To the Graduate Council:

I am submitting herewith a dissertation written by Nancy Stevens Kuemmerle entitled "Characterization of Two Solitary Long Terminal Repeats and Other Murine Leukemia Virus-Related Elements in the Genome of the RFM/Un Mouse." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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CHARACTERIZATION OF TWO SOLITARY LONG TERMINAL REPEATS AND OTHER MURINE LEUKEMIA VIRUS-RELATED ELEMENTS IN THE GENOME OF THE RFM/Un MOUSE

A Dissertation

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ABSTRACT

The genome of the RFM/Un mouse harbors many copies of elements related to murine leukemia viruses (MuLVs). Not all of these elements are capable of producing infectious virus particles without undergoing some modification or rearrangement. Nonetheless, these non-producing elements may have a profound effect upon the host organism.

Two MuLV-related elements have been characterized in detail. These elements were found in the chromosomal DNA of the RFM/Un mouse as solitary long terminal repeats (LTRs). Southern blotting experiments demonstrated that both solitary LTRs and the unique-sequence regions on either side of them were present in most inbred mouse strains examined; the flanking sequences were represented in several feral Mus species and Mus musculus subspecies, although the solitary LTRs were not necessarily present in these mice. Restriction of genomic DNA from liver and spleen of two inbred mouse strains with PstI and the methylation-sensitive endonuclease SmaI, followed by hybridization of the resulting DNA fragments to the unique-sequence probes from the region 3′ to each solitary LTR, revealed that neither element is detectably methylated at its LTR SmaI site in at least one tissue in both mouse strains. Neither solitary LTR, however, appeared to be particularly sensitive to DNAse I digestion, leaving unresolved the possibility that such elements may be promoting the transcription of adjacent cellular sequences.

A similar approach was employed in the determination of DNAse I sensitivity and methylation status of MuLV LTR-related elements in DNA obtained from the spleen and liver of the RFM/Un mouse. Although there was no correlation between hypomethylation at the LTR SmaI site and DNAse I sensitivity, Southern blotting experiments demonstrated a correlation between hypomethylation as detected by viral structural gene probes and the presence of an insertion into the U3 region of the LTR of MCF-type proviruses. Hence most of the MCF-type proviruses that harbor 170-200-bp insertions into their LTR U3 regions were relatively insensitive to SmaI restriction as determined from use of the LTR probe; use of gag, gag-pol, and env gene probes, however, revealed that some of these MCF-type elements, unlike most endogenous ecotropic and xenotropic proviruses, were relatively hypomethylated at SmaI sites within the structural genes. Although several members of this class of endogenous proviruses are known to have suffered deletions in their structural genes, it may be that they are responsible for a substantial portion of the MuLV-related transcripts observed in the RFM/Un mouse.

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CHAPTER I

INTRODUCTION

Retroviruses

A distinguishing feature of retroviruses is their diploid, single-stranded ribonucleic acid (RNA) genome. This genetic constitution makes obligatory in their life cycle a reverse transcription step. Once the genetic material of a retrovirus has entered the cytoplasm of a permissive host cell, its RNA is reverse-transcribed into double-stranded deoxyribonucleic acid (DNA), which can then enter the nucleus and become integrated into the chromosomal DNA of the host. These processes are mediated primarily by virus-encoded enzymes, particularly reverse transcriptase, RNAse H, and endonuclease, although the host cell frequently contains enzymes which can perform parallel functions, as well as factors which can facilitate or inhibit the viral enzymes. The integrated provirus, like a cellular gene, is transcribed and replicated by constituents of the host systems for these processes. Processing of the transcripts and the protein precursors they specify is accomplished by cellular and viral enzymes. Newly synthesized viral genomic RNAs can serve as substrates for reverse transcription and further integration events; alternatively, they can be assembled, along with viral structural proteins and enzymes and host-derived small RNA passenger molecules, into particles which bud from the cytoplasm (115).

Retroviral particles are classified into four categories on the basis of their morphology (109). Type A particles, represented by

intracisternal A particles, have electron-lucent centers surrounded by a double shell. Although they are abundant (about 650 to 1800 copies per cell) and increase in association with certain tumors, they have not been shown to be infectious. Type B particles, typified by murine mammary tumor virus (MMTV), contain electron-dense nucleoids that are eccentrically located within the particle. In the laboratory inbred mouse, MMTV-related sequences generally occur in about 5 to 15 copies per diploid genome. The type B viruses are transmitted horizontally as well as vertically, and they have been implicated as causative agents in more than one type of tumor. Mature type C retroviral particles, exemplified by Moloney murine leukemia virus (Mo-MuLV), are characterized by a crescent-shaped, electron-dense, centrally located core surrounded by a membrane which may have discernible spikes. MuLV-related sequences are more abundant in the mouse genome than are MMTV-related sequences; similarly, they are transmitted both vertically and horizontally. Type D retroviruses, which include Mason-Pfizer monkey virus, contain an eccentrically located, electron-dense nucleoid and exhibit shorter surface spikes than MMTV viruses. Only a few isolates have been described; these are not present in the mouse genome.

Endogenous Type C Retroviruses of the Mouse

The genome of the laboratory inbred mouse harbors multiple copies of MuLV-related, type C retroviruses. The endogenous proviruses, as they appear at their chromosomal integration sites, have the general structure of 5′-LTR-gag-pol-env-LTR-3′, that is, the coding regions

for viral internal structural proteins and the first four amino acids of the protease (gag), protease, reverse transcriptase, RNAse H, and endonuclease (pol), and envelope glycoproteins (env) are flanked by long terminal repeats (LTRs) (22,23). The endogenous proviruses are structurally similar to newly integrated proviruses in that they are flanked by 4-bp direct repeats of cellular DNA and they have lost two basepairs from each terminus. As any other stably integrated chromosomal element, they are transmitted in a Mendelian fashion (60); these elements reside at defined loci characteristic for each laboratory inbred mouse strain.

Endogenous type C retroviruses, while widespread in their distribution, have been extensively studied in inbred mice. As for infectious retroviruses, they have been divided into classes on the basis of tropism (109): ecotropic proviruses are potentially capable of replicating only in the host species; xenotropic proviruses are capable of replication only in species other than the host; polytropic proviruses, also known as mink cell focus-forming (MCF) proviruses, can replicate in both homologous and heterologous hosts. Viruses and proviruses of the last class are env gene recombinants, and as such are neutralized by antisera to gp70, the major envelope glycoprotein from both ecotropic and MuLV-related MCF viruses. While the amphotropic viruses, which can also replicate in more than one species, can be propagated in cells from inbred mice, they have thus far been isolated only from feral mice, and hence do not constitute a class of MuLV-related endogenous proviruses of inbred mice.

The expression of the endogenous proviruses frequently differs from that of the corresponding exogenous viruses, and levels of expression also vary (17). Some of the endogenous proviruses are incapable of producing infectious particles without undergoing some type of rearrangement, modification, or recombination. Their RNA transcripts and/or protein products may nonetheless be expressed; production of infectious particles is not a prerequisite for generating an effect upon the host. The single inducible ecotropic provirus of the RFM/Un mouse is expressed in hemopoietic tissues throughout the life of the animal without apparent effect (71); upon induction by ionizing radiation or chemical treatment, however, the virus undergoes multiple reintegrations and possibly other modifications which may be involved in the induction of thymic lymphomas or myeloid leukemias (3,111). The endogenous proviruses of AKR and C58 mice are involved in the high spontaneous incidence of leukemias observed in these strains. The xenotropic viruses are rarely expressed in high titers and have not frequently been associated with pathological changes. MCF viruses, however, have been isolated from a number of spontaneous and induced hematopoietic neoplasias of various inbred mouse strains (110). Portions of the protein products of endogenous MuLV-related genomes are expressed in a tissue-specific pattern (17); partial and complete proviral transcripts have been detected in tissues of untreated inbred mice (24,56,68,69). The consequences of partial expression of endogenous MuLV-related elements are not understood at this time.

Other Retrovirus-related Elements

The chromosomes of mice and other species harbor a number of other retrovirus-related elements. Among these are the VL30 sequences of rodents, found in 20 to 50 copies per cell (17). They share several structural features with the type C endogenous proviruses: long terminal repeats containing putative promoter and polyadenylation signals flank intervening genetic information, and a potential tRNA primer binding site has been identified (42). Although dimers of the 30S RNA can be packaged into helper virions (47), the particles are not known to be infectious, except that particles packaged with a type C helper virus have been found to be infectious in bats (97).

Members of the murine ETn family of middle-repetitive genes are also structurally similar to type C endogenous proviruses (6,7). The 6-kb element includes directly repeated LTRs 148 bp in length, with 19-bp imperfect inverted repeats flanking a 110-bp region having the organization U3-R-U5 and containing a polyadenylation signal and site. The RNA transcript encoded by an ETn element is colinear, as determined by heteroduplex formation, and is expressed in early mouse embryogenesis.

Another family of murine repetitive sequences, termed MuRRs, (95) is related to endogenous MuLV-type retroviruses by virtue of the identity of an element contained therein with the 190-bp insertion into the LTRs of MCF-type proviruses (57,86). These 5.7 kb elements, present in about 50 to 100 copies in the haploid mouse genome, include LTRs about 600 bp in length and intervening genetic information with sequence homology to type C retroviral gag, pol, and env genes. Like

ETn sequences, these elements are prevalent among Mus species, but do not show homology to human or rat sequences. Some members of this family hybridize to RNA transcripts (56).

The copia transposable elements of Drosophila also exhibit relatedness to retroviral elements (62). Long terminal repeats bounded by short inverted repeats contain polypurine tracts; putative primer binding sites are observed adjacent to the 5′ LTRs. The 5-kb copia element is transcribed; particles isolated from cultured Drosophila cells contain copia RNA and reverse transcriptase activity (98).

Yeast Tyl transposable elements also bear structural resemblances to retroviruses (25). Directly repeated delta sequences, or LTRs, of about 335 bp in length flank 5.3 kb of internal sequence which contain a putative primer binding site and regions homologous to the gag and pol genes of retroviruses. Transposition of Tyl elements occurs via an RNA intermediate (2), and reverse transcriptase activity has been demonstrated in virus-like particles associated with yeast cells that have been induced to transpose (29). Like IAPs, these particles do not appear to be infectious. Some of the Tyl elements are defective, analogous to endogenous type C proviruses.

Retroviral Elements as Insertional Mutagens and Modulators of Cellular Transcription

Retroviruses have been exploited experimentally as insertional mutagens to facilitate the study of essential genes. Jaenisch and coworkers (43,45,46) have derived a series of single integrations of Mo-MuLV into the genomes of BALB/c and other inbred mice. One of these

insertions was into the alpha-1 collagen gene, and its lethality demonstrated an essential role in embryogenesis (5,33,44,72,96). Another retroviral insertion into an abundantly transcribed gene has recently been shown to result in early embryonic death of mice homozygous for the insertion (102).

Naturally occurring retroviral insertions have also proven useful in elucidating gene function. A retroviral insertion at the dilute locus on chromosome 9 of the DBA/2 mouse was shown to be responsible for that mutant phenotype (51); its excision, leaving behind a solitary LTR, effected reversion to the normal distribution of pigment granules (20). Similarly, a retroviral element at the agouti locus is associated with the lethality of the yellow mutation in homozygous state; it is probable that the nonlethal yellow phenotype results from the excision of all but a solitary LTR (21).

Retroviruses, by virtue of the elements contained in their LTRs, have been shown to modulate the transcription of adjacent cellular sequences in such a way that normal cellular control mechanisms are obviated and disease is manifested. In the majority of bursal lymphomas of chickens, the insertion of the avian leukosis virus LTR into a region near myc, the cellular homolog of the oncogene of myelocytomatosis viruses, promotes or enhances the expression of myc, which is believed to be involved in oncogenic processes (35,79,87,88). The insertion of an endogenous type A retrovirus is involved in the activation of the cellular homolog of the mos oncogene in a mouse plasmacytoma (8). A copy of the gibbon leukemia virus is integrated at the 3′ end of the IL-2 gene in a gibbon leukemia cell line that

constitutively produces interleukin 2 (14). Thus the role of aberrant viral integrations in pathologic processes is well established.

Solitary LTRs

Solitary LTRs are distinguished from viral LTRs by their lack of association with viral structural genes. Additionally, both termini are flanked by direct repeats of a short cellular sequence. As in the case of endogenous proviruses, solitary LTRs can also be present as stably inherited chromosomal elements (61). Some of these elements, including an avian solitary LTR (100) and a VL30 solitary LTR (93), are without apparent phenotypic effect. Moreover, the solitary LTR remaining after excision of the ecotropic provirus at the *dilute* locus on chromosome 9 of DBA/2 mice (51) does not prevent reversion to the wild-type distribution of pigment granules affecting coat color (20). Parallel examples include the excision of all but a solitary *copia* LTR from the *white-apricot* locus of *Drosophila*, resulting in reversion to wild-type eye color (12), and the excision of the internal portion of the yeast transposable element Ty 1, which leaves a single copy of its directly repeated delta sequence, causing restoration of the wild-type phenotype at the *his4* locus (26). That the solitary LTRs alter transcription of adjacent cellular genes is demonstrated by the finding that a solitary LTR inserted into the DNA genome of an insect baculovirus promotes bidirectional transcription of adjacent viral genes (27). Transcription from the solitary LTR of MuLV type described by Wirth and coworkers (59,70) has been demonstrated in an *in vitro* system; it is possible that this and other solitary LTRs also modulate

the transcription of adjacent cellular sequences in vivo.

Aims of This Dissertation

The relative stability of ecotropic endogenous MuLV-related proviruses among inbred laboratory mouse strains has been well documented. The existence of an insertion-containing solitary LTRs in the chromosomes of laboratory inbred mice and in Mus cookii and Mus caroli (94) has been described, and the persistence of an ecotropic-type solitary LTR after excision of the intervening viral genetic material at the dilute locus of the DBA/2 mouse has been detailed (20). Neither of these solitary LTRs has an apparent effect upon phenotype. Because retroviruses have been shown to function as insertional mutagens and as modulators of cellular transcription in murine systems, it was of interest to determine whether solitary LTRs were stable elements in the mouse chromosome and might be capable of altering the transcription of adjacent cellular sequences in the absence of an identifiable phenotype.

In the course of these studies, it was noted that one species of MCF-related LTR was relatively undermethylated at its LTR SmaI restriction site in the DNA of the RFM/Un mouse. Although retrovirus-related transcripts have been detected in DNA obtained from normal mouse livers, the endogenous sequences responsible for this transcriptional activity have not been identified. Thus it was of interest to determine whether LTRs of this type lay in transcriptionally active regions of chromatin.

CHAPTER II

SOLITARY LTR ELEMENTS

Introduction

The solitary LTRs described thus far are notable for their lack of phenotypic effect or for their reversal of a demonstrable mutant phenotype. The avian endogenous solitary LTR (100), the VL30 solitary LTR (93), and the insert-containing murine solitary LTR (120) were detected after screening genomic DNA libraries with whole-virus or LTR probes. Investigation of revertants of a coat- color mutation in mice (20), an eye-color mutation in Drosophila (12), and a histidine prototroph in yeast (26) led to the discovery of those solitary LTRs. The exception is the solitary LTR remaining in the mutant nuclear polyhedrosis virus after excision of the copia-related retroposon TED: the solitary LTR apparently causes the same mutant phenotype observed in the presence of the inserted retrotransposable element (27). Additionally, transcripts originate from this solitary LTR in both directions. Thus it was conceivable that there existed in the mouse genome solitary LTRs lacking apparent phenotypic effect, but which nonetheless modulated transcriptional activity in their vicinity in the chromosome.

In somatic cells, the presence of actively transcribed genetic information is strongly correlated with alterations in chromatin conformation. Experimentally, these altered conformations are detected as undermethylation and DNAse I hypersensitvity in the vicinity of the actively transcribed gene (32). Whereas altered chromatin constitutes

an indication of transcriptional activity, ultimate proof of transcription resides in the detection of specific transcripts. In order to characterize solitary LTRs in the mouse genome, a library of murine DNA in phage lambda was screened. This chapter details the isolation and characterization of three such elements. Restriction endonuclease digestions and hybridization data indicated that these elements were not cloning artifacts or parts of incomplete proviruses. Methylation status and chromatin conformation in the vicinity of the solitary LTRs were examined, and the transcriptional activition of sequences adjacent to the solitary LTRs was probed. Their distribution among laboratory inbred mouse strains and in several feral Mus species was ascertained, and their relationship to other endogenous murine proviral LTRs is discussed. A possible origin for these elements is postulated.

Materials and Methods

Recombinant DNA clones

High-molecular-weight DNA extracted from the spleen of a male RFM/Un mouse was digested with HindIII and ligated into the HindIII site of bacteriophage lambda Charon 9 by Paul Glover under the direction of L. R. Boone (4,80,81). The resulting library was screened first with a molecular probe derived from the ecotropic-specific region of the env gene (3,4,80,81). After removal of clones hybridizing to the env probe, the remainder of the library was screened with a probe prepared from the PstI-SmaI fragment of the LTR from the RFM endogenous ecotropic MuLV (71). Of the 20 plaques selected for hybridization to

the ecotropic MuLV LTR probe, three failed to hybridize to a probe derived from the ecotropic MuLV gag-pol genes. The insert DNAs from these clones were transferred to pBR322 and the plasmids were designated pRFM2, pRFM7, and pRFM8. Plasmids were maintained in Escherichia coli C600 or E. coli HB101; pUC9 subclones derived from these plasmids (116) were propagated in E. coli JM103.

Preparation of plasmid DNAs

Overnight cultures (25 ml) in L-broth (74) were inoculated from single colonies of the plasmid-containing host bacterium and grown to high density. After removal of 1.5 ml for use in ascertaining that the culture contained the plasmid, the overnight culture was diluted into 500 ml of L-broth containing ampicillin at a concentration of 50 ug/ml. This large culture was incubated at 37^{o} with aeration until its optical density at 580 nm reached 0.6. At that time, chloramphenicol was added to a concentration of 170 ug/ml in order to inhibit further growth of the bacterial host while permitting replication of the plasmid; the incubation was continued overnight.

Cells were harvested by centrifugation at 8000 rpm in the GSA rotor in a Sorvall RC-2 refrigerated centrifuge. Pellets were rinsed with ice-cold phospate-buffered saline and resuspended in 10 ml of 15% sucrose/50 mM EDTA/50 mM Tris (pH 7.8). After addition of 5 mg of lysozyme, the cells were held on ice for 5 minutes. A 10-ml volume of 0.5% Triton X-100/50 mM EDTA/50 mM Tris (pH 7.8) was added and mixed gently on ice for 10 minutes. The lysate was divided into two centrifuge tubes, and most of the chromosomal DNA was pelleted at

30,000 rpm in a 50 Ti rotor in a Beckman L-2 ultracentrifuge at 0^{o} for 90 min.

A one-tenth volume of 10% sodium dodecyl sulfate (SDS) was added to the plasmid-containing supernatant, which was heated at 60^{o} for 5 minutes. One-half volume of 5 M NaCl was then added and mixed by inversion. The mixture was held on ice for at least two hours, after which it was centrifuged for 30 min at 0^{o} at 24,000 rpm in a 50 Ti rotor in an ultracentrifuge. To each ml of supernatant was added 0.11 g of polyethylene glycol (PEG 6000). This solution was held on ice for at least one hour, then centrifuged for 20 minutes at 20,000 rpm at 0^{o} in a 50 Ti rotor in a Beckman ultracentrifuge. The supernatant was discarded, and the pellet, now relatively free of RNA and host chromosomal DNA, was drained and dried in the lyophilizer.

Plasmid DNA preparations were further purified by chromatography on hydroxylapatite (18). Pellets were resuspended in 4 ml of 8 M urea and 0.8% SDS dissolved 0.24 M sodium phosphate buffer (pH 6.8), cooled to room temperature, and applied to a column of 0.7 g hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) which had been washed with water and equilibrated with the same buffer. After adsorption of the DNA to hydroxylapatite, columns were washed sequentially with the equilibration buffer, the same buffer without SDS, and 10 mM phosphate buffer (pH 6.8). Plasmid DNA was eluted from the column with 0.3 M phosphate buffer (pH 6.8). Fractions containing DNA, as determined spectrophotometrically from absorbance at 260 nm, were pooled and dialyzed at 0^{o} against 3 changes of 500 volumes of 10 mM Tris (pH 7.5)/1 mM EDTA. DNA samples were stored at 4^{o} or -20^{o}.

Rapid isolation of small amounts of plasmid DNA

A 1.5 ml portion of an overnight culture was pelleted for 2 min in a microfuge. The pellet was washed with 0.1 ml of H_2O and resuspended in 0.1 ml of 15% sucrose/0.5 mM EDTA/50 mM Tris (pH 8.0). The suspension was held on ice for 10 min after the addition of 0.005 ml of lysozyme (20 mg/ml), then frozen at -80°. The lysate was thawed at 37°, and cellular debris and host chromosomal DNA were pelleted by centrifugation in a microfuge for 2 min. The supernatant was extracted sequentially with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1). Plasmid DNA was precipitated with a one-tenth volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol at 0° for 15 min and recovered by centrifugation in a microfuge for 10 min. The amount of DNA recovered using this procedure was usually sufficient for two restriction enzyme digestions.

Restriction endonuclease characterization of subclones

Restriction endonuclease mapping of pRFM2, pRFM7, and pRFM8 and the pUC9 subclones derived from them was accomplished by the partial digestion method (101) and confirmed by inspection of ethidium-bromide-stained fragments from single- and multiple- enzyme digests resolved on agarose or polyacrylamide gels. In general, stained gels contained 0.5 to 1 ug of DNA fragments per lane; partial digestion reactions contained 500-2000 cpm of ^{32}P per lane, and were evaluated after 6-60 hours of exposure of the dried gel to X-ray film.

To prepare fragments for end-labeling reactions, 10-50 ug of plasmid DNA was digested with 0.5 to 1 unit of the appropriate

restriction enzyme(s) per microgram of DNA at a concentration of 0.05-0.1 ug/ul for at least 1 hour, or until digestion was complete. Fragments resolved through agarose gels were recovered by electroelution or by the freeze-squeeze method (106). Stained fragments were excised from the gel and placed in 0.4 ml microfuge tubes which had been punctured and contained a plug of glass wool. These tubes were placed inside 1.5 ml microfuge tubes and filled with 0.3 M sodium acetate (pH 7.0)/1 mM EDTA and equilibrated at room temperature for approximately 15 minutes, depending upon the size of the fragment. After freezing at -80^{o}, the nested tubes were spun in a microfuge at room temperature for 10 minutes. To the DNA-containing buffer in the larger microfuge tubes were added 0.01 volume of 1 M $MgCl_2$/10% acetic acid and 2.5 volumes of 95% ethanol. Pellets recovered after freezing at -80^{o} and centrifugation for 10 min in the microfuge were reprecipitated with sodium acetate and ethanol and rinsed several times with 80% ethanol. Fragments resolved on polyacrylamide gels were excised, crushed with a pestle in a microfuge tube, and homogenized with a suitable volume of extraction buffer consisting of 0.5 M ammonium acetate, 1 mM EDTA, 5 mM magnesium acetate, and 0.1% SDS. The mixture was incubated with occasional vortexing at 37^{o} for 0.5-18 hr, depending upon the size of the fragment. After freezing at -80^{o} and pelleting the acrylamide by centrifugation in the microfuge for 10 min, the DNA-containing supernatant was subjected to an additional centrifugation to remove residual acrylamide. DNA was precipitated by addition of 0.1 volume of 20% potassium acetate and 2.5 volumes of ethanol. The pellet recovered

after freezing at -80^{o} and centrifugation in the microfuge was washed several times with 80% ethanol.

Fragments for end-labeling reactions were dephosphorylated with calf intestinal alkaline phophatase and subjected to sequential extraction with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1). Termini were labeled using T4 polynucleotide kinase in the presence of [gamma-^{32}P]ATP. After cleavage by a restriction endonuclease and resolution by electrophoresis, fragments were recovered from acrylamide as described above.

Partial digestion reactions of approximately 1000 cpm of end-labeled fragments were carried out in the presence of 0.05-0.1 unit of restriction endonuclease in 0.045 ml of the appropriate buffer. The products of partial digestion reactions, obtained by removing aliquots from incubation mixtures at various time intervals, were characterized by electrophoresis through agarose or acrylamide gels.

Subcloning

Portions of the insert DNAs from pRFM2, pRFM7, and pRFM8 were subcloned into pUC vectors (116). In general, plasmid and vector DNAs were digested with the appropriate enzymes and isolated from agarose or acrylamide gels. When necessary, termini were dephosphorylated with calf intestinal alkaline phosphatase to prevent self-ligation. After ligation of vector and insert DNAs, the mixture was used to transform E. coli RR1. By plating on media containing ampicillin to select for cells transformed by the antibiotic-resistant plasmid and IPTG as an

inducer and Bluo-Gal^R as an indicator, it was possible to select as candidates for plasmids which had acquired the desired insertion into the partially deleted lactose operon those colonies which were white or colorless; blue colonies harbored plasmids which had not acquired an insert. Candidate colonies were purified by restreaking onto another plate; several representatives of the transformation reaction were then grown in liquid medium. Plasmid DNA isolated from the candidate cultures was cut with the appropriate restriction enzymes and subjected to electrophoresis in order to ascertain whether the desired insert DNA was present.

The subclones derived from pRFM7 and pRFM8 are depicted in Figure 1. Subclones derived from pRFM7 included: 7a1, a 2.6 kb BamHI fragment containing an LTR and cloned into the BamHI site of pUC9; 7c5, a 1.8 kb HindIII-BamHI fragment immediately 5′ to 7a1, cloned into the pUC9 HindIII and BamHI sites; 7b4, a 2.6 kb internal BamHI fragment adjacent to 7a1 and cloned into the BamHI site of pUC9; 7d5, a 1.7 kb HindIII-BamHI fragment immediately 3′ to 7b4, cloned into the HindIII and BamHI sites of pUC9; 7a1/0.9, a 900-bp PstI fragment whose 3′ terminus is approximately 50 bp 5′ to the LTR PstI site, cloned into the pUC9 PstI site; 7a1/1.2, a 1.2 kb PstI fragment including most of the LTR, cloned into the PstI site of pUC9; 7a1/SmaI, a 550-bp SmaI fragment beginning 5′ to the LTR and terminating at its SmaI site, cloned into the SmaI site of pUC9; and A1, a 100-bp BglII-PstI fragment downstream from the LTR, inserted into pUC9 BamHI and PstI sites.

Figure 1. Map locations of the subclones derived from pRFM7 and pRFM8. The map locations of subclones derrived from pRFM7 and pRFM8 are shown. Restriction enzymes employed in the isolation of their inserts inchluded: B, BamHI; BII, BglII; H, HindIII; Hc, HincII; P, PstI; S, SmaI.

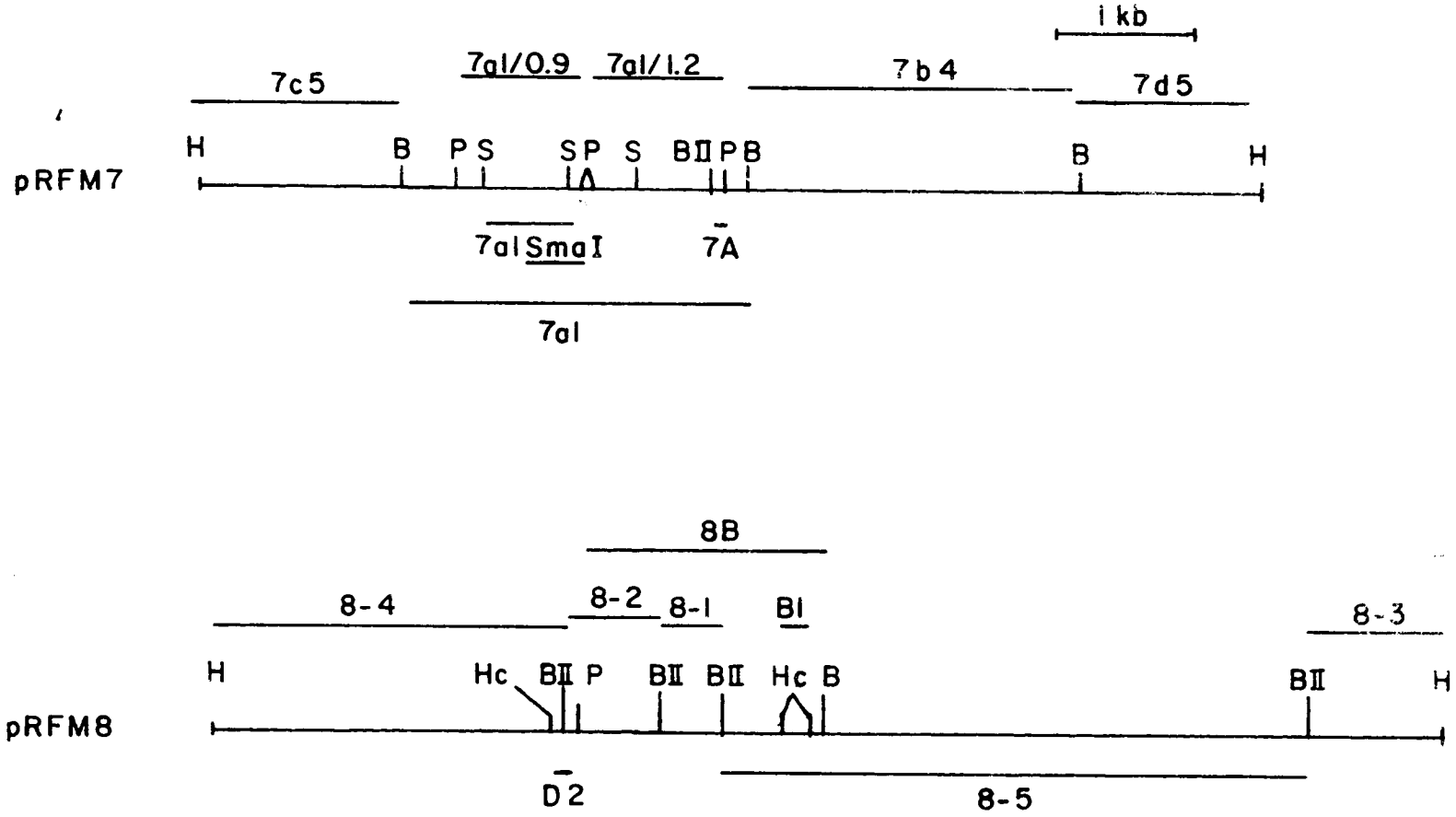

1 kb
7c5
7a1/0.9
7a1/1.2
7b4
7d5
pRFM7
H
B
P S
S P
S
BII P B
B
H
7a1 SmaI
7A
7a1
8B
8-4
8-2
8-1
BI
8-3
pRFM8
H
Hc
BII
P
BII
BII
Hc
B
BII
H
D2
8-5

Figure 1

Subclones derived from pRFM2 and pRFM8 include: 2B and 8B, 1.95 kb fragments beginning at the LTR PstI site and including most of the LTR, terminating at the BamHI site 3′ to the LTR, cloned into the PstI and BamHI sites of pUC9; 8-2 and 2-2, 0.8 kb BglII fragments harboring the LTR, cloned into the BamHI site of pUC9; 8-1 and 2-1, 0.6 kb BglII fragments immediately 3′ to 8-2 and 2-2, also cloned into the BamHI site of pUC9; 8-3 and 2-3, the 3′terminal 1.1 kb BglII-HindIII fragments of pRFM8 and pRFM2, cloned into the BamHI and HindIII sites of pUC9; 8-4 and 2-4, the 5′ terminal 3.2 kb HindIII-BglII fragments adjacent to 8-2 and 2-2, also cloned into pUC9 HindIII and BamHI sites; 8-6 and 2-6, the 5.5 kb internal BglII fragments of pRFM8 and pRFM2 contiguous with 8-1 (2-1) and 8-3 (2-3), cloned into the BamHI site of pUC9; B1, a 250-bp KpnI-HincII fragment derived from the region 3′ to the pRFM8 LTR and inserted into pUC 19 KpnI and HincII sites; and D2, a 60-bp HincII-BglII fragment immediately 5′ to the pRFM8 LTR, cloned into the HincII and BamHI sites of pUC 19.

DNA sequencing

The chemical degradation method (75) was employed for DNA sequencing reactions, performed by C. K. Koh, as previously described (61). Sequence analyses were facilitated by the computer programs made available by Mount and Conrad (78).

Preparation of genomic DNA from murine tissues

DNA from mouse livers and other tissues was obtained by a minor modification of the method described by Jenkins et al. (52). Portions

of mouse tissues (0.2 - 1 g) frozen at -70° were homogenized without thawing in 5-10 ml disruption buffer [0.25 mg of pronase (Boehringer-Mannheim) per ml, 0.5% sodium dodecyl sulfate, 0.15 M NaCl, 0.015 M sodium citrate, 0.001 M EGTA]. The homogenizer was rinsed with an additional 5-10 ml disruption buffer, and the homogenate was incubated at 37° for 2-6 h. An equal volume of phenol saturated with 1 X SSC (0.15 M NaCl plus 0.015 M sodium citrate) was added, and the mixture was allowed to stand at room temperature for 5 min. The same volume of chloroform:isoamyl alcohol (24:1) was added and mixed; separation was effected by centrifugation at 2000 rpm in a refrigerated International centrifuge. The aqueous phase was transferred to a clean polypropylene centrifuge tube, extracted with an equal volume of chloroform:isoamyl alcohol, and collected on a glass rod or polystyrene pipette after addition of two volumes of 95% ethanol to the aqueous phase.

The DNA was redissolved in 4-10 ml of 10 mM Tris (pH 7.6), 0.1 M NaCl, 10 mM EDTA. The solution was incubated at 37° for 1 h after the addition of 0.1 ml of 10-mg/ml RNAse A (Sigma). Sodium dodecyl sulfate was then added to a final concentration of 1%, and the incubation was continued for 1-2 h after the addition of 0.2 mg/ml of pronase. After addition of NaCl to a concentration of 0.5 M, the solution was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol as described above. DNA was precipitated from the aqueous solution by the addition of two volumes of ice-cold 95% ethanol.

Restriction endonuclease digestion of genomic DNA

High-molecular-weight DNAs from mouse tissues were digested to completion under conditions recommended by the suppliers of the restriction endonucleases (BRL and New England Biolabs). When multiple digestions involved SmaI or KpnI, the DNAs were restricted first with the other enzyme(s), e.g., PstI or BamHI, extracted with phenol and chloroform:isoamyl alcohol (24:1), and precipitated before digestion with SmaI or KpnI.

Electrophoresis, transfer, and hybridization of DNA

DNA fragments, generally 5 ug per lane, were fractionated by electrophoresis through 0.6 - 1.5% agarose in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 2 mM EDTA). Gels were routinely stained with ethidium bromide and photographed under ultraviolet light using a red photographic filter.

To improve the efficiency of transfer of high-molecular-weight fragments to membranes, the DNA in the gels was depurinated with two 500-ml washes of 0.25 N hydrochloric acid for 15 minutes with agitation. After several brief rinses in water, the DNA contained in the gels was then denatured for 20 minutes with 0.5 N NaOH in the presence of 1.5 M NaCl. Transfer to Zetabind (AMF Cuno) nylon membranes was effected by 0.5 N NaOH containing 5 M NaCl.

Upon completion of transfer, membranes were floated for one minute on the surface of 500 ml of 0.05 M phosphate buffer (pH 6.5), then submerged and agitated briefly. This procedure was followed by two 15-min washes in 2X SSC (SSC is 0.15 M NaCl and 0.015 M sodium

citrate). After blotting on filter paper and air-drying, the membrane was baked in vacuo at 80° for 2 hours. Proteins, agarose, and ethidium bromide were removed from the membrane in a one-hour incubation at 70° with 0.1X SSC and 1% SDS. Brief agitation in a large volume of 2X SSC removed most of the SDS; the membrane was then equilibrated in 6X SSC prior to prehybridization.

Membranes were prehybridized for at least 2 hours at 42° in hybridization buffer, which consisted of 0.25% nonfat dry milk in 6X SSC and 50% formamide (53). The prehybridization solution was replaced with 1-2 ml of hybridization buffer containing 1.2 x 10^6 cpm of the appropriate nick-translated probe (specific activity 2-10 x 10^8 cpm/ug DNA), and incubation was continued for at least 24 h. Membranes were washed three times at room temperature in 2 X SSC containing 0.1% sodium dodecyl sulfate, followed by three 15-min washes at 53°. Optimum exposure to Kodak XAR film and DuPont Lightning Plus intensifying screens usually required 2-4 days at -70°.

Membranes were deprobed after one hour at 70° in a solution containing 98% deionized formamide, 0.02 M Tris (pH 7.4), and 2 mM EDTA. To insure removal of material which might contribute to background, membranes were treated for 1 hour at room temperature with 0.5 N NaOH and 1.5 M NaCl, then neutralized at 42 o with 0.2 M Tris (pH 7.5), 0.5% SDS, and 0.1X SSC. After removal of SDS by a brief rinse in 2X SSC, the membrane was equilibrated in 6X SSC and prehybridized.

DNAse I sensitivity

The protocol employed for assessment of DNAse I sensitivity was adapted from that used for determination of nuclease-sensitive sites in the chicken lysozyme gene (28,50). Tissues (1 g) were homogenized in the cold with 15ml of homogenization buffer [0.15 mM spermine, 0.5 mM spermidine, 60mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 15 mM Tris (pH 7.4), 0.5 M sucrose, 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma; stock solution was 0.1 M in ice-cold isopropanol], and the homogenate was filtered through four layers of gauze. Nuclei were pelleted at 2000 x g for 10 min (Sorvall SS-34 rotor, 4400 rpm at 4^{o}) and washed in equal volumes of homogenization buffer containing 0.5% Triton X-100 until nearly white (usually 3 washes). Detergent was removed with two additional washes in wash buffer containing 0.35 M sucrose without Triton, and nuclei were resuspended at a DNA concentration of 200-1000 ug/ml in resuspension buffer [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 (pH 7.4), 1 mM PMSF]. Aliquots (0.1 ml) were dispensed into sterile microfuge tubes.

Five mg of DNAse I (Cooper Biomedical, Worthington DPFF, 2333 units per mg) was dissolved in 0.29 ml of 50% glycerol-15 mM NaCl such that the stock solution contained 40,000 units per ml. This stock solution was diluted five-fold into 0.16 ml of resuspension buffer containing 10 mM $MgCl_2$. Half of the resultant solution was added to the first tube of nuclei; the remainder was serially diluted two-fold into equal volumes of resuspension buffer containing 10 mM $MgCl_2$ before addition to the remaining aliquots of nuclei. DNAse I

concentrations were thus 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 0 units per ml. Nuclei were digested with DNAse I at 0^{o} (ice-water bath) for 10 min; reactions were terminated with EDTA at a concentration of 10 mM. Digested nuclei were spun for 1 min at 2000 x g (5500 rpm in the Sorvall centrifuge with adapters for the SS-34 rotor) and washed once or twice in homogenization buffer. Alternatively, 0.2 ml of Buffer C (containing pronase) was added to each tube immediately after termination of the reaction with EDTA; pronase digestion proceeded with no washing of the nuclei.

Pellets were resuspended in 0.2 ml of 10 mM Tris (pH 7.6), 100 mM NaCl, 10 mM EDTA, and 0.2 ml of Buffer C [0.2 M Tris (pH 7.5), 0.44 M NaCl, 2% SDS, 25 mM EDTA] containing 400 ug/ml of pronase (Calbiochem; stock solution was 20 mg/ml). Incubation was at 37^{o} for 4-6 h, followed by extraction with phenol and chloroform-isoamyl alcohol as described above. The isolated nuclease-treated DNA samples were incubated at -20^{o} overnight with one-half volume of 7.5 M ammonium acetate and 3.75 volumes of 95% ethanol. DNA pellets were recovered by centrifugation for at least 10 min in a microfuge.

Samples (usually 50-100 ug of DNA) were resuspended in 0.1-0.2 ml of water, and 6 ul portions were diluted to 1.2 ml for the purpose of obtaining optical density readings at 260 and 280 nm in order to determine concentration. A portion of each sample was digested with PstI or another restriction endonuclease. After extraction and back-extraction with phenol and chloroform-isoamyl alcohol, samples were precipitated with ammonium acetate and ethanol and recovered by centrifugation. Samples were resuspended in 100 ul of H_2O, and the

concentration of DNA in each sample was again determined spectrophotometrically. DNA fragments, usually 7.5 ug per lane, were resolved by electrophoresis at 20-50 V through 0.6-1.5% agarose gels for 24-60 hours, and transfer and hybridization were performed as detailed above.

Restriction endonuclease sensitivity

Techniques for the examination of the sensitivity of isolated nuclei to restriction endonuclease cleavage were developed by others in this laboratory; methods were based on the protocols for determination of DNAse I sensitivity described above. One gram of fresh tissue was homogenized in 7.5 ml of homogenization buffer [0.15 mM spermine, 0.5 mM spermidine, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 15 mM Tris (pH 7.4), 0.35 M sucrose, 1 mM PMSF] and filtered through 4 layers of cheesecloth. Homogenate was removed from cheesecloth by pouring an additional 2.5 ml of homogenization buffer through it. The homogenate was spun at 2000 rpm for 10 min in a refrigerated IEC centrifuge; the supernatant was removed, and the pellet was resuspended in 5 ml of homogenization buffer containing 0.35 M sucrose. This suspension was layered atop 5 ml of homogenization buffer containing 0.5 M sucrose and spun again at 2000 rpm for 10 min. The resultant pellet was resuspended in 5 ml of homogenization buffer containing 0.35 M sucrose, and 0.15 ml of 20% Triton X-100 was added. The mixture was allowed to stand on ice for 5 min, then spun at 2000 rpm for 10 min. The Triton wash was repeated, yielding nuclei. These nuclei were resuspended in 5 ml of homogenization buffer containing 0.35 M sucrose and pelleted at

2000 rpm for 5 min. Washed nuclei were then resuspended in 0.5 ml of 10 mM Tris (pH 7.5), 10 mM NaCl, 1 mM EDTA. The resuspended nuclei were incubated with 250 units of PstI for 30 min on ice. One-tenth volume of 10X medium-salt buffer (74) was added, and the incubation was continued at 37° for 2-4 hours. The reaction was terminated by addition of 35 ul of 0.25 M EDTA and the dropwise addition of 65 ul of 10% SDS.

One-half of the digested nuclei (0.3 ml) was added to each of two tubes containing 0.6 ml of Hirt solution [10 mM Tris (pH 7.6), 10 mM EDTA, 1% SDS] at 65°. A 0.25 ml volume of 5 M NaCl was added to each tube of nuclear lysate; the mixture was agitated and incubated at 65° for 5 min, then held at 4° overnight.

Chromosomal DNA was pelleted by centrifugation in the adapters of the Sorvall SS-34 rotor at 12,000 rpm for 30 min. The supernatant (approximately 1 ml) was removed to another tube, and pronase was added to a concentration of 1 mg/ml. Digestion required about 2 hours at 5°. DNA was extracted with phenol and chloroform:isoamyl alcohol (24:1) in the usual manner and precipitated by the addition of one volume of isopropanol. Centrifugation was at room temperature in a microfuge for 10 minutes. The pellets recovered after extraction and precipitation were resuspended in 0.2 ml of 10 mM Tris (pH 7.6), 1 mM EDTA and precipitated with 0.1 ml of 7.5 M ammonium acetate and 0.75 ml of 95% ethanol after overnight incubation at -20°. Finally, the DNA was resuspended in 0.2 ml of Tris-EDTA, and concentration was determined from optical density readings of a diluted sample. Portions were digested with SmaI or KpnI in volumes of 0.2 ml for 4 h. Fresh enzyme

in 0.1 ml 1X buffer was then added, and the incubation was continued for an additional 2-3 h. To facilitate recovery, transfer RNA (1 ug/tube) was added to the sodium acetate and ethanol in the final precipitation reaction. Electrophoresis, transfer, and hybridization were performed by the procedures described above.

Isolation of RNA from murine tissues

RNA was obtained from fresh tissues by a modification of the guanidine thiocyanate-cesium chloride method (15,16,31). Animals were killed by cervical dislocation, and organs were removed with sterile instruments to sterile beakers containing ice-cold phosphate-buffered saline (PBS) without Ca^{+2} or Mg^{+2}; tissues to be used at some future time were plunged into liquid nitrogen and were stored under liquid nitrogen in sterile polypropylene tubes. Fresh tissues were immediately homogenized (1 g tissue per 15 ml) in guanidinium thiocyanate buffer [5 M guanidine thiocyanate (Eastman Kodak), 0.5% N-lauroyl sarcosine, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.1% Antifoam A (Sigma)]. Homogenates were transferred to sterile 30-ml polypropylene tubes, and cell debris was pelleted at 9500 rpm for 10 min in the Sorvall at 10^{o}. Approximately 7 ml homogenate was layered over 3.5 ml of 5.8 M cesium chloride solution in 0.1 M EDTA in new 9/16" x 3.5" polyallomer tubes. RNA was pelleted by centrifugation in the SW41 rotor in a Beckman ultracentrifuge at 35,000 rpm for 22 h at 21^{o}.

RNA was isolated from frozen tissues by a further modification of the same procedure (23). Tissues were pulverized and ground to a fine

particle size with a sterile mortar and pestle in the presence of liquid nitrogen, then transferred to a homogenizer. As the liquid nitrogen evaporated, guanidine thiocyanate solution was added, and the tissue was homogenized and handled as described above.

After removal of the supernatant, samples were dissolved in ice-cold 1 mM EDTA and transferred to 10-ml polypropylene tubes. Insoluble material was removed by centrifugation in the Sorvall SS-34 rotor for 20 min at 9500 rpm and 4^{o}; it was re-extracted two or more times. One-fourteenth volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol were added to the recovered material, and the samples were held at -20^{o} overnight. RNA was recovered by pelleting at 10,000 rpm for 30 min at 0^{o} in the Sorvall SS-34 rotor.

In order to exchange salts and eliminate any contaminating DNA, samples were emulsified in 3 M sodium acetate (pH 5.2) and left on ice for 30 minutes. Following recentrifugation to pellet the RNA while leaving the DNA in solution, this procedure was repeated. The pellets were resuspended in 1 mM EDTA; one-fourteenth volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol were again added, and the samples were held at -20^{o}. Samples were reprecipitated in this manner at least one more time prior to selection of polyadenylated RNA on oligo(dT)-cellulose. Polyadenylated RNA was selected by two cycles of oligo(dT)- cellulose chromatography (74). Samples were dissolved in 2 ml of water by heating briefly to 65^{o}. After removal of an amount to be used for spectrophotometric determination of concentration, an equal volume of 2X loading buffer [loading buffer was 20 mM Tris (pH 7.6), 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS] was added, and the samples were

heated to 65° for 5 minutes, then cooled to room temperature. Samples were applied to 1-ml columns of oligo(dT)-cellulose (Collaborative Research) which had been washed with water, 5 mM EDTA in 0.1 N NaOH, and more water, and equilibrated with loading buffer. The pass-through was collected, heated to 65°, cooled, and reapplied to the column, which was then washed with 5-10 volumes of loading buffer. Ribosomal and other non-polyadenylated RNAs were eluted with loading buffer containing 0.1 M NaCl. Finally, poly(A)-containing RNAs were eluted from the column with 10 mM Tris (pH 7.5), 1 mM EDTA, 0.05% SDS. After adjustment of NaCl concentration to 0.5 M, samples were heated to 65°, cooled, and reapplied to columns (washed with 0.1 N NaOH in 5 mM EDTA and reequilibrated) for an additional cycle. Fractions of interest were precipitated with a one-fourteenth volume of 3 M sodium acetate or 4.5 M ammonium acetate and 2.5 volumes of 95% ethanol; they were reprecipitated twice and rinsed with 70% ethanol before gel electrophoresis.

Electrophoresis, transfer, and hybridization of RNA

RNA was subjected to electrophoresis under denaturing conditions (67,112). Lyophilized RNA pellets, containing 1-5 ug of polyadenylated or 20-50 ug of total cytoplasmic RNA, were resuspended in 7.5 ul of sterile water. To each sample was added 22.5 ul of 1.5X sample buffer [0.3 ml deionized formamide, 0.108 ml 37% formaldehyde, 0.006 ml of 1 M sodium phosphate buffer (pH 6.5)], which was then denatured for 15 minutes at 53°. Samples were quickly chilled on ice, and one-tenth volume of 10X loading buffer was added (50% glycerol, 0.4% bromophenol

blue, 0.4% xylene cyanol, 1 mM EDTA) and the samples were applied to 1 mm wells.

Gels, usually 1.2% agarose, contained 10 mM sodium phosphate buffer (pH 6.5) and 2.2 M formaldehyde in a total volume of 300 ml. The running buffer was 10 mM sodium phosphate buffer (pH 6.5), which was recirculated. Generally, electrophoresis was carried out at 100 V for approximately 6 hours.

Following electrophoresis, gels were soaked for 15 minutes in 15X SSC. They were then transferred to either nitrocellulose or nylon (Zetabind; AMF Cuno) membranes, using 15X SSC as the transfer buffer. Nitrocellulose membranes were rinsed in 6X SSC prior to vacuum drying at 80^{o} for 2 hours. Nylon membranes were handled as described above for DNA transfers. Prehybridization and hybridization procedures were identical to those described for DNA-containing membranes.

Probes

The LTR probe employed in these studies was a 0.37 kb PstI-SmaI fragment derived from pRFM7. The unique-sequence probe 5′ to the pRFM7 LTR was the 0.9 kb PstI fragment of subclone 7a1/0.9, while the 3′ unique-sequence probe was the BglII-PstI insert of subclone A1. The unique-sequence probe immediately 5′ to the pRFM8 LTR was the 60-bp HincII-BglII fragment of subclone D2; and the probe derived from the region 3′ to that solitary LTR was the 250-bp HincII fragment of subclone B1. pTE201, a gift from Carmen Cadilla, was used as a probe of a unique-sequence gene (gene 33) which is transcribed in the liver. All probes were prepared by nick translation, except that a random

oligonucleotide-primed probe was used for detection of RNA.

Results

Presence of LTRs in both clones

Two lines of evidence suggested that both pRFM7 and pRFM8 contained LTRs. From restriction endonuclease characterization of the two subclones, each contained a PstI site separated from partially overlapping SmaI/KpnI sites by approximately 0.37 kb (Fig. 2). Additionally, each subclone contained a unique PstI fragment which hybridized to the LTR probe (Fig. 3). These fragments were consistent in size with the putative LTR-containing fragments, 1.3 and approximately 8 kb, predicted from the restriction maps of pRFM7 and pRFM8, respectively.

Solitary LTRs

In the course of isolating these clones from the lambda Charon 9 library derived from RFM/Un mouse spleen DNA, the possibility that these clones contained complete endogenous proviruses was eliminated by their failure to hybridize to an ecotropic-specific env gene probe and to a probe derived from the gag-pol region of a MuLV-related retrovirus. However, the possibility that the clones represented partial or defective MuLV-related proviruses could not be dismissed. The restriction endonuclease sites in the regions flanking the LTRs in pRFM7 and pRFM8 did not coincide with sites characteristically associated with known endogenous MuLV-related proviral structural genes

Figure 2. Restriction endonuclease maps of pRFM7 and pRFM8. (A) The inserts and their orientations in pBR322 are shown. The probes derived from these subclones are delineated by brackets. They include 7A, a 0.9 kb PstI fragment; 7B, a 100-bp BglII-PstI fragment; 8A, a 60-bp HincII-BglII fragment; 8B, a 250-bp HincII fragment; and LTR, a 0.37 kb PstI-SmaI fragment derived from pRFM7. (B) Sequencing strategy is indicated for each LTR. A, AvaI; AII, AvaII; B, BamHI; BI, BglI; BII, BglII; E, EcoRI; H, HindIII; Hc, HincII; Hf, HinfI; K, KpnI; P, PstI; PI, PvuI; PII, PvuII; S, SmaI; X, XhoI; Xb, XbaI. An asterisk denotes a fragment labeled at a subclone vector site.

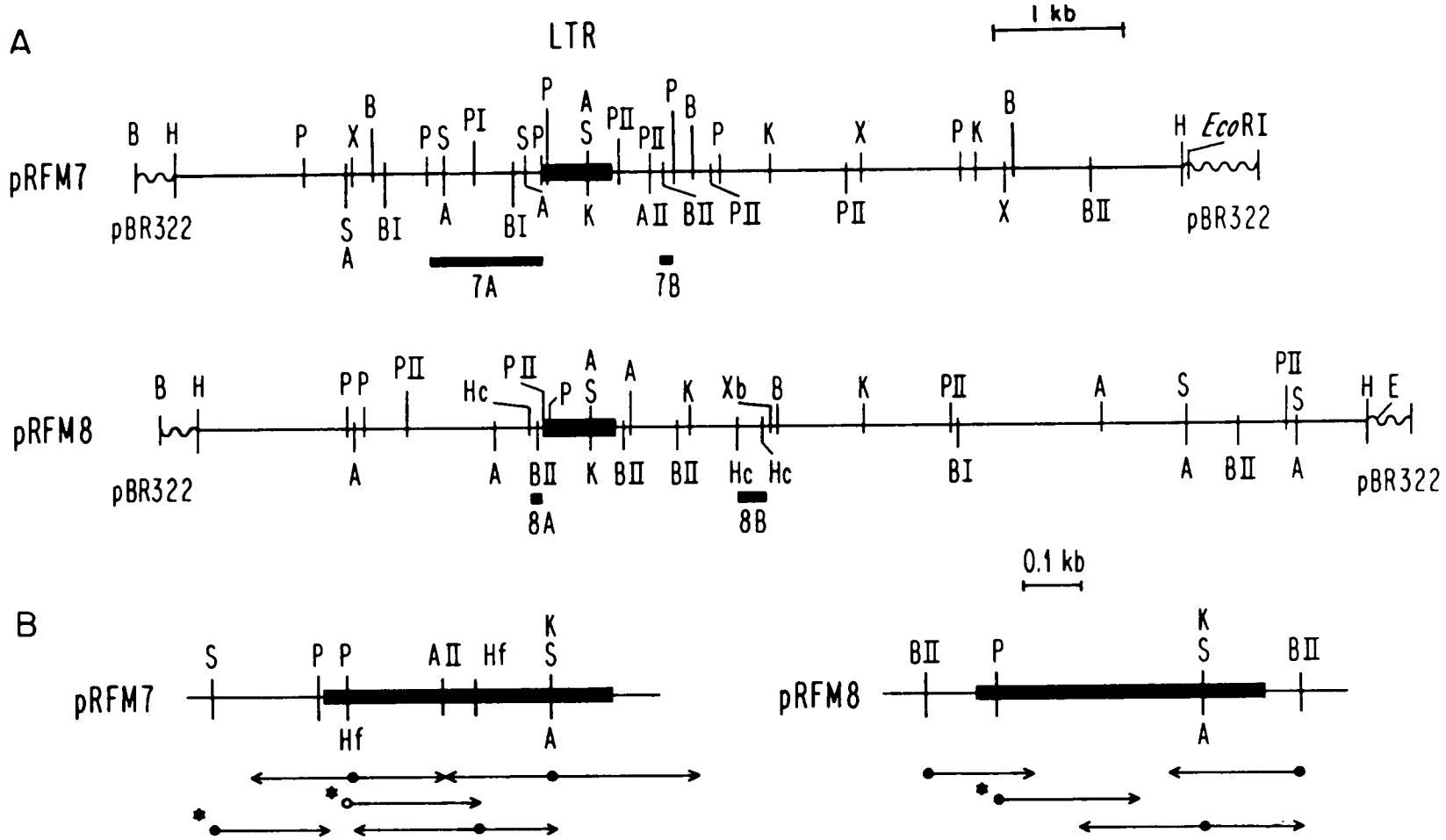
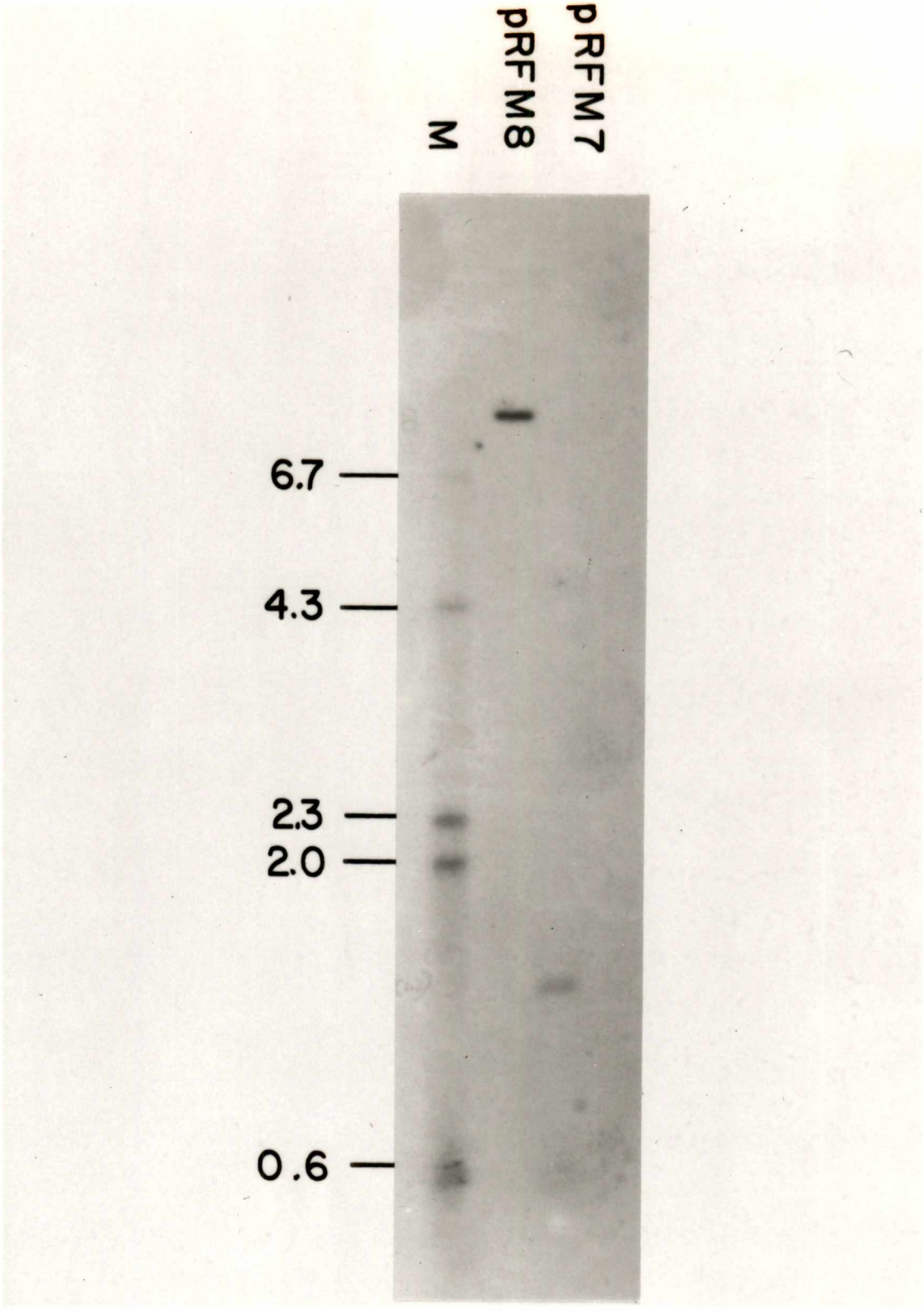

A
LTR
1 kb
pRFM7
pBR322
pBR322
EcoRI
7A
7B
pRFM8
pBR322
pBR322
8A
8B
B
0.1 kb
pRFM7
pRFM8

Figure 3. Hybridization of the LTR probe to PstI fragments of pRFM7 and pRFM8. PstI-digested DNA fragments from pRFM7 and pRFM8 were fractionated by electrophoresis through 0.6% agarose, transferred to a nylon membrane, and hybridized to the LTR probe. The autoradiogram of his membrane is shown. Each lane of plasmid DNA contains approximately 50 pg. M, ^{32}P-end-labeled HindIII fragments of bacteriophage lambda.


M
pRFM8
pRFM7
6.7
4.3
2.3
2.0
0.6

(1,9,10,39,52,80,81,84,85). Thus it appeared that these LTRs were not associated with any intact viral structural genes.

In order to determine whether these elements were independent structures, it was necessary to determine whether tRNA primer binding sites adjoined the 3′ ends of these LTRs, and whether the LTRs were bounded by 4-bp direct repeats of cellular DNA sequence. Both of these questions were addressed by nucleotide sequence analysis of the LTRs and their flanking regions (61). Neither subclone harbored a sequence recognizable as a primer binding site in the vicinity of the LTR (61; Fig. 4), precluding the possibility that the pRFM7 and pRFM8 LTRs represented the 5′ LTRs of incomplete proviruses. Moreover, the sequence CAAG immediately 5′ to the pRFM7 LTR was repeated in the sequence adjacent to its 3′ 11-bp inverted terminal repeat, and the sequence ACCT was likewise found in the regions immediately flanking the pRFM8 LTR (61). Additionally, two basepairs had been deleted from each of the 13-bp inverted terminal repeats normally characteristic of the LTRs of unintegrated, infectious viruses of the MuLV type (99); the deletion of two basepairs from each terminus is a consequence of the retroviral integration process (114). Hence both LTRs were found to be independent elements, or solitary LTRs, rather than constituents of incomplete or defective proviruses.

Nucleotide sequence analysis also demonstrated in the two solitary LTRs several of the canonical features of murine retroviral LTRs: a "CAAT" box, "TATA" box, polyadenylation signal and site, and a putative cap site (115). The sequences of the two LTRs were very similar (98% homologous), and closely resembled the LTR of RFv (87% homologous), the

Figure 4. Nucleotide sequences of the solitary LTRs derived from pRFM7 and pRFM8 compared to the LTR of RFv. Flanking sequence duplications and inverted terminal repeats are boxed; putative transcriptional regulatory sequences are indicated. Numbering begins at the first T in the inverted terminal repeat and ends with the A in the 3´ inverted repeat.

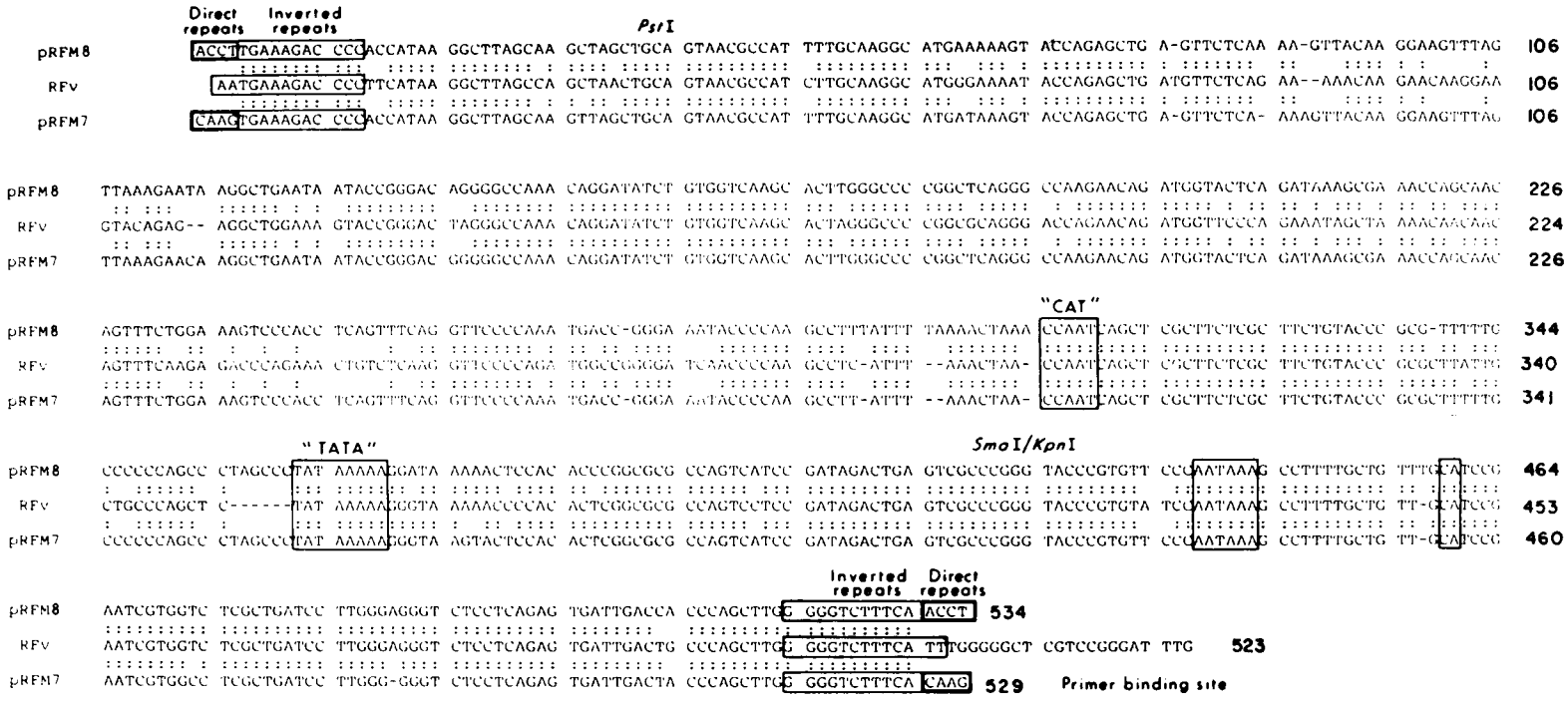

Direct repeats
Inverted repeats
PstI
pRFM8
RFv
pRFM7
"CAT"
"TATA"
SmoI/KpnI
Inverted repeats
Direct repeats
Primer binding site

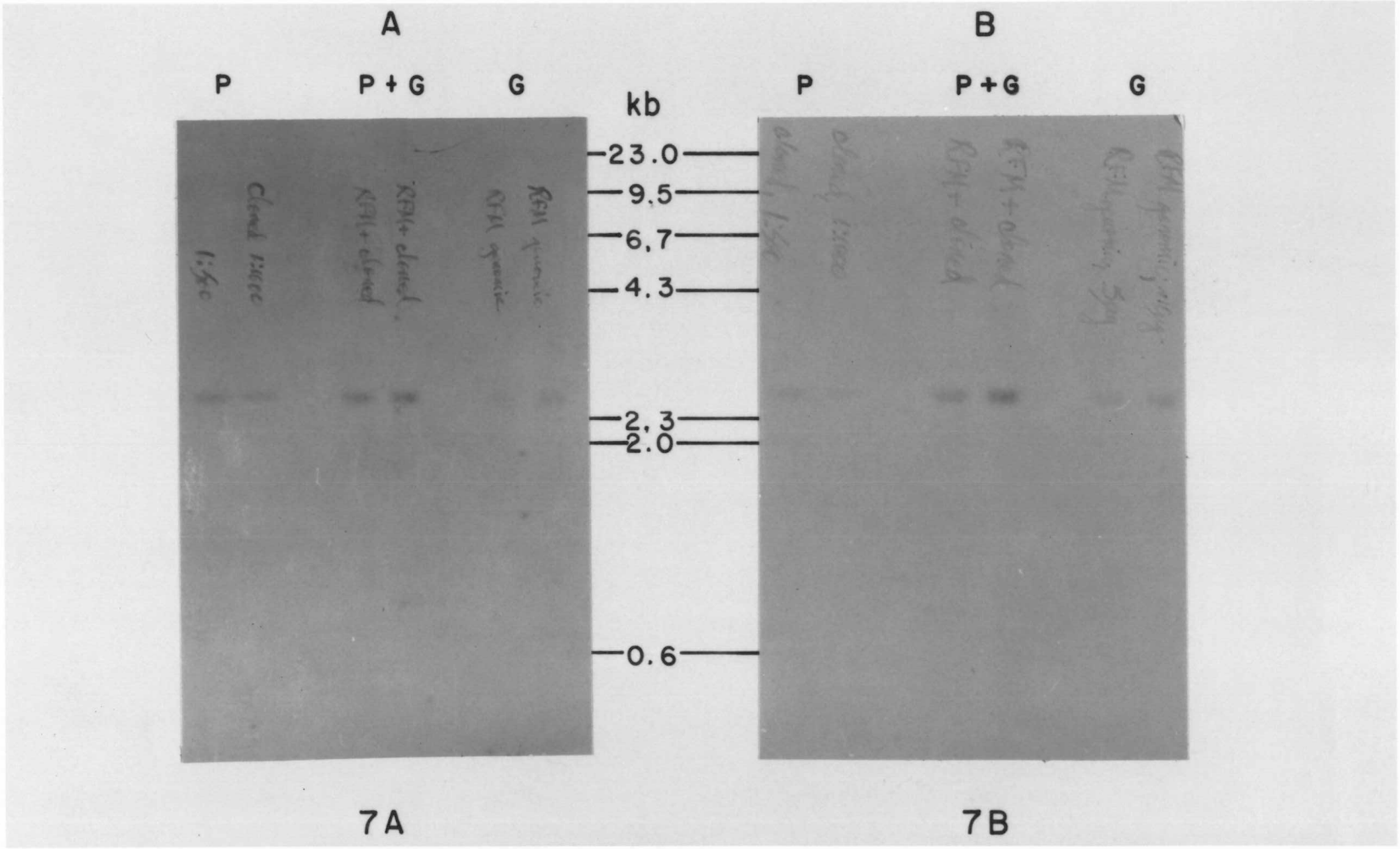
Figure 4

single inducible ecotropic provirus of the RFM/Un mouse (71). However, the LTRs of pRFM7 and pRFM8 contain two distinctive features which are absent in the RFv LTR. One is a duplication of CAGCCC immediately preceding the "TATA" box; the other is the 14-bp sequence TAGTTAAAGAA(T/C)AA in positions 104-117.

Presence of the solitary LTRs in the chromosomal DNA of the normal RFM mouse as single-copy elements

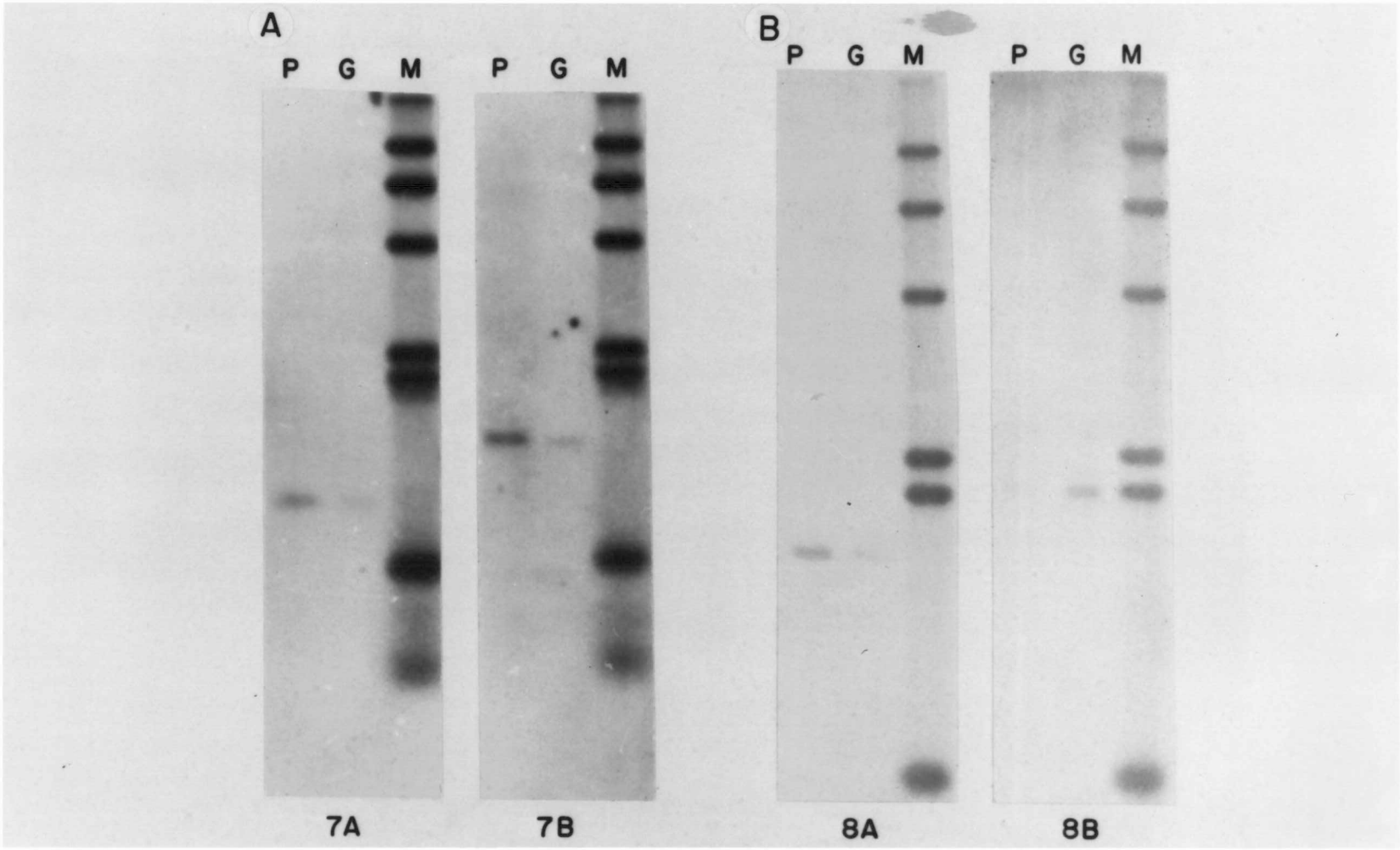
DNA obtained from the livers of normal, male RFM mice was digested with BamHI. After electrophoresis through 0.8% agarose gels, the DNA fragments were transferred to nitrocellulose or nylon membranes. Hybridization to probes derived from unique-sequence regions on either side of the LTR from pRFM7 (see Fig. 2) revealed a single 2.6 kb band which comigrated with the LTR-containing fragment observed in BamHI-restricted pRFM7 (Fig. 5). Digestion of genomic DNA with PstI followed by electrophoresis, transfer, and hybridization to the same probes demonstrated 0.9 and 1.3 kb fragments which comigrated with the corresponding fragments from PstI-digested pRFM7 (Fig. 6A). Similarly, digestion of liver DNA with PstI and hybridization to a probe derived from the unique-sequence region 5′ to the LTR from pRFM8 resulted in the appearance of a 1.5 kb band consistent in size with that predicted from the restriction map of pRFM8; digestion with PstI and BamHI followed by hybridization to a probe derived from the region 3′ to the LTR yielded a 2.0 kb fragment that comigrated with the LTR-containing PstI-BamHI digestion product of pRFM8 (Fig. 6B). These results are consistent with the presence of the solitary LTRs and their

Figure 5. Hybridization of unique-sequence probes from pRFM7 to BamHI fragments of male RFM/Un mouse liver. DNAs prepared from the livers of male RFM/Un mice were digested to completion with BamHI, and the fragments were resolved by electrophoresis through 0.8 % agarose gels, as were the corresponding digestion products of pRFM7. Each lane of plasmid DNA contained approximately 50 pg; genomic DNA lanes contained approximately 5 or 10 ug. After transfer to a nylon membrane, hybridizations, washes, and autoradiography were performed as detailed in the text. (A) Hybridization of the probe derived from the region 5′ to the pRFM7 LTR. (B) Hybridization of the probe derived from the region 3′ to the pRFM7 LTR after deprobing of the membrane shown in Frame A. P, plasmid; G, genomic DNA; M, marker DNA fragments.



7A 7B

Figure 6. Hybridization of unique-sequence probes to genomic DNA of the RFM/Un mouse. DNAs prepared from the livers of male RFM/Un mice were digested to completion with BamHI, PstI, or both, and the fragments were resolved by electrophoresis through 0.8 % agarose gels, as were the corresponding digestion products of pRFM7 and pRFM8. Each lane of plasmid DNA contained approximately 50 pg; genomic DNA lanes contained approximately 5 ug. However, intensity differences should not be construed as quantitative differences. After transfer to nylon or nitrocellulose membranes, hybridizations, washes, and autoradiography were performed as detailed in the text. (A) hybridization of 7A and 7B to PstI-digested fragments; (B) detection of PstI digestion products by 8A and PstI-BamHI digestion products by 8B. HindIII fragments of bacteriophage lambda were employed as markers. P, plasmid DNA; G, genomic DNA; M, marker.

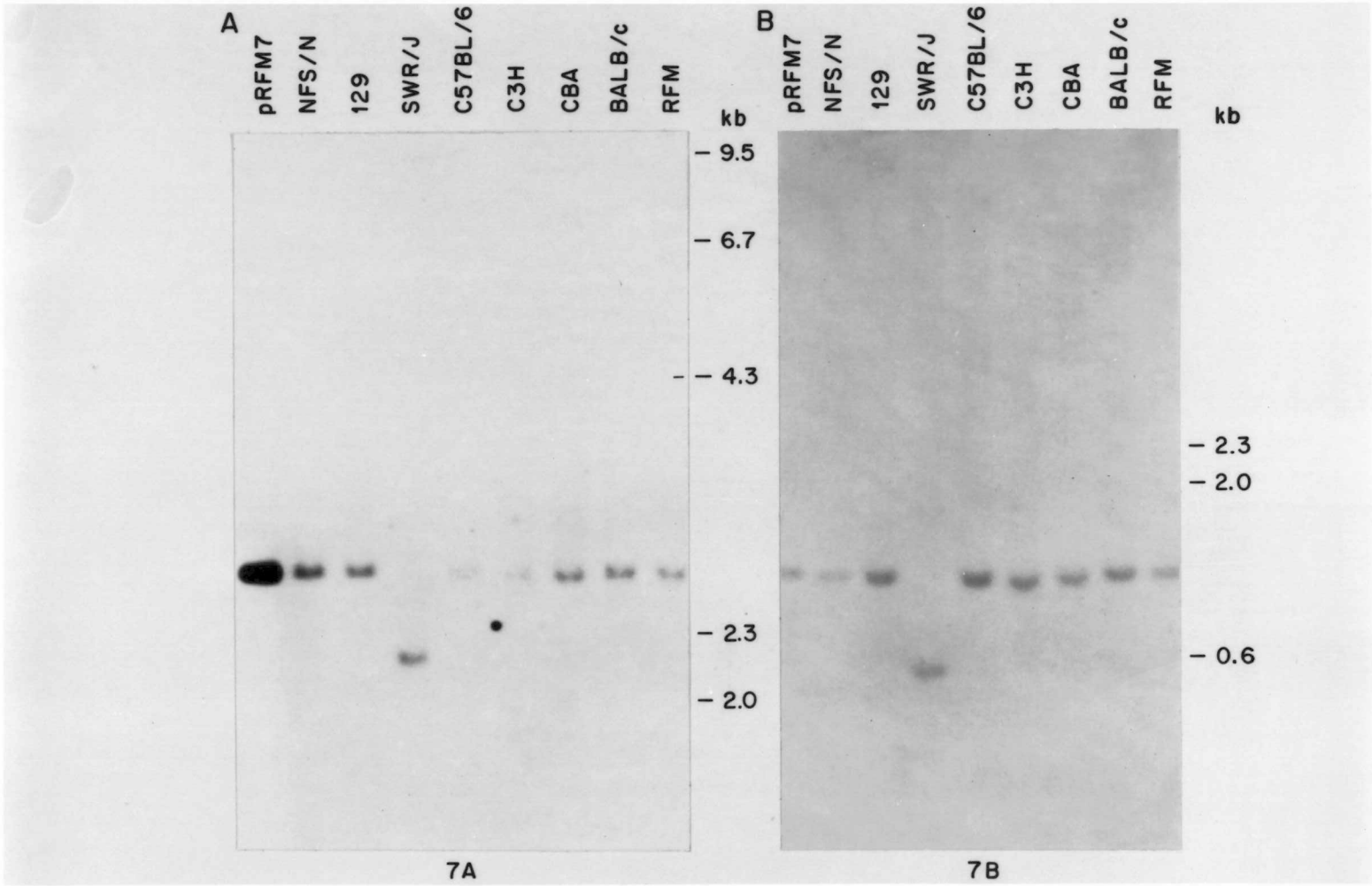

A
P G M
P G M
B
P G M
P G M
7A
7B
8A
8B

flanking regions in the chromosomal DNA of the normal RFM mouse. The absence of additional fragments hybridizing to the unique-sequence probes in the above and other restriction endonuclease digestions of chromosomal DNA (data not shown) suggests that each of these solitary LTRs is probably a single-copy element in the haploid mouse genome.

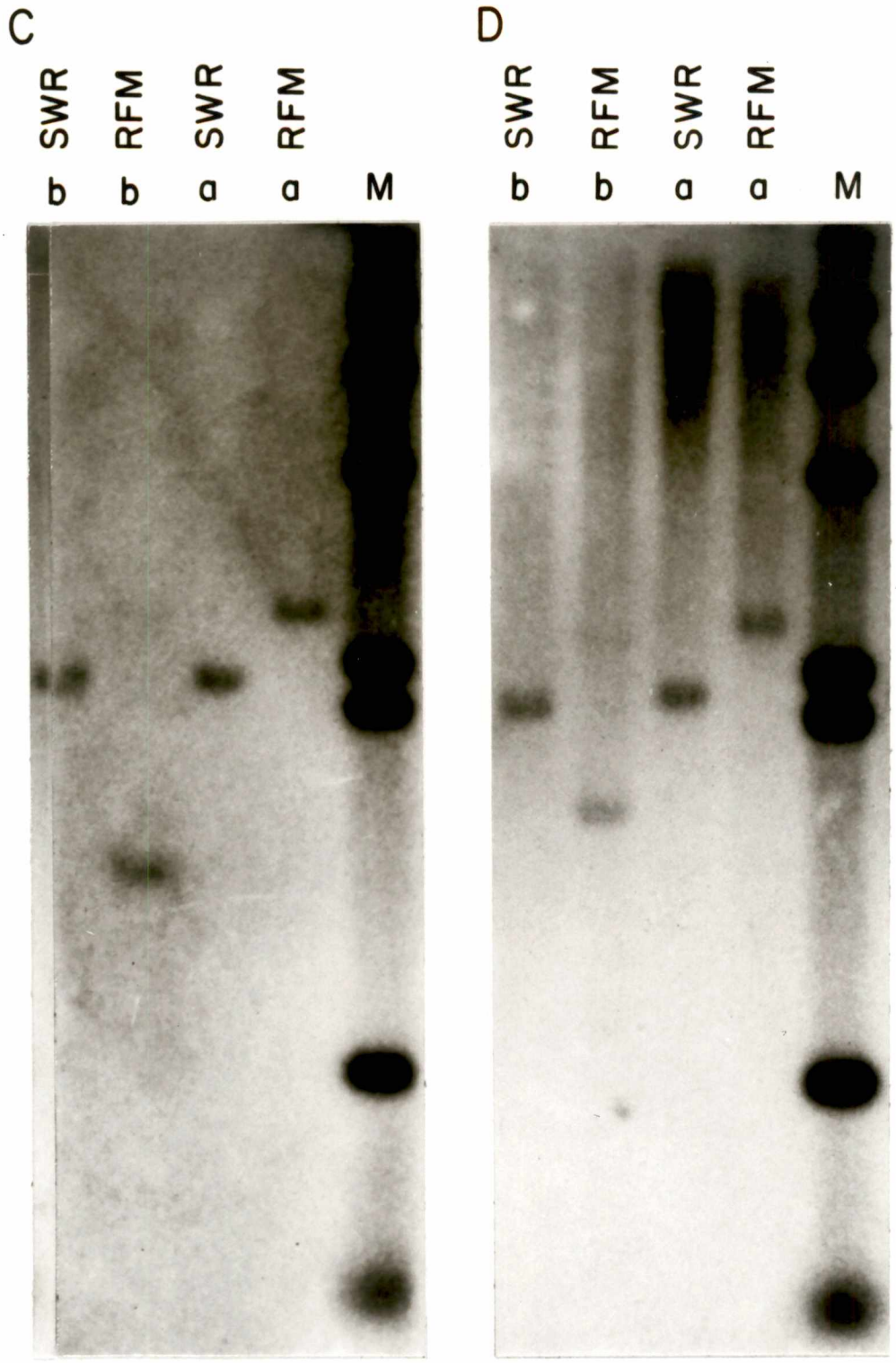
Presence of the solitary LTRs and their flanking sequences in a number of inbred mouse strains

Chromosomal DNA was prepared from the livers of male 129, CBA, C3H, C57BL/6, BALB/c, SWR/J, RFM/Un, and NFS/N mice. After digestion with BamHI, PstI, or BamHI and PstI and electrophoresis through 0.8% agarose gels, the DNA fragments were transferred to nitrocellulose or nylon membranes and hybridized to the unique-sequence probes derived from the regions flanking the LTRs from pRFM7 and pRFM8. Autoradiography (Fig. 7) revealed the presence in these mouse strains of some or all of the four fragments predicted from the restriction maps of pRFM7 and pRFM8. Only one polymorphism with respect to pRFM7 was generated by these restriction enzymes. The 2.1 kb BamHI fragment and 0.8 kb PstI fragment observed in the DNA of the SWR/J mouse upon hybridization to the probes derived from pRFM7 were approximately 0.5 kb smaller than the fragments from other inbred mouse strains, suggesting that the solitary LTR and flanking regions represented in pRFM7 are present in all but the SWR/J mouse (Fig. 7A,B). Digestions with BamHI in the presence or absence of KpnI revealed that the SWR/J DNA lacks the LTR KpnI site (Fig. 7C,D); furthermore, the sizes of the BamHI and BamHI-KpnI fragments were consistent with the absence of the

Figure 7. Characterization of sequences adjacent to the solitary LTRs in a number of inbred mouse strains. High-molecular-weight DNAs isolated from the livers of male RFM/Un, BALB/c, CBA, C3H, C57BL/6, 129, SWR/J, and NFS/N mice were digested with BamHI (A), PstI (B,E), BamHI alone (C and D, lanes a) or in the presence of KpnI (C and D, lanes b), or with PstI and BamHI (F). Fragments were resolved and transferred as described in the legend to Fig. 3. Hybridizations were to probes 7A (A,C); 7B (B,D); 8A (E), and 8B (F). End- labeled HindIII fragments of bacteriophage lambda were employed as markers. These data should be interpreted qualitatively, as the observed variations in intensity were not reproducible.

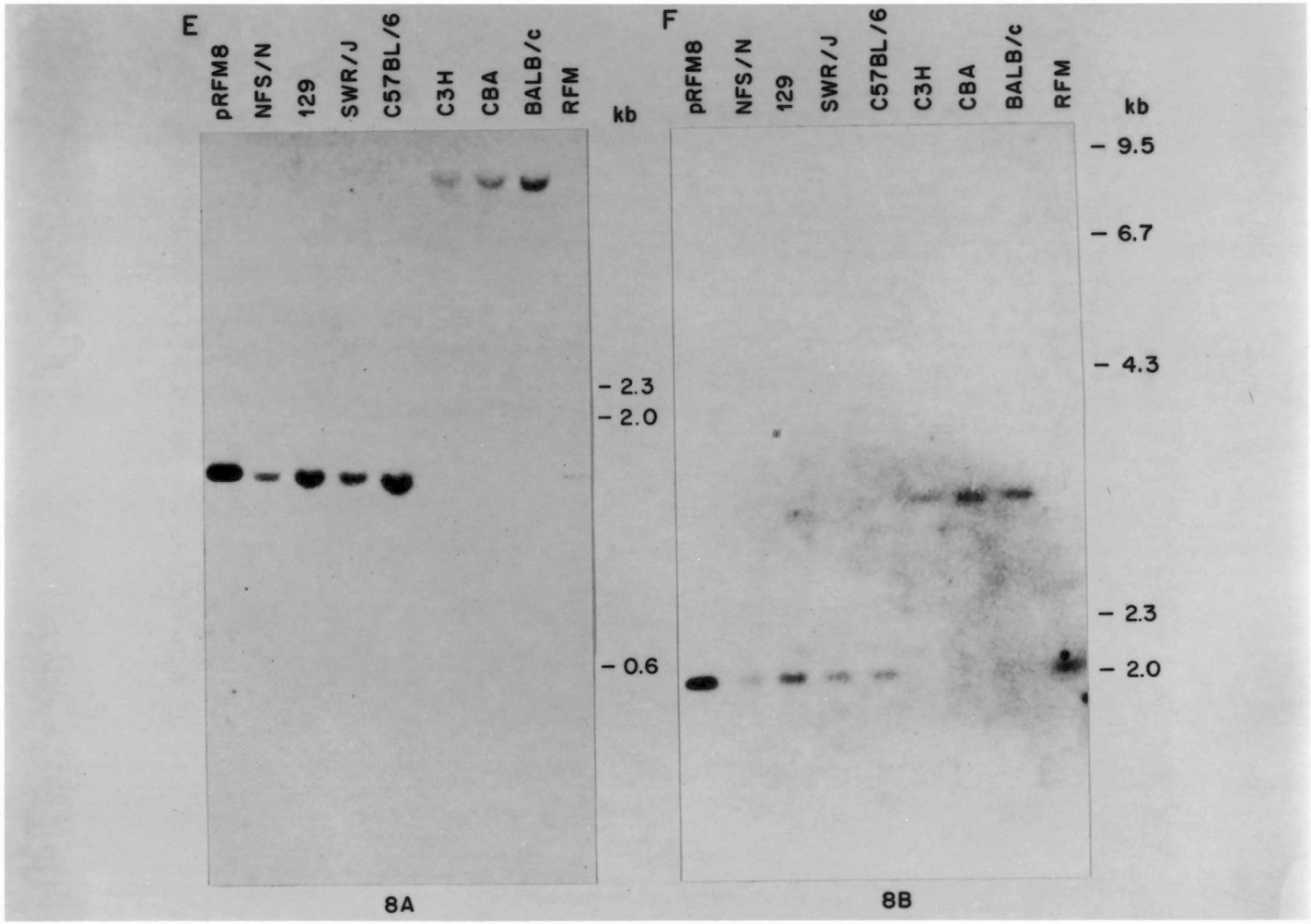

A
pRFM7
NFS/N
129
SWR/J
C57BL/6
C3H
CBA
BALB/c
RFM
kb
9.5
6.7
4.3
2.3
2.0
B
pRFM7
NFS/N
129
SWR/J
C57BL/6
C3H
CBA
BALB/c
RFM
kb
2.3
2.0
0.6

7A 7B



7A

7B


E
pRFM8
NFS/N
129
SWR/J
C57BL/6
C3H
CBA
BALB/c
RFM
kb
- 2.3
- 2.0
- 0.6
F
pRFM8
NFS/N
129
SWR/J
C57BL/6
C3H
CBA
BALB/c
RFM
kb
- 9.5
- 6.7
- 4.3
- 2.3
- 2.0

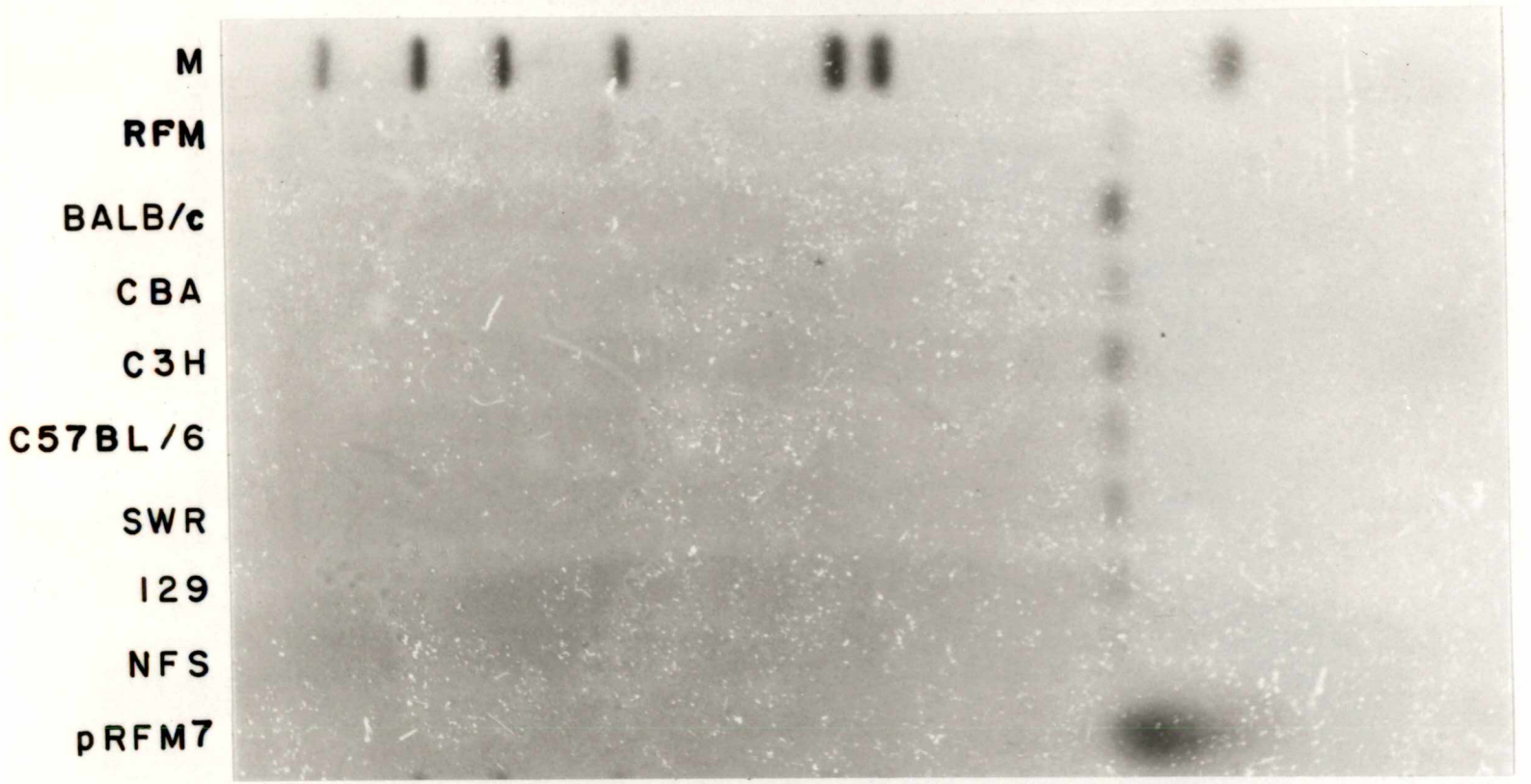
8A 8B

pRFM7 solitary LTR, but not its flanking sequences, in SWR/J DNA. The solitary LTR represented in pRFM8 appeared to be absent in three closely related strains, BALB/c, CBA, and C3H (Fig. 7E,F). In these three strains, the large PstI fragments detected by the probe 5′ to the pRFM8 LTR would be consistent with the absence of the LTR PstI site (see Fig. 2), since this probe detected 1.5 kb PstI fragments in strains harboring the pRFM8 solitary LTR. This was further supported by using the probe derived from the region 3′ to the pRFM8 LTR for gel blot analysis of chromosomal DNA doubly digested with BamHI and PstI. As predicted, this probe detected 2.8 kb PstI-BamHI fragments in the BALB/c, CBA, and C3H mouse DNAs, indicating the absence of an LTR, in contrast to the 2.0 kb fragments detected in the DNAs of those mouse strains harboring the pRFM8 solitary LTR. Moreover, digestion with PvuII and hybridization to the pRFM8 5′ probe revealed conservation of those sites and fragments among the eight mouse strains (Fig. 8A); hybridization of the pRFM8 3′ probe to the PvuII-restricted DNAs yielded fragments consistent with the absence of the pRFM8 solitary LTR from BALB/c, CBA, and C3H DNAs (Fig. 8B); and digestion with KpnI generated no polymorphisms in the region 3′ to the pRFM8 solitary LTR detected by that probe (data not shown). Hybridization of the probe derived from the region 5′ to pRFM7 to PstI-cleaved genomic DNAs revealed no polymorphisms in that 0.9 kb segment of DNA (Fig. 9). Thus the regions flanking the two solitary LTRs have been conserved, irrespective of the presence of the LTRs.

Figure 8. Hybridization of probes from pRFM8 to PvuII fragments of male mouse liver DNAs. DNAs prepared from the livers of male inbred mice were digested to completion with PvuII, and the fragments were resolved by electrophoresis through 0.8 % agarose gels, as were the corresponding digestion products of pRFM8. Each lane of plasmid DNA contained approximately 50 pg; genomic DNA lanes contained approximately 5 ug. However, intensity differences should not be construed as quantitative differences. After transfer to nylon membranes, hybridizations, washes, and autoradiography were performed as detailed in the text. (A) hybridization of 8A to PvuII-digested fragments; (B) detection of PvuII digestion products by 8B. HindIII fragments of bacteriophage lambda were employed as markers.

M
RFM
BALB/c
CBA
C3H
C57BL/6
SWR
129
NFS
pRFM8
B
8B
M
RFM
BALB/c
CBA
C3H
C57BL/6
SWR
129
NFS
pRFM8
A
8A

Figure 9. Hybridization of the probe from the region 5′ to pRFM7 to PstI fragments of male inbred mouse liver DNAs. DNAs prepared from the livers of male inbred mice were digested to completion with PstI, and the fragments were resolved by electrophoresis through 0.8% agarose gels, as were the corresponding digestion products of pRFM7. Each lane of plasmid DNA contained approximately 50 pg; genomic DNA lanes contained approximately 5 ug. However, intensity differences should not be construed as quantitative differences. After transfer to nylon membranes, hybridizations, washes, and autoradiography were performed as detailed in the text. HindIII fragments of bacteriophage lambda were employed as markers.


M
RFM
BALB/c
CBA
C3H
C57BL/6
SWR
129
NFS
pRFM7

Detection of the solitary LTRs and/or their flanking sequences in the DNAs of several feral mouse species

DNAs extracted from livers of Mus musculus domesticus (Centreville, MD), Mus musculus musculus (Czech II), Mus musculus domesticus (Lake Casitas, CA) and from Mus dunni cells in tissue culture were digested with PstI, PstI and BamHI, or PvuII and subjected to Southern gel blot analysis as described above for DNAs obtained from inbred mouse strains. The DNA of M. dunni, which contains sequences which hybridize only weakly to probes derived from MuLV (66), appeared to lack sequences which hybridized to either of the flanking sequence probes derived from pRFM7 (data not shown). However, it did harbor polymorphic fragments which were detected by both pRFM8 probes; the fragment hybridizing to the pRFM8 3′ probe was large enough to include the solitary LTR (Fig. 10).

Both M. m. domesticus (Centreville) and M. m. musculus (Czech II) DNAs contained segments which hybridized without apparent polymorphism to the unique-sequence probes from pRFM7 (Fig. 10C) and pRFM8 (Fig. 10D). Both of these subspecies contained fragments homologous to the 3′ probe from pRFM7 which were too small to accommodate that solitary LTR (Fig. 10B). A similar result was observed in the Centreville mouse with respect to the pRFM8 3′ probe. The Czech II mouse, however, harbored a DNA segment hybridizing to the pRFM8 3′ probe which, although probably polymorphic, was sufficently large to include the pRFM8 solitary LTR (Fig. 10D). The Lake Casitas mouse, a M. m. domesticus subspecies, harbors an endogenous, ecotropic, neurotropic retrovirus which is quite similar to the MuLV-related proviruses of the

Figure 10. Hybridization of probes from the regions flanking the pRFM8 solitary LTR to DNAs prepared from feral Mus species. DNAs prepared from the livers of male RFM/Un (R), Mus musculus domesticus Lake Casitas (LC), M. m. domesticus Centreville (C), M. m. musculus Czech II (Cz) mice and from Mus dunni cells in tissue culture were digested with PvuII, PstI, or BamHI and PstI, and the fragments were resolved by electrophoresis through 0.8 % agarose gels, as were the corresponding digestion products of pRFM8. Each lane of plasmid DNA contained approximately 50 pg; genomic DNA lanes contained approximately 5 ug. After transfer to nylon membranes, hybridizations, washes, and autoradiography were performed as detailed in the text. (A) Hybridization of 8A to PstI-digested fragments; (B) detection by 8B of the products of double digestion by BamHI and PstI; (C and E) hybridization of 8A to PvuII fragments; (D and F) detection of PvuII fragments by 8B. End-labeled HindIII fragments of bacteriophage lambda were employed as markers.

kb
23
9.5
6.7
4.3
2.3
2.0
0.6
M
LC
R
pRFM8
M
R
LC
pRFM8
M
R
C
Cz
pRFM8
M
R
C
Cz
pRFM8
M
R
pRFM8
pRFM8
M. dunni
M
R
pRFM8
pRFM8
M. dunni
F
E
D
C
B
A

inbred M. musculus musculus strains; it lacks endogenous xenotropic proviruses (11,34,38,66,89). It does, however, contain DNA sequences which hybridize to all four flanking probes without apparent polymorphism, and thus may very possibly harbor both the pRFM7 and pRFM8 solitary LTRs (Fig. 11E,F). These data are summarized in Table 1.

Methylation status of the solitary LTRs

Hypomethylation within the LTRs of avian endogenous viruses has been correlated with transcriptional activation and gene expression (32). The LTRs of most murine type C retroviruses contain common SmaI/KpnI restriction endonuclease sites approximately 110 bp

Table 1. Distribution of Two Solitary LTRs and Their Flanking Sequences Among Feral Mus Species.[a]

	5′ pRFM7	LTR	3′ pRFM7	5′ pRFM8	LTR	3′ pRFM8
Mus dunni	-	-	-	+(p)	?	+(p)
M. m. domesticus (Centreville)	+	-	+(p)	+	-	+(p)
M. m. musculus Czech II	+	-	+(p)	+	?	+(?p)
M. m. dom. Lake Casitas	+	+	+	+	+	+

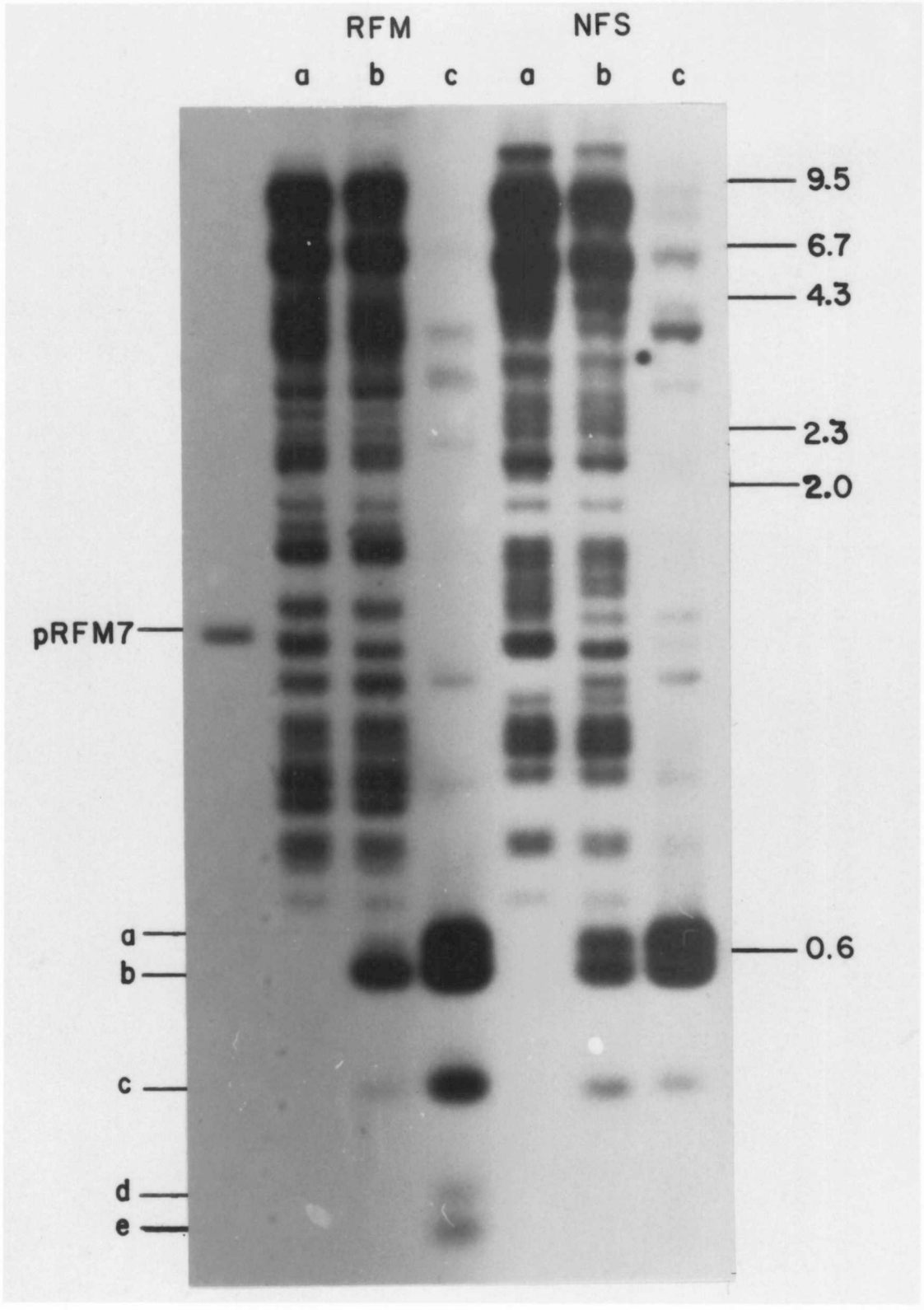
[a]Absence of a fragment is indicated by a minus sign, and its presence is indicated by a plus sign. Polymorphic fragments are indicated (p). A question mark denotes those fragments for which definitive assignments could not be made.

Figure 11. Hybridization of probes from the regions flanking the pRFM7 solitary LTR to DNAs prepared from feral Mus species. DNAs prepared from the livers of male RFM/Un (R), Mus musculus domesticus Lake Casitas (LC), M. m. domesticus Centreville (C), M. m. musculus Czech II (Cz) mice were digested with PstI, and the fragments were resolved by electrophoresis through 0.8% agarose gels, as were the corresponding digestion products of pRFM7. Each lane of plasmid DNA contained approximately 50 pg; genomic DNA lanes contained approximately 5 ug. After transfer to nylon membranes, hybridizations, washes, and autoradiography were performed as detailed in the text. (A and C) Hybridization of 7A to PstI-digested fragments; (B and D) detection of PstI digestion products by 7B. HindIII fragments of bacteriophage lambda were employed as markers.

kb
23
9.5
6.7
4.3
2.3
2.0
0.6
M
R
LC
pRFM7
D
M
R
LC
pRFM7
C
M
R
C
Cz
pRFM7
B
M
R
C
Cz
pRFM7
A

downstream from the "CAAT" box, 50 bp downstream from the "TATA" box, and 30 bp downstream from the cap site, as well as a common PstI site about 38 bp from the 5′ end. Double digestion of cellular DNA with PstI and KpnI generates LTR-related fragments (Fig. 12, bands a-e) whose sizes vary according to the elements contained in their U3 regions and hence reflect the several classes of endogenous proviruses (10,86). In order to examine the methylation status of the solitary LTRs, advantageous use was made of the sensitivity to methylation of the restriction enzyme SmaI, for which there is a single recognition site in the LTR. The enzyme KpnI, whose LTR recognition site partially overlaps that of SmaI, is insensitive to methylation. By cleaving genomic DNA with PstI and KpnI or SmaI, it should be possible to estimate the extent of methylation at the LTR SmaI/KpnI site by comparing the intensities of the PstI-KpnI and PstI-SmaI bands detected by a probe derived from the corresponding region of the LTR. In the RFM mouse the two ecotropic and numerous xenotropic proviral LTRs, as well as any solitary LTRs and LTR-containing defective proviruses of this type, contribute to the 0.37 kb PstI-SmaI/KpnI fragment (Fig. 12, band c). In the NFS/N mouse, the only contributions to the 0.37 kb PstI-SmaI/KpnI band arise from solitary LTRs of this type and its single xenotropic provirus (85), and possibly other similar LTR-containing incomplete proviruses. Therefore, both of these mouse strains have been employed in order to assess the methylation status of the solitary LTRs. Fig. 12 shows that in the NFS mouse the intensity of the 0.37 kb PstI-KpnI band (band c) is comparable to that of the corresponding PstI-SmaI band, indicating that at least one of the

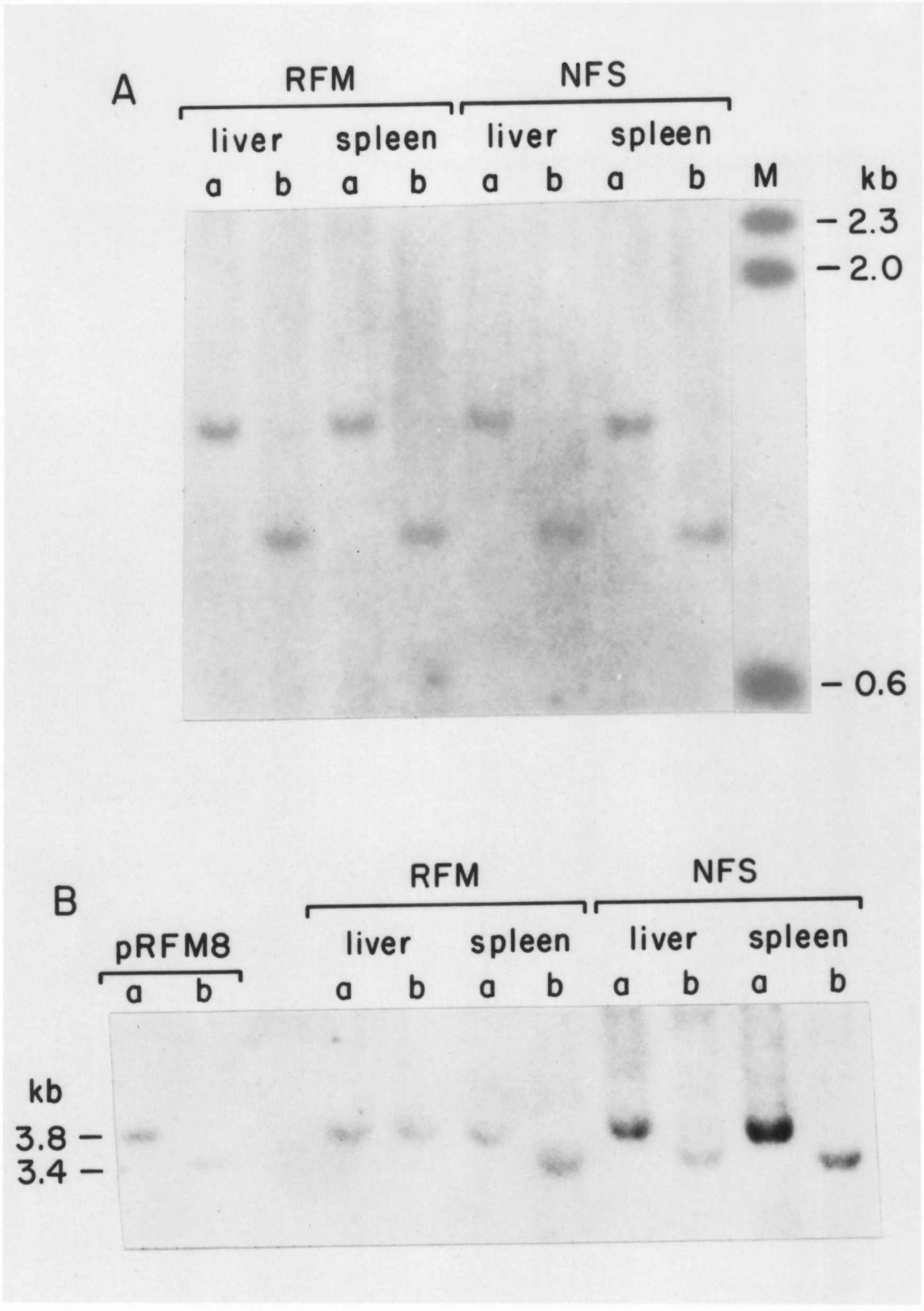
Figure 12. Methylation at LTR SmaI sites. Genomic DNAs from male RFM/Un and NFS/N livers were cleaved with PstI (lanes a) and SmaI (lanes b) or KpnI (lanes c), fractionated through 1.2% agarose, and transferred to a nylon membrane. Hybridization was to a probe derived from the LTR of pRFM7. Markers are HindIII fragments of bacteriophage lambda; the left-most lane contains PstI-digested pRFM7.


RFM
NFS
a
b
c
a
b
c
9.5
6.7
4.3
2.3
2.0
0.6
pRFM7
a
b
c
d
e

contributors to this band is not methylated at the SmaI site. Persistence of the PstI bands harboring other endogenous proviruses after SmaI digestion (Fig. 12, lanes b) indicates that SmaI cleavage is incomplete and that these endogenous MuLV-related LTRs are methylated at the LTR SmaI site in both strains. Hence the hypomethylation suggested by the generation of the 0.37 kb PstI-SmaI fragment must be attributable to a lack of methylation in one or more solitary LTRs. Whereas the intensities of the 0.37 kb PstI-SmaI bands (band c) are similar in RFM/Un and NFS/N mice, the intensity of the 0.37 kb PstI-KpnI band is considerably greater in the RFM strain. These data suggest that the majority of LTRs contributing to this fragment are relatively heavily methylated in the RFM mouse. It is noteworthy that in both strains the fragments defining the larger endogenous LTRs (0.59 and 0.62 kb, bands b and a) also appear to be methylated at the SmaI site.

A more direct approach to examining the methylation status at the SmaI sites within the solitary LTRs involves the use of the unique-sequence flanking regions as probes. Cleavage of genomic DNA with PstI and SmaI should yield a 0.9 kb fragment detectable by the probe derived from the region 3′ to the LTR of pRFM7 if the SmaI site is unmethylated. If that site is methylated, however, the 1.3 kb PstI fragment should persist, and this fragment would then be detected by either the flanking-sequence probe or the LTR probe. The former result has been observed (Fig. 13A), indicating that this solitary LTR is almost completely unmethylated. Similarly, cleavage of genomic DNA with PvuII and SmaI should yield a 3.4 kb fragment detectable with a

Figure 13. Methylation status at the SmaI sites of the solitary LTRs. DNAs extracted from livers and spleens of male RFM/Un and NFS/N mice were restricted with PstI (A, lanes a) or PvuII (B, lanes a) with (lanes b) or without subsequent digestion by SmaI. Fragments resolved by electrophoresis through 0.8% agarose were transferred to nylon membranes and hybridized to 7B (A) or 8B (B) as described in the legend to Fig. 3. End-labeled HindIII fragments of bacteriophage lambda or pRFM8 digested with PvuII in the absence or presence of SmaI served as markers.


A
RFM
NFS
liver
spleen
liver
spleen
a
b
a
b
a
b
a
b
M
kb
2.3
2.0
0.6
B
pRFM8
a
b
RFM
NFS
liver
spleen
liver
spleen
a
b
a
b
a
b
a
b
kb
3.8
3.4

probe derived from the region 3′ to the LTR of pRFM8 in the absence of methylation; the 3.8 kb PvuII fragment would persist if this site is methylated. As shown in Fig. 13B, the SmaI site corresponding to that in pRFM8 is almost completely methylated in RFM liver DNA, and not detectably methylated in RFM and NFS spleen DNAs or NFS liver DNA. Hence the pRFM7 LTR, but not the pRFM8 LTR, is a primary component of the 0.37 kb PstI-SmaI band from RFM liver DNA which hybridizes to the LTR probe, while both the pRFM7 and pRFM8 LTRs contribute to the 0.37 kb band (Fig. 12, band c) from NFS liver DNA.

DNAse I sensitivity

The sensitivity of isolated nuclei to digestion by DNAse I, as well as hypomethylation, have been correlated with chromatin conformations observed in the vicinity of actively transcribed genetic information (32). Since the SmaI sites of the solitary LTRs were not detectably methylated in certain mouse tissues (vide supra), it was of interest to determine whether chromatin in the vicinity of the solitary LTRs was sensitive to the activity of DNAse I. By isolating nuclei in the presence of the stabilizing agents spermine and spermidine and the chelating agents EDTA and EGTA, and subjecting these nuclei to digestion with exogenously added DNAse I in the presence of Mg^{+2} (rather than Ca^{+2}, for which DNAse I has a strong but not absolute requirement), it is possible to observe DNAse I sensitivity over a wide range of concentrations and, in effect, to generate a high-resolution DNAse I sensitivity pattern under conditions which minimize the contributions of endogenous nucleases (28,50). Experiments of this

type were performed using nuclei isolated from spleen and liver of male RFM mice. In order to assess the DNAse I sensitivity of DNA in regions containing MuLV-related sequences, the DNAs isolated from such nuclei after DNAse I digestion were restricted with PstI, fractionated by electrophoresis through agarose, transferred to membranes, and hybridized to the LTR probe. Figure 14 shows that most of the LTR-related sequences in the liver are moderately to relatively resistant to the action of DNAse I. In order to determine specifically whether the solitary LTRs lay in DNAse I-sensitive domains, the flanking sequence probes were hybridized to membranes obtained from the same experiments described above. Figure 15 shows the DNAse I sensitivity patterns of three of the four unique-sequence probes, those derived from pRFM7 and that derived from the region 5′ to pRFM8. While they appear to be slightly more sensitive to DNAse I than are LTR-related sequences in general, they certainly do not appear to be hypersensitive. Hybridization of a probe derived from a gene which is known to be transcribed in the liver to the DNAse I-treated DNAs served as a control. As shown in Fig. 16, one of the three PstI fragments detected by the gene 33 probe is very sensitive to DNAse I degradation.

Restriction endonuclease sensitivity

A complementary technique for examining chromatin conformation involves determining the accessibility of regions of chromatin to digestion by restriction endonucleases. In this analysis, it is postulated that transcriptionally active regions would be more

Figure 14. Hybridization of the LTR probe to PstI digests of liver nuclei treated with DNAse I. Nuclei isolated from the livers of male RFM mice in the presence of spermine and spermidine were incubated with concentrations of DNAse I ranging from 0 to 4000 units/ml for 10 min at 0^{o}. After phenol extraction and precipitation from ethanol, samples were digested with PstI and subjected to electrophoresis through (A) 1.2% or (B) 0.6% agarose. Transfer to nylon membranes and hybridization to the LTR probe was as described in the text. Each lane contained approximately 5 ug of DNA.

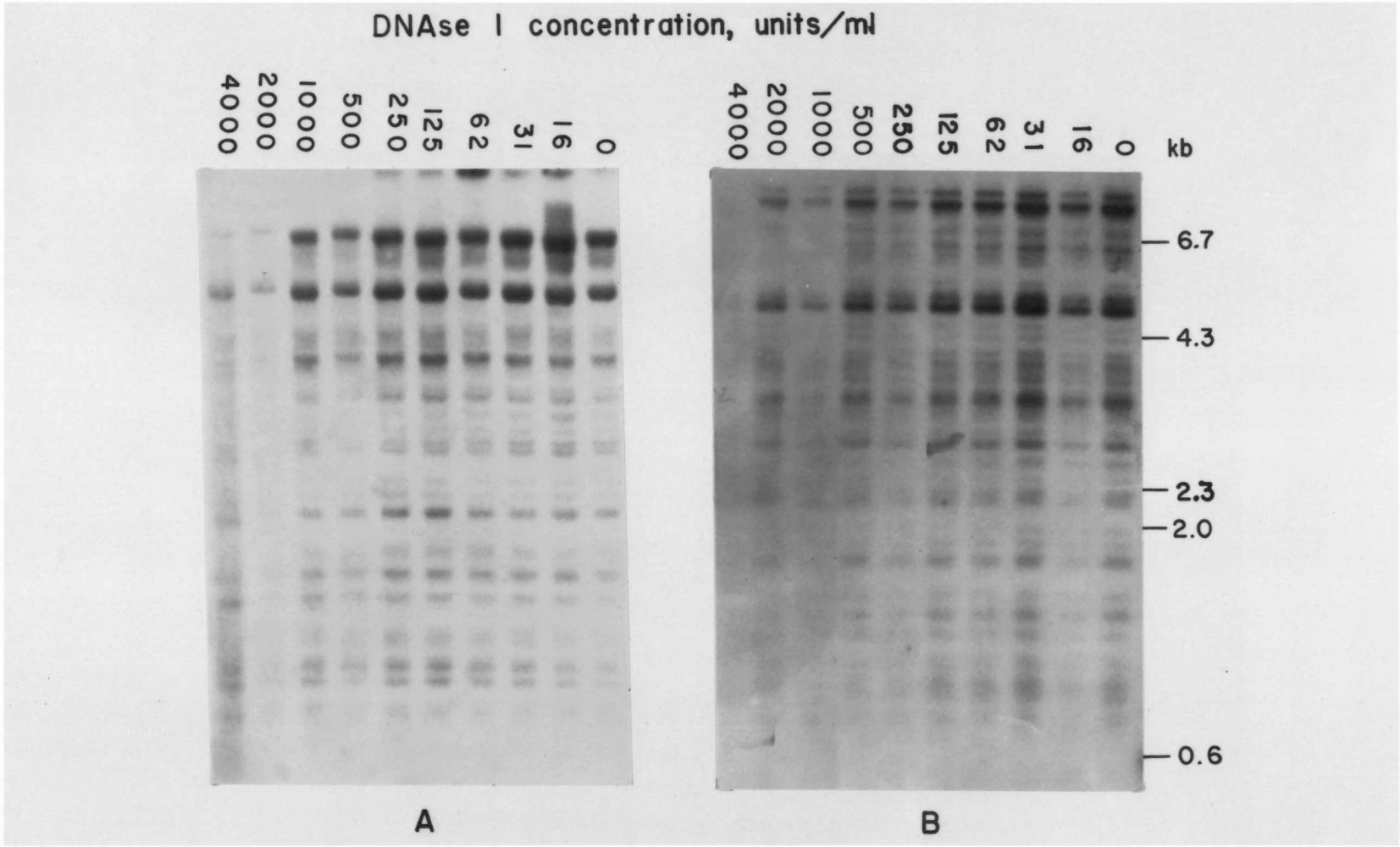

DNAse I concentration, units/ml
4000
2000
1000
500
250
125
62
31
16
0
kb
6.7
4.3
2.3
2.0
0.6
A
B

Figure 15. Hybridization of flanking-sequence probes to PstI digests of male RFM liver nuclei treated with DNAse I. The membranes described in the legend to Figure 14 were deprobed and hybridized to probes 7A, 7B, and 8A. The position of the PstI fragment recognized by each probe is indicated by the position of the PstI-restricted plasmid band.

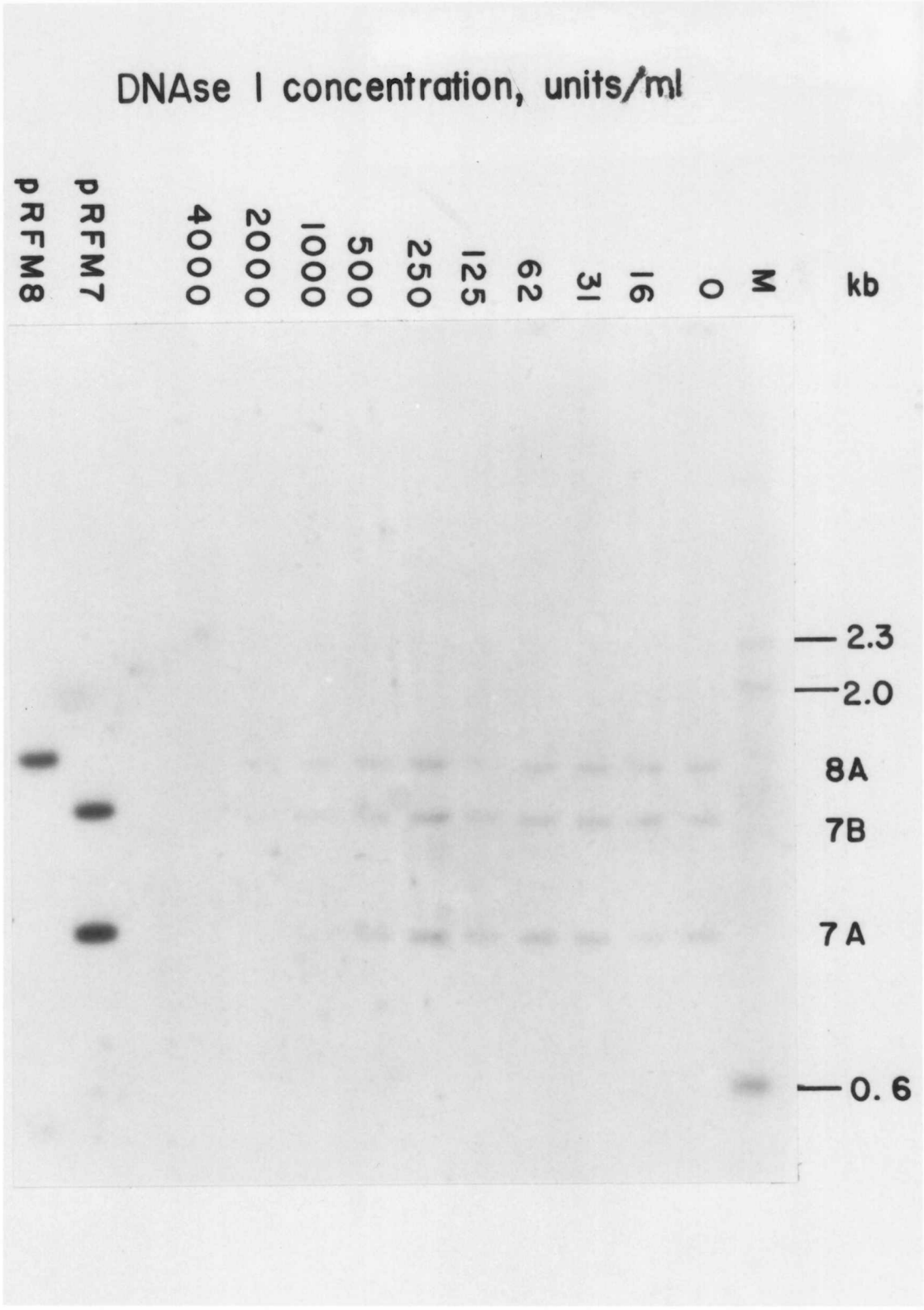
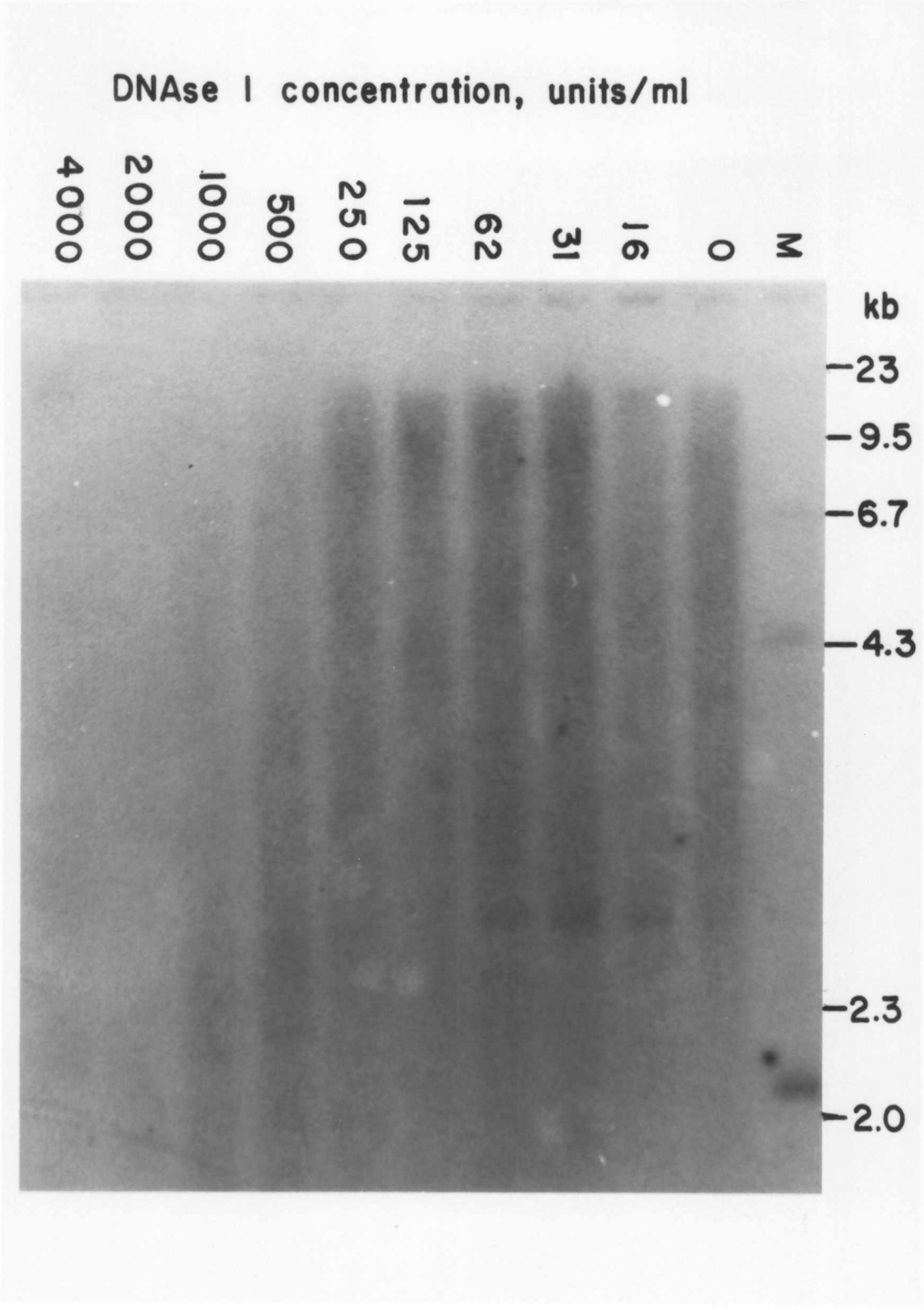

DNAse I concentration, units/ml
pRFM8
pRFM7
4000
2000
1000
500
250
125
62
31
16
0
M
kb
2.3
2.0
8A
7B
7A
0.6

Figure 16. Hybridization of a probe derived from a gene transcribed in the liver to PstI fragments of DNAse I-treated DNAs from male RFM mouse liver. The membranes described in Figure 14 were deprobed and hybridized to pTE201, which represents the hormone-inducible gene 33 and is transcribed in mouse liver.


DNAse I concentration, units/ml
4000
2000
1000
500
250
125
62
31
16
0
M
kb
23
9.5
6.7
4.3
2.3
2.0

susceptible to endonucleolytic cleavage than would inactive regions. Nuclease-sensitive regions would be detected as those released from the bulk, undigested chromatin. Nuclei isolated from male RFM liver and spleen by the method described above for DNAse I sensitivity experiments were incubated with PstI. After lysis of the nuclei, DNA released from bulk chromatin was associated primarily with the Hirt supernatant fraction. Following digestion of portions of these DNA samples with KpnI, SmaI, or PstI, DNA isolated from these experiments was fractionated by electrophoresis through agarose gels and transferred to membranes. Hybridization to the LTR probe (Fig. 17) demonstrated that some of the MuLV-related sequences in liver and spleen had been released from the bulk chromatin by endonuclease digestion, but not as many LTR-related sequences were present as were observed after digestion of genomic DNA with PstI (compare Fig. 12, lanes c). Deprobing of this membrane and sequential rehybridization with each of the unique-sequence probes revealed that sequences in the vicinity of the pRFM7 solitary LTR, but not those neighboring the pRFM8 solitary LTR, had been released from the bulk chromatin (Figs. 18 and 19).

RNA transcription in the vicinity of the solitary LTRs

In order to determine whether transcripts homologous to the unique-sequence regions flanking the pRFM7 and pRFM8 solitary LTRs could be detected in tissues of normal mice, poly(A)-containing RNAs were isolated from various tissues from RFM, NFS, C3H, and SWR mice. After electrophoresis and transfer, hybridization (Figure 20) to each

Figure 17. Hybridization of the LTR probe to PstI fragments released from nuclei isolated from liver and spleen of male RFM mice. Nuclei isolated from male RFM mouse liver and spleen were incubated with (a) 0, (b) 60, (c) 125, or (d) 250 units of PstI. Hirt supernatant DNAs were extracted with phenol and precipitated with ethanol. Approximately 5 ug of DNA per lane was subjected to electrophoresis through 0.6% agarose. Transfer to a nylon membrane and hybridization to the LTR probe were as described above.

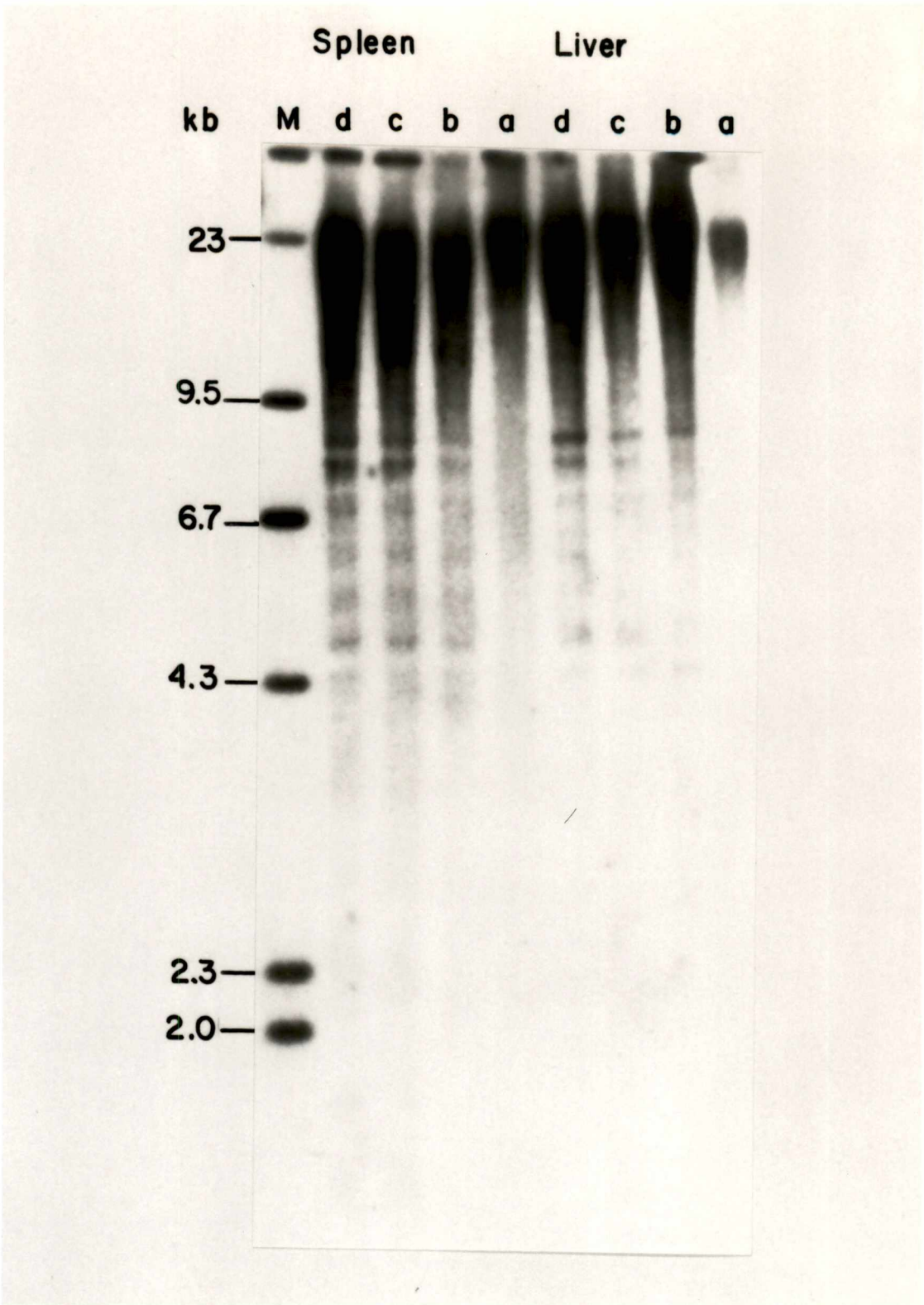

Spleen
Liver
kb
M
d
c
b
a
d
c
b
a
23
9.5
6.7
4.3
2.3
2.0

Figure 18. Hybridization of probes 7A and 8B to preparations of nuclei incubated with PstI. The membrane described in Figure 17 was deprobed and rehybridized to (A) 7A. It was deprobed again and rehybridized to (B) 8B.

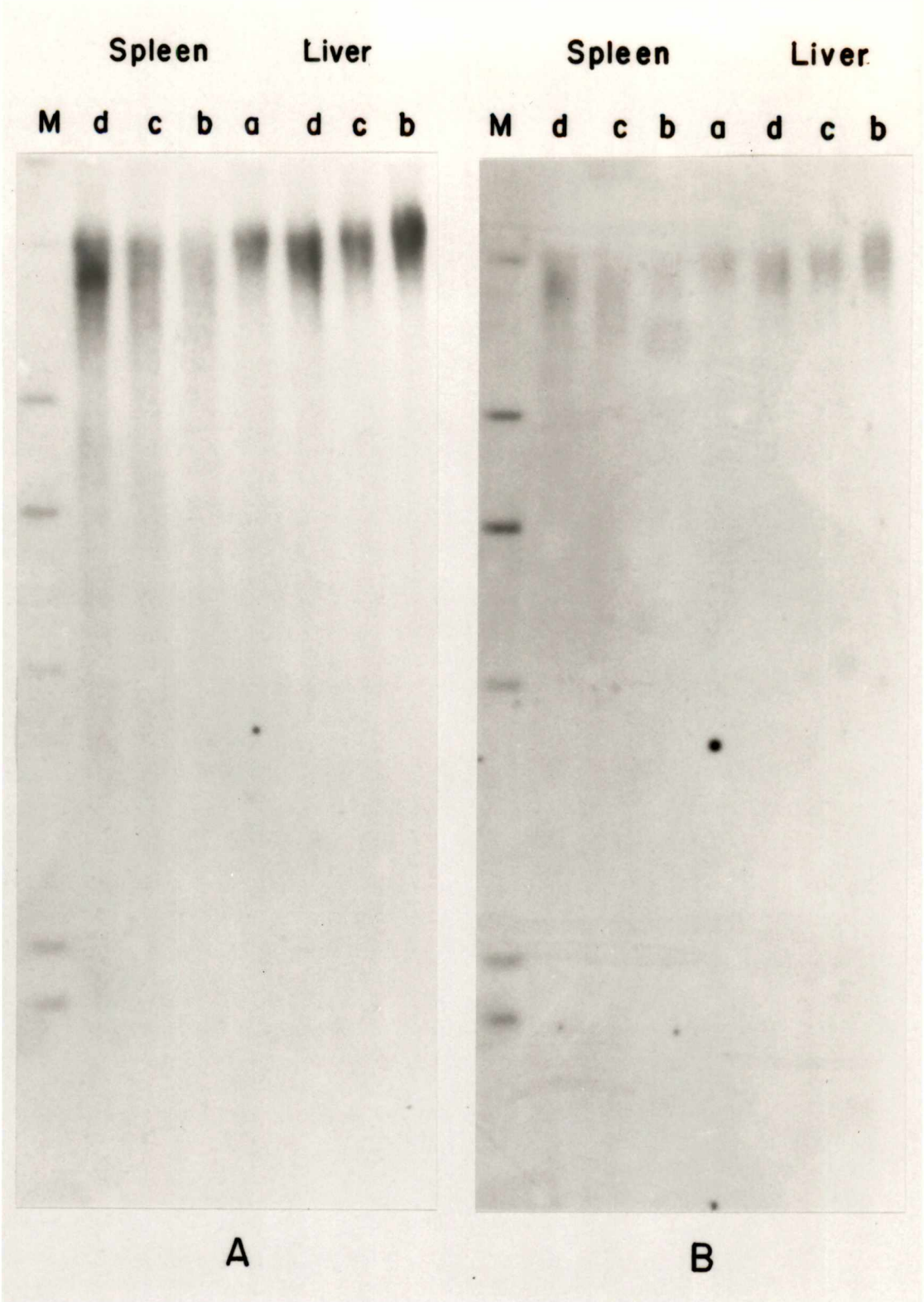

Spleen
Liver
M d c b a d c b
A
Spleen
Liver
M d c b a d c b
B

Figure 19. Hybridization of probes 7B and 8A to preparations of nuclei incubated with PstI. DNAs from lanes d of the experiment described in Figure 17 were fractionated through 1.2% agarose after (a) no additional treatment, (b) restriction with SmaI, (c) restriction with KpnI, or (d) recutting with PstI. A preparation of genomic DNA obtained from male RFM liver was restricted with the same enzymes. Transfer was to a nylon membrane. (A) Hybridization to the probe derived from the region 3′ to the pRFM7 solitary LTR; (B) hybridization of the probe derived from the region 5′ to the pRFM8 solitary LTR after deprobing the membrane shown in Frame A. End-labeled HindIII fragments of bacteriophage lambda were employed as markers.

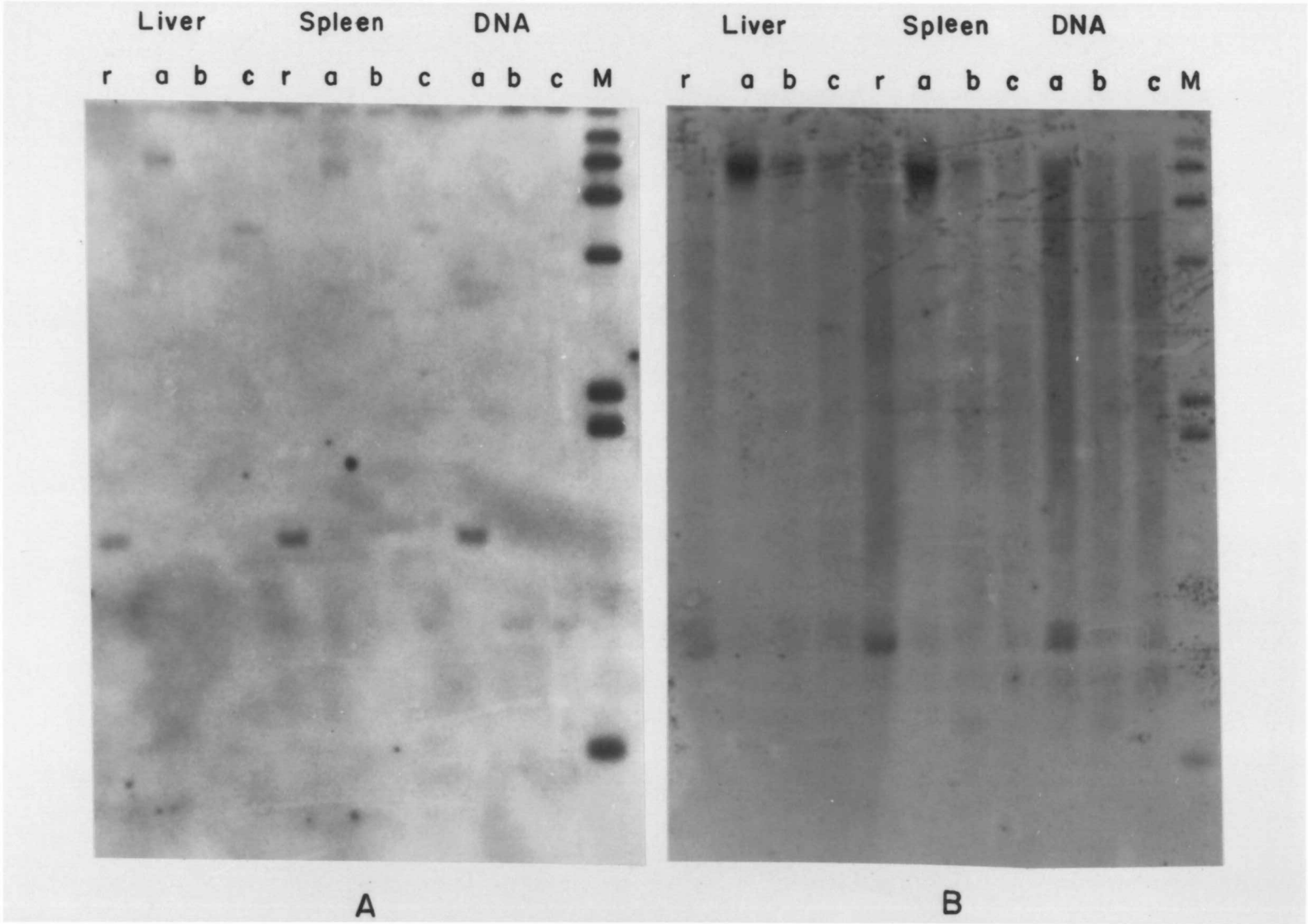
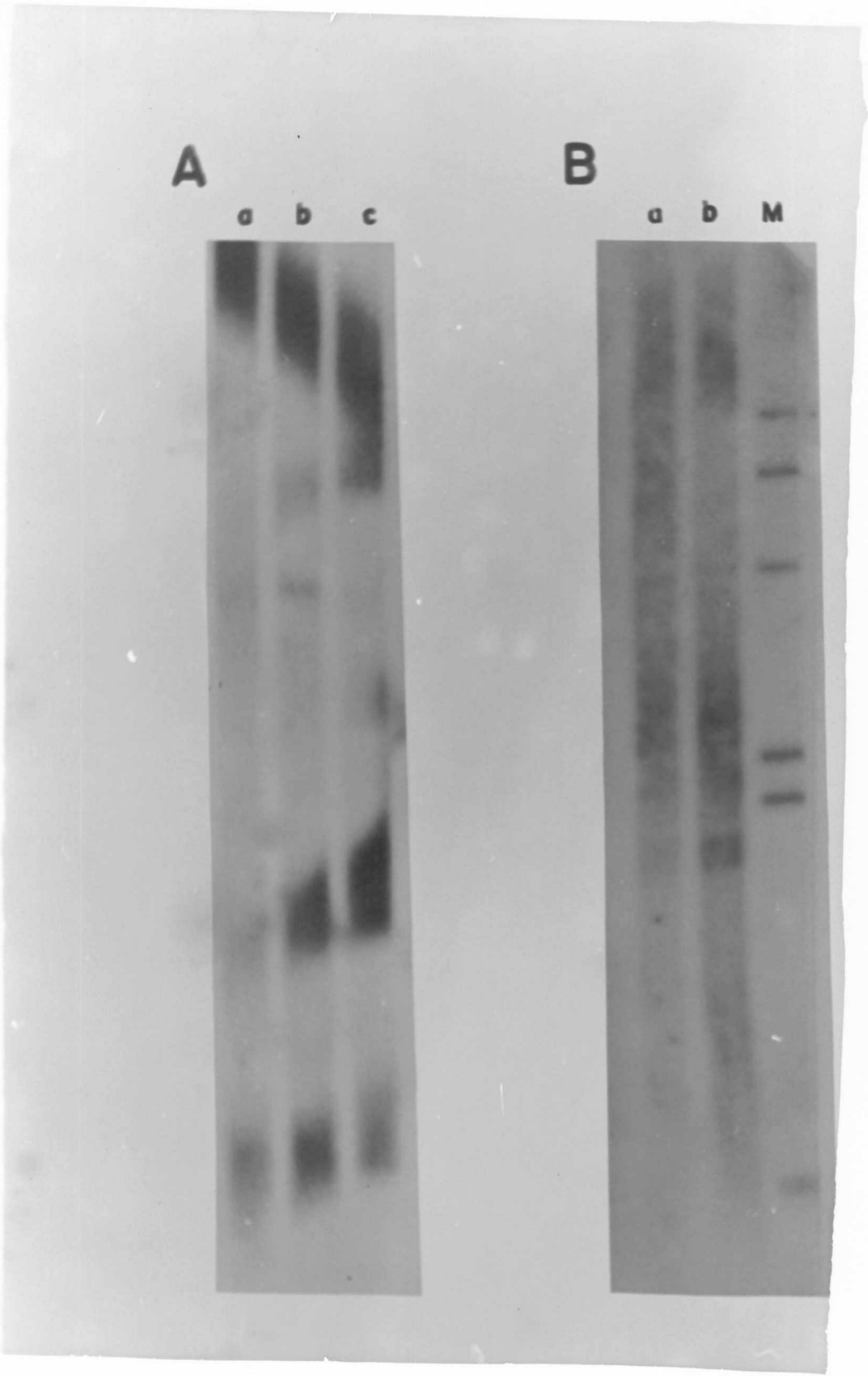

Liver
Spleen
DNA
r a b c r a b c a b c M
Liver
Spleen
DNA
r a b c r a b c a b c M
A
B

Figure 20. Hybridization of probe 7B to RNA. A, Hybridization of oligonucleotide-primed probe to poly(A)-containing RNAs from male RFM spleen (a,b) and liver (c). B, hybridization of nick-translated probe to poly(A)-containing RNA isolated from male RFM liver. M, end-labeled HindIII fragments of lambda.


A
a b c
B
a b M

of the flanking probes yielded no definitive evidence for LTR-promoted transcription. However, preliminary results suggested that the probe from the region 3′ to the pRFM7 LTR (probe 7B) detected a transcript larger than 4 kb.

Discussion

The isolation of three clones containing solitary, RFv-like LTRs from a lambda library derived from RFM/Un mouse spleen DNA was unexpected, particularly since the RFM/Un mouse harbors a single ecotropic provirus and 20 to 50 additional MuLV-related proviruses. On the basis of their representation in this library, one might expect solitary LTRs of this type to constitute a major proportion of the MuLV and related LTR sequence population in the mouse genome. However, the intensities of the 0.37 kb bands in Fig. 12 indicate that there are probably few, if any, additional solitary LTRs of this type present in the RFM and NFS genomes. This finding is consistent with the observation of several VL30 LTRs in the BALB/c genome, which harbors 100-200 copies of complete VL30 elements, and the apparent overrepresentation of VL30 solitary LTRs in a BALB/c genomic library (93).

The genetic mechanism by which these solitary LTRs were introduced into the mouse germline is obscure. The presence of the pRFM7 solitary LTR in two inbred mouse strains (129 and NFS/N) and the presence of the pRFM8 solitary LTR in those two strains plus SWR/J, all of which harbor xenotropic and MCF-type endogenous proviruses but lack endogenous ecotropic proviruses, would argue for a xenotropic origin for these elements. However, the discovery of both of these solitary LTRs in the

feral Lake Casitas mouse, a domesticus subspecies which lacks endogenous xenotropic proviruses (89), negates this argument.

The sequence similarity of the two LTRs (98% homology), at least in the RFM genome, would imply a common origin. Comparisons of the sequence similarity of these two LTRs (98% homology), at least in the RFM genome, would imply a common origin. Comparisons of the nucleotide sequences of these solitary LTRs with those reported for ecotropic MuLVs, xenotropic MuLVs, and MuLV-related endogenous proviral elements are informative. Like endogenous ecotropic and xenotropic MuLV LTRs, the RFM solitary LTRs lack an insert into their U3 enhancer regions of approximately 200 bp of the type found in MuLV-related proviral sequences such as AL10 (86), pRFM16 (81), pRFM6 (80), and several other MCF-like proviruses (10). If the insertion sequences were to be deleted from AL10, MCF247, and pRFM16, the solitary LTRs would share 81%, 82%, and 87% homology, respectively. Similarly, sequence homologies of 85%, 83%, and 87% are observed between the solitary LTRs and the LTRs of the endogenous ecotropic MuLVs BL/Ka(B) (58), AKv (36), and RFv (71), respectively. Perhaps more significantly, the pRFM7 and pRFM8 solitary LTRs share approximately 95% homology with the sequences of the xenotropic LTRs which have been reported (49,57,84). In addition, two particular features are useful in determining the genetic origins of LTRs. Unlike ecotropic viral LTRs, the solitary LTRs contain one copy of the 14-bp sequence TAGTTAAAGAA(T/C)AA in the U3 region (positions 104-117, Fig. 4) which is absent from ecotropic proviruses but present in one or two copies in xenotropic and MCF proviruses (57,80,81,84,86; reviewed in 13 and 115). Also unlike

ecotropic LTRs, they contain a duplication of the 6-bp sequence CAGCCC immediately preceding the "TATA" box (57,84). Thus, on the basis of nucleotide sequence homology and structural features, the mouse genomic solitary LTRs discovered in the present study are most probably of xenotropic origin. However, whereas the avian solitary LTR closely resembles the LTR of an endogenous avian provirus (100), and the endogenous ecotropic proviruses of RFM, C57BL/Ka, and AKR mice differ from each other by only a few base-pair changes except for their inserts (36,58,71), and the sequences of the endogenous xenotropic proviruses likewise differ by only a few base-pair changes (49,57,84), the unambiguous assignment of the pRFM7 and pRFM8 solitary LTRs to a particular class of endogenous proviruses remains to be determined by isolation and characterization of xenotropic proviruses from the RFM/Un mouse genome.

Considering the apparent capability for mobility of the endogenous MuLV and related proviral sequences, and the analogy of proviruses vs. solitary LTRs and transposons vs. IS elements (62), it might be tempting to speculate that the solitary LTRs were independently mobile at one time. However, since our results indicate that the mouse genomic solitary LTRs and their flanking sequences appear to be highly conserved among inbred mouse strains, it is more probable that they are remnants of a recombinational event between the LTRs of a provirus. Such an event would have resulted in excision of the intervening viral genetic material, just as deletion of the provirus from the dilute locus of DBA/2 mice left a solitary LTR (20), and excision of a copia element from the white-apricot locus of Drosophila left a solitary

copia LTR (12). The restriction sites flanking the proviral integration at the dilute locus of DBA/2 mice (20) do not correspond to those observed in the regions flanking the pRFM7 and pRFM8 solitary LTRs. Also, the apparent stability of these LTRs is in sharp contrast to the induced mobility of the RFM/Un ecotropic provirus, which undergoes multiple re-integration events in hematopoietic neoplasias (3). Furthermore, it is interesting to note that the solitary LTR represented by pRFM7, the more widely distributed of the two solitary LTRs, is also hypomethylated to a greater extent in the two tissues examined in two strains of mice. Although the DNAse I sensitivity of chromatin in the vicinity of the solitary LTRs is not particularly informative, it is noteworthy that the regions adjacent to the pRFM7 LTR are susceptible to endonucleolytic cleavage (Fig. 19). If this solitary LTR is serving to promote transcription of adjacent cellular sequences, as might be implied from its methylation status and accessibility to endonuclease, its persistence in the genome might confer some evolutionary advantage.

CHAPTER III

OTHER MURINE LEUKEMIA VIRUS-RELATED ELEMENTS IN THE RFM GENOME

Introduction

Although the genome of the RFM/Un mouse harbors a single ecotropic endogenous provirus and 20-50 copies of other MuLV-related endogenous sequences, including xenotropic and MCF-type sequences, not all of these elements represent complete, intact proviruses capable of directing the production of infectious particles. Non-producing proviruses are nonetheless capable of exerting a profound effect upon the host: they might act as insertional mutagens to abolish gene function, or, by virtue of the transcriptional processing signals contained in their LTRs, they might promote or otherwise modulate the transcription of adjacent genetic regions; additionally, they may serve as parental sequences for the generation of recombinant viruses. Two of these elements have been characterized (80,81). It was of interest to determine what proportion of MuLV-related proviruses in the RFM genome were defective, and which of these resided in transcriptionally active regions of chromatin.

The correlation between hypomethylation of DNA in somatic tissues and transcriptionally active chromatin, and that between nuclease sensitivity of chromatin and transcriptional activation, have been well documented. The demonstration that globin genes were sensitive to DNAse I digestion in the tissues in which they were expressed, but not in other tissues, suggested that the conformation of transcribed DNA differed from that of DNA not being transcribed (118). These

observations have been confirmed and extended in various systems; the heat shock protein loci of Drosophila are among the most thoroughly characterized (55). The nuclease-sensitive sites 5′ to transcribed regions of chromatin reside in nucleosome-free regions, which are nonrandomly distributed (64,122); it is not known whether the nucleosome-free status is attributable to the presence of proteins involved in transcription (55) or to the binding of the nonhistone high-mobility-group proteins 17 and 14 (119). The region of chromatin sensitive to nuclease digestion, although less susceptible than the region immediately 5′ to the transcribed gene, has been found to span several kilobases (28,50,121). A decrease in the number of methyl groups associated with cytosine residues in CpG dinucleotides has also been correlated with transcriptional activity (reviewed in 91) and the presence of nuclease-sensitive sites (63). It is not clear whether methyl moieties interfere with transcription by preventing the binding of RNA polymerase and regulatory proteins, or whether they confer an effect upon nucleosome phasing and higher-order packaging of chromatin which renders it inaccessible to regulatory proteins and the enzymatic machinery of transcription (19). The chromatin structural alterations detectable as sensitivity to nucleases and loss of methylated restriction enzyme sites have been associated with the changes in gene expression which accompany developmental processes (reviewed in 55; 108), environmental stimulation (19), and virus induction (32,82,105).

In the chicken, the endogenous type C retroviral proviruses which are expressed reside in regions of chromatin which are hypomethylated and sensitive to DNAse I (32). Treatment of cells containing silent

proviral genomes with 5-azacytidine, which results in generalized undermethylation of chromatin, effects a change in the conformation of DNA, rendering it sensitive to DNAse I digestion, and activating expression of the proviral sequences (32). In leukemic BALB/Mo mice, whose genome harbors a single copy of Moloney murine leukemia virus, the proviral sequences were found to be sensitive to nuclease digestion only in the target tissue; in older BALB/Mo mice harboring multiple copies of the provirus, only one copy was DNAse I-sensitive, whereas the remaining copies were as resistant to DNAse I digestion as the bulk chromosomal DNA (5). Additionally, the progeny of Mov-7 and Mov-10 mice, each of which carries a mutant Moloney murine leukemia provirus, generated infectious virus following treatment with 5-azacytidine (46), consistent with the notion that hypomethylation is prerequisite but not necessarily sufficient for transcription. Studies of retroviral genomes introduced into embryonal carcinomna cells (48,104) provide particularly strong evidence for the role of methylation in the regulation of gene expression. De novo methylation of exogenously introduced retroviral genomes by F9 embryonal carcinoma cells coincided with loss of viral infectivity upon transfection into permissive cell lines; infectious virus particles were produced when the recipient cells for transfection were treated with 5-azacytidine (104). It should be noted, however, that inactivation of virus replication and expression in early mouse embryonal cells and in teratocarcinoma cells is due to some mechanism other than methylation, as de novo methylation of the integrated virus does not occur before 8 days post-infection (30,83). Moreover, once methylation of the provirus had occurred,

virus expression could not be activated by induction of differentiation, treatment with 5-azacytidine, or transfection of the DNA into permissive cells (30,83), suggesting that methylation may be important for the maintenance of repression. Also, the finding that *in vitro* methylation by bacterial methylases failed to significantly reduce the infectivity of cloned proviral DNAs emphasizes the importance of the specific sites at which methylation occurs (37); this finding implies that hypomethylation, unlike the alteration in chromatin conformation which confers nuclease sensitivity, need not span a large region of the chromosome.

Although murine endogenous proviruses tend to be more refractory to the action of DNAse I than the bulk chromatin (5,77), new integrations tend to occur in regions of chromatin near DNAse I-sensitive sites (92,117). In an analysis of three integrations in retrovirus-induced tumors of chicken and rat and four unselected integrations, both the integration sites and the acceptor sites for integration mapped to within 0.5 kb of a DNAse I-hypersensitive site (117). By infecting NIH3T3 murine fibroblasts with a suppressor-tRNA-containing Moloney murine leukemia virus and recovering regions of the genome containing newly integrated copies of the virus as molecular clones in phage lambda grown on a suppressor-free host, a random sampling of integration sites could be obtained (92). Although 80% of the new integrations selected in this manner were excluded from further analysis because of their content of repetitive sequences, the remaining 20% were found to have integrated into sites not more than a few hundred base pairs from a DNAse I-hypersensitive site (92). While

neither of these studies is conclusive, both suggest that the conformation of chromatin, and thus its accessibility to host factors and viral proteins such as the endonuclease, may be important for the integration process. These observations, coupled with the correlation of DNAse I sensitivity and transcriptional activation, further imply that new integrations would tend to occur in regions of consequence to the host.

The specific DNAse I sensitivities of regions within the LTR have been examined in detail (90,113). The 5′ LTR of Moloney murine leukemia virus integrated into NIH3T3 fibroblasts was found to contain two strong DNAse I-hypersensitive sites, corresponding to the enhancer region and the cap site (113). The 3′ LTR, which does not serve as the initiation site for viral transcription, was found to contain only the strong DNAse I-hypersensitive site corresponding to the enhancer region (113). Similar results were obtained when the bacterial neomycin resistance gene was substituted for most of the coding region of Moloney murine leukemia virus (90). The significance of these findings is suggested by the recent demonstration that six distinct nuclear transcription factors and DNA-binding proteins interact with the enhancer region of the LTR of Moloney murine leukemia virus (103).

This chapter describes the examination of methylation status and DNAse I sensitivity of MuLV-related sequences in the genome of the male RFM/Un mouse. The potential for transcription of the several classes of endogenous MuLV-related proviral elements characterized in the liver and in the spleen is discussed.

Materials and Methods

DNAse I sensitivity

The sensitivity of nuclei isolated from liver and spleen of male RFM/Un mice to digestion by DNAse I was determined by methods described in Chapter II. Densitometric analysis was performed using a Model 2202 LKB Ultroscan laser densitometer with Gelscan software for the Apple IIe computer. Graphics and other further analyses were facilitated by Ability^{R} software for the IBM personal computer.

Methylation status

The methylation status of MuLV-related sequences in the spleen and liver of male RFM/Un mice was examined by restricting genomic DNA with PstI in the absence and presence of SmaI and, as a control, KpnI. The enzyme PstI generally has a recognition site approximately 40 bp in from the 5´ end of the LTR. SmaI, which is sensitive to methylation, recognizes a specific CCCGGG site commomly located immediately downstream from the promoter sites and other transcriptional processing signals in the LTR. KpnI, whose recognition site GGTACC partially overlaps that of SmaI in the LTR, is insensitive to methylation. Restriction endonuclease digestions, electrophoresis, transfer to membranes, and hybridization were performed as detailed in Chapter II.

RNA transcription

Procedures for the isolation and detection of polyadenylated RNA were described in Chapter II.

Probes

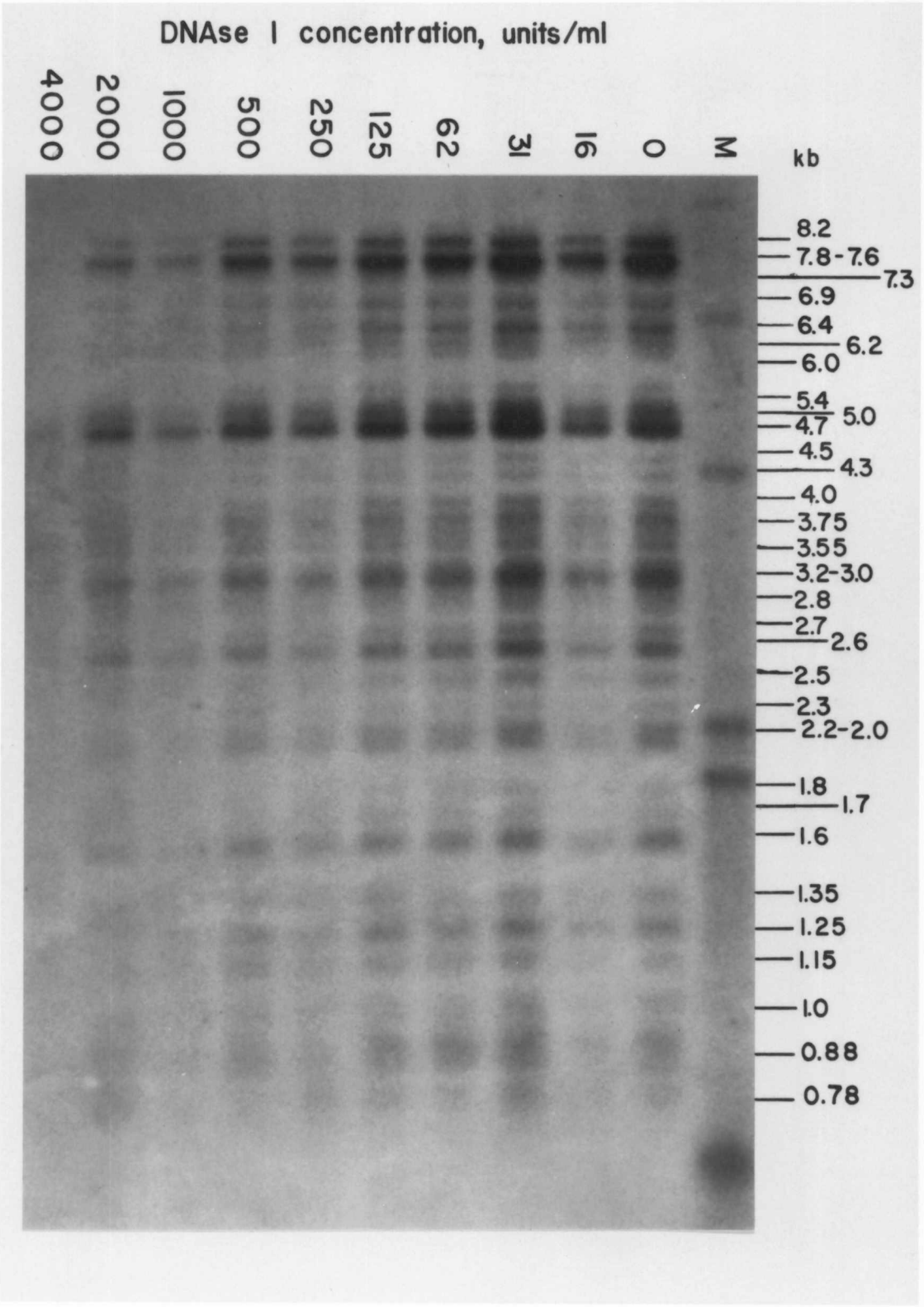
The LTR probe described in Chapter II, a 0.37 kb PstI-SmaI fragment derived from the pRFM7 LTR, was employed in these studies. The gag probe utilized here was a BglII fragment of approximately 2 kb derived from AL10 (86). A 700-bp AvaI-XhoI fragment from pRFM16 which brackets the region deleted from some defective MuLV-related proviruses constituted the gag-pol deletion probe; this probe included the 3′ region of the gag gene and the middle and 3′ portions of the pol gene (81). Derived from the 3′ portion of the envelope gene, the env probe employed in these studies was not specific for detection of ecotropic, xenotropic, or MCF-type proviruses. The IS probe encompassed the 130-bp HindIII-EcoRI segment which is part of the insertion into the U3 region of the AL10 LTR (86). pTE201, a gift from Carmen Cadilla, was used as a probe of a unique-sequence gene, the hormone-inducible gene 33, which is transcribed in the liver of the mouse.

Results

DNAse I sensitivity of LTR-containing MuLV-related fragments

Twenty-six PstI fragments of male RFM/Un liver DNA, many of which contained more than a single individual copy of sequences hybridizing to the LTR probe (Fig. 21, lane 0) have been assigned approximate molecular weights. Although both 0.6% and 1.2% gels were employed for the fractionation of DNA fragments at low voltage, the resolution obtained under these conditions was not sufficient to allow discrimination of individual bands greater than 5 kb. Fragments

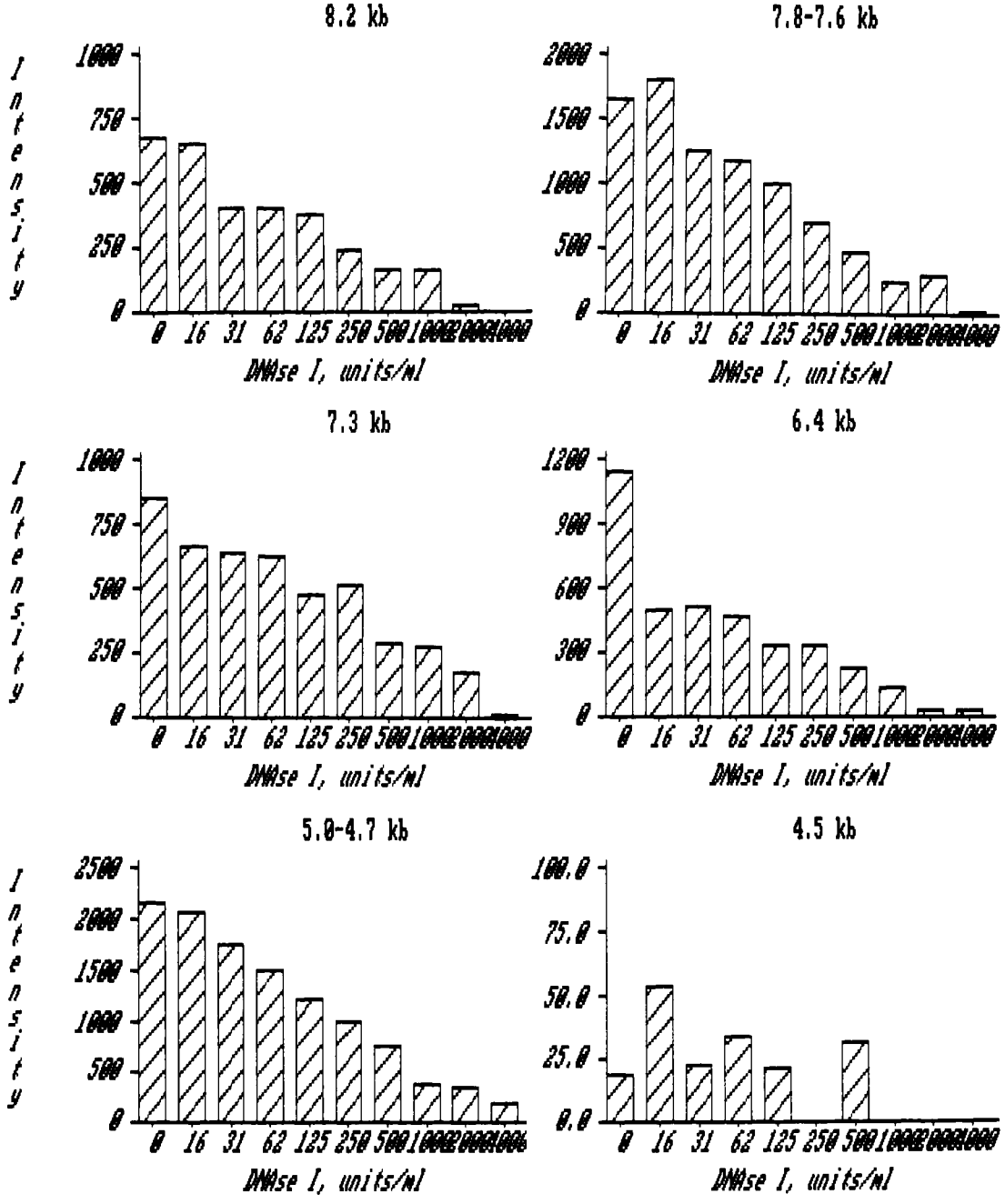
Figure 21. Approximate molecular weights of fragments of male RFM mouse liver DNA after digestion with PstI following incubation with DNAse I. The experiment was performed as described in the legend to Figure 14 (B). End-labeled HindIII fragments of bacteriophage lambda served as the basis for assignment of an approximate molecular weight to each fragment detected by the LTR probe.


DNAse I concentration, units/ml
4000
2000
1000
500
250
125
62
31
16
0
M
kb
8.2
7.8-7.6
7.3
6.9
6.4
6.2
6.0
5.4
4.7
5.0
4.5
4.3
4.0
3.75
3.55
3.2-3.0
2.8
2.7
2.6
2.5
2.3
2.2-2.0
1.8
1.7
1.6
1.35
1.25
1.15
1.0
0.88
0.78

generated by treatment at each concentration of DNAse I (Fig. 21, lanes representing 16 to 4000 units of DNAse I per ml) were scanned densitometrically, and the relative intensities were plotted as a function of DNAse I concentration (Fig. 22). Intensity profiles represent average values for one or more autoradiographic exposures of two separate experiments, except that peaks which were not resolved in one experiment or the other were not considered in these analyses. On the basis of this analysis, four classes of LTR-containing fragments could be distinguished: relatively resistant (represented by the intensity profiles shown for fragments of approximately 4.5, 2.8, 2.6, 1.7, 1.15, 1.0, and 0.78 kb), moderately resistant (as seen in the intensity profiles of fragments of approximately 7.3, 4.3, 3.8, 3.6, 2.1, 1.6, 1.35, 1.25, and 0.88 kb), of intermediate sensitivity (typified by the profiles for fragments of about 8.2, 7.8, 5.0, 3.2, 2.5, and 1.8 kb), or relatively sensitive (as seen in the profiles for fragments of approximately 6.4, 4.0, 2.3, and 0.67 kb) to digestion by DNAse I. The fragments which are classified as relatively sensitive to DNAse I degradation are slightly less sensitive than the PstI fragments detected by pTE201, the probe derived from gene 33, which is known to be transcribed in the liver (Fig. 16).

The sensitivity pattern for spleen nuclei incubated with DNAse I and cleaved with PstI (Fig. 23) differed from that observed for liver nuclei. Although the fragments from spleen nuclei appear to be generally more sensitive to DNAse I than those from liver nuclei, this difference is probably attributable to the lower density of spleen nuclei in these experiments. Densitometry (Fig. 24) revealed that

Figure 22. Intensity profiles of individual fragments as a function of DNAse I concentration. The autoradiograms of Figure 14 were scanned densitometrically, and computer-assisted curve-fitting routines allowed estimation of the intensity of individual bands. Intensity values for individual fragments are plotted as a function of DNAse I concentration. Each value represents one or two autoradiographic exposures from two experiments over most of the range examined. Approximate molecular weights of the fragments are indicated in each frame.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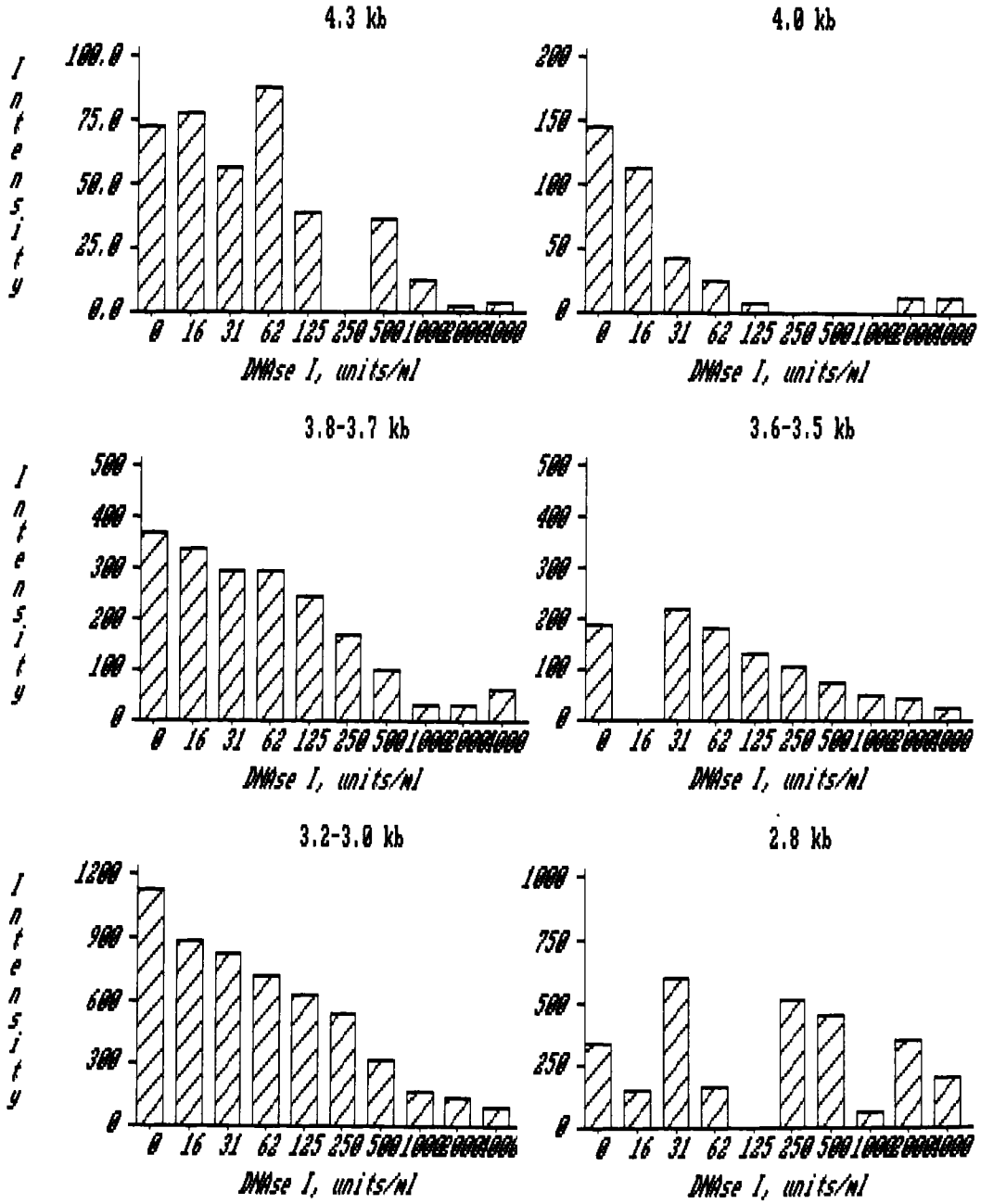
7.8-7.6 kb
7.3 kb
6.4 kb
5.0-4.7 kb
4.5 kb
Intensity
DNAse I, units/ml
0
16
31
62
125
250
500
1000
2000
4000
8000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DNAse I, units/ml
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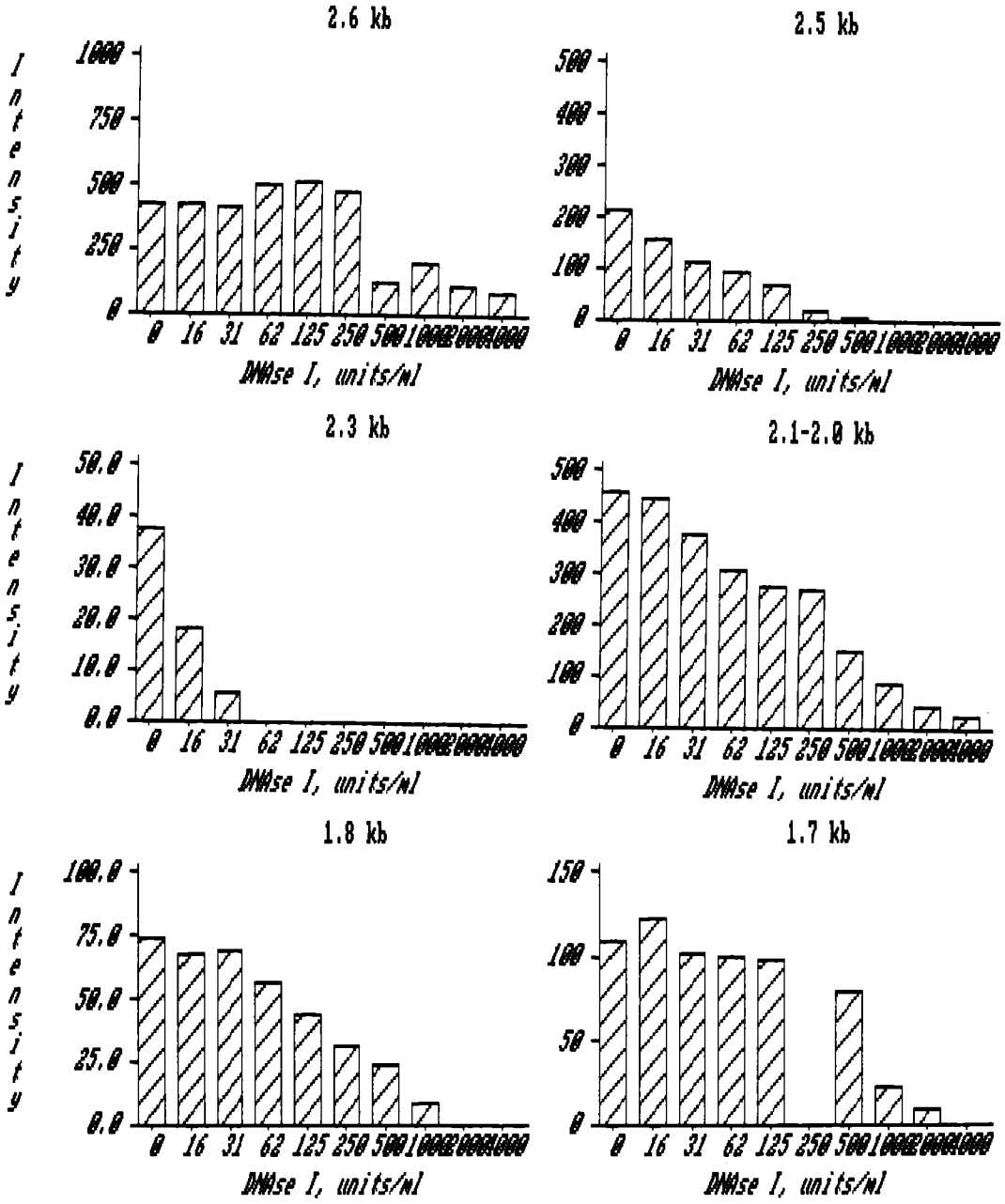
31
62
125
250
500
1000
2000
4000
8000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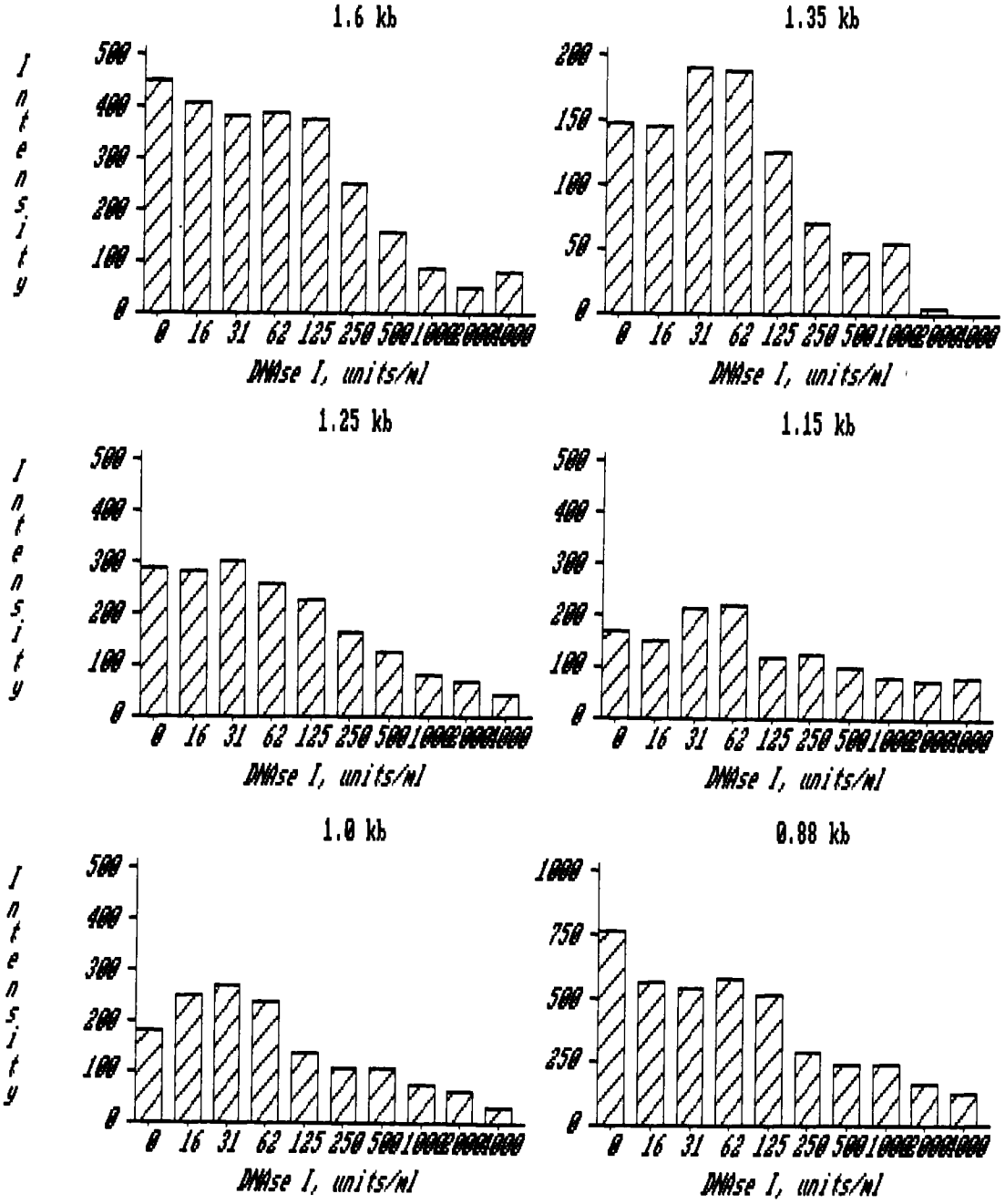
2.1-2.0 kb
1.8 kb
1.7 kb
Intensity
DNAse I, units/ml
0
16
31
62
125
250
500
1000
2000
4000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Intensity
DNAse I, units/ml
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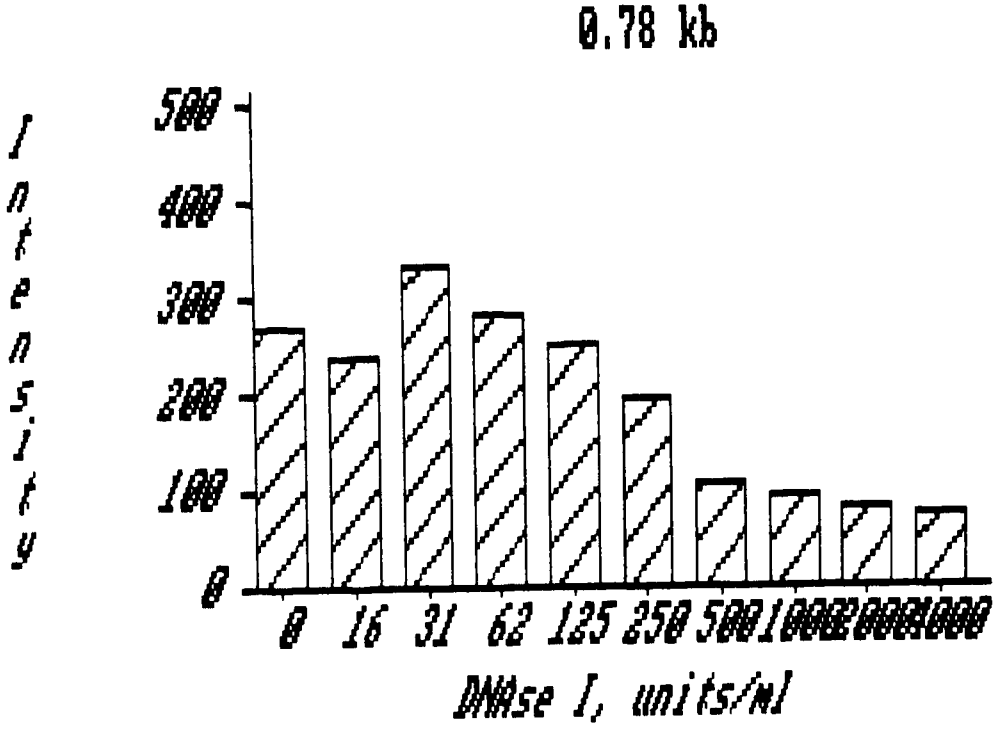
16
31
62
125
250
500
1000
2000
4000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500
400
300
200
100
0
0 16 31 62 125 250 500 1000 2000 4000
DNAse I, units/ml

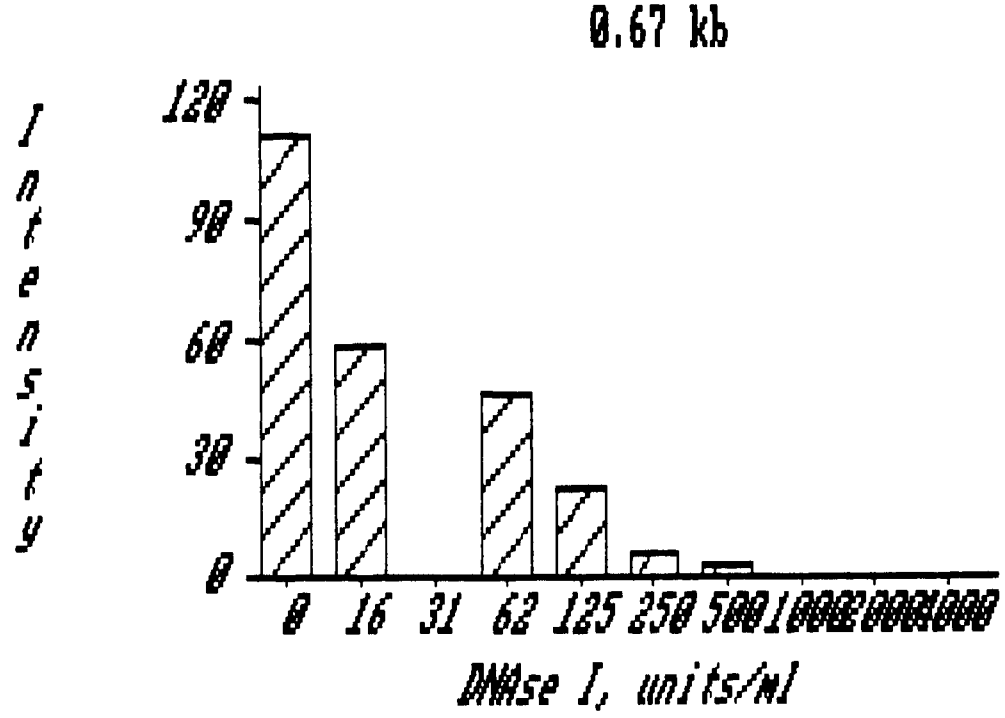

0.67 kb
Intensity
120
90
60
30
0
0 16 31 62 125 250 500 1000 2000 4000
DNAse I, units/ml

Figure 23. Hybridization of the LTR probe to PstI digests of spleen nuclei treated with DNAse I. Nuclei isolated from the spleens of male RFM mice in the presence of spermine and spermidine were incubated with concentrations of DNAse I ranging from 0 to 4000 units/ml for 10 min at 0^{o}. After phenol extraction and precipitation from ethanol, samples were digested with PstI and subjected to electrophoresis through (A) 0.6% or (B) 1.2% agarose. Transfer to nylon membranes and hybridization to the LTR probe was as described in the text. Each lane contained approximately 5 ug of DNA.

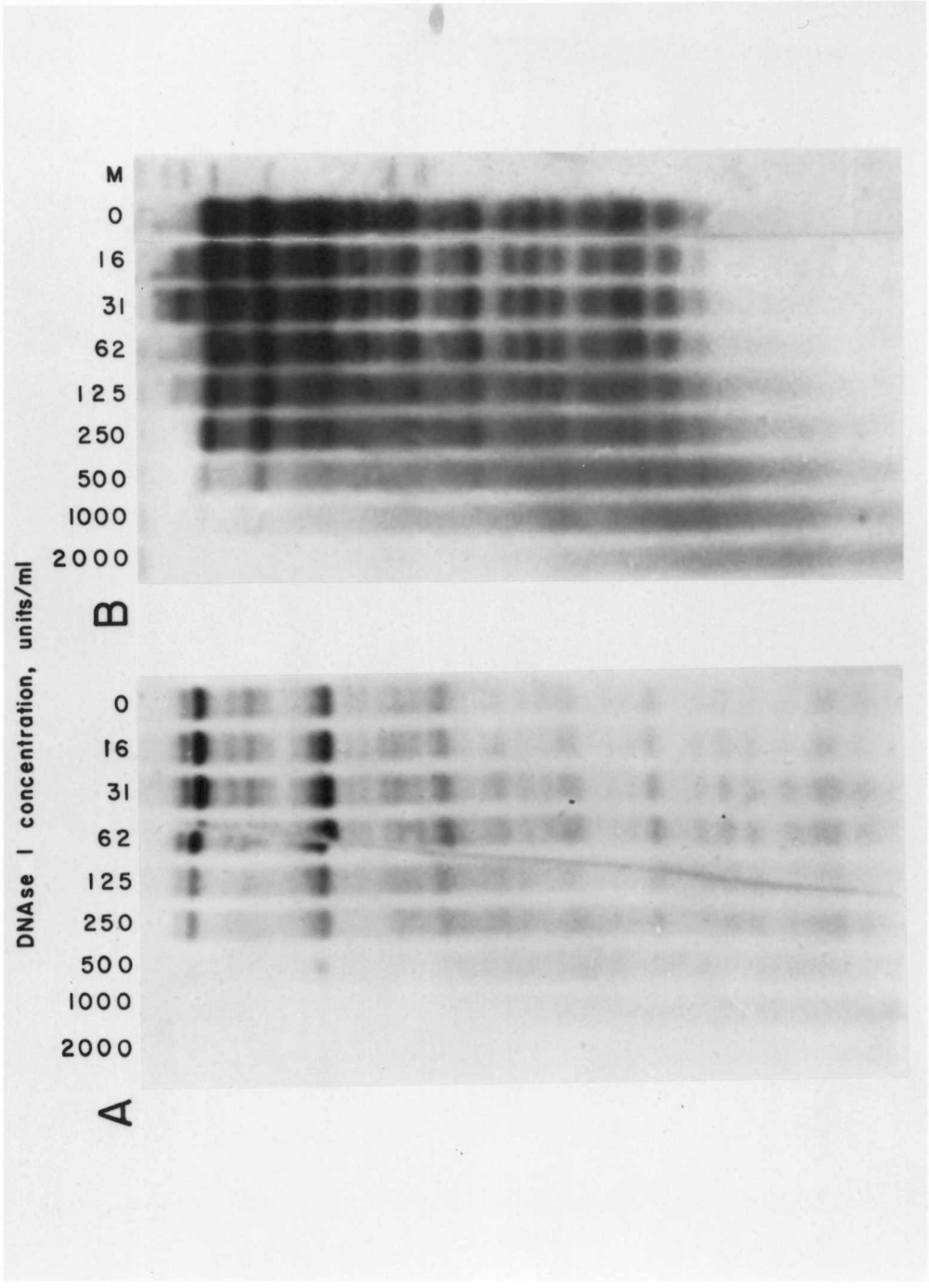
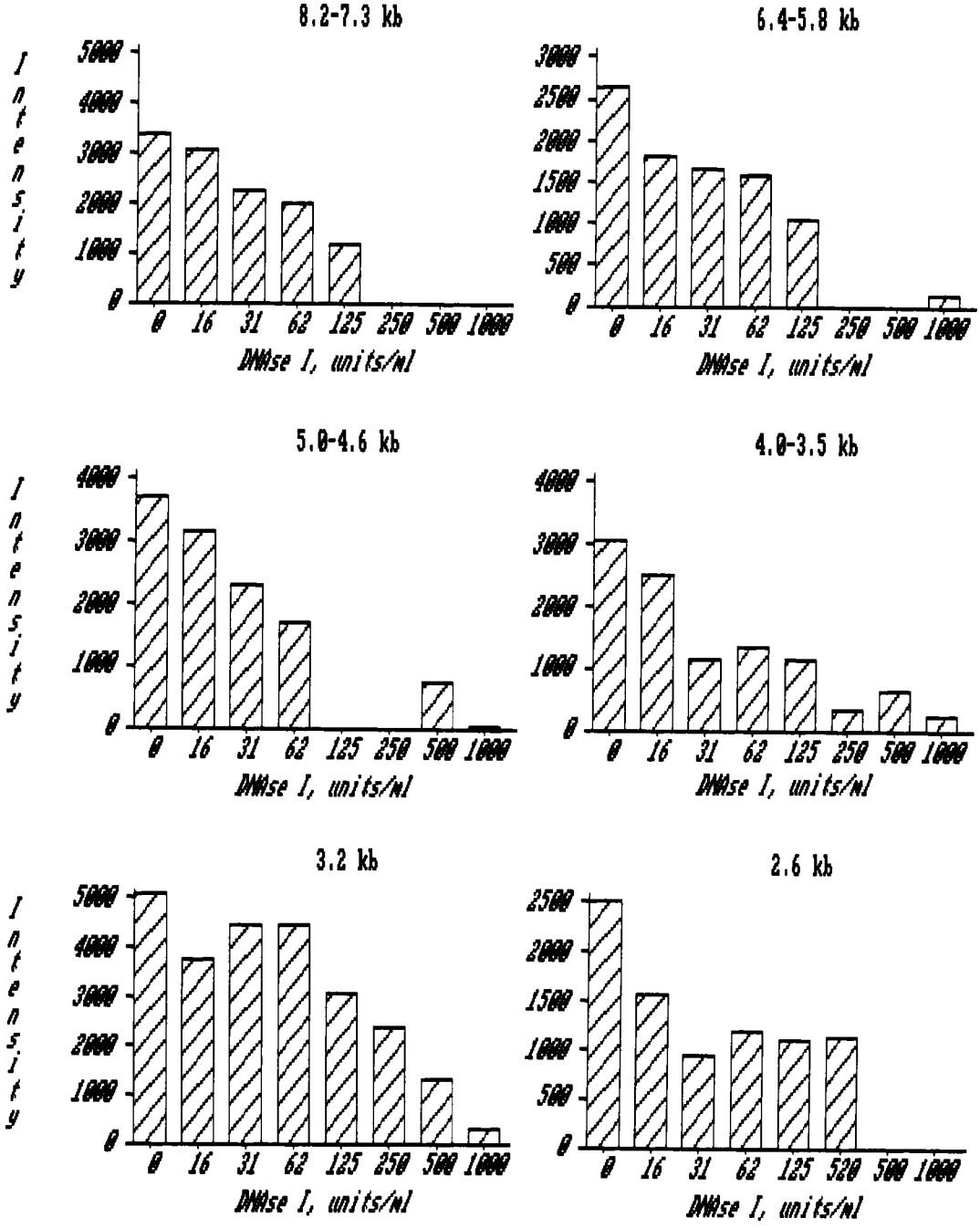

DNAse I concentration, units/ml
M
0
16
31
62
125
250
500
1000
2000
B
0
16
31
62
125
250
500
1000
2000
A

Figure 24. Intensity profiles of individual PstI fragments of male RFM spleen DNA as a function of DNAse I concentration. The autoradiogram of Figure 23 (B) was scanned densitometrically, and computer-assisted curve-fitting routines allowed estimation of the intensity of individal bands. Intensity values for individual fragments are plotted as a function of DNAse I concentration. Approximate molecular weights of the fragments are indicated.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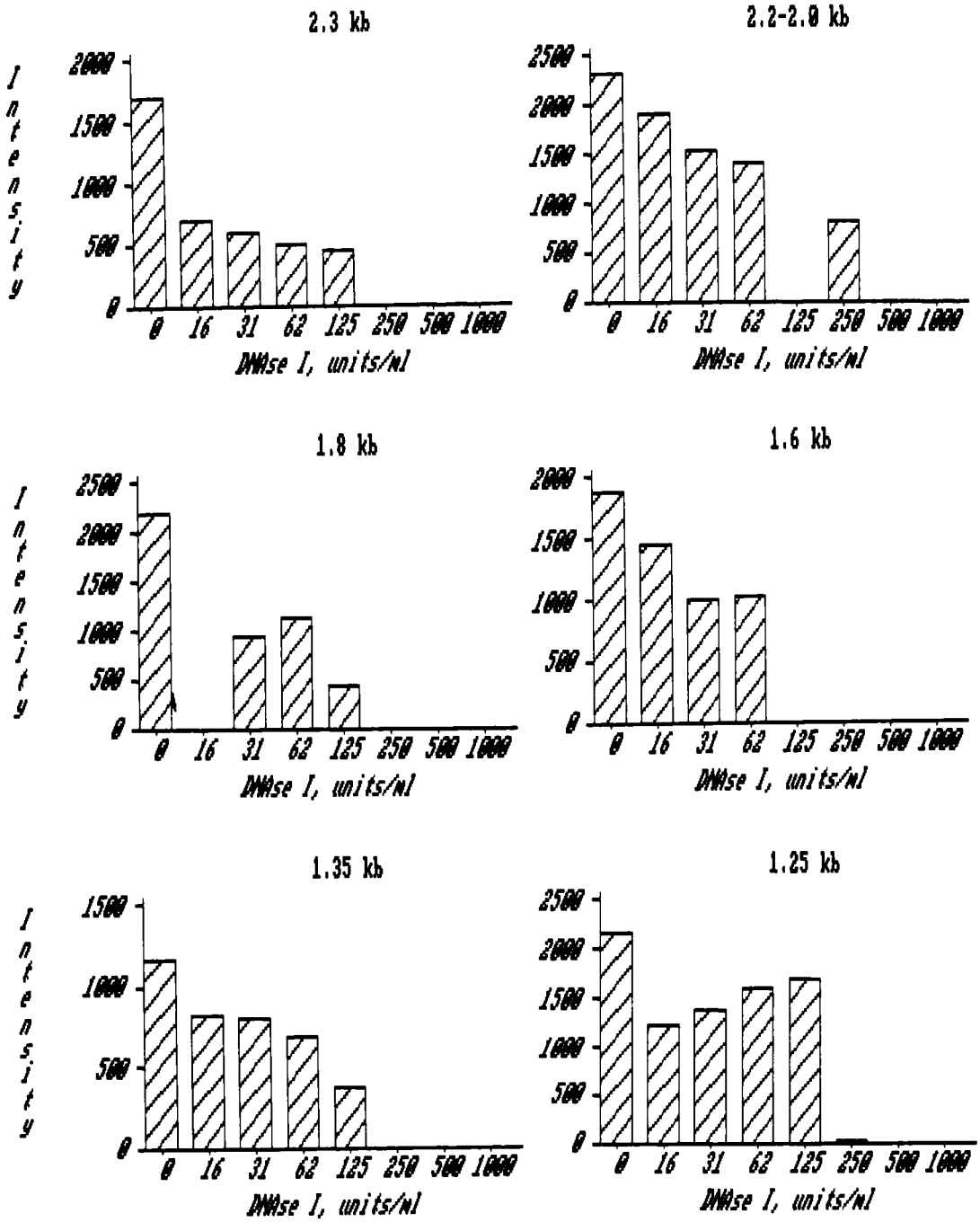
16
31
62
125
250
500
1000
520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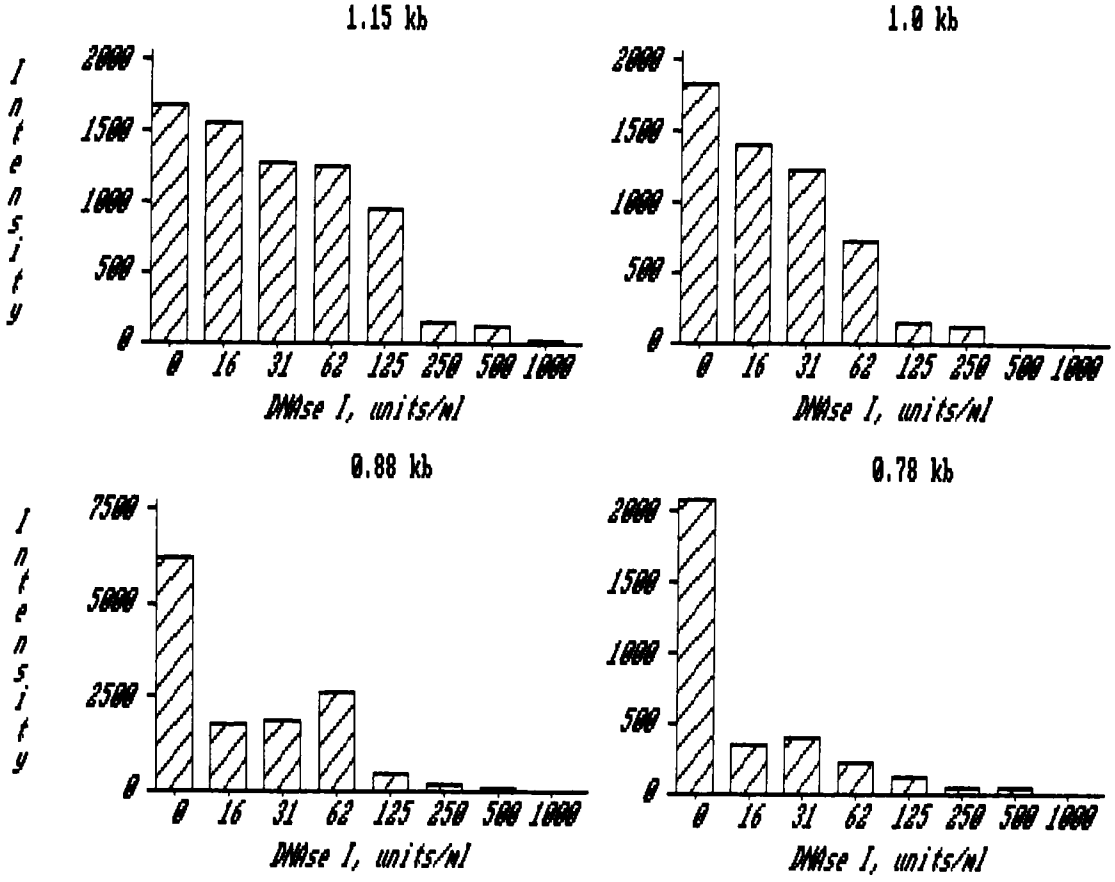
Intensity
DNAse I, units/ml
0
16
31
62
125
250
500
1000


1.15 kb
1.0 kb
0.88 kb
0.78 kb
Intensity
2000
1500
1000
500
0
7500
5000
2500
0 16 31 62 125 250 500 1000
DNAse I, units/ml

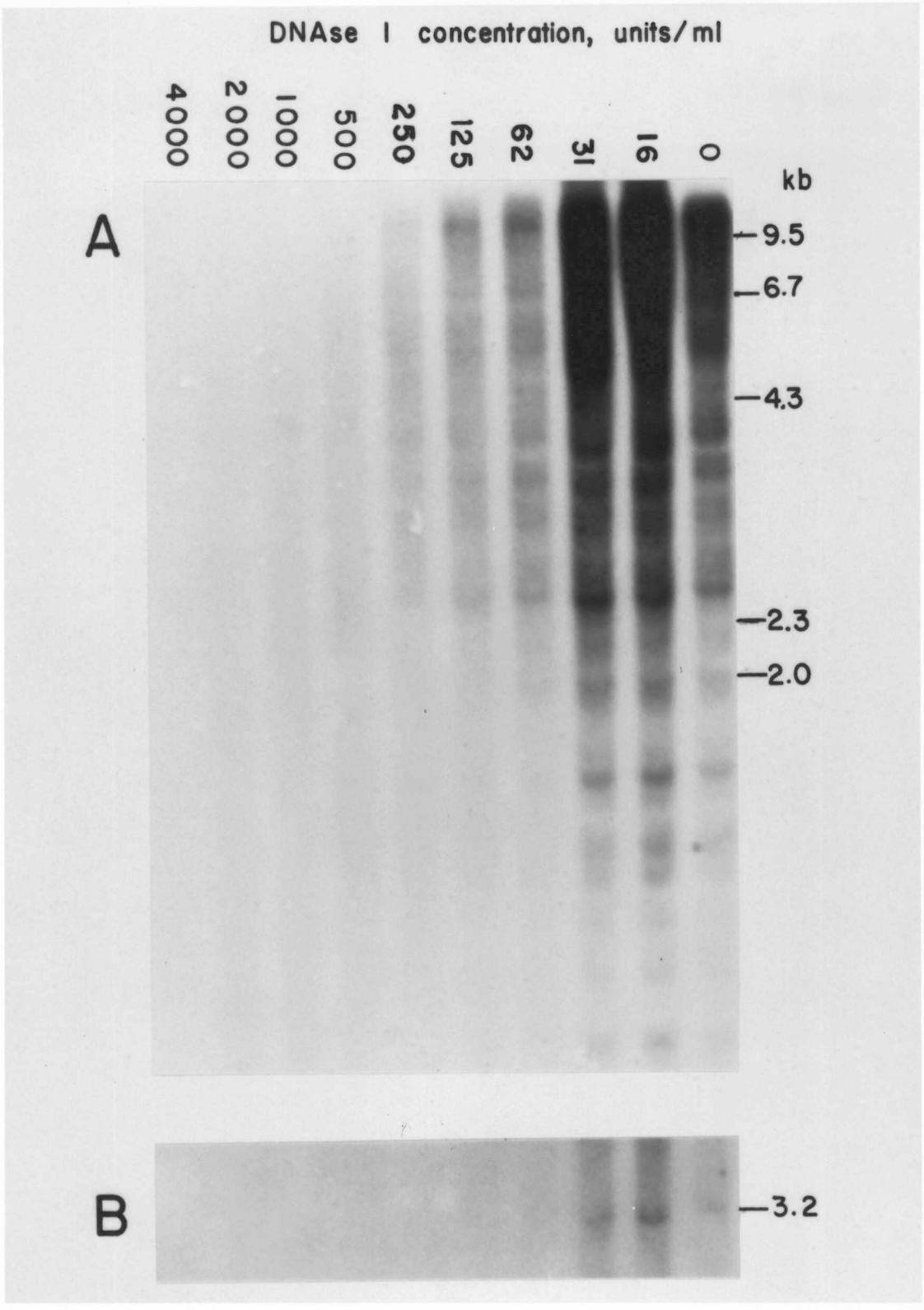
the PstI fragments of spleen DNA most resistant to DNAse I digestion were those of approximately 3.2 and 1.25 kb, which in the liver appeared to be of intermediate sensitivity and moderately resistant, respectively. Among the most sensitive PstI fragments of spleen DNA (5.0-4.6, 4.0-3.5, 2.3, 1.0, 0.88, and 0.78 kb) were two (4.0 and 2.3 kb) of corresponding sensitivity in the liver and two (1.0 and 0.78 kb) which in the liver appeared to be relatively resistant to DNAse I degradation. Other fragments were classified as moderately resistant (approximately 2.6, 1.8, and 1.6 kb) or of intermediate sensitivity (approximately 8.2-7.3, 6.4-5.8, 2.2-2.0, 1.35, and 1.15 kb).

Hybridization of the IS probe to HindIII-digested DNA fragments obtained from spleen nuclei incubated with DNAse I (Fig. 25A) indicated that the IS-containing fragments were generally more sensitive to DNAse I than were LTR-containing fragmnents. Despite intensity differences between the two probes, the IS fragments did not disappear as rapidly as the 3.2 kb HindIII fragment detected by the gene 33 probe, pTE201 (Fig. 25B).

Methylation of MuLV-related sequences in the RFM genome

Although the endogenous type C retroviral proviruses are classified according to their host range, a property determined by the gp70 product of the envelope gene, each class can be distinguished on the basis of sizes of characteristic fragments generated by restriction endonuclease cleavage. However, the RFM/Un genome, as well as those of several other inbred mouse strains, contain MuLV-related fragments not corresponding in size to fragments predicted for intact ecotropic,

Figure 25. Hybridization of the IS and gene 33 probes to HindIII digests of spleen nuclei treated with DNAse I. Nuclei isolated from the spleens of male RFM mice in the presence of spermine and spermidine were incubated with concentrations of DNAse I ranging from 0 to 4000 units/ml for 10 min at 0^{o}. After phenol extraction and precipitation from ethanol, samples were digested with HindIII and subjected to electrophoresis through 1.2% agarose. Transfer to a nylon membrane was as described in the text. Each lane contained approximately 5 ug of DNA. Hybridization was to (A) the IS probe. After deprobing, the membrane was hybridized to a probe derived from gene 33 (B).


DNAse I concentration, units/ml
4000
2000
1000
500
250
125
62
31
16
0
kb
A
9.5
6.7
4.3
2.3
2.0
B
3.2

xenotropic, or MCF-type proviruses (9,10,39,40,52,54,84). While some of these fragments may be attributable to restriction-site polymorphisms, many are possibly due to proviruses which contain deletions within their structural genes. By employing probes derived from different regions of the provirus, it should be possible to recognize such proviruses. Additional information regarding the methylation status of proviruses with or without sequence deletions could be gained by use of a pair of restriction enzymes with characteristic sites in MuLV-related sequences.

An intact ecotropic provirus is 8.8 kb; the distance between its two PstI sites is 8.2 kb (52,54). The LTR probe should hybridize to two fragments generated by PstI cleavage of each ecotropic provirus: a segment 8.2 kb which includes the 5′ LTR, and a segment unique to each integration which includes the 3′ LTR and the adjacent cellular DNA. Probes derived from viral structural genes would be expected to detect only the 8.2 kb fragment. If the methylation-sensitive restriction enzyme SmaI cleaves at some or all of its sites located 0.4, 5.2, and 6.7 kb from the 5′ end of the provirus (46), probes derived from the gag and pol regions would detect some or all of these fragments (summarized in Fig. 25 and Table 2): 4.8, 5.2, 6.3, 6.7, 7.8, and 8.2 kb; probes from the env gene would hybridize to fragments of 1.7, 3.2, 7.8, and 8.2 kb. DNAs cleaved with PstI and KpnI would yield a 2.9 kb fragment hybridizing to a gag probe; a pol probe would detect the same fragment and a 4.1-kb internal fragment; the env gene probe would detect a 1.0 kb fragment (and the 4.1 kb fragment if it included the 5′ region of the env gene).

Figure 26. Relevant restriction endonuclease sites of an endogenous ecotropic murine type C provirus.[a,b] (A) The restriction sites for PstI (P), SmaI (S), and KpnI (K) typical of most endogenous ecotropic proviruses are summarized. (B) The approximate boundaries of the LTR and the gag, pol, and env genes are indicated. The probes used in this study are shown below.

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[a]Joseph, D. R. 1981. Comparison of the restriction endonuclease maps of unintegrated proviral DNAs from four xenotropic murine leukemia viruses. J. Virol. 40:963-970.

[b]The fragments generated by cleavage of an ecotropic provirus with these enzymes are summarized in Table 2.

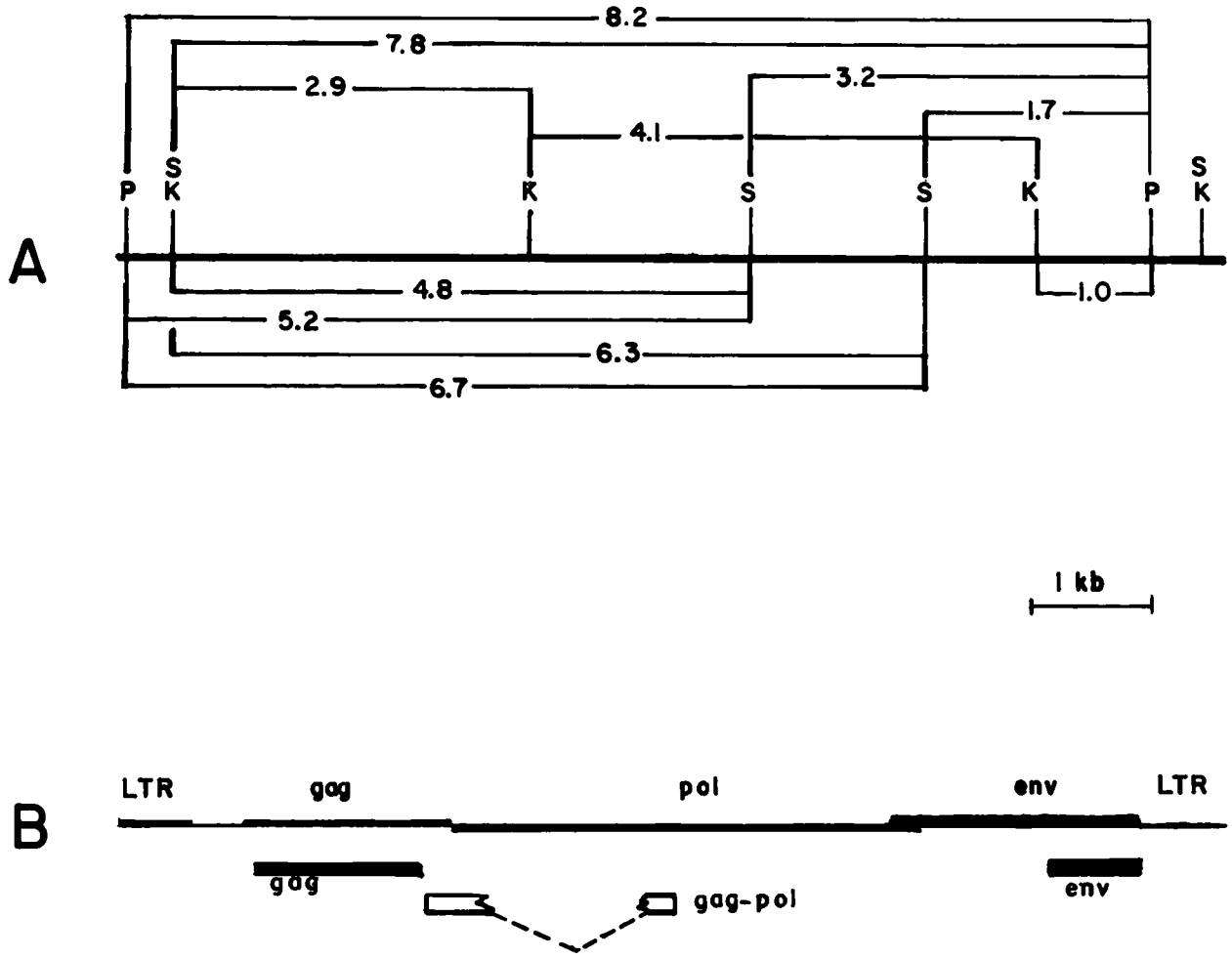
A
8.2
7.8
2.9
3.2
1.7
4.1
P
S
K
K
S
S
K
P
S
K
4.8
1.0
5.2
6.3
6.7
1 kb
B
LTR
gag
pol
env
LTR
gag
gag-pol
env

Table 2. Restriction Fragments of Ecotropic Proviruses Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from			
	LTR	gag	gag-pol	env
PstI	8.2 unique	8.2	8.2	8.2
PstI + SmaI	8.2 6.7 5.2 0.4	8.2 7.8 6.7 6.3 5.2 4.8	8.2 7.8 6.7 6.3 5.2 4.8	8.2 7.8 3.2 1.7
PstI + KpnI	0.4	2.9	2.9 4.1	1.0

[a]These data are derived from Figure 26.

[b]Joseph, D. R. 1981. Comparison of the restriction endonuclease maps of unintegrated proviral DNAs from four xenotropic murine leukemia viruses. J. Virol. 40:963-970.

Xenotropic proviruses are of the two general types shown in Figure 27 (39,40,54). The structure in Frame A, that of AKR-6, represents the inducible provirus encoded by the chromosome 1 locus Bxv-1, which is present in a number of inbred mouse strains, including BALB/c, C58, and AKR (40). The structure depicted in Frame B is that of the xenotropic provirus originating in NZB mice (10,40,54). While this virus may actually be a recombinant of a xenotrope and the envelope gene sequences specified by Nfxv-1 (40), it is presented here because its components are presumably represented in the genomes of NFS, NZB, and other inbred mouse strains; it is similar to AKR-40, a xenotropic

Figure 27. Relevant restriction endonuclease sites of two types of endogenous xenotropic murine type C proviruses.[a] The restriction sites for PstI (P), SmaI (S), and KpnI (K) characteristic of subclass A[b] and subclass B[c] endogenous xenotropic proviruses are summarized.

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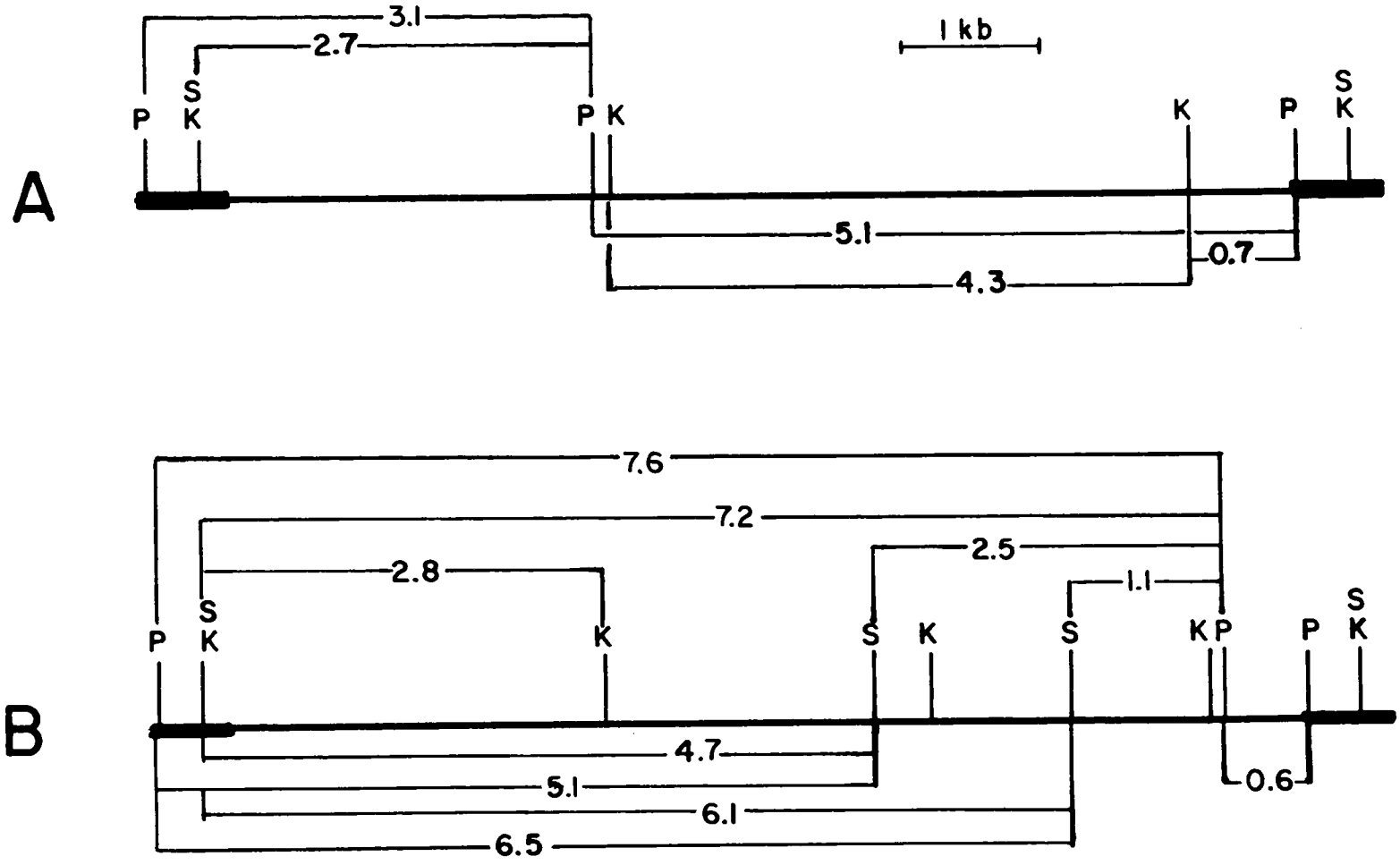
[a]The fragments generated by cleavage of xenotropic proviruses with these enzymes are summarized in Tables 3 and 4.

[b]Hoggan, M. D., R. R. O'Neill, and C. A. Kozak. 1986. Nonecotropic murine leukemia viruses in BALB/c and NFS/N mice: characterization of the BALB/c Bxv-1 provirus and the single NFS endogenous xenotrope. J. Virol. 60:980-986.

[c]Joseph, D. R. 1981. Comparison of the restriction endonuclease maps of unintegrated proviral DNAs from four xenotropic murine leukemia viruses. J. Virol. 40:963-970.


A
3.1
2.7
1 kb
P
S
K
P K
K
P
S
K
5.1
0.7
4.3
B
7.6
7.2
2.8
2.5
1.1
P
S
K
K
S
K
S
K P
P
S
K
4.7
5.1
6.1
6.5
0.6

virus from the AKR inbred mouse strain which is quite possibly also a recombinant (40). An intact xenotropic provirus is about 8.8 kb; the distance between the PstI site in the 5′ LTR and the internal PstI site is 3.1 kb (Fig. 27, frame A) or 7.6 kb (Fig. 27, frame B). The LTR probe would detect these fragments, as well as the 3′ LTR-containing PstI fragments unique to each integration. Subsequent SmaI cleavage would generate 0.4 kb fragments hybridizing to the LTR probe; methylation of some of these sites would result in the same fragments as described for PstI cleavage, and, in the case of the second type of xenotropic provirus, additional fragments of 6.5 and 5.1 kb. Restriction with PstI and KpnI would yield only 0.4 kb fragments homologous to the LTR probe. The gag gene probe would be expected to hybridize to the 3.1 kb PstI fragment described above for the first type of xenotropic provirus; subsequent cleavage by KpnI or successful cutting by SmaI would result in hybridization to a 2.7 kb fragment. The second type of xenotropic provirus contains a 7.6 kb PstI fragment which would be expected to hybridize to the gag probe; subsequent restriction with SmaI could result in fragments of 7.6, 7.2, 6.5, 6.1, 5.1, and 4.7 kb, whereas restriction with KpnI would yield a single, 2.8 kb fragment hybridizing to this probe. The gag-pol deletion probe would detect the same fragments which hybridize to the gag probe. In the case of the first type of xenotropic provirus, it would also detect a 5.1 kb PstI fragment and 0.1 and 4.3 kb PstI-KpnI and KpnI fragments. The only fragment of the second type of provirus which hybridizes to the gag-pol deletion probe but not to the gag probe is a 2.3 kb internal KpnI fragment. These fragment sizes, together with the sizes

predicted for fragments hybridizing to the env gene probe, are summarized in Tables 3 and 4.

Table 3. Restriction Fragments of Xenotropic Subclass A Proviruses Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from			
	LTR	gag	gag-pol	env
PstI	3.1 unique	3.1	3.1 5.1	5.1
PstI + SmaI	0.4 3.1	3.1 2.7	3.1 2.7 5.1	5.1
PstI + KpnI	0.4	2.7	2.7 0.1 4.3	0.7

[a]These data are derived from Figure 27.

[b]Hoggan, M. D., R. R. O'Neill, and C. A. Kozak. 1986. Nonecotropic murine leukemia viruses in BALB/c and NFS/N mice: characterization of the BALB/c Bxv-1 provirus and the single NFS endigenous xenotrope. J. Virol. 60:980-986.

Several examples of MCF-type proviruses present in the genome of the RFM/Un mouse have been characterized by restriction endonuclease digestion in this laboratory (80,81). The salient features of a subclass L provirus containing a large insertion in the U3 region of its LTR (86) are shown in Figure 28; two examples of MCF-type proviruses harboring smaller U3 insertions (86) are depicted in Figure 29, along with pRFM17, another example of a provirus harboring the larger U3 insertion (86). The restriction fragments generated by

Figure 28. Relevant restriction endonuclease sites of the MCF- type proviral clone pRFM1.[a,b] The restriction sites for PstI (P), SmaI (S), and KpnI (K) determined from the map of pRFM1 are shown.

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[a]Nikbakht, K. N., C.-Y. Ou, L. R. Boone, P. L. Glover, and W. K. Yang. 1985. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. J. Virol. 54:889-893.

[b]The fragments generated by cleavage of pRFM6 with these enzymes are summarized in Table 5.

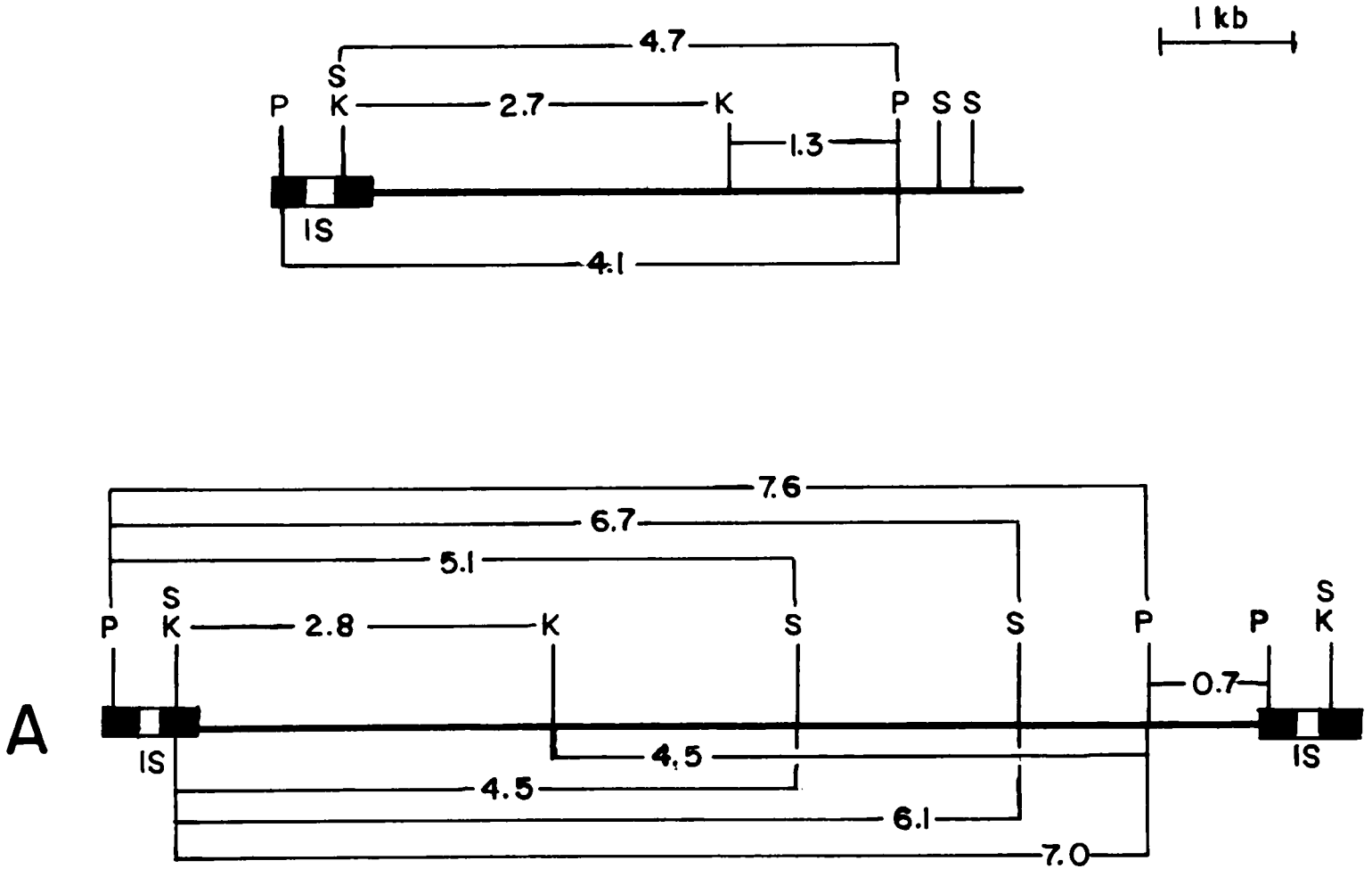
1 kb
4.7
S
P
K
2.7
K
P
S
S
1.3
IS
4.1
A
7.6
6.7
5.1
S
P
K
2.8
K
S
S
P
P
S
K
0.7
IS
IS
4.5
4.5
6.1
7.0

Figure 29. Relevant restriction endonuclease sites of three MCF- type proviral subclones.[a,b,c] The restriction sites for PstI (P), SmaI (S), and KpnI (K) of (A) pRFM6, (B) pRFM16, and (C) pRFM17 are shown.

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[a]Nikbakht, K. N., L. R. Boone, P. L. Glover, F. E. Myer, an W. K. Yang. 1987. Characterization of a molecular clone of RFM/Un mouse chromosomal DNA that contains a full-length endogenous murine leukemia virus-related proviral genome. J. Gen. Virol. 68:683-693.

[b]Nikbakht, K. N., C.-Y. Ou, L. R. Boone, P. L. Glover, and W. K. Yang. 1985. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. J. Virol. 54:889-893.

[c]The fragments generated by cleavage of pRFM6, pRFM16, and pRFM17 are summarized in Tables 6, 7, and 8, respectively.

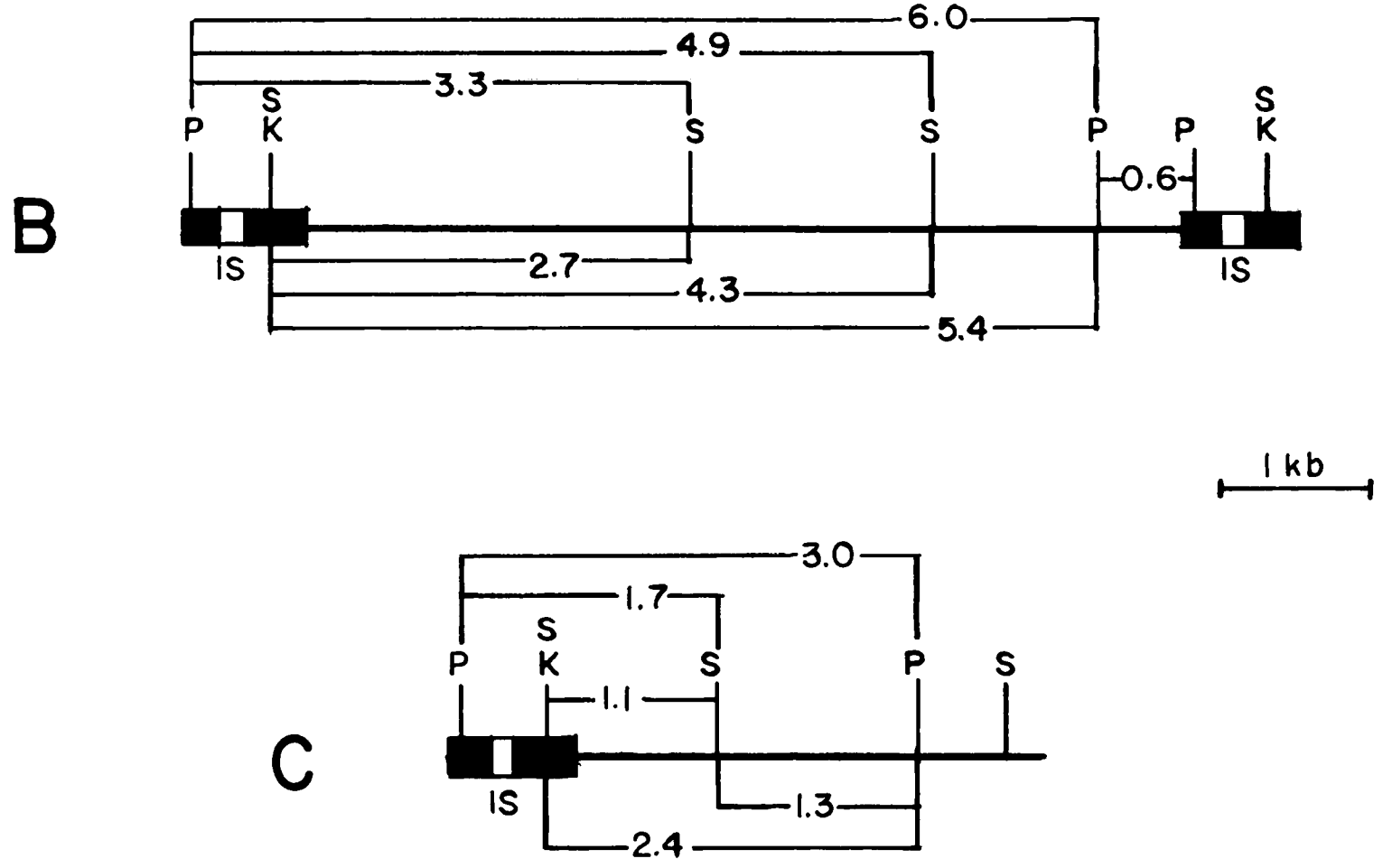

B
6.0
4.9
3.3
S
P
K
S
S
P
P
S
K
0.6
IS
2.7
4.3
5.4
IS
1 kb
C
3.0
1.7
S
P
K
S
P
S
1.1
IS
1.3
2.4

Table 4. Restriction Fragments of Xenotropic Subclass B Proviruses Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from			
	LTR	gag	gag-pol	env
PstI	7.6	7.6	7.6	0.62
PstI + SmaI	0.4	7.6	7.6	0.62
	7.6	7.2	7.2	
	6.5	6.5	6.5	
	5.1	6.1	6.1	
		5.1	5.1	
		4.7	4.7	
PstI + KpnI	0.4	2.8	2.8	0.62
			2.3	

[a]These data are derived from Figure 27.

[b]Joseph, D. R. 1981. Comparison of restriction endonuclease maps of unintegrated proviral DNAs from four xenotropic murine leukemia kemia viruses. J. Virol. 40:963-970.

digestion of these proviral clones with PstI in the absence and presence of SmaI or KpnI are summarized in Tables 5 through 8.

Comparison of the LTR-hybridizing digestion products of PstI and SmaI (Fig. 12, lane b) with those of PstI and KpnI from male RFM liver (Fig. 12, lane c) and with those of PstI (Fig. 12, lane a) indicates that many PstI fragments harboring MuLV-type LTRs are refractory to cleavage by SmaI, and are therefore methylated at their LTR SmaI sites. Furthermore, only a small fraction of LTRs containing large insertions (Fig. 12, bands a) or no insertions (Fig. 12, bands c) in their U3 enhancer regions are generated by SmaI cleavage of the PstI fragments, whereas a large proportion of the LTRs containing the smaller subclass

Table 5. Restriction Fragments of the MCF-type Subclass L Provirus Represented by pRFM1 Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from			
	LTR	gag	gag-pol	env
PstI	4.7 unique	4.7	4.7	nd[c]
PstI + SmaI	0.62 4.7	4.7 4.1	4.7 4.1	nd
PstI + KpnI	0.62	2.7	2.7 1.3	nd

[a]These data are derived from Figure 28.

[b]Nikbakht, K. N., C.-Y. Ou, L. R. Boone, P. L. Glover, and W. K. Yang. 1985. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. J. Virol. 54:889-893.

[c]Not determined.

L insertions in their U3 enhancer regions (Fig. 12, bands b) are sensitive to SmaI digestion and hence are unmethylated at these sites. Comparison of the intensities of the PstI fragments (Fig. 12, lane a) with the intensities of their counterparts in the adjacent lane after an additional incubation with SmaI reveals few striking differences. There is a slight diminution in the intensities of unresolved fragments in the size ranges from 8.2 to 7.6 kb, 6.2 to 5.8 kb, 5.1 to 4.6 kb, 3.8 to 3.6 kb, and 3.2 to 3 kb. Fragments of approximately 6 kb and 1.25 kb show pronounced intensity differences after SmaI cleavage; the latter fragment is that on which the pRFM7 solitary LTR resides.

Table 6. Restriction Fragments of the MCF-type Subclass M Provirus Represented by pRFM6 Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from			
	LTR	gag	gag-pol	env
PstI	7.6 1.0	7.6	7.6	0.75
PstI + SmaI	0.59 7.6 6.7 5.1	7.6 7.0 6.7 6.1 5.1 4.5	7.6 7.0 6.7 6.1 5.1 4.5	0.75
PstI + KpnI	0.59	2.8	2.8 4.5	0.75

[a]These data are derived from Figure 29.

[b]Nikbakht, K. N., L. R. Boone, P. L. Glover, F. E. Myer, and W. K. Yang. 1987. Characterization of a molecular clone of RFM/Un mouse chromosomal DNA that contains a full-length endogenous murine leukemia virus-related proviral genome. J. Gen. Virol. 68:683-693.

Deprobing of the membrane from the experiment depicted in Fig. 12 and rehybridization with the unique-sequence probe derived from the region 3′ to the pRFM8 solitary LTR demonstrate that, in the RFM liver, the diminution in intensity after SmaI restriction of the fragments in the range 8.2 to 7.6 kb is not attributable to SmaI cleavage of that solitary LTR from the PstI fragment which harbors it (Fig. 30B). In general, most of the MuLV-type LTR-related sequences of the male RFM/Un liver appear to be relatively heavily methylated.

Figure 30. Hybridization of probes to male RFM liver DNA restricted with PstI in the absence or presence of SmaI or KpnI. The membrane described in Figure 12 was deprobed and hybridized sequentially to (B) the unique-sequence probe from the region 3′ to the pRFM8 solitary LTR; (C) a probe encompassing most of the viral gag gene; (D) the gag-pol deletion probe derived from pRFM16; (E) a probe from the viral env gene; and (F) the IS probe, derived from the insertion into the U3 region of AL10. Hybridization of the LTR probe (A) is shown to facilitate comparison. DNAs in lanes a were digested with PstI; those in lanes b were incubated with SmaI following the PstI digestion; those in lanes c were restricted with KpnI after PstI digestion. The positions of HindIII fragments of bacteriophage lambda are indicated.

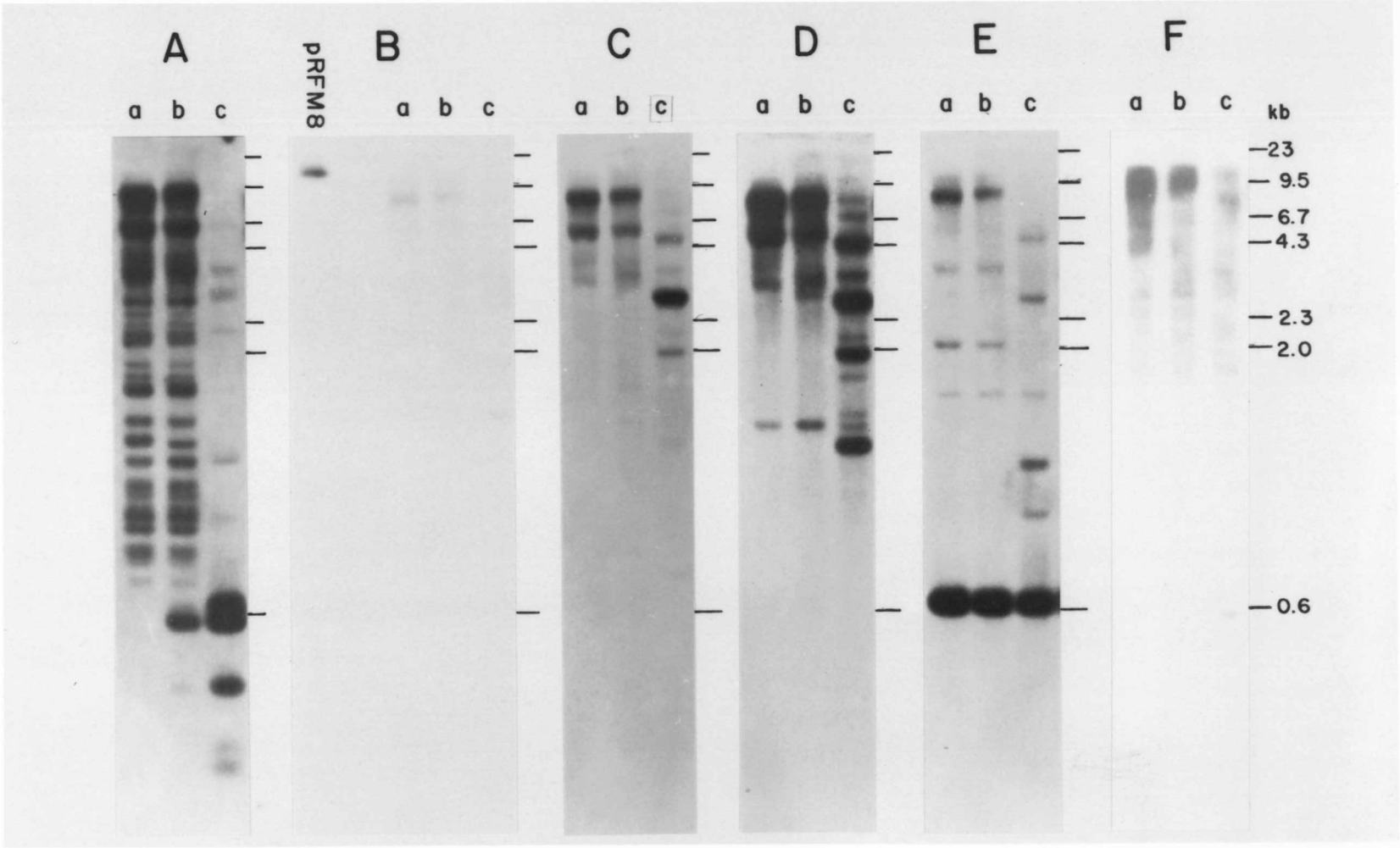

A
a b c
pRFM8
B
a b c
C
a b c
D
a b c
E
a b c
F
a b c
kb
23
9.5
6.7
4.3
2.3
2.0
0.6

Table 7. Restriction Fragments of the MCF-type Subclass M Provirus Represented by pRFM16 Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from			
	LTR	gag	gag-pol	env
PstI	6.0 unique	6.0	6.0	0.65
PstI + SmaI	0.59 6.0 4.9 3.3	6.0 5.4 4.9 4.3 3.3 2.7	6.0 5.4 4.9 4.3 3.3 2.7	0.65
PstI + KpnI	0.59	5.4	5.4	0.65

[a]These data are derived from Figure 29.

[b]Nikbakht, K. N., C.-Y. Ou, L. R. Boone, P. L. Glover, and W. K. Yang. 1985. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. J. Virol. 54:889-893.

Further insight into the nature of heavily methylated and relatively undermethylated MuLV-related sequences can be gained by analysis of methylation status using probes derived from the structural genes of endogenous proviruses. The membrane from the experiment of Fig. 12 was deprobed and rehybridized to a probe representing the gag gene (Fig. 30C). The prominent PstI bands at 8.2 to 7.6 kb represent the endogenous ecotropic and xenotropic proviruses and MCF-type proviruses related to pRFM6. There is only a slight decrease in the intensity of fragments comigrating with these species after restriction with SmaI, indicating that the majority of these proviruses are

Table 8. Restriction Fragments of the MCF-type Subclass L Provirus Represented by pRFM17 Which Hybridize to Four MuLV-related Probes.[a,b]

Enzyme(s)	Probe derived from: LTR	gag	gag-pol	env
PstI	3.0 unique	3.0	3.0 nd	nd[c]
PstI + SmaI	0.62 1.7 3.0	3.0 2.4 1.7 1.3 1.1	3.0 2.4 1.3 1.1 nd	nd
PstI + KpnI	0.62	2.4	2.4 nd	nd

[a]These data are derived from Figure 29.

[b]Nikbakht, K. N., C.-Y. Ou, L. R. Boone, P. L. Glover, and W. K. Yang. 1985. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. J. Virol. 54:889-893.

[c]Not determined.

relatively highly methylated at SmaI sites. The 6 kb fragment, corresponding to proviruses of the type represented by the defective MCF-type pRFM16, virtually disappears upon SmaI cleavage, indicating that proviruses of this type are not detectably methylated at all SmaI sites. Consistent with this assignment is the appearance of a band at approximately 3.3 kb after cleavage with SmaI, although the existence of this band could be ascribable to other proviral species not yet described; the absence of a band at 2.7 kb indicates partial methylation of these proviruses or an inconsistency with other data

regarding this assignment. The prominent bands from 5 to 4.6 kb undergo pronounced diminution in intensity following SmaI restriction; one contribution to these bands is probably proviruses represented by the MCF-type proviral clone pRFM1. The absence of a detectable band at 4.1 kb suggests that this particular provirus is a minor component of the 5-4.6 kb band, or that it is not the component which is undermethylated at that site. The PstI fragments at approximately 3.8 to 3.6 kb do not correspond to any endogenous MuLV-related moieties which have been characterized at this time; however, they do not appear to be fully methylated, and must comprise a significant population. The remaining prominent gag-related PstI band, that at 3.2 to 3.0 kb, is probably attributable to xenotropic and pRFM17-like proviruses of the MCF type. It appears to be largely hypomethylated, suggesting that the pRFM17-like entitities are the major contibutors to this band. Consistent with this interpretation is the appearance of bands at approximately 1.7 and 1.3 kb after SmaI digestion; these bands could arise from the MCF-type provirus but not from the xenotrope. Since a 1.1 kb fragment is not observed, it appears that the pRFM17-like LTR is methylated at its SmaI site, but the SmaI site in the gag gene is not fully methylated. Partial undermethylation at gag-related SmaI sites thus appears to be associated primarily with MCF-type proviruses, particularly those resembling pRFM1, pRFM16, and pRFM17, and to a category of MuLV-related proviruses not yet characterized which include PstI fragments of approximately 3.8 to 3.6 kb.

The interpretation of these data is reinforced by analysis of fragments hybridizing to the gag-pol deletion probe. Most of the same

fragments are detected, with several notable exceptions. The 1.7 kb PstI-SmaI fragment associated with pRFM17-like gag regions is not detected by the gag-pol deletion probe, which is consistent with the prediction. Two bands not detected by the gag probe become apparent upon hybridization to the gag-pol deletion probe: one of approximately 0.9 kb, the other of approximately 0.62 kb (Fig. 30D, lanes a and b). Neither of these fragments corresponds to PstI fragments predicted from known proviral species, although they could have arisen from regions of proviruses which have been only partially characterized.

Analysis of fragments hybridizing to an env gene probe provides further insights (Fig. 30E). The major band at 0.62 kb corresponds to that predicted for one population of endogenous xenotropic proviruses, as well as MCF-type viruses represented by pRFM6 and pRFM16. The absence of novel fragments arising from SmaI digestion of the PstI fragments suggests that the endogenous ecotrope is very heavily methylated at all of its internal SmaI sites and the 5′ LTR SmaI site; it is detected only as an 8.2 kb fragment hybridizing to the env gene probe. The weak band at approximately 5 kb is presumably due to another population of xenotropic proviruses; the decreased intensity of the bands in this region compared to the strong bands observed with LTR, gag, and gag-pol probes strongly suggests that pRFM1-like proviruses are the major contributors to this region of bands. The weak bands at 1.6 and 3.2 kb, along with the stronger bands at 2.3 and 3.8 kb, do not correspond to fragments predicted from the endogenous proviruses listed in Tables 2 through 8.

The fractionation of these DNAs digested with PstI in the absence and presence of SmaI through 0.6% agarose provides additional information about LTR- and gag-containing fragments of high molecular weight (Fig. 31). The 6.9 kb band detected by both probes appears to be fully methylated; it persists at 2000 units/ml of DNAse I (Fig. 21). The 6.4 kb band detected by both probes, however, appears somewhat sensitive to SmaI cleavage, and is among the most sensitive to DNAse I. Neither the 6.2 kb nor the 6.0 kb bands detected by both probes appear to be undermethylated; both appear to be at least as sensitive to DNAse I as the 6.4 kb band. Thus the lack of correlation between DNAse I hypersensitivity and relative hypomethylation prevails in the analysis of individual PstI fragments of high molecular weight; these fragments include a large portion of the population of gag-containing PstI fragments observed in the liver DNA of the male RFM mouse.

The methylation patterns of spleen and kidney DNAs cleaved by PstI and SmaI as detected by the LTR probe do not differ greatly from that observed in liver DNA (Fig. 32). However, there are subtle differences. The PstI band at 4.8 kb, for example, appears to be more heavily methylated in the liver and kidney than in the spleen, whereas the 3.2 kb band appears to be more heavily methylated in the liver and spleen than in the kidney. The band at about 1.6 kb is more fully methylated in the spleen and liver than in the kidney. These differences have not been correlated with tissue-specific differences in the expression of MuLVs; use of available flanking-sequence probes would clarify whether there is any correlation.

Figure 31. Hybridization of the LTR and gag probes to digests of male RFM liver DNA resolved through 0.6% agarose. DNA extracted from the liver of a male RFM mouse was digested with PstI (lanes a) or PstI and SmaI (lanes b). Fragments were resolved by electrophoresis at low voltage through 0.6% agarose and transferred to a nylon membrane. Hybridization was to the LTR probe (A). The membrane was deprobed and rehybridized to the gag probe (b). M, end-labeled HindIII fragments of bacteriophage lambda.

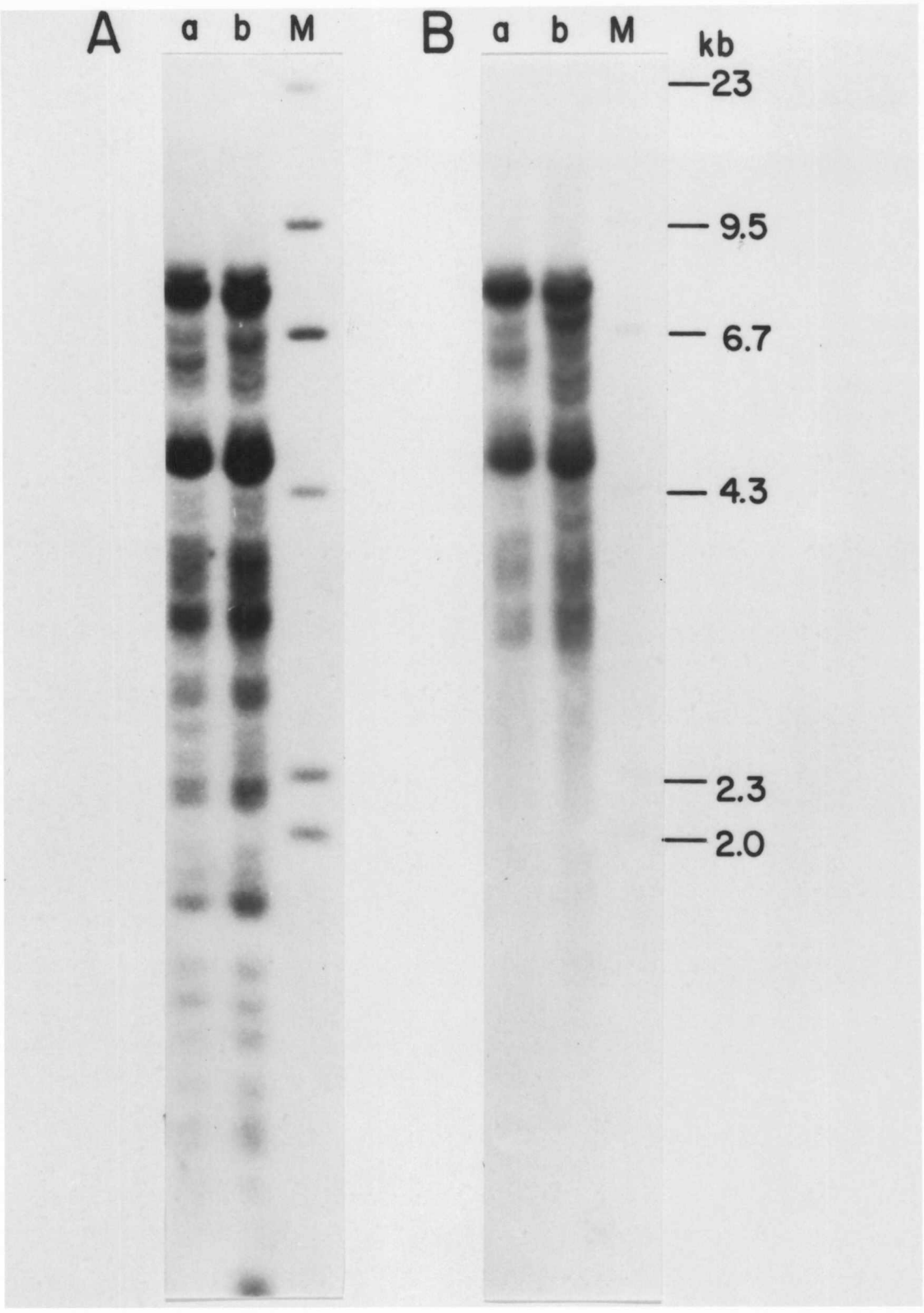
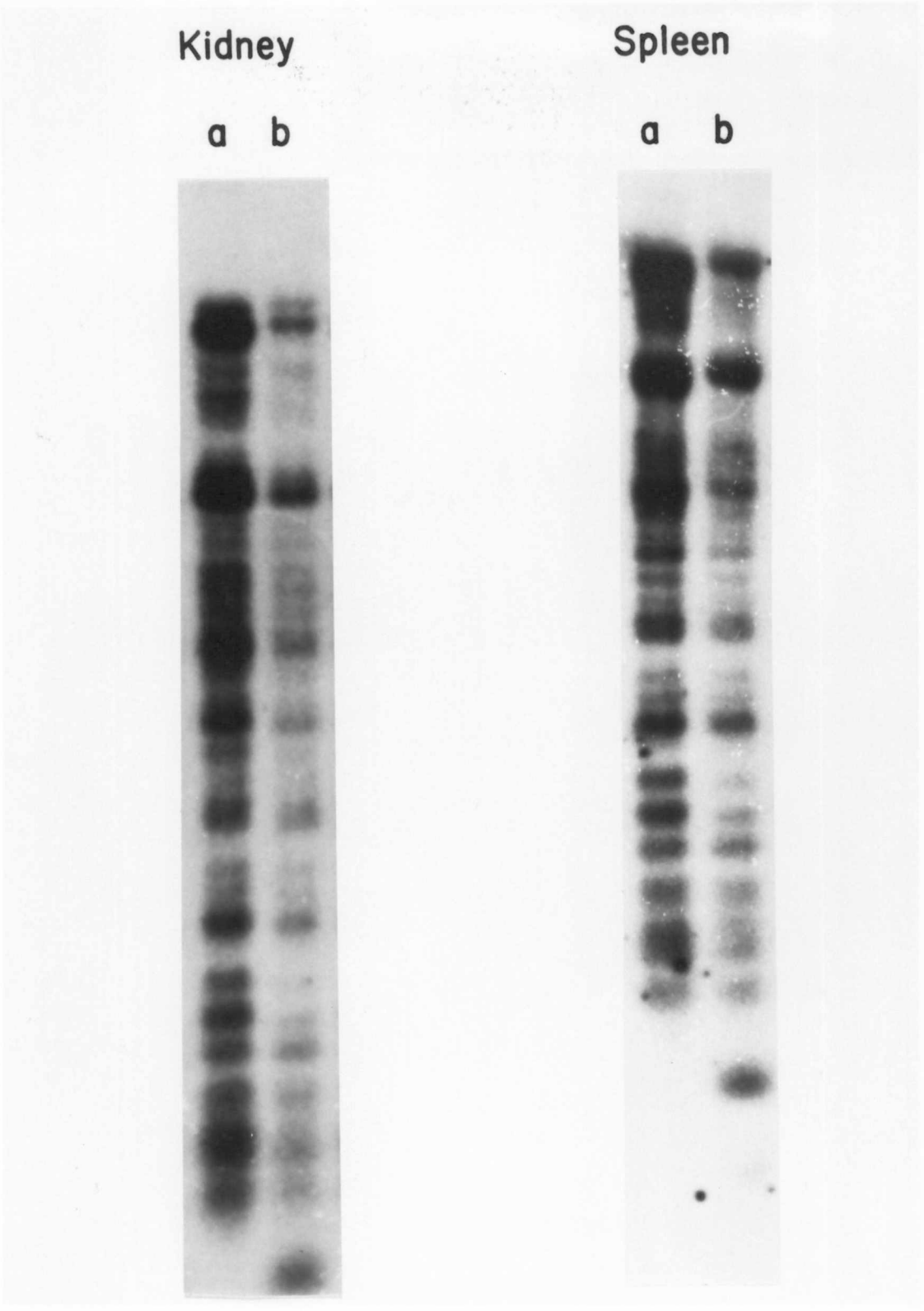

A
a
b
M
B
a
b
M
kb
23
9.5
6.7
4.3
2.3
2.0

Figure 32. Methylation status of LTR-hybridizing fragments of male RFM kidney and spleen DNAs. DNAs extracted from kidney and spleen of male RFM mice were digested with PstI (lanes a) or PstI and SmaI and fractionated through 0.6% and 1% agarose, respectively. After transfer to nylon membranes, the fragments were hybridized to the LTR probe.

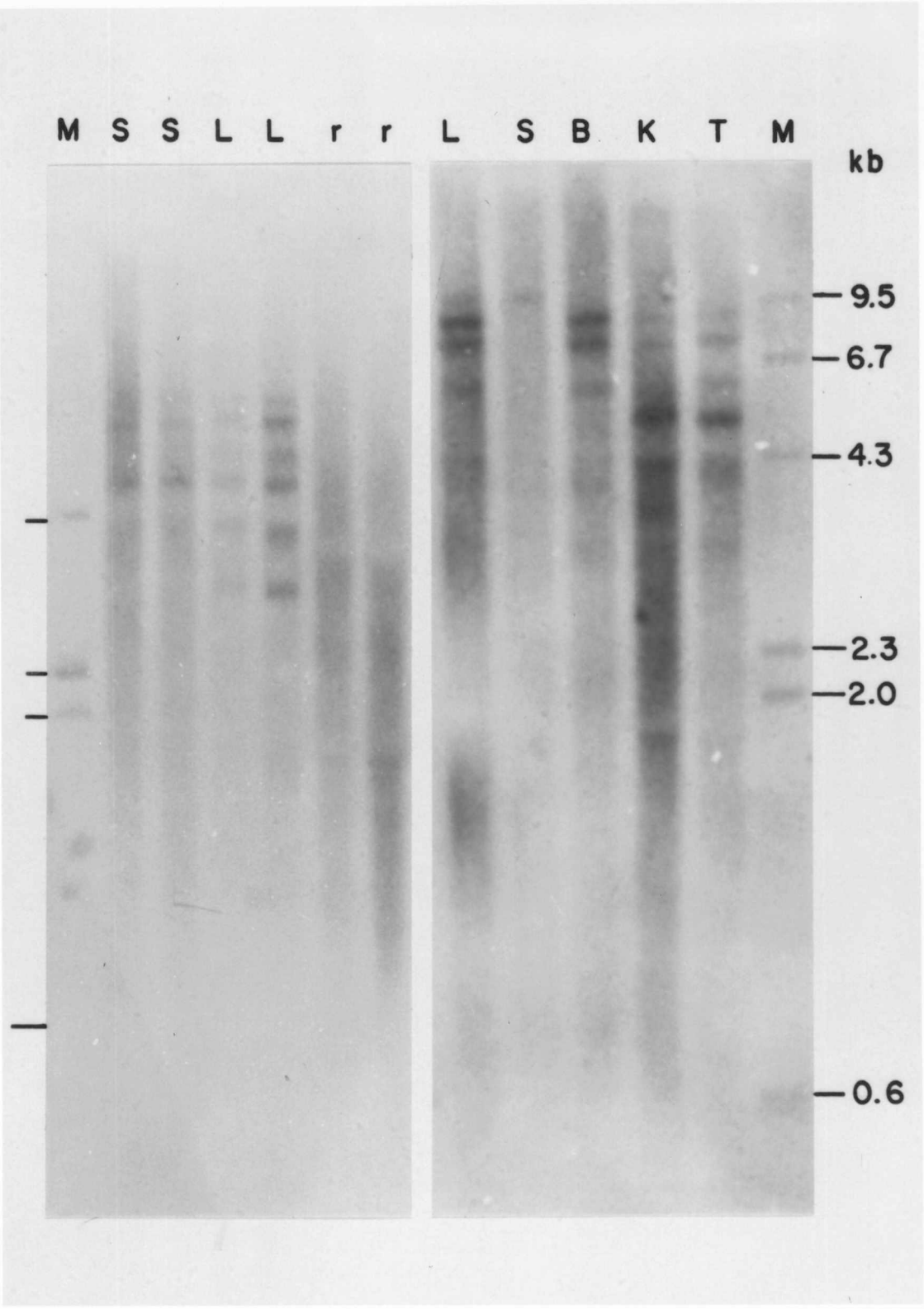

Kidney
a b
Spleen
a b

While the IS probe, derived from the insertion into the U3 enhancer regions of MCF-type proviruses (80,81,86) hybridizes to too many bands to be diagnostic for these studies, it reveals several interesting features (Fig. 30F). First, it recognizes fragments known to be associated with MCF-type proviruses; it also hybridizes to fragments not related to any MuLV-type endogenous proviruses. Secondly, the intensities of the PstI fragments ranging from about 4.8 to 3.2 kb are greatly diminished after SmaI restriction; a comparable decrement in intensity of IS-containing bands of smaller size is not observed. Finally, although the IS probe hybridizes strongly to two LTR fragments generated by cleavage of male RFM liver DNA with PstI and KpnI, it hybridizes strongly only to the PstI-SmaI LTR fragment containing the smaller insertion into its U3 enhancer (Fig. 12, lanes b and c, band b). These data suggest that only the LTR with the smaller insertion is significantly undermethylated, and further, that these LTRs are associated only with proviruses of the type represented by pRFM6 and pRFM16. Many of these proviruses, unlike pRFM6, have suffered major deletions in their structural genes.

RNA transcription of endogenous proviruses

RNA transcripts hybridizing to the gag gene probe have been detected in the liver, spleen, brain, kidney, and testis of the RFM/Un mouse (Fig. 33). The transcripts detected by this probe were approximately 10, 8.2, 7.4, 6, 5.2, 4.3-4.1, 3.2, and 2.6 kb. Although the function and origin of the 10 kb transcript are not clear, it is probable that the 8 kb transcript represents the full-length mRNA; the

Figure 33. Transcripts related to the MuLV gag gene in tissues of the male RFM mouse. RNA was isolated from various male RFM mouse tissues by centrifugation through guanidinium thiocyanate and cesium chloride; polyadenylated species were selected twice by chromatography on oligo(dT)-cellulose. After electrophoresis through 1.2% agarose under denaturing conditions and transfer to nylon membranes, RNAs were hybridized to a probe derived from the gag gene. Each lane contained approximately 5 ug of polyadenylated RNA from liver (L), spleen (S), kidney (K), brain (B), or testis (T); two lanes containing 50 ug each of ribosomal and other non-polyadenylated RNAs (r) are also shown. End-labeled HindIII fragments of bacteriophage lambda were denatured in the same manner as the RNA samples and served as approximate indicators of molecular weight.


M S S L L r r L S B K T M
kb
9.5
6.7
4.3
2.3
2.0
0.6

7.4 kb transcript probably corresponds to the 7.2 kb spliced RNA which would also be detected with an env gene probe (68,69); and transcripts ranging from 4 to 6 kb probably include both gag and pol genetic information. It has not been determined which classes of endogenous proviruses encode these transcripts, or whether some include adjacent cellular genetic information. However, transcripts of approximately 8.4, 7.2, 6.0, 3.0, and 1.8 kb have been detected with an MCF-specific complementary oligonucleotide probe derived from the env gene (56).

Discussion

Although the relative resistance of most of the endogenous MuLV-related proviruses of the RFM/Un mouse to DNAse I digestion is consistent with published reports (5,77), the only data available are those from experimentally introduced retroviral integrations. This work presents a comparison of the relative susceptibilities of naturally occurring endogenous proviruses. Because of the established correlation between nuclease sensitivity and hypomethylation, the finding that most of the endogenous MuLV-related proviruses of the RFM/Un male mouse liver are rather fully methylated at LTR and internal viral SmaI sites is likewise not surprising. However, the failure to find correlation between hypomethylation and DNAse I sensitivity at individual proviral sequences was unanticipated.

Several explanations for the discrepancy between DNAse I sensitivity and methylation status can be advanced. In the liver, the DNAse I-hypersensitive fragment of approximately 6.4 kb is apparently well resolved and distinguishable from other bands in that experiment,

whereas it is not separable from other fragments of similar size in the experiment designed to study methylation status. Thus it is conceivable that the fragment is not fully methylated; however, its contribution to that band cannot be discerned from this experiment, and its methylation status remains undetermined. This argument prevails for the DNAse I-hypersensitive fragment of about 4 kb; a shorter exposure of the membrane to film, however, provides some indication that SmaI has cleaved this fragment, albeit to a limited extent (data not shown). Whereas the 2.3 kb fragment appears to be hypomethylated in the liver, the 0.67 kb DNAse I-hypersensitive fragment does not appear to be at all diminished in intensity by incubation with SmaI.

The most prominent examples of PstI fragments whose LTR SmaI recognition sites are hypomethylated are those of 2.5, 2.3, 1.8, and 1.25 kb. Among these, only the 2.3 kb fragment was classified as DNAse I-hypersensitive. The 2.5 and 1.8 kb fragments were considered relatively sensitive to the action of DNAse I, while the 1.25 kb fragment was moderately resistant. This last result was the most enigmatic, in view of the failure of the unique-sequence probe derived from the region 3′ to the pRFM7 LTR to detect any evidence of methylation in DNAs from male RFM liver and spleen (Fig. 12). Inspection of the 1.25 kb PstI band in Fig. 12 (lane a) reveals that only the upper portion of that band disappears after cleavage with SmaI (lane b). Thus it is conceivable that closely migrating fragments were not resolved by electrophoresis of the DNAse I-treated samples, and the DNAse I sensitivities of the two components could not be distinguished.

The notion that hypomethylation is a prerequisite, but not sufficient, condition for active transcription of genetic material has been widely accepted (reviewed in 91 and 108). The thinking that a DNA conformation sensitive to nucleases is a necessary but not sufficient condition for transcription has likewise been widely accepted (reviewed in 55). Demonstrations of the correlations between hypomethylation and nuclease sensitivity, hypomethylation and transcriptional activity, and nuclease sensitivity and transcriptional activity are abundant (32,46,48,63,82,104,118). However, there are notable exceptions to these dogmas in addition to those discussed with regard to methylation of retroviral DNA in embryonal cells (30,83). Transcription and level of expression of the alpha2(I) collagen gene in five tissues was found to be independent of methylation (76). Use of the methylation-sensitive restriction enzymes AvaI, HpaII, MspI, and SmaI in conjunction with three region-specified probes demonstrated that the DNA around the start site for transcription was unmethylated regardless of whether collagen is synthesized in those cells; DNA from the central and 3′ coding regions of the gene is methylated to about the same extent regardless of whether the cells synthesize collagen (76). The relationship between hypomethylation and DNAse I sensitivity was uncoupled by the demonstration that the lack of methylated sites in the nontranscribed spacer region of Xenopus borealis ribosomal DNA (rDNA) genes are not sufficient to confer DNAse I sensitivity or ensure transcription (73). The transcribed regions of X. laevis rDNA genes, on the other hand, were heavily methylated and hypersensitive to DNAse I (73). Although the rDNA genes are not transcribed by RNA polymerase

II, this example serves to emphasize that transcriptional activity is governed by factors other than methylation status. Hence the failure to observe correlation between hypomethylation and DNAse I sensitivity in this work may not be totally artifactual. It is conceivable that, whereas conformational changes accompanying transciption encompass relatively large areas of chromatin, methylation and demethylation need affect only limited numbers of very specific sites within a region of chromatin.

Use of the gag and gag-pol deletion probes revealed hypomethylation at SmaI restriction sites in the structural genes of the non-producing MCF-type proviruses represented by pRFM1, pRFM16, and pRFM17 (Fig. 30C,D). This conclusion was corroborated by use of the IS probe (Fig. 30F). All three of these proviral LTRs contain inserts into their U3 enhancer regions of the types shown in band a and b of Fig. 12; the b type of LTR, unlike those of xenotropic and ecotropic proviruses (Fig. 12, band c) or those containing the larger insertions into their U3 regions (Fig. 12, band a), is largely undermethylated. Interestingly, the 5′ LTR of pRFM17-like proviruses appears to be methylated. The methylation status of 3′ LTRs was not addressed in these studies, as the use of unique-sequence probes from the regions 3′ to the integration sites would have been required for the combinations of restriction enzymes employed here. It may be that the methylation status of the 3′ LTR is more relevant to DNA conformation, nuclease sensitivity, and transcriptional activity, as it is the 3′ LTR which would serve as the promoter/enhancer and initiation site for transcription of adjacent cellular genes. The 3′ LTR of a provirus

harboring the larger insertion in its U3 region has been shown to function as a promoter in an expression vector based on transient assay of chloramphenicol acetyl transferase activity (61). Tissue-specific transcripts of MCF-type proviruses associated with the LTR bearing the larger insertion in its U3 region have been detected in AKR mice using a synthetic oligonucleotide probe complementary in sequence to a 16-bp region of the envelope gene specific for MCF proviruses (56). The possibility that MCF-specific transcripts not identifiable with this LTR may have arisen from proviruses associated with the LTR harboring the smaller insertion in its U3 region is not precluded (56). Whether these MCF LTRs also promote transcription of adjacent cellular genes is not clear.

The failure to observe correlation between DNAse I hypersensitivity and hypomethylation may be due to the inability to examine 3′ LTRs as a class; the combinations of restriction enzymes employed in these studies permitted the study of 5′ LTRs as a class, but 3′ LTRs were detected as unique-sequence fragments hybridizing to the LTR probe. It is conceivable that the DNAse I-hypersensitive fragments represent actively transcribed 3′ LTRs and their flanking regions; it is also possible that the few fragments hypomethylated at their LTR SmaI sites represent actively transcribed 3′ LTRs and their flanking regions. Use of viral structural genes as probes of methylation status permitted the study of ecotropic, xenotropic, and MCF-type proviruses as classes. Whether the greater extent of hypomethylation associated with proviruses containing structural defects can be correlated with greater transcriptional activity

remains to be determined. RNA transcripts related to the gag gene occur in a tissue-specific manner in the RFM mouse; the classes of endogenous proviruses responsible for these transcripts have not yet been identified.

CHAPTER IV

SUMMARY

The genome of the RFM/Un mouse harbors many copies of elements related to MuLVs. Not all of these elements are capable of producing infectious virus particles without undergoing some modification or rearrangement. Nonetheless, these non-producing elements may have a profound effect upon the host.

Two approaches have been employed in these studies of non-producing MuLV-related elements. The first approach entailed the characterization of two very specific moieties, the two solitary LTRs represented in subclones obtained from a library of RFM/Un spleen DNA in phage lambda. The second approach involved the use of probes derived from viral structural genes to examine the population of MuLV-related elements present in the RFM genome and to assess their potential for transcriptional activity.

The two solitary LTRs and their flanking sequences were present in liver DNA preparations obtained from RFM/Un, BALB/c, CBA, C3H, C57BL/6, NFS/N, 129, and SWR/J inbred mice. The pRFM7 solitary LTR was absent from the DNA of the SWR/J mouse; the pRFM8 solitary LTR was apparently absent from three closely related strains, BALB/c, CBA, and C57BL/6. Whereas the pRFM7 solitary LTR was not detectably methylated in RFM or NFS liver or spleen DNAs, the pRFM8 LTR was apparently hypomethylated in NFS liver and spleen and in RFM spleen DNAs, but not detectably undermethylated in RFM liver DNA. These results suggested that both elements resided in transcriptionally active regions of chromatin in

certain tissues. However, none of the PstI fragments harboring the solitary LTRs appeared to be unusually sensitive to DNAse I digestion. Only the pRFM7 LTR was released from isolated nuclei obtained from the spleen and liver of male RFM mice by incubation with the restriction enzyme PstI; if the accessibility of a segment of chromatin to endonucleolytic cleavage is an indication of its transcriptional activity, then perhaps this solitary LTR resides in a transcriptionally active domain of chromatin. Thus at the present time, there is no clear indication that these solitary LTRs are transcribed or promote the transcription of adjacent cellular sequences, and the reason for their persistence in the genomes of inbred mice remains obscure.

Strategies similar to those described above were employed for the general study of MuLV-related sequences endogenous to the RFM/Un mouse. The methylation status of all LTR SmaI sites in the RFM genome was determined by cleaving DNA first with PstI to generate restriction fragments of defined size, then incubating the PstI digest with the methylation-sensitive enzyme SmaI. Most of the PstI fragments detected by the LTR probe persisted after SmaI cleavage, indicating that the preponderance of these sites were methylated. However, one class of LTRs generated by the above double digestion was conspicuously undermethylated. That class of LTRs contained the smaller of two insertions into the U3 region of the LTR, and has been associated with the MCF-type endogenous proviruses which have suffered major deletions in their structural genes. Use of probes derived from the gag, gag-pol, and env viral structural genes revealed little evidence of hypomethylation among endogenous ecotropic and xenotropic proviruses,

but provided additional indications that both classes of MCF-type endogenous proviruses were hypomethylated at SmaI sites outside the LTR. Thus studies of methylation status suggested that in the liver of the male RFM mouse, non-producing proviral elements were more likely to reside in transcriptionally active regions of chromatin than were the full-length ecotropic or xenotropic proviruses.

Although the endogenous proviruses were generally relatively resistant to DNAse I degradation, there was a gradient of DNAse I sensitivity among the PstI fragments detected by the LTR probe. The most sensitive fragments, however, were not those which were greatly undermethylated. This apparent lack of correlation between nuclease sensitivity and hypomethylation could be due to several factors. The design of this experiment permitted the facile detection of fragments harboring 5′ LTRs and viral structural genes; the contributions of 3′ LTRs and their adjacent sequences were more difficult to assess. If the conformation, and thus the nuclease sensitivity, of the 3′ LTRs is the more valid indicator of potential transcriptional activity, which would be expected if these elements serve to promote the transcription of adjacent cellular genes, then the lack of correlation between hypomethylation and DNAse I sensitivity as detected by the LTR probe is not unreasonable. Use of restriction enzymes which do not cleave within the provirus, in conjunction with additional viral and flanking sequence probes, would clarify these results and predictions.

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