

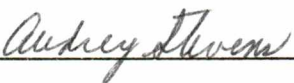
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I am submitting herewith a thesis written by Kaori Tano entitled "Nuclease Hypersensitivity of Endogenous Murine Leukemia Virus-Related Gene Sequences In Isolated Nuclei". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

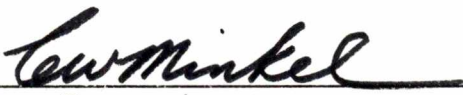

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Vice Provost
and Dean of The Graduate School

NUCLEASE HYPERSENSITIVITY OF ENDOGENOUS MURINE
LEUKEMIA VIRUS-RELATED GENE SEQUENCES
IN ISOLATED NUCLEI

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ABSTRACT

Inbred strains of mouse contain 40-60 copies of DNA sequences homologous to murine leukemia virus (MuLV) in various chromosomal locations; and, the majority of those sequences belong to the non-ecotropic and non-xenotropic MuLV-related proviral sequences or the polytropic classes. MuLV-related specific RNA transcripts were detected in the liver and kidney of RFM/Un and NFS/N mice. In order to investigate how many and which members of MuLV-related proviral sequences are responsible for the expression of the RNA transcripts, I investigated the nuclease hypersensitivity of MuLV-related proviral sequences in the nuclear chromatin of a few tissues of these mouse strains.

The indirect end-labeling method using DNaseI was able to detect several strong DNaseI-hypersensitive sites in the LTRs (long terminal repeats) of MuLV-related proviral sequences. Hypersensitive sites were mapped to immediately upstream and downstream of the 14-bp repeat negative regulatory element of the 5' and 3' LTRs, cap (transcription initiation) site and the "CAAT" box of the 5' LTR, and the U3 region immediately upstream of the IS (insertion sequence) of the 3' LTR.

Sensitivity to restriction endonuclease PstI was also studied in the isolated nuclei because MuLV and related proviral sequences contain a common PstI site in the LTR and DNaseI-hypersensitive sites are located near the PstI site.

The PstI site within the LTR of certain proviral sequences in the nuclei was sensitive to cleavage by this restriction endonuclease while the PstI sites in the structural gene region of the same proviral sequences were not. In addition, the PstI sensitivity was presented mainly in a minor population of MuLV-related proviral sequences, which contain sequence deletion(s) in the structural gene regions.

It is known that chromatin structure involved in gene regulation is also associated with DNA methylation. The SmaI site in the LTR partially overlaps with the KpnI site, therefore methylation status at the SmaI site can be detected by comparing the intensity of double digestion fragments between PstI/SmaI and PstI/KpnI. Most of MuLV-related proviral sequences were methylated; however, in the liver the minor population of proviral LTR that were relatively sensitive to PstI digestion in the nuclei was digested by SmaI, suggesting that these LTRs were relatively undermethylated in the DNA. In the testis, no such correlation between PstI hypersensitivity and SmaI digestion was seen, suggesting that the proviral LTRs were relatively methylated, regardless of their nuclease hypersensitivity.

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CHAPTER I

INTRODUCTION

Retroviruses have been biologically of significant interest because some of them are known to produce cancer in the animal, and they can be transmitted as stably integrated proviruses through many generations in the germline of animal hosts. The virion of retroviruses contains two 35S single-stranded genomic RNA molecules about 9 to 10 kb long. Each RNA strand is polyadenylated at the 3' end and capped at the 5' end (Weiss et al., 1955a). After absorption of the virion to specific host cells, the viral RNA genome serves as a template to synthesize viral DNA in the cytoplasm. The viral DNA subsequently enters the nucleus and becomes integrated into various sites of the host chromosome and transmitted as a Mendelian gene (Coffin, 1988).

The integrated provirus, which characteristically carries two identical long terminal repeats (LTR) at the 5' and 3' ends as well as gag, pol and env genes in between the two LTRs, can serve as an efficient template for mRNA synthesis by RNA polymeraseII (Coffin, 1988). The gag, pol and env genes code, respectively, for the virion structure proteins, the enzymes necessary for replication (reverse transcriptase, pronase and endonuclease) and the viral envelop proteins (Weiss et al., 1955a). The LTR is divided into three

regions, U3 (3' unique region), R (the directly repeated sequence) and U5 (5' unique region). The U3 region contains the "enhancer" as well as the "promoter" elements---"CAAT" box and "TATA" box. The transcription initiation site, or the "cap" site is located at the R region (Coffin, 1988). Because the LTR contains regulatory elements for expression, it is very important to investigate the activity of the LTR in the endogenous proviral sequences.

Although retrovirus replication is usually not lethal to the infected cells, malignant transformation of infected host cells may occur either due to a transduced oncogene carried by the virus genome or from activation of a cellular oncogene (c-onc or protooncogene) by insertion of the provirus near or within the c-onc. In the first case of acutely transforming viruses, all or part of a cellular oncogene may be inserted in the viral genome without affecting viral replication function (e.g. Rous sarcoma virus with sac gene) or may be replaced a position of the viral genome (e.g. Abelson murine virus with abl gene and simian sarcoma virus with sis gene).

The second type of activation of a cellular oncogene is by integration of a provirus into or near the gene. For example, integration with the c-myc gene is found in B-cell lymphomas induced by avian leukosis virus (ALV) and in T-lymphomas induced by murine leukemia virus (MuLV). Four different mechanisms seem to be responsible for this type of activation of cellular oncogene: (1) The 3' LTR can act as a

newly inserted promoter of the downstream gene (e.g. c-myc gene in ALV-induced lymphoma), (2) The 5' LTR inserted in the same transcriptional orientation as the oncogene provides the initiation site for transcription (e.g. c-erbB activation in ALV-induced erythroleukemia), (3) The enhancer of the inserted LTR regulates the expression level of the cellular oncogene although the transcription seems to be initiated at its own correct promoter, (4) A provirus is integrated downstream of the cellular oncogene, resulting in termination insertion (e.g. pim-1 gene in MuLV-induced lymphoma). Activation of the cellular oncogene can be represented by (1) elevation of the level of transcription (and hence of the level of the protein product), (2) alteration of the transcript and/or protein product or (3) increased stability of the mRNA leading to a substantially greater level of the product (Coffin, 1988).

Transposable gene elements (transposons) are genetic elements capable of movement to a new nonhomologous location in the genome (e.g. copia element of Drosophila and Ty element of yeast). Retrotransposons, defined as the transposable element using a retrovirus-like reverse transcription mechanism for transposition, are widely distributed, including the mammalian Alu sequences, the Drosophila F elements, murine L1 sequences and processed pseudogenes, lack LTRs (Boeke, 1988). Since endogenous proviruses contain the sequences necessary for gene retrotransposition and gene expression, it

is possible that insertion of the elements at new sites could result in altered expression of the gene either by inactivation of the gene due to integration of a retroviral provirus within the gene or by activation of the gene due to the regulatory effect of integrated retrovirus in or near the gene.

Endogenous proviruses, derived presumably from past retrovirus infection of the germline, are located at various chromosomal sites all over the genome. In mice, endogenous proviruses or virus-like elements comprise about 0.5 % of the genome (Coffin et al., 1989). They are classified into five groups: (1) endogenous type C sequences (related to murine leukemia virus (MuLV)), (2) endogenous type B sequences (related to mouse mammary tumor virus (MMTV)), (3) Intercisternal A Particles (IAP) genes, (4) Virus like 30 S RNA (VL30) genes and (5) MboI repeat LTR (MRL) elements. The LTR of MMTV is glucocorticoid responsive. IAP RNAs are 30S to 38S size but never found outside the cell, so never infectious. Analyses indicate that the haploid Mus musculus genome contains about 1000 copies of IAP genes (Weiss et al., 1955b). VL30 RNA is infectious and inducible by halogenated pyrimidines; however, simultaneous helper MuLV infection is required for horizontal transmission of VL30 (Weiss et al., 1955b). MRL elements are also called IS-type elements or MURRS (murine retrovirus-related sequences) (Wirth et al., 1983; Wirth et al., 1984). At least 2000 to 5000 copies of

these elements are found in the mouse genome, and about 95 % of these LTRs are solitary. The nucleotide sequence analyses showed that the cloned MRL proviral structures contain sequences corresponding to the gag and pol genes but not the env gene. Certain MRL proviruses can be found only in the male mice (the male-specific MRL elements) (Yang et al., 1989).

Inbred strains of mice contain 40-60 copies of DNA sequences homologous to MuLVs in a variety of chromosomal locations (Stoye and Coffin, 1987). These sequences can be divided into four classes on the basis of the env gene property (Stoye and Coffin, 1987; Stoye and Coffin, 1988): (1) ecotropic (encoding an env gene product capable of infecting only mouse cells), (2) xenotropic (encoding an env gene product capable of infecting only the cells other than mouse cells), (3) polytropic and (4) modified polytropic. The latter two types of proviruses are designated MuLV-related sequences and have mink cell focus-forming (MCF) virus determinants in the env gene (Chattopadhyay et al., 1981; Stoy and Coffin, 1987; Nikbakht et al., 1987). The MCF viruses are leukemogenic and presumably generated by recombination of three different endogenous proviruses, an ecotropic virus, a polytropic virus and a xenotropic virus (Coffin et al., 1989; Haetley et al., 1977).

MuLV-related proviral sequences can be distinguished from ecotropic and xenotropic MuLV by some characteristics.

(1) MuLV-related sequences have Fv-1 B-tropism determinant in the gag-30 region (Boone et al., 1988). The major virion protein p30 is the target for the host Fv-1 gene restriction (N- and B-tropism) which governs the replication of viral DNA and integration into the host chromosome. (2) The primer binding site for initiation of reverse transcription of MuLV-related sequences is complementary to the glutamine tRNA or UGA-suppressor glutamine tRNA, while MuLV utilizes proline tRNA as the primer (Nikbakht et al., 1985; Ou et al., 1983).

(3) The LTR of MuLV-related proviruses has a 190-200 bp insertion sequence (IS) between the U3 "enhancer" region and "promoter" region (Khan and Martin, 1983; Ou et al., 1983).

The IS was found to be a potential enhancer by the transient expression assay of chloramphenicol acetyltransferase (CAT) gene (Ch'ang et al., 1989a). In addition, MuLV-related LTRs contain 14 bp repeat negative regulatory element immediately upstream of the "enhancer" region (Ch'ang et al., 1989a).

(4) The specific sequence deletions, a 1487 bp gag-pol sequence, a 113 bp pol sequence and 1811 bp env sequence, are found (Ch'ang et al., 1989b).

The polytropic and modified polytropic MuLV-related proviral sequences can be distinguished by physical characteristics of restriction site (e.g. modified polytropic MuLV-related sequences contain a HindIII site and a PstI site within the pol region whereas polytropic MuLV-related sequences do not) (Stoy and Coffin, 1987), by the size of LTR

(i.e. polytropic and modified polytropic MuLV-related LTRs are about 700 bp and 750 bp long, respectively, and latter contains an extra 37-40 bp long sequence immediately upstream of the IS segment) (Ch'ang et al., 1989a), and by the sequence polymorphism in the env gp-70 coding region. Coffin et al. defined the copy number of individual non-ecotropic class sequences and made the chromosomal linkage map by using class specific oligonucleotide probes based on the difference of sequence in gp-70 (Stoye and Coffin, 1988).

The endogenous ecotropic loci have been designated emv (ecotropic murine leukemia virus locus), and there are only a small number of copies (0-6) in any mouse strain (Weiss et al., 1955b). The majority of endogenous proviruses related MuLV belongs to non-ecotropic type (Stoye and Coffin, 1987).

Ecotropic and xenotropic MuLV proviruses can be induced to produce infectious viruses by the chemical treatment or spontaneously; while, little is known about the inducibility and virus expression of MuLV-related proviral sequences. Some mouse strains spontaneously express the ecotropic MuLV in certain stages of their life; and the MuLV expression level appears to correlate with incidence of leukemia. Thus, the mouse strains which show a high incidence of leukemia also express high levels of the endogenous ecotropic viruses (Gelb et al., 1973; Rowe, 1973; Stephenson and Aaronson, 1973; Berns and Jaenish, 1976; Lowy et al., 1974). The expression of RNA transcripts from three families of endogenous retrovirus-

related sequences, Mo-MuLV, IAP and VL30, was recognized by treatment of TCPOBOP or CCl₄ (Dragani et al., 1987). Recently, some experiments have supported the possibility of expression of MuLV-related proviruses in a tissue specific manner. In 129G_{IX}⁺ mice and AKR mice, the RNA transcripts detected by mink cell focus forming (MCF) env specific probe were found in liver, kidney and other tissues (Levy et al., 1985; Khan et al., 1987). The MuLV-related specific probes could detect several RNA transcripts in the liver and kidney of RFM/Un and NFS/N mice, although most of the retroviral RNAs were ecotropic and xenotropic MuLV specific in the spleen and thymus of RFM/Un mice (Yang et al., 1989). The in situ hybridization analyses revealed that the MuLV-related RNA transcripts were located mainly in hepatocytes and epithelial cells of renal tubules (Yang et al., 1989).

In order to know how many and what types of members of MuLV-related proviral sequences are responsible for the distinctive expression of MuLV-related type RNA transcripts, we have investigated nuclease sensitivity of these sequences.

In chromatin, nucleosome-free regions known as nuclease hypersensitive sites are believed to be associated with cis-acting DNA sequences which are accessible to trans-acting factors necessary for transcription, replication, recombination and other important biological functions (Gross and Garrard, 1988). The method most commonly used to map nuclease hypersensitive sites is indirect end-labeling using

DNaseI (Wu, 1980; Nedospasov and Georgiev, 1980). Weintraub and Groudine (Weintraub and Groudine, 1976) found that actively transcribed or potentially active chromatin was preferentially digested with DNaseI, and such sensitive regions extended over many kilobases. DNaseI-hypersensitive sites exhibit one order more sensitivity than the sensitive region. The presence of DNaseI-hypersensitive sites was shown by generation of specific DNA fragments following mild digestion of nuclei with DNaseI. Control experiments showed that no such band was seen by selective digestion of purified DNA with DNaseI, demonstrating that the hypersensitive sites exist as a consequence of the chromatin structure.

A number of hypersensitive sites have been mapped by an indirect end-labeling technique using DNaseI first applied by Wu (Wu, 1980). In this study, constitutive hypersensitive sites were found at or near the 5' end of Drosophila major heat-shock proteins (hsps), and in the upstream active sequence (UAS) of the GAL1-10 locus in yeast (Gross and Garrard, 1988). Some nuclease hypersensitive sites exist in a tissue-specific fashion. It was reported that a hypersensitive site was observed close to the 5' end of the embryonic β -globin gene in chick embryonic red blood cells but not in the adult red blood cells, while two hypersensitive sites were observed upstream from the 5' side of the adult β 2-globin gene but not in the embryonic cells. No such sites were seen in chromatin from brain cells (Stalder et al., 1980;

Elgin, 1981). Similar results were obtained in the α -globin gene cluster, and hypersensitive sites of the rat preproinsulin gene were observed in a pancreatic β -cell tumor (Wu and Gilbert, 1981). From many DNaseI-hypersensitive site studies accumulated, it might be concluded that a DNaseI-hypersensitive site at or near the 5' end of a gene is necessary, but not sufficient, for gene expression.

The regulation of gene expression in eukaryotes is likely to involve specific DNA-protein interactions, that is, the interactions of cis-acting DNA element with trans-acting factors. DNA methylation at specific sequences may modulate the interactions. Usually DNA methylation of C residues (5^mC) in specific sequences is associated with inactivation of the gene. Numerous studies have demonstrated the correlation between the level of gene expression and the degree of methylation. The methylation patterns are tissue-specific and clonally heritable. Once the specific DNA methylation patterns are established, they are passed on to all of the cells of a given lineage by the DNA methylase that is specific for half-methylated sequences. Thus, activation of tissue-specific genes from the repression state may involve demethylation of the gene. The treatment with the drug 5-azacytidine, which is a base analog of cytosine and which can not be methylated, results in the expression of genes which normally are not expressed in the tissues (Doerfler, 1983; Jaenisch and Jahner, 1984; Cedar, 1988).

Chromatin structure that plays an important role in the gene regulation is also associated with DNA methylation. Chromatin regions containing unmethylated DNA have a DNaseI sensitive structure while fully methylated sequences in inactive genes are resistant to mild DNaseI digestion (Keshet et al., 1986). Methylation of DNA might considerably alter the local conformation of DNA. The regions of DNA that contain repetitive CG sequences can form a left handed Z structure (Z-DNA). Since C residues of CG sequences are the main sites of eukaryotic methylation, methylation of those sites might be involved in altering or stabilizing Z-DNA structure. The possible involvement of Z-DNA in regulating gene expression is suggested by the fact that certain regulatory proteins possess structures for binding to the major groove of Z-DNA (Garrard et al., 1988). Although the possible involvement of DNA methylation in the control of gene expression in eukaryotes is based on indirect evidence, it is of interest to compare the relationship of the DNA methylation status with nuclease hypersensitivity in the regulatory regions of endogenous MuLV and related sequences.

CHAPTER II

MATERIALS AND METHODS

Preparation of nuclei. Liver was removed from NFS/N and RFM/Un mice at the age of 12-24 weeks and stored in the freezer at -70°C . The frozen livers were homogenized in 5 volumes (v/w) of buffer A (0.15 mM spermine, 0.5 mM spermidine, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 15 mM Tris.HCl pH 7.4) supplemented with 0.35 M sucrose and freshly added 1 mM PMSF (Jantzen et al., 1986) and centrifuged at 2000 rpm for 10 minutes in the Sorvall RC-2B centrifuge after filtration through four layers of gauze. The pellet was suspended in the same buffer in the presence of 0.5 % TritonX-100 and allowed to stand in ice for 5 minutes before centrifugation at 2000 rpm for 5 minutes. The pelleted nuclei were washed twice with buffer A supplemented with 0.35 M sucrose and 1 mM PMSF. All processes were carried out in the cold.

DNA preparation. DNA for the control was prepared from the isolated nuclei. The nuclei were suspended in 10 mM Tris.HCl, 10 mM EDTA and 50 mM NaCl, and then lysed by addition of 2 volumes of Hirt solution (10 mM Tris.HCl pH 7.6, 10 mM EDTA, 1 % SDS, warmed at 65°C). To the mixture 100 $\mu\text{g/ml}$ proteinase K was added, and it was incubated overnight at 37°C . After adjusting to contain 0.5 M NaCl, DNA was extracted once or twice with phenol, once with phenol:chloroform:isoamyl alcohol (24:24:1), and once with

chloroform:isoamyl alcohol (24:1). DNA in the aqueous phase was precipitated with 2.5 volumes of 95 % ethanol. The restriction enzyme digestion was performed at a concentration of 40 µg/ml according to the supplier's protocols (Bethesda Research Laboratories, Inc.).

DNaseI digestion and extraction of DNA fragments.

Isolated nuclei were suspended in buffer B (0.15 mM spermine, 0.5 mM spermidine, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 15 mM Tris.HCl pH 7.4) supplemented with 1 M PMSF (Jantzen et al., 1986) to give a DNA concentration of 400-600 µg/ml and digested with 1000 or 3000 units of DNaseI (Boehringer Mannheim) at 0° C for 10 minutes. Digestion was started by the addition of 5 mM MgCl₂ and terminated by the addition 10 mM EDTA. After digestion, the nuclei were lysed with Hirt solution at 65° C. The whole DNA or the small sized DNA fragments were prepared for further restriction enzyme digestion. The whole DNA was prepared using the same procedure for the control DNA extraction. To prepare the small size DNA fragments, the Hirt solution of lysed nuclei was adjusted to contain 1.25 M NaCl and incubated overnight at 0° C to precipitate the high-molecular weight chromatin DNA. After centrifugation at 12,000 rpm for 30 minutes, DNA in the supernatant was isolated by phenol/chloroform extraction and ethanol precipitation.

Restriction endonuclease digestion of nuclei and DNA extraction. Nuclei were prepared from fresh tissues of

NFS/N mice or from frozen liver of young C3H mice and suspended at 0.5-1.0 mg DNA per ml of the core buffer (50 mM Tris.HCl pH 8.0, 10 mM MgCl₂, 50 mM NaCl) for digestion with various concentration of restriction endonucleases at 37° C for 1 hour. After digestion, chromatin was eluted with Hirt solution at 65° C followed by precipitation with 1.25 M NaCl at 0° C overnight or with 8 mM Tris.HCl pH 7.6, 10 mM EDTA, 0.4 M NaCl at 0° C for 30 minutes. The supernatant was separated by centrifugation at 12,000 rpm for 30 minutes and digested with proteinase K (100 µg/ml) at 37° C overnight. The DNA fragments digested by restriction endonucleases in the nuclei were extracted with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and then precipitated with 2.5 volumes of 95 % ethanol.

Analysis of methylation. The control DNA after PstI digestion and the DNA fragments digested by PstI in isolated nuclei were extracted with phenol and phenol:chloroform:isoamyl alcohol (24:24:1) and precipitated with 2.5 volumes of 95 % ethanol. After washing with 70 % ethanol twice and drying, the DNA pellet was suspended in water and divided into two samples. One was digested with SmaI and the other was with KpnI, and their digestion patterns were compared by electrophoresis, Southern transfer and DNA hybridization.

DNA gel blot analysis. DNA was separated by electrophoresis in a horizontal agarose gel (BRL) and then

transferred to nylon membrane (Zetabind, Cuno Inc.) in alkaline solvent (Southern, 1975; Reed and Mann, 1985). The concentration of agarose was 0.7 % or 1.5 %, and Tris-borate (TBE) buffer was used in the electrophoresis. Ten to 15 µg of DNA sample was applied to each gel well. After electrophoresis, the gel was treated twice with 0.25 N HCl for 15 minutes and rinsed briefly in water. The DNA was transferred to the nylon membrane in 500 ml of 0.4 N NaOH overnight. Then the membrane was washed twice with 2x SSC (0.3M NaCl, 0.03 M sodium citrate) by shaking and dried at room temperature. The membrane was plated into a plastic bag and washed with 0.1x SSC, 0.5 % SDS, 0.5 % nonfat dry milk for 1 hour at 65° C. I employed the company's protocol for hybridization (Zetabind, Cuno Inc.). The prehybridization solution (5x SSC, 0.5 % nonfat dry milk, 0.02 M NaPO₄ pH6.7, 500 µg/ml sonicated denatured salmon sperm DNA, 5 % dextran sulfate, 50 % formamide) was added to the bag, and the membrane was incubated overnight at 42° C. After removal of prehybridization solution, the hybridization solution (the same solution for prehybridization except that the concentration of dextran sulfate was 10 % instead of 5 %) containing radioactive probe ($2-10 \times 10^6$ cpm/100 cm² membrane) was added to the bag and the membrane was incubated overnight at 42° C. Then the membrane was washed as follows: 50 ml of 10 x SSC, 50 % formamide (once at room temperature), 30 ml of 2 x SSC, 0.1 % SDS (5 times at room temperature), 100 ml of

0.2 x SSC, 0.1 % SDS (once at 50° C for 30 minutes) and 50 ml of 2 x SSC (once at room temperature). The membrane was dried, wrapped in plastic wrap and exposed to an X-ray film (Kodak) at -80° C.

Preparation of radioactive probes and size markers.

The following fragments from cloned MuLV sequences were nick translated using [α -³²P]dCTP and served as the probes: 0.4 kb PstI/SmaI fragment from LTR of pRFM8 (Kuemmerle et al., 1987), 0.4 kb PstI/SmaI fragment from LTR of pWN41 (Boone et al., 1983), 0.7 kb BglIII/BglIII gag gene fragment from pRFM16 (Nikbakht et al., 1987), 0.13 kb HindIII/EcoRI fragment from pUC19 clone that carries Sau3A IS insert (Ou et al., 1983), 0.16 kb BssHII/BamHI R-U5 fragment from pFBR-1 (Charles et al., 1984) and 0.35 kb PstI/NcoI p15E env region fragment from pRFM16 (Ch'ang et al., 1989b). The fragments were isolated from polyacrylamide gel by the crushing method. Radio active labeled probes were prepared using the nick translation kit (Amersham). The specific radioactivity was $1-2 \times 10^8$ cpm/ μ g DNA. Lambda HindIII fragments labeled with [γ -³²P]ATP by the end-labeling method or 123 bp ladder (BRL) labeled with [α -³²P]dCTP by the fill-in method were used as DNA molecular weight standard.

CHAPTER III

RESULTS

I. DNaseI-hypersensitive sites in the endogenous MuLV-related LTRs.

DNaseI-hypersensitive sites were mapped by the indirect end-labeling method (Wu, 1980). In this method, isolated nuclei are incubated with a high concentration of DNaseI for 10 minutes at 0°C. I used this limited digestion condition instead of the usual procedure with diluted DNaseI concentration, since it was reported that under long incubation time and/or at high incubation temperatures, endogenous nuclease activity was apparent, and therefore the result became ambiguous (Fritton et al., 1983). After DNaseI digestion, DNA was extracted from the nuclei and subsequently digested with a reference restriction enzyme; then, the specific DNA fragment pattern was analyzed by Southern blotting and hybridization with an appropriate radioactive labeled probe. If there are DNaseI-hypersensitive sites between the restriction enzyme recognition sites, the appropriate probe will detect the smaller fragments which are defined on one side by the DNaseI cut in the nuclei and on the other side by the reference enzyme. Therefore the location of DNaseI-hypersensitive sites can be estimated by measuring the size of the specific DNA fragments. In order to study DNaseI-hypersensitive sites in the endogenous MuLV-related LTRs, I used RFM/Un and NFS/N mouse strains. Both strains

contain more than 30 copies of MuLV-related proviral sequences in contrast with small copy number on MuLV sequences. RFM/Un mouse genome has one ecotropic and a few xenotropic MuLV proviruses; and NFS/N mouse genome has no ecotropic and one xenotropic MuLV provirus. And, the transcripts of MuLV-related proviral sequences were detected in the liver and kidney of these mouse strains (Yang et al., 1989).

DNaseI-hypersensitive sites in the 5' and 3' LTRs.

Nuclei were prepared from mouse livers and digested with 1000 units of DNaseI for 10 minutes at 0° C. From the digested nuclei, whole DNA was isolated and digested with KpnI. The LTR has a KpnI recognition site within the R region, and DNaseI-hypersensitive sites upstream of the KpnI site can be detected by using a LTR probe, as shown in Fig.2. The five LTR fragments generated by combined DNaseI and KpnI digestion are marked by arrows (K-1 through K-5) (Fig.1A). The DNaseI-hypersensitive sites represented by K-1, K-2, K-3, K-4 and K-5 fragments were about 320 bp, 360 bp, 420 bp, 460 bp and 500 bp long, respectively from the 3' end generated by KpnI digestion. The K-3 fragment did not hybridize with the IS probe so it does not contain the IS segment of the LTR (Fig. 1B). This result suggests that the ecotropic- and/or xenotropic-type LTRs have one DNaseI-hypersensitive site near 5' end of U3 region, and the MuLV-related -type LTRs have four DNaseI-hypersensitive sites at the IS and at the 5' portion of U3 region (Fig. 2). The hybridization intensity of the

Figure 1. Autoradiograms showing DNaseI-hypersensitive sites in the 5' and 3' LTRs. Isolated mouse liver nuclei were treated with DNaseI (1000 U/ml, 0° C, 10 min.). High molecular weight DNA was then prepared and digested with KpnI. The digests were analyzed by electrophoresis in a 1.5 % agarose gel and by blot-hybridization with the 0.4 kb PstI/SmaI LTR fragment probe derived from ecotropic or xenotropic type LTRs (panel A) and the 0.13 kb Sau3A/BglII IS sequence-specific probe (panel B). The KpnI-digested DNA from NFS/N mouse liver nuclei without DNaseI treatment (lane 1); NFS/N mouse liver nuclei with DNaseI treatment (lane 2); RFM/Un mouse liver nuclei without DNaseI treatment (lane 3); and RFM/Un mouse liver nuclei with DNaseI treatment (lane 4). Lane M represents the λ -HindIII molecular size marker. The five fragments produced by combined DNaseI and KpnI digestion (indicated by (.), K-1, K-2, K-3, K-4 and K-5 from the smallest fragment) were detected by the LTR probe (Fig. 1A) and the four fragments (K-1, K-2, K-4 and K-5) were detected by the IS probe (Fig. 1B)

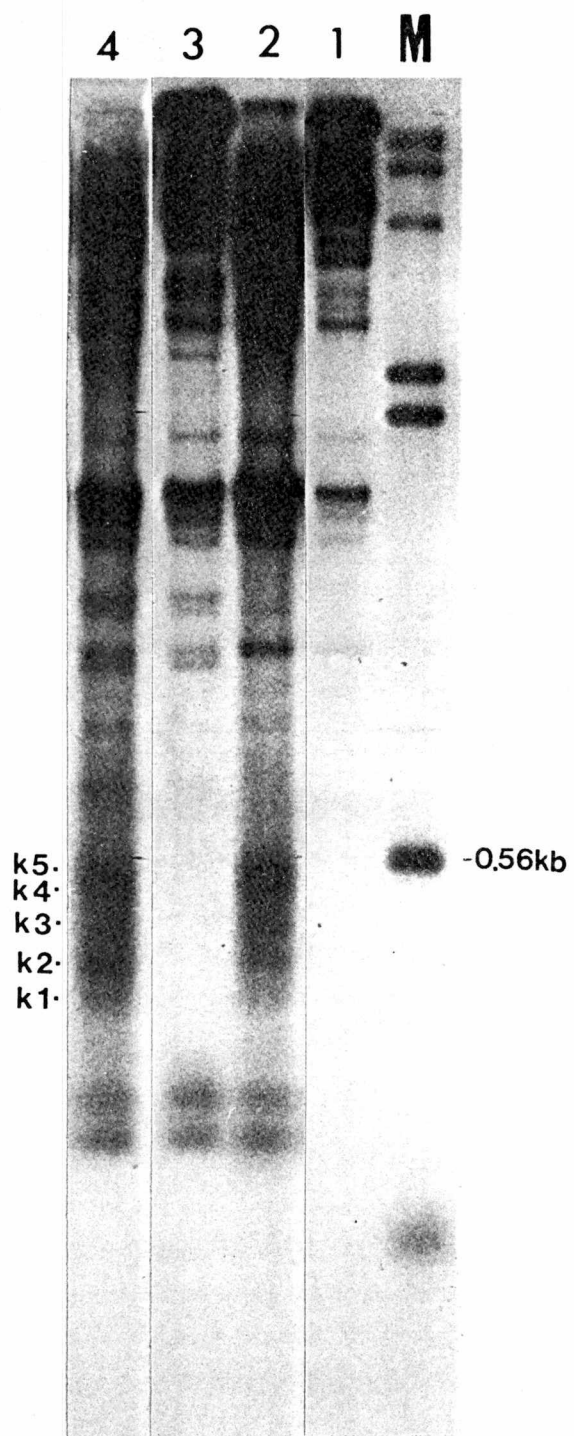
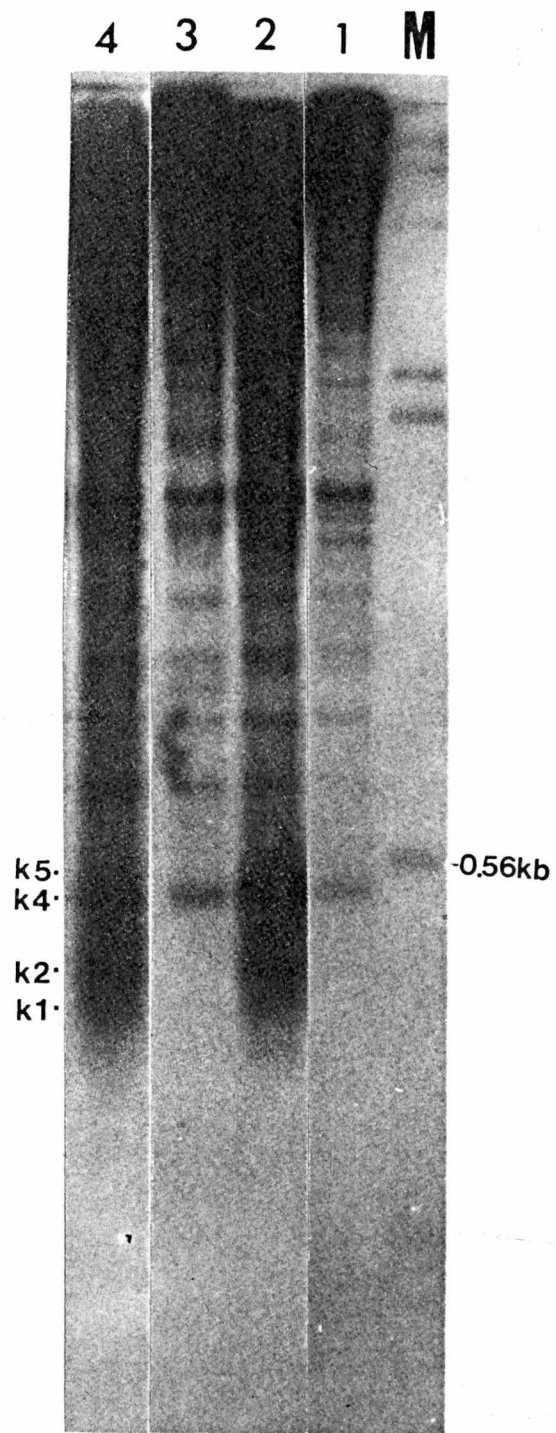
A**B**

Figure 1

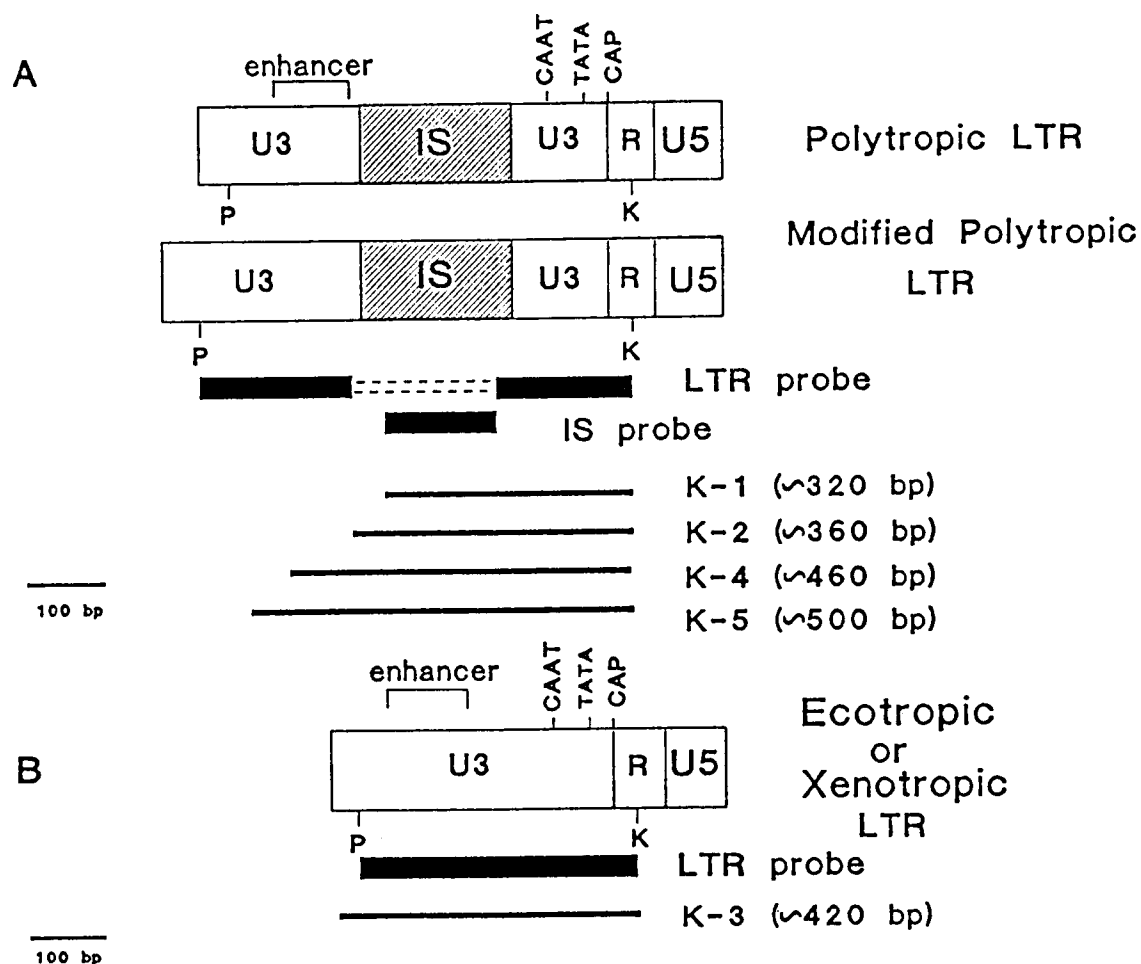


Figure 2. Maps of DNaseI-hypersensitive sites in the 5' and 3' LTRs based on Fig. 1. Panel A depicts the structures of polytypic MuLV-related LTR and modified polytypic MuLV-related LTR. The "enhancer" region and the "promoter" elements are indicated. The size of the polytypic LTR is about 700 bp long while that of the modified polytypic LTR is about 750 bp long due to the additional sequences in the U3 region. The solid bars under the LTR structure indicate the region hybridized with each probe. The open bars under the LTR structure indicate DNaseI-hypersensitive sites, and the number of bars corresponds to that of fragments detected by DNaseI and KpnI digestion. The four fragments, K-1, K-2, K-4 and K-5 were mapped on MuLV-related type LTRs. Panel B depicts the structure of ecotropic or xenotropic MuLV type LTR. The regulatory elements are shown. The K-3 LTR fragment not detected by the IS segment probe is generated from this type of LTR. The number after the name of fragment shows the approximate size of the fragment. Abbreviations: P, PstI; and K, KpnI.

K-2, K-4 and K-5 fragments are relatively stronger than the other two fragments. The DNaseI-hypersensitive site represented by K-5 is located at immediately upstream and downstream of the tandem 14-bp repeat, the presumed negative regulatory element, immediately upstream of the "enhancer region". The intensity of the K-2 fragment in RFM/Un mice was relatively stronger than in NFS/N mice. The hypersensitive site of K-2 was located in the 5' portion of the IS region.

DNaseI-hypersensitive sites in the 5' LTRs. The previous experiments using KpnI as a reference restriction enzyme detected DNaseI-hypersensitive sites in both 5' and 3' LTRs but could not distinguish between them. Therefore, BglII was used as a reference restriction enzyme and the LTR R-U5 sequence was used as a probe in the analysis to detect DNaseI-hypersensitive sites in the 5' LTRs. Xenotropic, polytropic and modified polytropic proviral sequences have common BglII site in 5' portion of gag region. Five fragments, B-1 (~305 bp), B-2 (~380 bp), B-3 (~585 bp), B-4 (~630 bp) and B-5 (~660 bp) were detected (Fig. 3). Three of them, B-3, B-4 and B-5, whose 3' end was generated by BglII digestion were larger than the BglII/BglII LTR fragment of modified polytropic MuLV-related-type and hence were likely derived from the 5' LTR of the polytropic MuLV-related proviral class. The hypersensitive site represented by B-1 was mapped to the cap (transcription initiation) site, and

Figure 3. Autoradiogram showing DNaseI-hypersensitive sites in the 5' LTR. High molecular weight DNA isolated from DNaseI-treated nuclei was digested with BglII. The digestion pattern was analyzed by electrophoresis in a 1.5 % agarose gel and blot-hybridization with the LTR R-U5 region probe (the 0.16 kb BssHII/BamHI fragment from pFBR-1). The BglII DNA preparations analyzed include those from RFM/Un mouse liver nuclei without DNaseI treatment (lane 1); from RFM/Un mouse liver nuclei with DNaseI treatment (lane 2); from RFM/Un mouse kidney nuclei with DNaseI treatment (lane 3); and from RFM/Un mouse testis nuclei with DNaseI treatment (lane 4). Lane M indicates the 123 bp ladder DNA molecular size standard. The five LTR DNA fragments produced by combined DNaseI and BglII digestion were designated as B-1, B-2, B-3, B-4 and B-5 (B-1 being the smallest fragment). The strong 0.46 kb fragment seen in all lanes represents the BglII fragment generated from the modified polytropic class proviral structures which contain a BglII site in the 3' side of the 5' LTR and a BglII site at the 3' end of the IS segment. This indicates that the hypersensitive sites represented by B-3, B-4 and B-5 were mapped to the U3 region of polytropic type LTR.

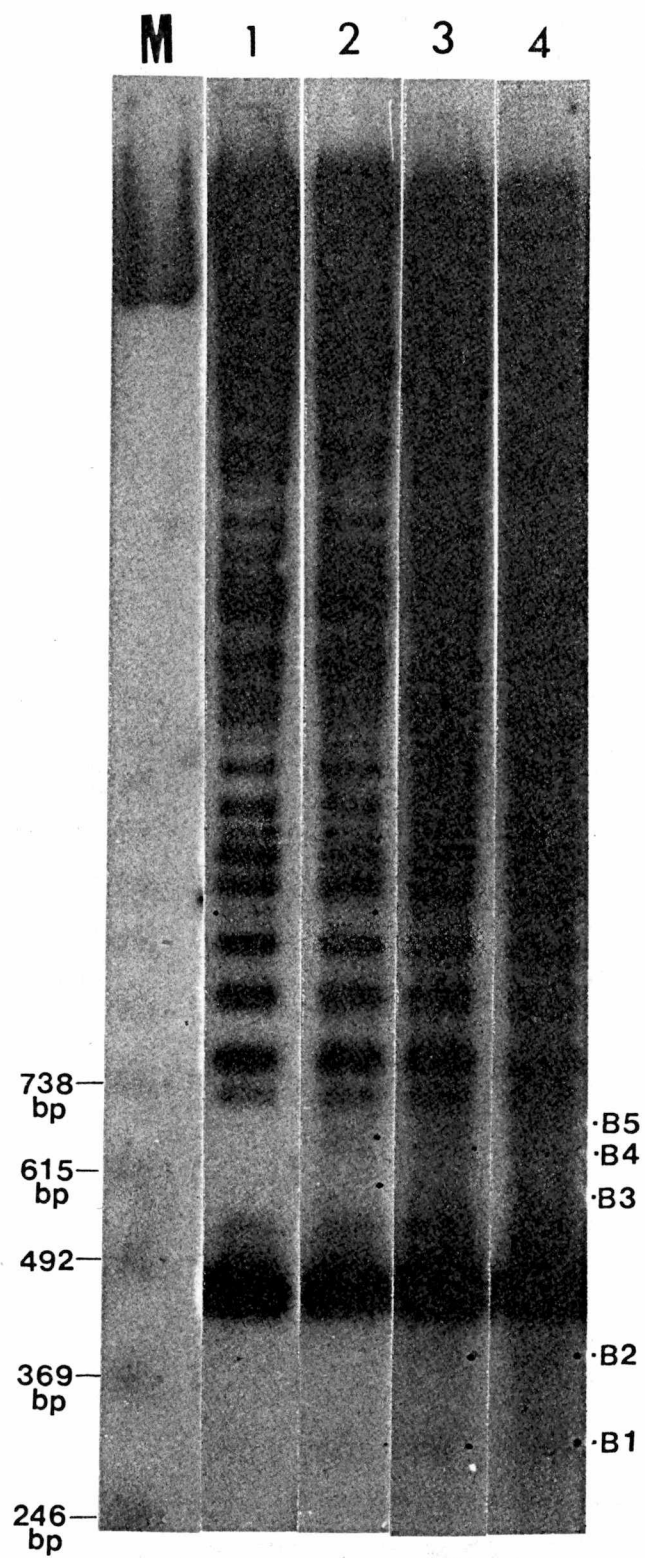


Figure 3

that of B-2 to near the "CAAT" box. The hypersensitive site of B-4 and B-5 were mapped to immediately upstream and downstream of the 14 bp repeat region (Fig. 5).

DNaseI-hypersensitive sites in the 3' LTRs. In order to detect DNaseI-hypersensitive sites in the 3' LTR distinctively, I used SmaI, SstI and EcoRI as reference restriction enzymes and the 15E sequence as the molecular probe. Three fragments, S-1 (~1.8 kbp long), S-2 (~2.0 kbp long) and S-3 (~2.1 kbp long), whose 5' end was generated by SmaI digestion were detected by DNaseI and SmaI digestion (Fig. 4A). If I postulate that the DNaseI-hypersensitive sites are located in the LTR, those sites are mapped as indicated in Fig. 5. However, the precise location of the hypersensitive sites in the LTR could not be determined by SmaI because two SmaI sites are close in the env region: in addition, since SmaI is methylation sensitive enzyme, methylation status of the recognition site should influence detection of DNaseI-hypersensitive sites. Next, SstI and EcoRI were used. Four SstI fragments, St-1 (~770 bp long), St-2 (~815 bp long), St-3 (~920 bp long) and St-4 (~960 bp long), whose 5' end was generated by SstI digestion and four EcoRI fragments, E-1 (~1.33 kbp long), E-2 (~1.43 kbp long), E-3 (~1.53 kbp long) and E-4 (~1.60 kbp long), whose 5' end was generated by EcoRI digestion were detected by the 15E probe (Fig. 4B and Fig. 5). Among four SstI fragments, the strongest fragment St-3 was mapped to the U3 region

Figure 4. Autoradiograms showing DNaseI-hypersensitive sites in the 3' LTR. High molecular weight DNA was extracted from nuclei after DNaseI treatment and digested with SmaI, SstI or EcoRI. The generated DNA fragments were analyzed by electrophoresis in a 1.5 % agarose gel and blot-hybridization with the 0.35 kb PstI/NcoI p15E fragment probe derived from the env region of pRFM16. Panel A shows the SstI fragment patterns from RFM/Un mouse liver nuclei without DNaseI treatment (lane 1) and with DNaseI treatment (lanes 2 and 3), as well as the EcoRI fragment patterns from RFM/Un mouse liver nuclei without DNaseI treatment (lane 4) and with DNaseI treatment (lane 5). The DNA molecular size standard (lane M) is 123 bp ladder marker. Four DNA fragments, St-1, St-2, St-3 and St-4 (St-1 being the smallest) were produced by combined DNaseI and SstI digestion. Combined DNaseI and EcoRI digestion also produced four DNA fragments, E-1, E-2, E-3 and E-4 (E-1 being the smallest). Panel B shows the SmaI fragment patterns from RFM/Un mouse liver nuclei without DNaseI treatment (lane 1); and from DNaseI-treated RFM/Un mouse liver nuclei (lane 2). Three DNA fragments generated by combined DNaseI and SmaI digestion (S-1, S-2 and S-3, S-1 being the smallest fragment) were detected by the p15E probe.

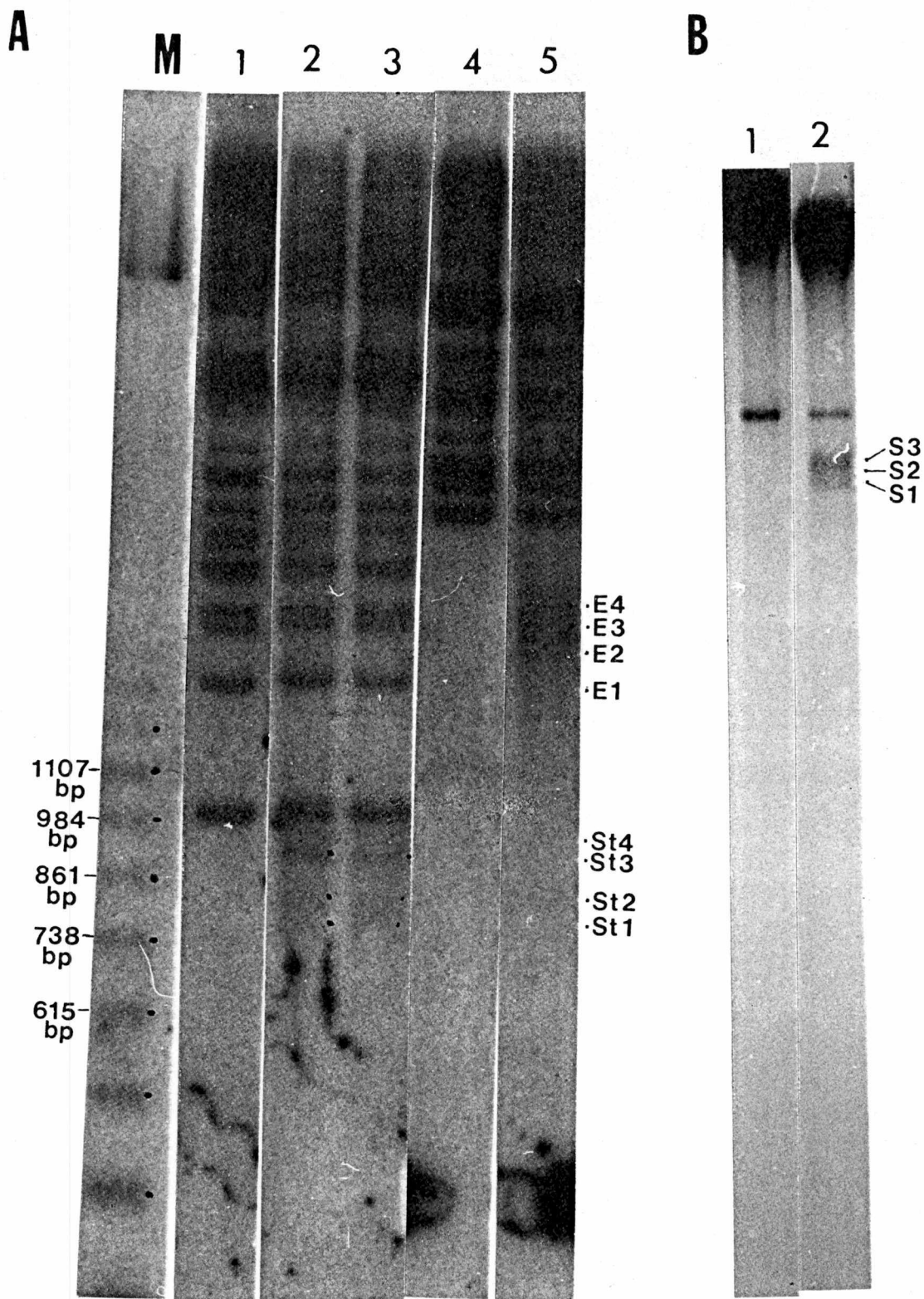


Figure 4

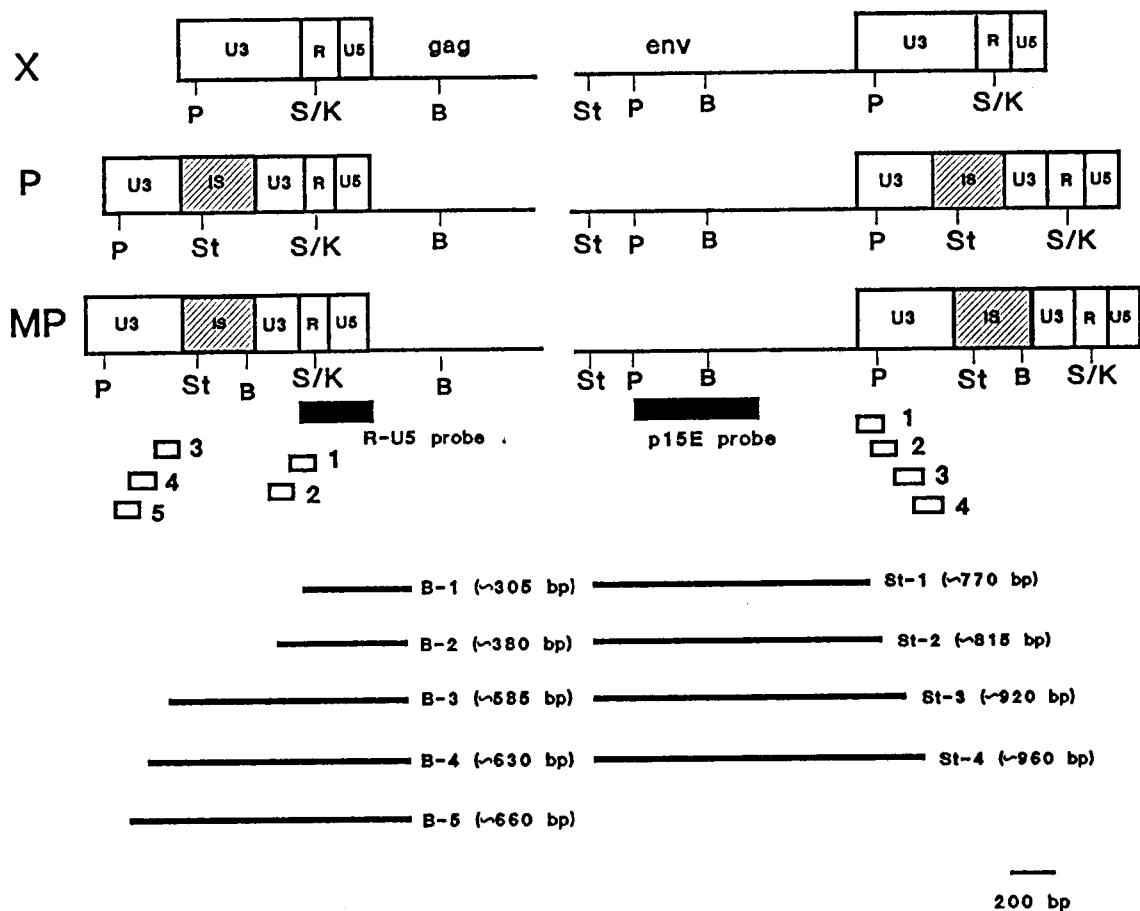


Figure 5. Map of DNaseI-hypersensitive sites in the 5' or 3' LTR, as summarized from the data of Fig.3 and Fig. 4B. The structures of three types of proviral sequences and the probes used (solid bars) were indicated. The open bars indicate DNaseI-hypersensitive sites, and the number of bars correspond to that of fragments detected by DNaseI and BglII or by DNaseI and SstI digestions. The number inside () represents the approximate size of the fragment. Abbreviations: P, PstI; S, SmaI; K, KpnI; B, BglII; St, SstI; X, xenotropic sequence; MP, modified polytropic sequence; and P, polytropic sequence.

immediately upstream of the IS sequence. The strong EcoRI fragment E-4 was mapped to the same region. And, the hypersensitive site represented by St-2 fragment was mapped to immediately upstream of the 14-bp repeat.

In the experiments using BglII, SmaI, SstI and EcoRI, the MuLV-type (ecotropic and xenotropic) and MuLV-related-type (modified polytropic and polytropic) LTRs have not been distinguished because the IS probe detects other types of endogenous provirus families and therefore shows quite complicated patterns by using those restriction enzymes.

It is known that in NFS/N mouse liver and kidney the RNA transcription level of MuLV and related sequences is high, but that there is little transcription in brain (Yang et al., 1989). The preliminary result of an experiment to investigate DNaseI hypersensitivity in liver and brain suggested that there was no distinct difference between those tissues.

II. Restriction endonuclease sensitivity of the endogenous MuLV-related proviral sequences.

DNaseI has been used most widely for research on chromatin structure because of its high selectivity for nucleosome free regions and relatively low DNA sequence specificity. However, it has the disadvantage that the analysis of DNaseI-hypersensitive sites is sometimes difficult due to the broad digestion region. In contrast, restriction enzymes show high sequence specificity and therefore can

generate distinct fragments. MuLV and MuLV-related sequences all contain a common PstI site within the 5' portion of the U3 region of their LTRs. The DNaseI study showed that hypersensitive sites were located around the PstI site (Fig. 5) and the genomic footprinting experiment and the electrophoretic mobility shift assay done by P. Hoyt suggested that one of the protein binding sites was located near the PstI site (personal communication). That is the reason I chose PstI sensitivity for the study of chromatin configuration in LTR.

Determination of optimal experimental conditions. In the initial experiment, two methods were used and compared for preparing nuclei from liver, which differ mainly in the buffer solution used for tissue homogenization and subsequent differential centrifugation of the nuclei. The buffer solution containing spermine and spermidine provided better results than the solution containing $MgCl_2$, as indicated by the extent of DNA degradation due to endogenous nuclease activity during incubation at 37° C in the restriction enzyme core buffer. And, nuclei should be prepared freshly from fresh tissues or frozen tissues to avoid DNA degradation. Isolated nuclei from either frozen or fresh tissues were suspended well in the core buffer at a DNA concentration of 300-500 µg/ml and digested with increasing amounts of PstI for an identical period or with identical amounts of PstI for various periods. When 1 unit of PstI per µg DNA was used, 15 minutes of

Figure 6. Autoradiogram showing PstI restriction endonuclease sensitivity of endogenous MuLV and related proviral sequences in NFS/N mouse liver with increasing incubation times. Isolated nuclei were digested with PstI (1 U/ μ g DNA) for various periods of time: lane 1, no incubation; lane 2, 15 min.; lane 3, 30 min.; lane 4, 60 min.; and lane 5, 120 min. DNA fragments generated by the PstI digestion in the nuclei were extracted by the 0.35 M NaCl elution method, and the patterns were analyzed by electrophoresis in a 0.7 % agarose gel and by blot-hybridization using an 0.4 kb PstI/SmaI LTR fragment probe. Lane C represents the PstI digestion pattern of the proviral sequences in purified genomic DNA hybridized with the LTR probe. The λ -HindIII fragments were employed as molecular size markers (lane λ H).

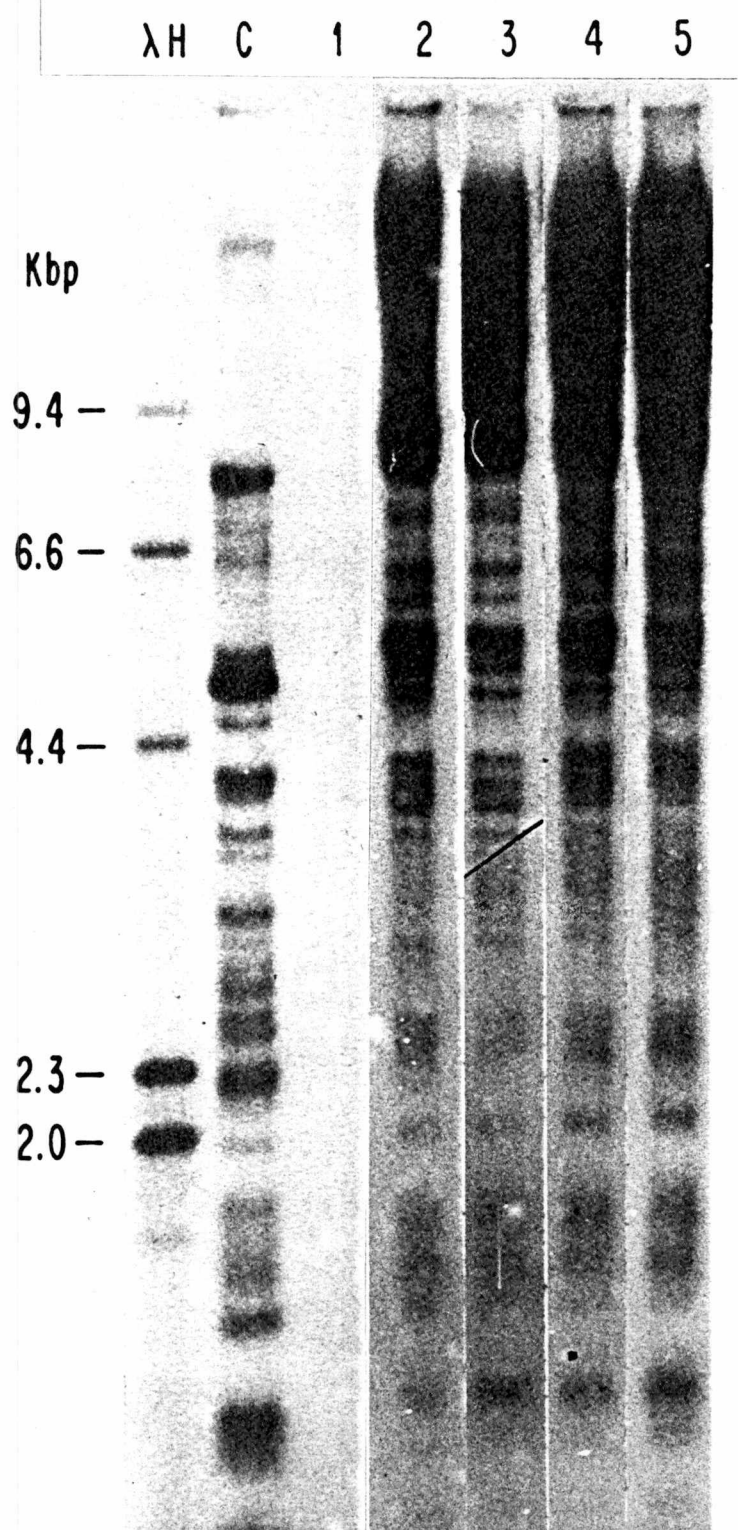


Figure 6

digestion period was sufficient (Fig. 6). When the nuclei were digested for 2 hours, 0.15 unit of PstI per μ g DNA was effective (Fig. 7). In order to isolate PstI-digested DNA fragments, three different methods were tried. The PstI-digested DNA fragments were expected to be relatively small size DNA and to be associated with proteins which were related to active chromatin configuration. The alkaline extraction method (Maniatis et al., 1982), in which small molecular DNA, such as the plasmid DNA, was extracted after lysis by alkali and neutralization, did not provide good results for this purpose. Therefore, I used the modified Hirt extraction method (Yang et al., 1980), which is used for isolation of small molecular weight DNA, such as free viral DNA, and the salt elution method (Weisbrod, 1982), in which nonhistone protein of high mobility group (HMG) associated with active nucleosomes was eluted in 0.35 M NaCl.

PstI sensitivity of endogenous proviral sequences.

The endogenous proviral sequences which were selectively digested by PstI in the nuclei were analyzed by electrophoresis, transferred to nylon membrane and hybridized with various MuLV probes. In isolated nuclei, a restriction enzyme presumably does not cleave chromatin DNA at all recognition sites except at the particular sites in nucleosome free regions. In the purified genomic DNA, the LTR probe and gag-pol probe recognized the two major expected PstI fragments of MuLV-related sequences, 4.8 kbp long fragment from modified

polytropic sequences and 7.7 kbp long fragment from polytropic sequences, (Fig. 7A,B, lane C and Fig 8), while in the PstI digested DNA in the nuclei those two bands were not major ones, and some new bands appeared (Fig. 7A,B, lanes 4, 5 and 6). For example, the LTR, gag-pol and p15E probes all detected new a 8.4 kbp fragment generated in the nuclei, and this fragment was confirmed to hybridize with the IS probe (data not shown). Therefore, it is suggested that this fragment was created in the nuclei by PstI digestion at its recognition site in the 5' LTR and in the 3' LTR but not those in the structural gene regions of MuLV-related sequences. The LTR and gag-pol probes detected two strong extra bands, 6.0 kbp and 5.5 kbp long, in the PstI digested DNA (Fig. 7A and 7B, lanes 4,5 and 6). The major fragment detected by the p15E probe in the purified genomic DNA was a 0.6 kbp long fragment which corresponds to the PstI fragment from the env region to the 3' LTR, whereas in the chromatin the major band was 8.4 kbp long fragment, and two new bands, 7.0 kbp and 5.5 kbp long, were detected (Fig. 7C, lanes 4, 5 and 6). According to the physical map about PstI shown in Fig.8, these fragments seem to derive from MuLV-related sequences which have internal deletion(s) because the IS probe could detect them (data not shown). The PstI restriction fragments derived from the MuLV-related sequences which have internal deletion(s) were preferentially detected in the nuclei in spite of their minor population, suggesting that these

Figure 7. Autoradiograms showing PstI restriction endonuclease sensitivity of endogenous MuLV and related proviral sequences in NFS/N mouse liver as indicated by increasing enzyme concentrations. Isolated nuclei were digested by various unit of PstI per μ g DNA at 37° C for 2 hours. Lane 1, no enzyme; lane 2, 0.015 U; lane 3, 0.075 U; lane 4, 0.15 U; lane 5, 0.75 U; and lane 6, 1.5 U. After PstI digestion of the nuclei, DNA was extracted by the modified Hirt extraction method and the patterns were analyzed by electrophoresis in a 0.7 % agarose gel and by blot-hybridization with the 0.4 kb PstI/SmaI LTR fragment probe (panel A), with the 0.7 kb BglII/BglII gag region fragment probe from pRFM16 (panel B) and with the 0.35 kb PstI/NcoI p15E env region fragment probe(panel C). Lane C represents PstI digestion pattern of purified genomic DNA. Lane λ H represents λ -HindIII molecular size markers.

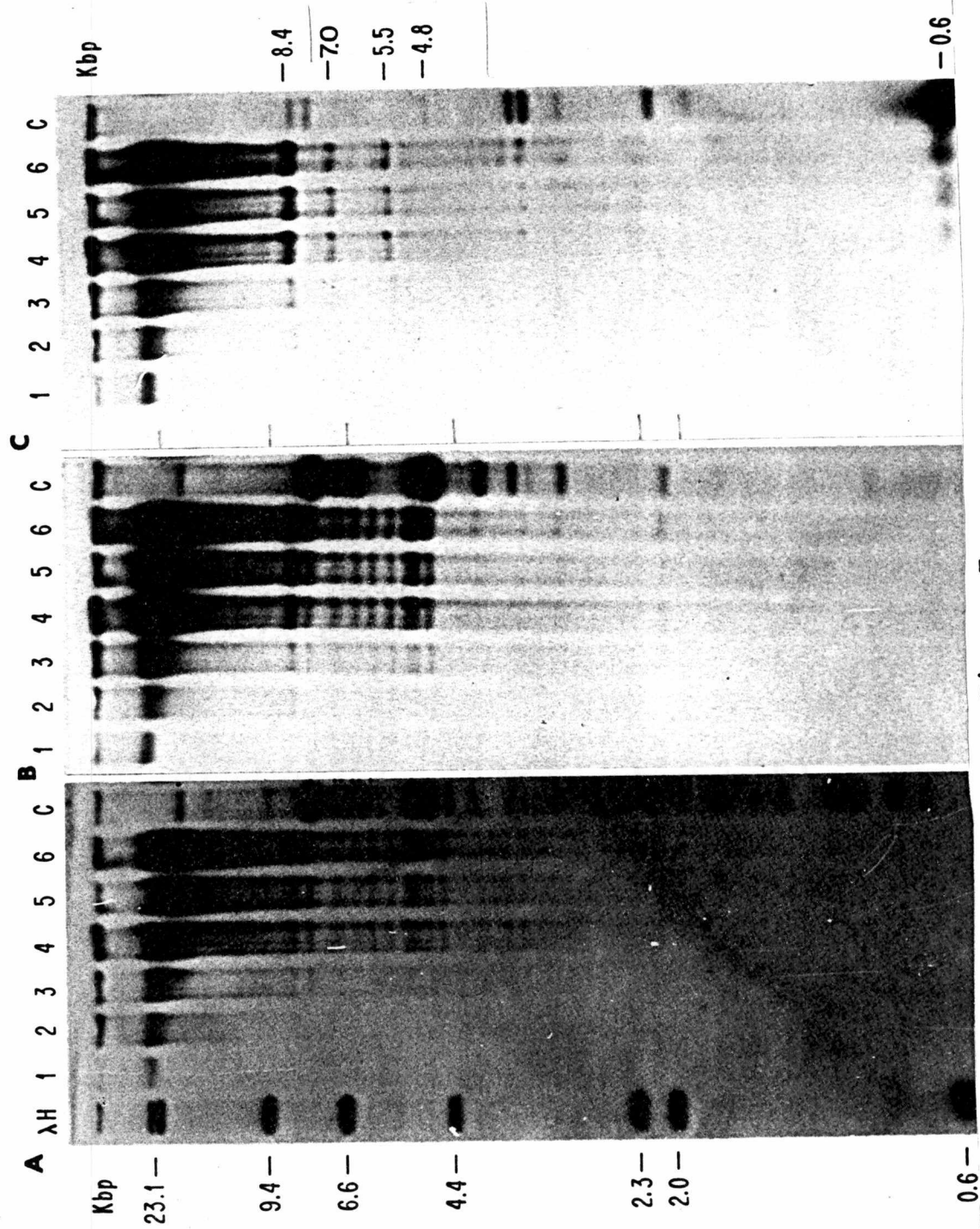


Figure 7

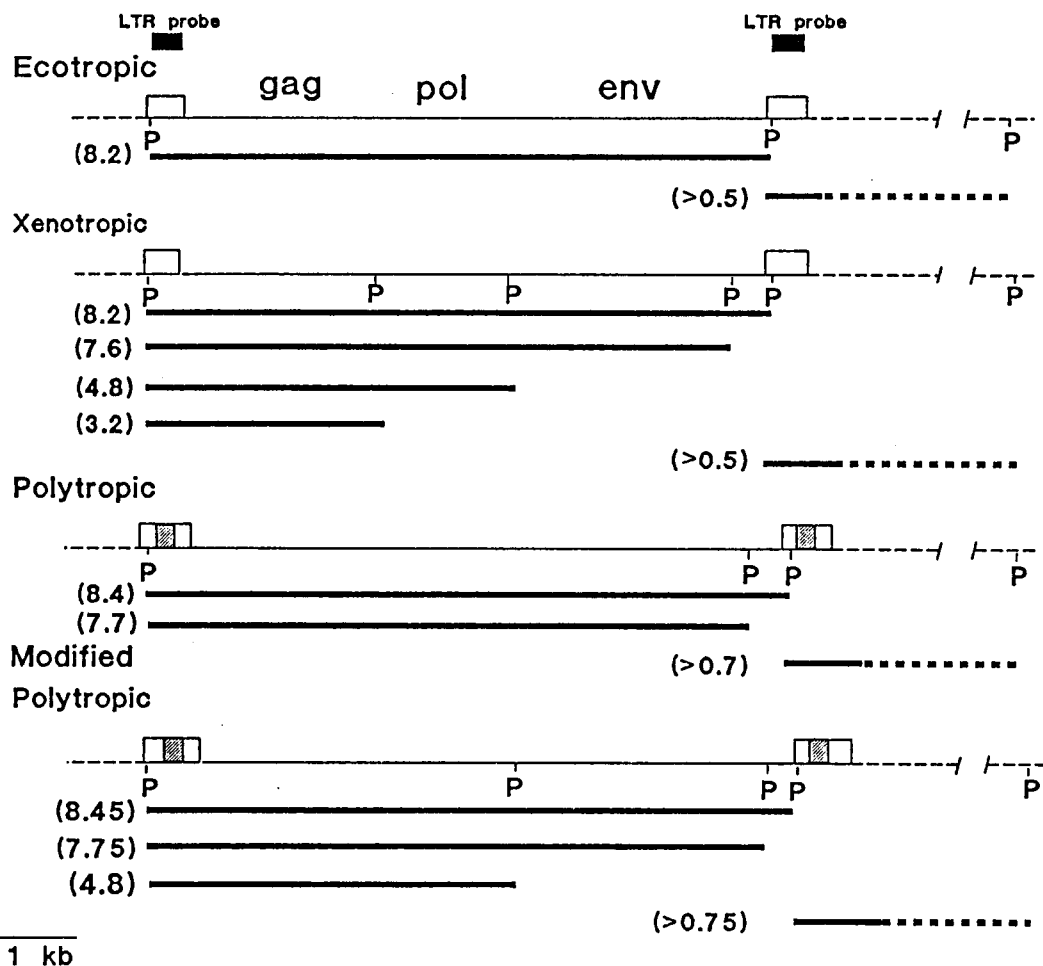


Figure 8. Physical map of four classes of endogenous MuLV and related sequences. The fragments shown under each map represent the expected PstI fragments detected by hybridization with the LTR fragment probe. The number inside () indicates approximate base pairs of the fragment.

specific sequences are active. This result is consistent with the previous molecular cloning studies reporting the high isolation frequency of the proviral sequences with deletions (Roblin et al., 1982; Khan et al., 1982). The results of the PstI sensitivity experiment indicated that the different restriction fragment pattern between the purified genomic DNA and the digested DNA in the nuclei was due to selective digestion of PstI only at its recognition site in the nucleosome free region. So, the MuLV-related sequences which were released from the isolated nuclei by restriction endonuclease represented a specific population expressing RNA transcripts detected in mouse liver (Yang et al., 1989). In subsequent experiments, PstI sensitivity of MuLV-related proviral sequences was investigated in other tissues. In the kidney and testis of RFM/Un mice, the DNaseI-hypersensitive sites could also be detected (Fig. 3), and the band pattern and intensity of PstI fragment digested in the nuclei of these tissues were the same as those of liver (data not shown).

It is known that male B6C3 mice and male C3Hf mice have a higher incidence of hepatoma than female of the same strain, and Dragani et al. reported that in male mice of these strains an increase in the expression of retrovirus-related sequences (Mo-MuLV, intracisternal A particle-related sequences and VL30-related sequences) in the liver after chemical treatment and in liver tumors (Dragani et al., 1986; Dragani et al., 1987). Considering these facts, I compared PstI sensitivity

Figure 9. Autoradiogram showing EcoRI and BamHI sensitivity of endogenous MuLV and related sequences in NFS/N mouse liver. Isolated nuclei were digested with EcoRI (lane 1) or BamHI (lane 3) (1 U/ μ g DNA, 1 hour), and digested DNA was extracted by the modified Hirt method. Total genomic DNA was digested with EcoRI (lane 2) or with BamHI (lane 4). The digestion patterns were analyzed by electrophoresis in a 0.7 % agarose gel and by blot-hybridization with the 0.4 % PstI/SmaI LTR fragment probe. The λ -HindIII fragment size marker was used. (lane λ H).

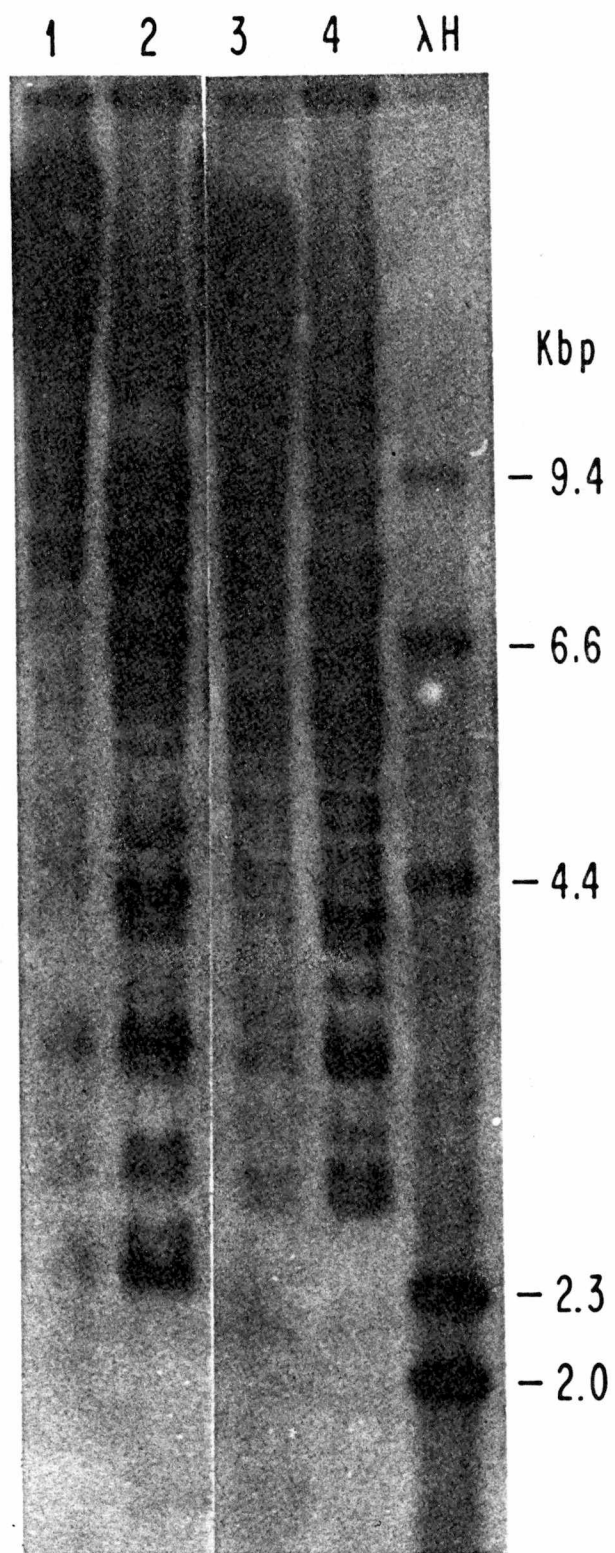


Figure 9

of MuLV and related sequences in the chromatin between C3H male and female mice in order to investigate whether the specific MuLV family could be detected only in male. So far the results indicate a quantitative difference of PstI sensitivity between young C3H male and female mice, that is, the intensity of PstI fragments digested in the nuclei of male C3H mice was stronger than that of female mice; nevertheless any qualitative difference could not be seen (data not shown).

The studies using other restriction enzymes such as EcoRI and BamHI were performed (Fig. 9), and those restriction digestion patterns in the nuclei were different from those obtained by using the purified genomic DNA because the restriction enzymes in the nuclei can cleave DNA only at the recognition sites which are accessible to the enzyme.

III. Methylation of the endogenous MuLV-related LTRs.

The LTRs of MuLV and related proviral sequences have common SmaI and KpnI recognition sites in the R region. SmaI is a methylation sensitive enzyme, so it can not cleave DNA if the cytosine residue of middle CG sequence in the recognition site is methylated; while KpnI is insensitive to methylation. The SmaI site in the LTR partially overlaps with the KpnI site, therefore the double digestion of mouse DNA with PstI and KpnI or PstI and SmaI generates distinctive internal LTR fragment which varies in the size according to the length of the U3 region. Ecotropic or xenotropic MuLV proviral sequences are expected to generate the 380 bp long

fragment (Fig. 11). In NFS/N mouse, one xenotropic and solitary LTRs contribute this size of PstI-SmaI/KpnI fragment; while in RFM/Un mouse, one ecotropic and a few xenotropic sequences as well as solitary LTRs contribute. The 550 bp and 590 bp long fragments are expected to be derived from endogenous MuLV-related polytropic and modified polytropic proviruses, respectively (Fig. 11). The extent of methylation at the SmaI site in individual type of LTR can be investigated by comparing intensities of the distinctive PstI-KpnI and PstI-SmaI bands.

In the liver of RFM/Un mice, polytropic MuLV-related proviral sequences were preferentially digested with PstI in the nuclei and the SmaI site in this type of LTR was undermethylated because the intensity of 550 bp fragment (band b), which was expected to be derived from polytropic LTRs, in subsequent SmaI and KpnI digestion (Fig. 10A, lanes 2 and 3) was stronger than the other two fragments, 590 bp and 380 bp (bands a and c), which were expected to be derived from modified polytropic-type LTR and MuLV-type LTR, respectively.

In the liver of NFS/N mice, the intensity of 380 bp PstI-KpnI fragment was comparable to that of 380 bp (band c) PstI-SmaI fragment in the genome, indicating undermethylation of this type of LTR at the SmaI site (Fig. 10C, lanes 5 and 6), and this result is consistent with the previous result (Kuemmerle et al., 1987). The SmaI site in the LTR defining MuLV-related LTRs (590 bp and 550 bp, bands a and b) were

relatively methylated in the NFS/N mouse genome (Fig. 10C, lanes 5 and 6), but if the PstI site of the LTR was cleaved in the nuclei, the SmaI site of the LTR seemed to be completely unmethylated (Fig. 10C, lanes 2 and 3). In the testis of RFM/Un and NFS/N mice, the SmaI site in all three types of LTRs were heavily methylated (Fig. 10B, 10D). The PstI sensitivity and methylation status of SmaI site in the LTR seemed not to have relationship in the testis. In contrast to the liver, in the testis MuLv-related LTRs were heavily methylated at the SmaI site even though they were digested with PstI in the nuclei; and the 590 bp LTRs (modified polytropic type, band a) were more methylated than 550 bp LTRs (polytropic type, band b) at the SmaI site.

Some extra bands which were not seen in the genomic DNA appeared in PstI-SmaI and PstI-KpnI lanes of the Hirt supernatant after PstI digestion of chromatin (Fig. 10, lanes 2 and 3). The larger fragment than 380 bp band was 430 bp long, and the smaller fragment seen in lane 2 of Fig. 10C was 330 bp long. Because these SmaI or KpnI bands were detected only after PstI digestion in the nuclei, their one end might be generated by endogenous endonuclease cleavage at the hypersensitive site during PstI digestion.

Figure 10. Autoradiograms showing DNA methylation patterns of endogenous MuLV and related LTRs in liver of RFM/Un mice (panel A), in testis of RFM/Un mice (panel B), in liver of NFS/N mice (panel C) and in testis of NFS/N mice (panel D). The Hirt supernatant DNA extracted from PstI-digested nuclei (1 U/ μ g DNA, 1 hour) was analyzed directly (lane 1) or following further digestion with SmaI (lane 2) or following further digestion with KpnI (lane 3). Paralleled experiments were performed in which total genomic DNA was digested with PstI (lane 4), PstI and SmaI (lane 5) or PstI and KpnI (lane 6). The patterns were analyzed by electrophoresis in 1.5 % agarose gel and by blot-hybridization with the 0.4 kb PstI/SmaI LTR fragment probe. Lambda phage HindIII DNA fragments (λ H) were used as DNA molecular size standard. The 0.59 kb (band a), 0.55 kb (band b) and 0.38 kb (band c) PstI/SmaI or PstI/KpnI LTR fragments are derived from modified polytropic type LTR, polytropic type LTR and ecotropic or xenotropic type LTR, respectively. The DNA methylation level of the SmaI site in these three classes of LTRs may be determined by comparing for relative intensity of these DNA fragments between lane 2 and 3 as well as between 5 and 6.

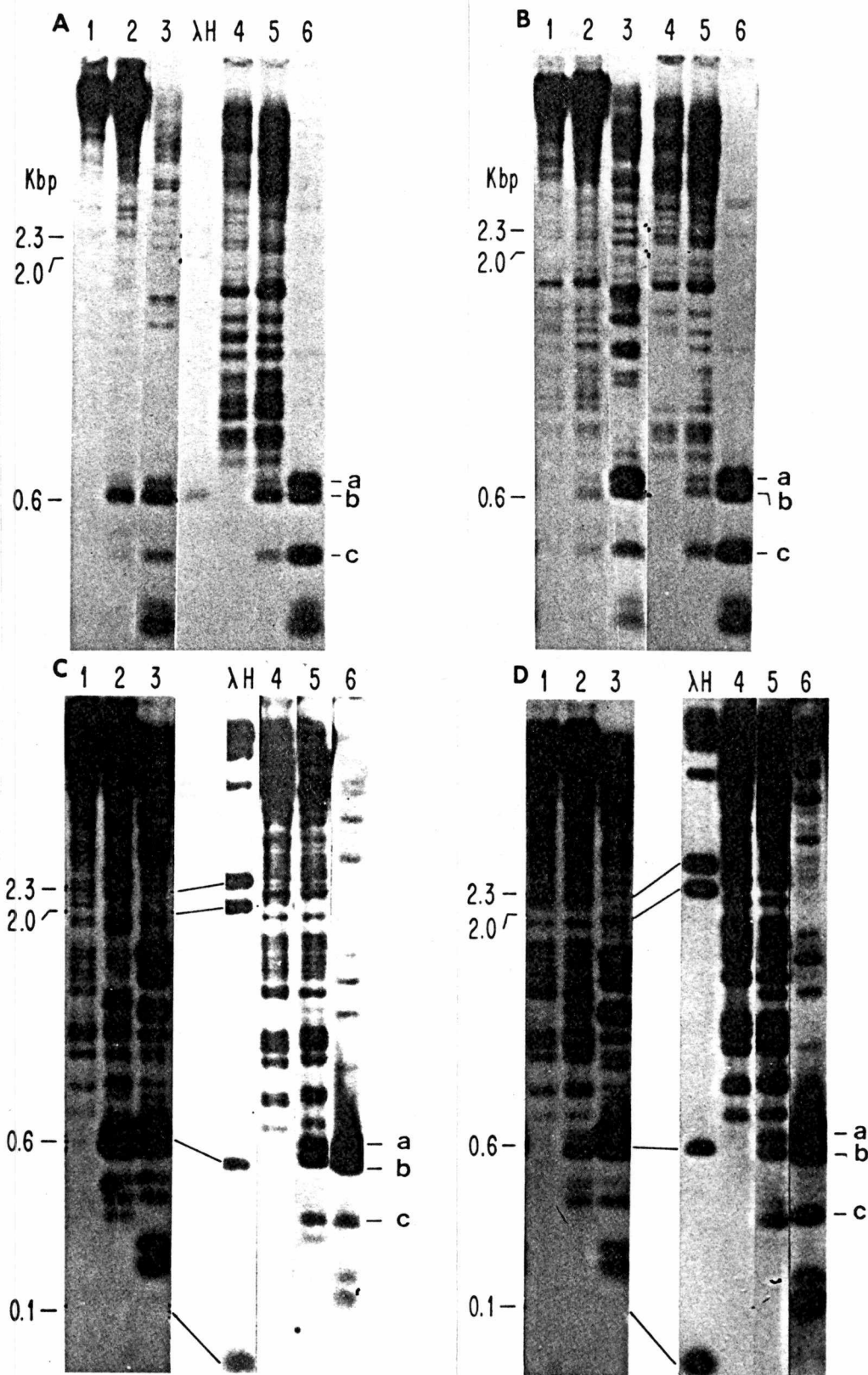


Figure 10

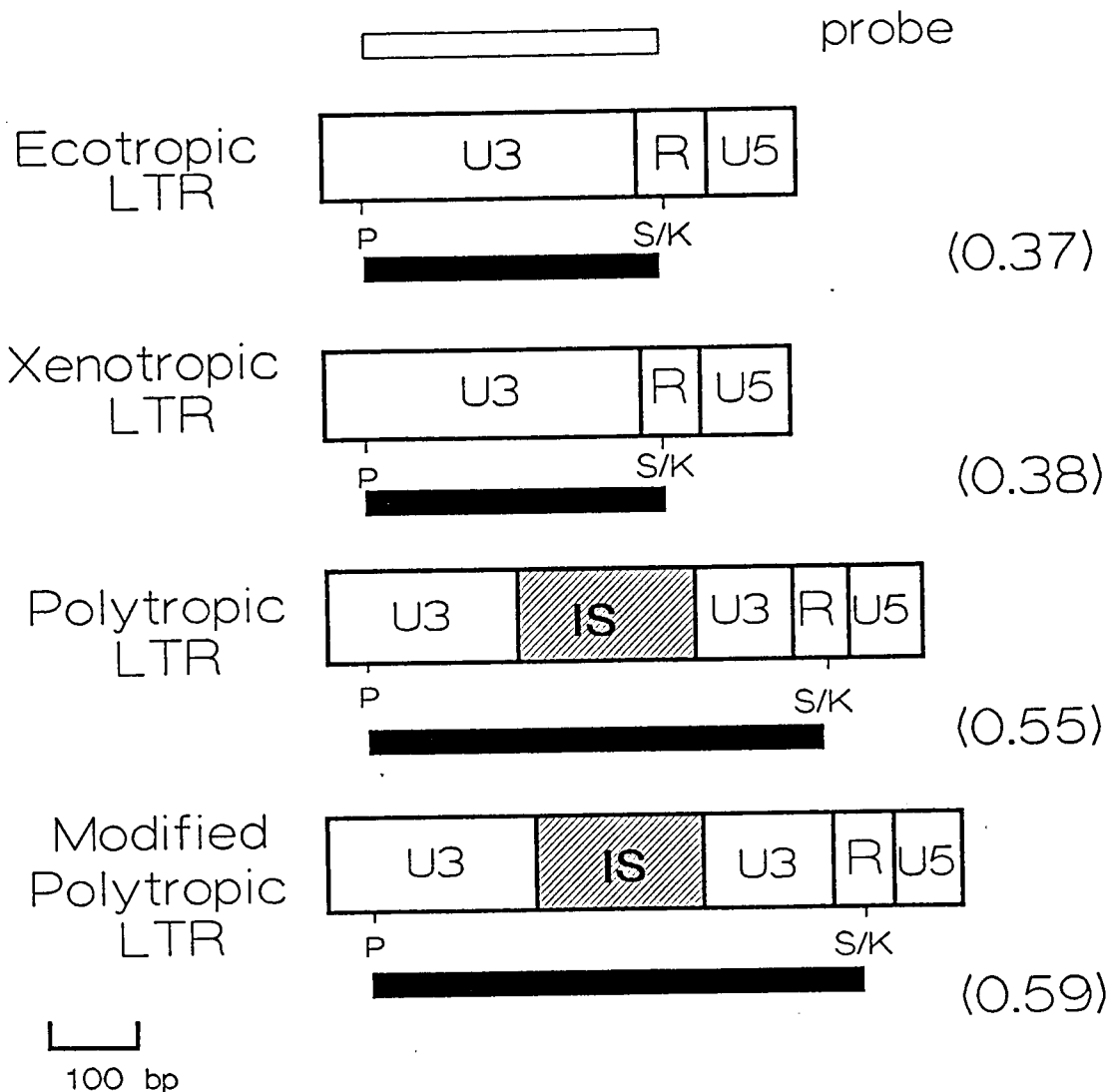


Figure 11. Map of four classes of MuLV and MuLV-related LTRs. The bar under the map shows the expected PstI/SmaI or PstI/KpnI fragments that can be detected by the LTR fragment probe. The probe used is shown in the top of the map. The number inside () represents approximate base pairs of the fragment. Abbreviations: P, PstI; S, SmaI; and K, KpnI.

CHAPER IV

DISCUSSION

In indirect end-labeling studies using DNaseI, MuLV-related proviral sequences showed several strong DNaseI-hypersensitive sites in the LTR, for example, at both the upstream and the downstream regions of the 14-bp repeat negative regulatory element in the 5' and 3' LTRs, "cap" (transcription initiation) site and "CAAT" box in the 5' LTR, and the U3 region immediately upstream of the IS segment in the 3' LTR.

Although DNA sequences of the 5' LTR and 3' LTR are the same, they may have different functions. For example, in the transcription of retroviral RNA, the 5' LTR provides the "promoter" functions while the 3' LTR provides the poly(A) addition signal functions. Therefore, due to differences in their function, the 5' and 3' LTRs of the same proviral sequence may be different in chromatin structure. The different DNaseI-hypersensitive sites in the 5' and 3' LTRs have been reported. The Moloney murine leukemia virus (Mo-MuLV)-related retroviral vector carrying the bacterial neomycin resistance gene was integrated in cultured cells and chromatin configuration of the integrated sequences was studied. Two strong DNaseI-hypersensitive sites in the 5' LTR were found to overlap the enhancer sequences and were close to the start site for viral transcription whereas one

hypersensitive site in the 3' LTR was found at the signal required for addition of a poly(A) trail (Rasmussen and Gilboa, 1987). In another experiment on the chromatin structure of integrated Mo-MuLV proviral DNA, strong DNaseI-hypersensitive sites in the 5' LTR were mapped to enhancers and the transcription initiation site while hypersensitive sites in the 3' LTR were located only in the enhancers (Thompson and Fan, 1985). My results also support the idea that the chromatin configuration of the 5' LTR and 3' LTR are different because DNaseI-hypersensitive sites were detected in different region between the 5' LTR and 3' LTR.

Since mouse cells contain multiple copies of endogenous MuLV and MuLV-related proviral sequences, the DNaseI hypersensitivity measured in these experiments represents the average sensitivity of multiple copies. In order to investigate DNaseI hypersensitivity of individual proviral sequences, preparation of cloned cellular flanking sequences as hybridization probe will be required.

There are a number of agents, useful for probing hypersensitive sites, which cleave DNA or modify bases within hypersensitive sites. One of the methods widely used to study chromatin structure is DNaseI digestion of nuclei followed by indirect end-labeling of the resulting fragments because DNaseI has high selectivity for nucleosome free regions and relatively low DNA sequence specificity (Dinwall et al., 1981; Drew, 1984); however, this technique has

limitations that may lead to ambiguity in positioning hypersensitive sites. First, the accuracy of hypersensitive site determination by large fragments (e.g. >1 kb) is relatively poor (+ 50 bp). Second, the migration of fragments which contain internal nicks by DNaseI digestion might be different from that of control DNA (without DNaseI treatment). To solve these problems, high-resolution techniques such as genomic footprinting, primer extension and exonuclease barriers can be used.

Two types of endogenous MuLV-related proviral sequences (polytropic and modified polytropic) are unable to be expressed directly into retroviruses in spite of the fact that most of the proviral sequences are not defective. The transient chloramphenicol acetyltransferase (CAT) expression assay revealed that the 3' portion of the MuLV-related LTRs was a functional promoter and the IS segment in the middle of the LTR was a potential enhancer while the 5' portion of these types of LTRs showed negative effects, presumably due to the 14-bp direct repeat sequence located immediately upstream of the "enhancer" region (Ch'ang et al., 1989a). This result suggests that the expression of MuLV-related proviral sequences are controlled by the negative regulatory element within the LTRs. The mechanism of this negative regulation is not clear yet; but it can be imagined that this cis-acting element has a chromatin structure accessible to trans-acting factor(s) for suppression of gene expression. My result

which showed DNaseI-hypersensitive sites surrounding the 14-bp direct repeat negative regulatory element in the MuLV-related proviral LTRs was supported by the studies using the genomic footprinting experiment and the electrophoretic mobility shift assay, in which it was reported that one of the protein binding sites was located in 14-bp direct repeat in polytropic and modified polytropic MuLV-related LTRs (P. Hoyt personal communication). Hypersensitive sites are associated with nucleotide sequences that play important functions in regulatory processes such as transcription, replication, recombination or chromatin segregation. Such functional sequences include enhancers, silencers, upstream activation sequences, promoters, terminators, replication origins, topoisomerase sites, recombination loci, centromeres and telomeres (Gross, 1988). It was also reported that 5'-hypersensitive sites can be experimentally induced in the absence of transcriptional activation of the chicken adult globin genes (Weintraub et al., 1982). Hence, hypersensitivity is necessary, but not sufficient for the DNA sequences to exert their function. Hypersensitivity is one of the steps to commit a sequence to be active in chromatin.

Since a restriction endonuclease carries high sequence specificity, it should have limitations for searching hypersensitive sites in chromatin. However, the sensitive sites can be definitely determined because of generation of distinct fragments. PstI was chosen as the restriction

endonuclease to study sensitive recognition sites of various types of endogenous MuLV-related proviral sequences in chromatin, because (1) MuLV and MuLV-related proviral sequences contain a common PstI site in their LTRs, (2) the DNaseI study revealed that hypersensitive sites were located around the PstI site in the LTR, (3) other studies (the genomic footprinting experiment and the electrophoretic mobility shift assay) showed that there was a protein binding site near the PstI site in the LTR, and (4) the physical map of each type of proviral sequences on PstI was available (Ch'ang et al., 1989b).

Restriction enzyme PstI should be able to enter the nuclei and cleave the chromatin DNA at the sensitive recognition sites because the molecular size of the enzyme is small (M.W. 37,400). No particular fragment can be detected without enzyme treatment (Fig. 7, lane 1), indicating that the effect by endogenous nuclease digestion during incubation is minimal. It is possible that the differences of restriction fragment patterns seen in the PstI-digested genomic DNA and the PstI-digested nuclei are due to selective digestion of the PstI sites in the open chromatin region of the isolated nuclei. However, further experimental tests are required to conclusively establish this point.

The majority of MuLV-related proviral sequences in the mouse genome are intact and contain no apparent internal deletion(s); thus, the polytropic and modified polytropic

class proviral sequences are expected to generate 7.7 kbp and 4.8 kbp PstI fragments, respectively, that are detectable by the LTR probe (Fig. 8). In contrast, the PstI-digestion of the nuclei generated a 8.4 kbp fragment corresponding to the full length (i.e. length extending from the PstI site in the 5' LTR to the PstI site in the 3' LTR). Also, it is likely that the 6.0 kbp and 5.5 kbp fragments detected by the LTR probe, the IS probe and the gag probe as well as 7.0 kbp and the 5.5 kbp fragments detected by the p15E probe are generated from the minor population of MuLV-related proviral sequences which contain internal deletion(s) (Fig. 7). From the present study, I could conclude that (1) the PstI site within the LTR of certain MuLV-related proviral sequences is sensitive to cleavage in the nuclei while the PstI sites in the structural gene regions of the same proviral sequences are not, and (2) only a minor population of MuLV-related sequences which contain internal deletion(s) are particularly sensitive to PstI digestion in the nuclei. Ch'ang et al (Ch'ang et al., 1989b) reported the specific sequence deletions in MuLV-related proviruses in the mouse genome. The cDNA cloning (Levy et al., 1985-a,b; Amanuma et al., 1988; Laigret et al., 1988) as well as RNA gel blot analyses (Khan et al., 1987; Yang et al., 1989) showed that the majority of RNA transcripts from MuLV-related proviruses were smaller than the full length and presumably carried specific sequence deletions. There are two possible explanations for these results. One is that

the transcripts from intact MuLV-related sequences are spliced to produce the shorter RNA products; and the other possibility is that the predominant MuLV-related RNA transcripts are transcribed directly from the MuLV-related proviruses that carry sequence deletion(s). My result as well as Ch'ang's result (Ch'ang et al., 1989b) support the latter possibility.

The expression of retrotransposable elements such as endogenous proviral sequences may cause genomic instability and consequently lead to the neoplastic state. It was reported that TCPOBOP or CCl₄ treatment induced expression of transcripts from endogenous retrovirus-related sequences (Mo-MuLV, IAP gene and VL30 gene) 2 days after treatment in the liver of male B6C3 mice which show high incidence of hepatoma (Dragani et al., 1987). The expression of RNA transcripts derived from the endogenous MuLV-related proviral sequences was stimulated by CCl₄ treatment in RFM/Un, NFS/N, B57Bl/6, BALB/c and C3H/Anf mice; and the response of male C3H/Anf mice was more prominent than that of female of the same strain (Yang et al., 1989). The restriction endonuclease sensitivity technique is useful to detect the particular type of endogenous proviral sequences that might be actively transcribed in the genome. The comparison of chromatin structure of proviral MuLV family between male and female C3H mice was carried out by using the PstI sensitivity technique. The result indicated that the endogenous proviral sequences of MuLV family in 2 weeks-old C3H male mice were more

sensitive to PstI digestion in the nuclei than in C3H female mice of the same age. Preliminary results of electrophoretic mobility shift assay showed that CCl₄ treatment of the mouse can lower the specific LTR binding activity of nuclear extracts from the liver (P. Hoyt personal communication). It is interesting to investigate whether or not CCl₄ treatment can cause specific changes in the chromatin structure of these proviral LTR sequences.

The degree of methylation at the SmaI site in the LTR was studied by comparing the intensities of PstI-KpnI and PstI-SmaI fragments of various classes of LTRs (those fragments from the MuLV, the polytropic and the modified polytropic can be distinguished by their sizes) (Fig. 11). Although in this study only the SmaI site was used to assess the DNA methylation status of the LTR, it is clear that the proviral LTR sequences showing sensitivity to PstI digestion in the nuclei were also digested by SmaI. In other words, when the PstI site in the LTR of a MuLV or a related proviral sequence is sensitive to PstI digestion in the liver nuclei, the SmaI site of the same proviral LTR appears not to be methylated.

In the testis, this correlation was not seen; and the SmaI site in most LTR was methylated and not digested by the enzyme. This difference probably reflects the characteristic DNA methylation properties of functional genes in the testis which is different from somatic tissues. In most somatic tissue DNA, methylation of regions proximal to the promoter

often appears to suppress the formation of hypersensitive sites; and demethylation of C residue in CG sequence in the regions is necessary, but not sufficient for the establishment of nuclease hypersensitive sites (Gross, 1988).

Genomic imprinting is the differential modification of the maternal and paternal genomic contributions. The molecular mechanism of initiation, maintenance and erasure of imprinting is unclear; however, differential DNA methylation during gametogenesis has been accepted to explain genomic imprinting by various transgene experiments (Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987; Hadchouel et al., 1987). It is possible that the chromatin structure of maternally derived and paternally derived genes may be different as a result of genomic imprinting. Because MuLV-related proviral sequences are widely distributed in the mouse genome, they might be used as probes to study genomic imprinting of endogenous genes. If the gene near a MuLV-related provirus is imprinted, the proviral sequence might be similarly imprinted. Therefore, genomic imprinting of endogenous genes may be studied by comparing the methylation status or chromatin structure (nuclease sensitivity) between maternally and paternally derived proviruses in the F1 mice whose parents have different restriction fragment patterns of the MuLV-related sequences. It has been difficult to study individual sequences of endogenous MuLV-related proviruses due to their large copy number and the lack of

specific hybridization probes to distinguish individual members of the group. Recently, Coffin's group prepared oligonucleotide probe specific for each group (ecotropic, xenotropic, polytropic and modified polytropic) (Stoye and Coffin, 1987; Stoye and Coffin, 1988). Although differences of restriction sites were used to distinguish two types of MuLV-related sequences (polytropic and modified polytropic) in the present study on chromatin structure and DNA methylation of MuLV-related proviral sequences, the specific oligonucleotide probes will be more useful for distinguishing each type of MuLV-related sequence and for obtaining more detailed information about the individual sequences.

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