemia viruses (MuLV).^{2),3)} Mice of certain inbred strains (e.g., AKR) express high titers of endogenous ecotropic MuLV and develop thymic lymphomas.⁴⁾ The ecotropic virus expression, however, is not sufficient for leukemia development,⁵⁾ and mink cell focus-inducing (MCF) viruses, which are recombinants between ecotropic and xenotropic MuLV *env* sequences, appear to be a more proximate cause of leukemia.⁶⁾ We report here, using a xenotropic MuLV-specific DNA sequence as a probe, that endogenous xenotropic or MCF virus-related sequences are carried by various rodents.

Materials and methods. Individual fresh liver specimens (from animals described later) were brought to DNA extraction.⁷⁾ The DNAs digested with a restriction enzyme *Hinc* II were subjected to agarose gel electrophoresis, transferred to a nitrocellulose filter⁸⁾ and hybridized with ³²P-nick-translated probes under non-stringent conditions.⁹⁾ Filters were washed with concentrations of SSC (6×, 0.8× or 0.1×SSC) at 55°C to assess homology degree between probes and sample cellular DNAs.

Hybridization probes are shown in Fig. 1. The sequence specific for the Friend strain of spleen focus-forming virus (SFFV), designated as SSS (Fig. 1A), was 0.75 Kb in size and subcloned into the plasmid vector pBR322 from molecularly cloned SFFV DNA.¹⁰⁾ This probe had nucleic acid sequence homology with the *env* region of xenotropic MuLV.^{10),11)} The second probe, long terminal repeat (LTR) of SFFV, was obtained as the internal *Kpn* I fragment from the two tandem LTRs of the cloned SFFV¹⁰⁾ (Fig. 1B) and could detect most of either type of LTR of MuLV.¹⁰⁾⁻¹²⁾ The third probe

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Fig. 1. Schematic representation of hybridization probes. Molecular cloning of unintegrated circular proviruses of both SFFV and F-MuLV has been described.¹⁰ Characterization and isolation of each hybridization probe are described in Materials and methods. A, SSS probe; B, LTR probe; C, F-MuLV probe.

was cloned, whole Friend MuLV genome, 9 Kb in size¹⁰) (Fig. 1C) and having extensive sequence homology with genomes of both other ecotropic MuLVs¹³⁾⁻¹⁵⁾ and of xenotropic MuLVs except *env* region.^{6),14)}

Results and discussion. *Hinc* II-digested DNAs from various rodents were brought to a Southern blotting analysis using a xenotropic *env*-related sequence (SSS) and LTR of SFFV as the probes. *Hinc* II cuts most of the murine xenotropic provirus DNAs at the 5' side of their *env* regions, giving rise to genomic DNA fragments tandemly composed of *env*, 3'LTR and cellular flanking DNA. Hence, if one *Hinc* II-digested fragment from a given animal DNA has sequence homology with both SSS and LTR, the fragment is presumed to contain endogenously a xenotropic MuLV-related sequence.

Multiple xenotropic MuLV genes in Mus musculus subspecies. The SSS hybridized with multiple bands of both inbred (AKR, Fig. 2A) and feral (*Mus musculus domesticus*, Fig. 2B; *M. m. molossinus*, Fig. 2C) mouse DNAs. Many of these bands were also hybridizable with LTR, indicating the presence of multiple copies of endogenous xenotropic MuLV-like sequences. The number of copies of such xenotropic MuLV-like sequences in AKR mouse was estimated as 20-30 per genome, agreeing with data previously obtained by liquid hybridization.¹⁶⁾ *M. m. domesticus* showed a hybridization pattern similar to that of AKR mouse (Fig. 2B), supporting the idea that inbred laboratory mice originated from the European mice.^{17),18)} *M. m. molossinus*, the Japanese feral mouse strain, showed quite a different pattern of hybridization with many more copies of xenotropic MuLV-related sequences (Fig. 2C). In all of these mice, however, both SSS and LTR remained hybridized with the multiple DNA

Fig. 2. Southern blot analysis of various rodent DNAs using SSS and LTR as probes. The DNAs were digested with *Hinc* II and subjected to Southern blot hybridization. In each panel (A-H), left and right lanes are for SSS and LTR probes, respectively (see panel A). Lanes numbered 1 are for low stringency ($6\times$ SSC wash), numbered 2 for medium stringency ($0.8\times$ SSC) and numbered 3 for high stringency ($0.1\times$ SSC). The arrowed bands are those detected by SSS but not by LTR probe. A, AKR mouse; B, *Mus musculus domesticus*; C, *Mus musculus molossinus*; D, rat (Sprague-Dawley); E, Syrian golden hamster; F, Chinese hamster; G, guinea pig (Hartley); H, squirrel (Korean chipmunk).

fragments even after stringent washing ($0.1\times\text{SSC}$) of the hybridized filters (no. 3 lanes). The *Mus musculus* subspecies thus appears to have closely related multiple copies of endogenous xenotropic MuLV.

Xenotropic MuLV-related sequences in rats. In the rat, about ten bands were detected by SSS probe above a high and smear background under non-stringent condition ($6\times\text{SSC}$ wash) (Fig. 2D). With stringent washing, most of these bands and the smear background became quite faint, showing a sharp contrast with the mouse subspecies results. With LTR as a probe, several faint bands were detected over an unexpectedly high background, some appearing to correspond to the bands detected by SSS. A few fragments (arrowed in Fig. 2D) detected by SSS probe, however, did not hybridize to the LTR. These results indicate the presence in the rat DNA of xenotropic MuLV-related sequences more distant from the probes than those in the mouse subspecies.

Xenotropic MuLV-related sequences in hamsters. Hamsters (Syrian golden and Chinese) showed several bands which could hybridize to both SSS and LTR (Fig. 2, E, F). Those detected in the Syrian golden hamster DNA were rather faint and hardly detectable after stringent washing (Fig. 2E). In the Chinese hamster (Fig. 2F), however, three of the several bands also hybridizable with LTR had strong intensities and still remained hybridized to SSS even after stringent washing, as in the mouse. The LTR probe hybridized strongly to both hamster DNAs under non-stringent conditions (LTR-probed first lanes in Fig. 2, E, F). These bands and the smear high background, however, became scarcely detectable after stringent washing, suggesting slight relatedness to the MuLV LTR. These animals, as well as mouse and rat, had a few bands (arrowed in Fig. 2, E, F) which could hybridize to SSS but not to LTR.

Xenotropic MuLV-related sequences in other rodents. In guinea pig and squirrel (Korean chipmunk), SSS hybridized to one prominent and a few other faint fragments (Fig. 2, G, H). These bands, quite strong in the guinea pig, became progressively faint by washing with decreasing concentration of SSC, as in the rat and Syrian golden hamster. In contrast to mouse, rat and hamster, LTR hybridized neither to these fragments nor to any others even under a nonstringent condition ($6\times\text{SSC}$ wash).

SSS-related sequences in rodent species. In all rodents examined, one or sometimes more bands could be detected which hybridized to SSS but not to LTR. DNAs digested with *Hinc* II were then hybridized to DNA of cloned F-MuLV which contained MuLV *gag*, *pol*, ecotropic *env* but not xenotropic *env* genes. In many of the animals, extra and very strong bands were detected besides

Fig. 3. Southern blot analysis of various rodent DNAs using F-MuLV DNA as a probe. The same experiments as in Fig. 2, except that the probe used was F-MuLV. The arrowed bands are those hybridized to SSS (but not to LTR, see Fig. 2) and to F-MuLV DNA.

the multiple SSS-probed bands. These are probably sequences homologous to *gag* or *pol* regions, the strong bands being the sum of internal fragments digested from the multiple copies of MuLV-related endogenous sequences. A comparison of the bands arrowed in Fig. 2 with those in Fig. 3 revealed that almost all bands detected by SSS but not LTR in Fig. 2 could again hybridize to whole F-MuLV DNA (for mouse, rat and two hamsters, Fig. 3, A-F), implying that these fragments are also derived from sequences related to xenotropic MuLV. In the guinea pig and squirrel, however, some of the bands detected by SSS but not LTR seemed not to hybridize to F-MuLV DNA (Fig. 3, G, H). These bands may suggest the presence of totally different endogenous viruses,¹⁰⁾ or of SSS-related sequences of cellular origin,²⁰⁾ or a difference of divergence rate between LTR and non-LTR sequences.

Evolution of xenotropic MuLV genes in rodents. We have looked for xenotropic MuLV-related sequences endogenous in various rodents and found that all those examined possess them. Among non-murine rodents, the Chinese hamster appeared to have the most highly related endogenous sequences. The rat and Syrian golden hamster seemed to carry sequences distantly related, while the guinea pig and squirrel bore only inert vestiges of xenotropic MuLV, or totally unrelated endogenous viruses. These findings contrast with other reports describing the absence of xenotropic MuLV-related endogenous sequences in non-murine rodents, a result obtained using the standard liquid hybridization technique.²¹⁾ Our success may depend on the usage of the Southern transfer technique under non-stringent hybridization conditions using molecularly cloned probes.

The presence of xenotropic MuLV-related sequences in all the rodents examined suggests that endogenous xenotropic MuLV has evolved from a common ancestral retrovirus carried in ancient rodents and amplified in the mouse and Chinese hamster. LTR sequences of the xenotropic MuLV might have evolved at a much higher rate than other viral genes because such sequences could hardly be detected in the evolutionally distant guinea pig and squirrel. The conservation of viral structural genes and the conversion of LTR sequences in retroviruses have been generally accepted.^{11),22)}

Possible leukemogenicity of endogenous MuLV-related genes in rodents. In the AKR strain of inbred mice, the preleukemic and leukemic stages are associated with MCF virus.⁶⁾ This leukemogenic virus is a recombinant between ecotropic MuLV and endogenous xenotropic MuLV *env* sequences. Similar situations may exist in naturally occurring leukemia in other rodents. Experiments to test this possibility are currently in progress in other vertebrates.

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References

- 1) Todaro, G. J., and Huebner, R. J.: Proc. Natl. Acad. Sci. U.S.A., **69**, 1009 (1972).
- 2) Levy, J. A.: Science, **182**, 1151 (1973).
- 3) Hartley, J. W., and Rowe, W. P.: J. Virol., **19**, 19 (1976).
- 4) Rowe, W. P.: Cancer Res., **33**, 3061 (1973).
- 5) Lilly, F., Duran-Reynals, M., and Rowe, W. P.: J. Exp. Med., **151**, 542 (1980).
- 6) Chattopadhyay, S. K. *et al.*: Nature, **295**, 25 (1982).
- 7) Onuma, M. *et al.*: Microbiol. Immunol. (in press).
- 8) Southern, E. M.: J. Mol. Biol., **98**, 502 (1975).
- 9) Martin, M. A. *et al.*: J. Virol., **39**, 835 (1981).
- 10) Sagata, N., and Ikawa, Y.: (submitted).
- 11) Chattopadhyay, S. K. *et al.*: Virology, **113**, 465 (1981).
- 12) Yamamoto, Y. *et al.*: Proc. Natl. Acad. Sci. U.S.A., **78**, 6893 (1981).
- 13) Bernstein, A. *et al.*: *ibid.*, **76**, 4455 (1979).
- 14) Bosselman, R. A. *et al.*: Virology, **102**, 234 (1980).
- 15) Rands, E. *et al.*: *ibid.*, **108**, 445 (1981).
- 16) Chattopadhyay, S. K. *et al.*: Cold Spring Harbor Symp. Quant. Biol., **39**, 1085 (1975).
- 17) Yonekawa, H. *et al.*: Japan. J. Genet., **55**, 289 (1980).
- 18) Ferris, S. D., Sage, R., and Wilson, A. C.: Nature, **295**, 163 (1982).
- 19) Besmer, P. *et al.*: J. Virol., **29**, 1168 (1979).
- 20) Temin, H. M.: Annu. Rev. Genet., **8**, 155 (1974).
- 21) Benveniste, R. E. *et al.*: J. Virol., **21**, 849 (1977).
- 22) Cohen, M. *et al.*: *ibid.*, **41**, 801 (1982).