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Characterization, Chromosome Assignment, and Segregation Analysis of Endogenous Proviral Units of Mouse Mammary Tumor Virus

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In the course of analyzing sites of proviral integration in tumors induced by mouse mammary tumor virus (MMTV), we have isolated recombinant DNA clones corresponding to the 5′ and 3′ ends of four endogenous MMTV proviruses present in BALB/c and BR6 mice. This has permitted the structural characterization of each locus by detailed restriction mapping and the preparation of DNA probes specific for the cellular sequences flanking each provirus. These probes have been used to trace the segregation patterns of the proviruses, designated *Mtv-8*, *Mtv-9*, *Mtv-17*, and *Mtv-21*, in a panel of inbred strains of laboratory mice and to map *Mtv-17* and *Mtv-21* to mouse chromosomes 4 and 8, respectively. The unambiguous resolution of these four proviruses on Southern blots has greatly facilitated the analysis of other endogenous MMTV proviruses in these inbred mice.

The DNAs of all common inbred strains and some feral colonies of *Mus musculus* contain sequences related to the genome of mouse mammary tumor virus (MMTV) (1, 3, 16, 33, 36, 38, 53). Most, though not all, of these sequences are organized into apparently complete proviral units that are integrated at different chromosomal sites and segregate independently during cross-breeding (2, 3, 6, 17, 19, 28, 34, 35, 45, 47, 48, 55). It is therefore likely that such endogenous proviruses arose through multiple, independent, but relatively rare infections of the germ line by MMTV and that they have subsequently become distributed in characteristic assortments among the various strains of laboratory mice in use today (reviewed in reference 48).

While the majority of the vertically transmitted MMTV proviruses probably remain transcriptionally silent throughout the lifespan of the animal, there are indications that some may be transiently expressed in certain tissues or in particular circumstances (27, 32, 37, 40, 50). Others, such as the *Mtv-8* locus, are demonstrably capable of directing RNA and protein synthesis and are modulated by glucocorticoid hormones when reintroduced into cells by transfection of cloned DNA (9, 21). Perhaps the most significant examples of transcriptionally active copies are the *Mtv-1* and *Mtv-2* loci which have been shown to express fully infectious MMTV particles and to contribute markedly to mammary tumor incidence in animals freed from horizontal infection by milk-borne MMTV (34, 51, 52, 54, 55). The endogenous MMTV proviruses have therefore commanded considerable attention as models for the control of gene expression, as indicators of essential functions in virus evolution, as chromosome-specific markers, and as potential cofactors in mammary tumorigenesis.

The majority of previous studies on endogenous MMTV proviruses have relied on Southern blotting analysis, exploiting the single cleavage site for the restriction enzyme *Eco*RI which occurs around the midpoint of each complete proviral unit (3, 4, 46). Cleavage of integrated MMTV DNA with *Eco*RI will therefore generate two virus-cell junction fragments. However, since such fragments tend to be rather homogeneous in length, their analysis has been hampered by inadequate resolution in agarose gels and by the failure in most instances to distinguish 5′ from 3′ junctions. In analyzing the sites of proviral integration in MMTV-induced mammary tumors (8, 39), we have obtained molecular clones comprising the 5′ and 3′ segments of four endogenous MMTV proviruses. Here we present comparative restriction maps of these proviruses and their flanking sequences. By preparing probes specific for the cellular DNA adjacent to each provirus, it was possible to discriminate unambiguously between the various endogenous units and to trace their patterns of segregation among a variety of inbred strains of mice. The same probes also facilitated the assignment of proviruses to particular mouse chromosomes and will therefore serve as chromosome-specific markers in future analyses.

MATERIALS AND METHODS

Inbred mouse strains. The following inbred strains of *M. musculus* held by the Imperial Cancer Research Fund (ICRF) Animal Breeding Unit were arbitrarily selected for analysis of endogenous MMTV proviruses: AKR, A/St, C57BL, CBA/Ca, DBA/2, DDSio, NZB, C3H/He, BALB/c and BR6. Some of these strains have been housed at ICRF for over 40 years. Thus, for example, the ICRF C57BL colony predates the separation between B6 and B10 derivatives. The BR6 strain was developed at ICRF as a cross between C57BL and RIII and has been maintained as an inbred colony since the late 1940s (15). Breeding females of this strain develop a very high incidence of spontaneous mammary carcinomas as a result of milk-borne MMTV

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(15, 26). Other tumors were obtained by inoculating newborn BALB/c mice with virus from the Mm5MT/C1 line (14, 39).

Extraction and analysis of tissue DNAs. Total cellular DNA was prepared from the freshly dissected spleens of uninfected mice or from MMTV-induced mammary tumors which had been rapidly frozen and stored at −70°C. Our procedures for the extraction of high-molecular-weight DNA, digestion with restriction enzymes, agarose gel electrophoresis, and Southern blotting were based on published protocols and have been adequately detailed elsewhere (8, 39). Hybridizations were perforemd in 50% formamide at 42°C as described previously (39), and the nitrocellulose filters were washed at a high stringency in 0.1× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C. The ^{32}P-labeled hybridization probes were prepared by nick translation of specific restriction fragments which had been excised from the appropriate cloned plasmids and recovered by preparative gel electrophoresis (8, 39). To expose the same Southern blots to multiple probes, the filters were rinsed briefly in 30 mM NaOH to remove the hybridized radioactive DNA, neutralized, and reprocessed.

Molecular cloning procedures and analysis of clones. Recombinant phage libraries were constructed by ligating *Eco*RI-digested mammary tumor DNA to the purified arms of the vector λgtWES·λB (31). Packaged phage particles were plated on *Escherichia coli* LE392 and screened by using a probe specific for the MMTV long terminal repeat (LTR) (see Fig. 2e). Recombinants containing the virus-cell junction fragments were plaque purified and rescreened with specific probes to distinguish between the 5′ and 3′ portions of the MMTV provirus (see Fig. 2e). Selected phages were then propagated in small-scale cultures for isolation of DNA, and the *Eco*RI inserts were excised and transferred into the plasmid vector pAT153 to facilitate larger-scale preparation and characterization. The methods used for these procedures were based largely on those described by Maniatis et al. (31) and those described in previous reports (8, 39).

The *Eco*RI fragments recovered from the recombinant clones were characterized by restriction enzyme digestion by using a selection of restriction endonucleases which were known to cleave MMTV proviral DNA. The sizes of the various fragments generated in these analyses were estimated relative to a series of plasmid DNA standards of known sequence, ranging from 6.5 kilobases (kb) down to about 150 base pairs. Maps were generally established by digestion with multiple combinations of enzymes and by blot hybridization with MMTV-specific probes. In some instances, specific fragments were isolated on preparative gels or subcloned in appropriate plasmid vectors to resolve otherwise intractable sections of the maps. Some cleavage sites could not be unambiguously mapped (see Fig. 2, dashed lines).

Chromosome mapping. A series of cell hybrids were generated by fusion of Chinese hamster cells (E36) with peritoneal cells or spleen cells of BALB/c, A/J, and NFS *Akv-2* congenic mice. The characterization of these hybrids and their use in chromosomal mapping of other cellular loci have been described elsewhere (22–25). High-molecular-weight DNA from each hybrid was digested to completion with either *Pst*I or *Eco*RI and subjected to agarose gel electrophoresis and Southern blotting. The filters were hybridized sequentially with cellular DNA probes specific for particular endogenous MMTV proviruses, each of which detected the appropriate mouse-specific restriction fragments with virtually no background hybridization to hamster DNA under the conditions used.

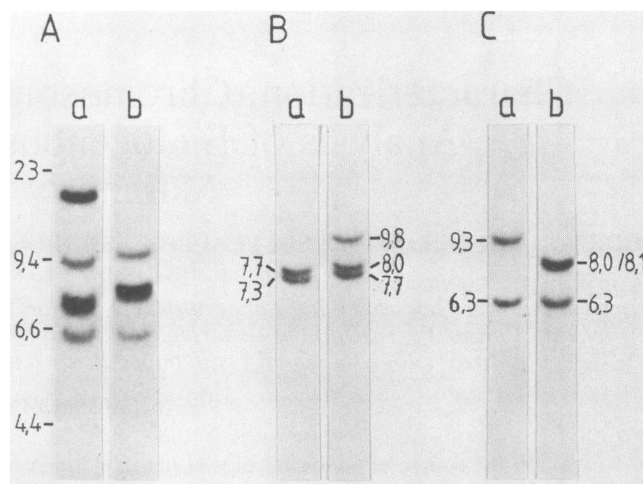

FIG. 1. Analysis of endogenous MMTV proviruses in BALB/c and BR6 mice. Samples (10 μg) of total cellular DNA from the spleen of either BALB/c (lanes a) or BR6 (lanes b) mice were digested with *Eco*RI, fractionated by electrophoresis in 0.8% agarose gels, and transferred to nitrocellulose. The filters were hybridized with ^{32}P-labeled fragments of DNA specific for the LTR (A), 5′ (B), or 3′ (C) region of the MMTV provirus. The derivation of these probes has been described previously (39) and is illustrated schematically in Fig. 2e. Each band visualized on the autoradiograph corresponds to a junction between viral and cellular DNA sequences. The sizes of the endogenous proviral fragments, estimated relative to *Hin*dIII-digested lambda DNA and from subsequent restriction mapping (Fig. 2), are indicated in kilobases, and their assignment to particular *Mtv* loci is discussed in the text.

RESULTS

Characterization of endogenous MMTV proviruses in BALB/c mice. BALB/c mice contain two complete endogenous MMTV proviruses, designated *Mtv-8* and *Mtv-9*, plus one partial proviral unit, *Mtv-6* (2, 3, 18). The latter is contained in an approximately 14.5-kb *Eco*RI fragment which reacts strongly with a probe for the MMTV LTR (Fig. 1A) and is just discernible with a probe specific for 3′ MMTV sequences (Fig. 1C). Specific attempts to clone the *Mtv-6* locus have so far proved unsuccessful. However, in the course of analyzing MMTV proviruses in BALB/c mammary tumors, we repeatedly cloned *Eco*RI fragments corresponding to the 5′ and 3′ virus-cell junctions of *Mtv-8* (7.7 and 6.3 kb, respectively) and of *Mtv-9* (7.3 and 9.3 kb, respectively) (Fig. 1).

Restriction maps derived from the cloned junction fragments of these proviruses are shown in Fig. 2a and b. Although they are presented as complete proviruses, it should be noted that each comprises a composite of two independent *Eco*RI fragments and that we have not rigorously established a map which crosses the *Eco*RI site. Since the maps are in close agreement with published data, there was little doubt that we had correctly matched the corresponding 5′ and 3′ ends. However, we confirmed these assignments by preparing probes for the flanking cellular DNA. These probes have been designated by using a logical convention; thus, *Mtv8-5′C* refers to cellular sequences at the 5′ end of the *Mtv-8* provirus and should be distinguished from the clone containing the complete *Eco*RI fragment,

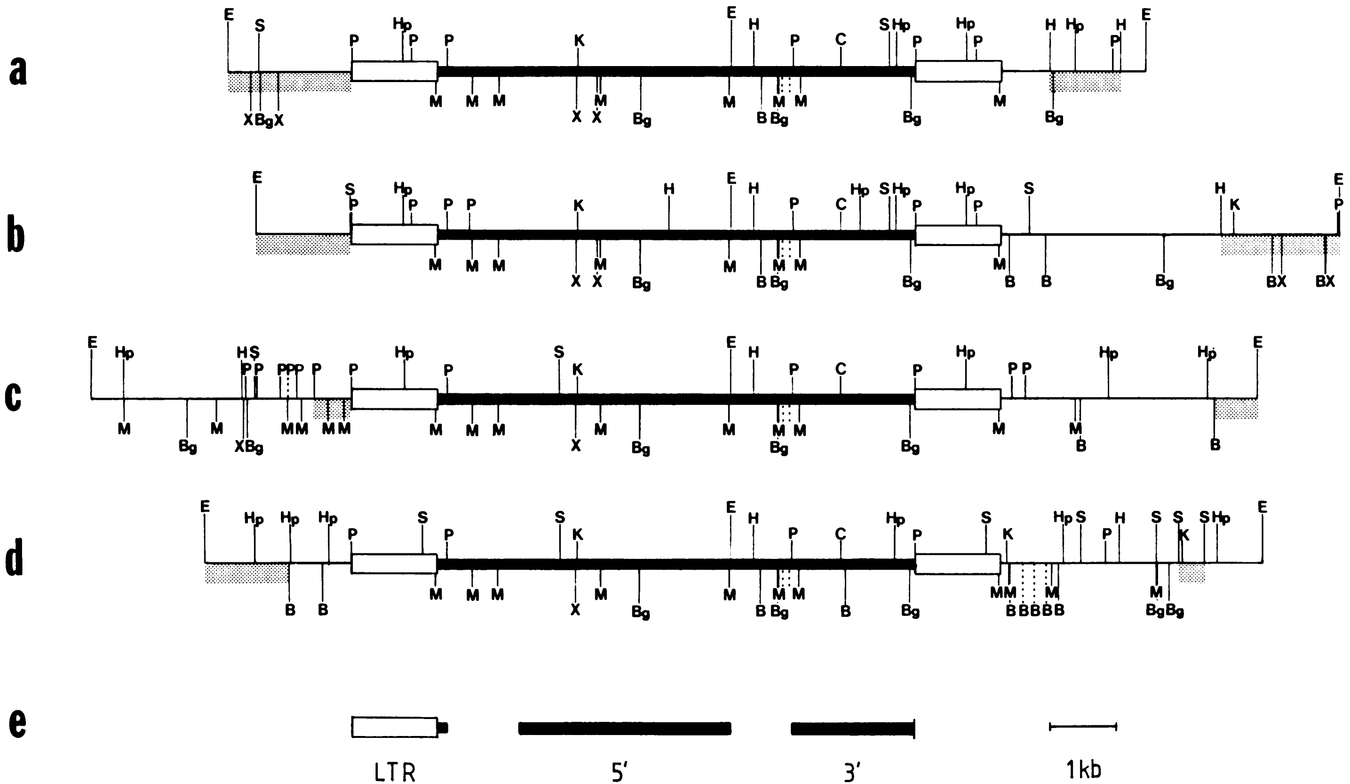

FIG. 2. Restriction maps of four endogenous MMTV proviruses. The various MMTV-specific *Eco*RI fragments recovered from recombinant phage libraries were characterized by digestion with the following restriction endonucleases: *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Hin*dIII (H), *Hpa*I (Hp), *Kpn*I (K), *Msp*I (M), *Pst*I (P), *Sac*I (S), and *Xba*I (X). Additional information on *Pvu*II and *Xho*I sites is available on request. The size of each digestion product was calculated relative to a series of standards generated from plasmid DNAs of known sequence, and fragments containing viral sequences were identified by blot hybridization with MMTV-specific probes. Symbols: ▬ Viral DNA; ——, cellular DNA; ▭, LTR regions; ▒, unique sequence probes isolated from the cellular DNA flanking each provirus. By using such probes to examine DNA from which the provirus was absent, it was possible to match the 5′ and 3′ ends of particular proviruses. Although these are shown as being linked at a single *Eco*RI site (E), we have not definitively mapped fragments which span this site. Other remaining ambiguities are shown by dotted lines. The multiple *Msp*I sites in the 3′ region are, however, likely to be correct by analogy to the sites predicted from the DNA sequence of the *env* gene of milk-borne MMTV (30, 44). The proviruses mapped here correspond to the *Mtv-8* (a), *Mtv-9* (b), *Mtv-17* (c), and *Mtv-21* (d) endogenous loci. The restriction fragments from cloned milk-borne MMTV that were used as 5′-, 3′-, and LTR-specific hybridization probes are indicated (e).

Mtv8-5′ (40). To facilitate the preparation and distribution of these probes, the various restriction fragments depicted by stippled bars in Fig. 2 have been subcloned into appropriate bacterial plasmids.

Distribution of *Mtv-8* and *Mtv-9* proviruses in inbred mice. Having prepared *Mtv-8*- and *Mtv-9*-specific probes, we used them to determine how widely these endogenous proviruses were distributed among a panel of inbred strains of *M. musculus*, particularly as *Mtv-8* is reportedly common to many strains (6, 47). The *Mtv-8* provirus was present in 8 of the 10 strains tested (Fig. 3a; see Table 4 for summary). Moreover, such analyses confirmed the linkage of the *Mtv8-5′C* and *Mtv8-3′C* probes, since both identified the same 4.2-kb *Eco*RI fragment corresponding to the unoccupied site in *Mtv-8*-negative DNA. The size of the fragment was exactly as predicted from the sum of the cellular sequences adjacent to the 5′ and 3′ ends of the *Mtv-8* provirus. Similarly, the combination of *Mtv9-5′C* and *Mtv9-3′C* confirmed the presence of the *Mtv-9* locus in 5 of the 10 strains of mice (Fig. 3b) and correctly identified an unoccupied site of 6.7 kb in the negative strains. The one exception occurred in BR6 mice, in which the *Mtv-9* probes reacted with an *Eco*RI fragment of approximately 12 kb. The most likely explanation for this result is that one of the *Eco*RI sites defining the boundaries of the 6.7-kb fragment may be absent in BR6 mice, but other possibilities cannot be discounted.

Characterization of endogenous MMTV proviruses in BR6 mice. Our particular interest in the BR6 mice stemmed from the fact that the spontaneous mammary tumors in this strain are frequently pregnancy dependent (15, 26). We therefore analyzed sites of MMTV provirus integration in a series of such tumors, screening a total of well over 5×10^6 recombinant phage in the process. BR6 mice contained a different assortment of endogenous MMTV proviruses as compared with BALB/c (Fig. 1). These were characterized by 5′ *Eco*RI junction fragments of 7.7, 8.0, and 9.8 kb (Fig. 1B), with corresponding 3′ fragments of 6.3 kb and a doublet at around 8.0 kb (Fig. 1C). As confirmed by hybridization to the *Mtv8-5′C* and *Mtv8-3′C* probes, the 7.7- and 6.3-kb fragments were attributable to the presence of the *Mtv-8* provirus (Fig. 3a) and could be readily identified among the MMTV-positive recombinant clones recovered. In addition, we isolated clones corresponding to both the 9.8- and 8.0-kb 5′ junctions, and to one of the 8.0-kb 3′ ends. The restriction maps of these *Eco*RI fragments are presented in Fig. 2c and d.

Confirmation that these fragments indeed represented endogenous MMTV proviruses was obtained by preparing

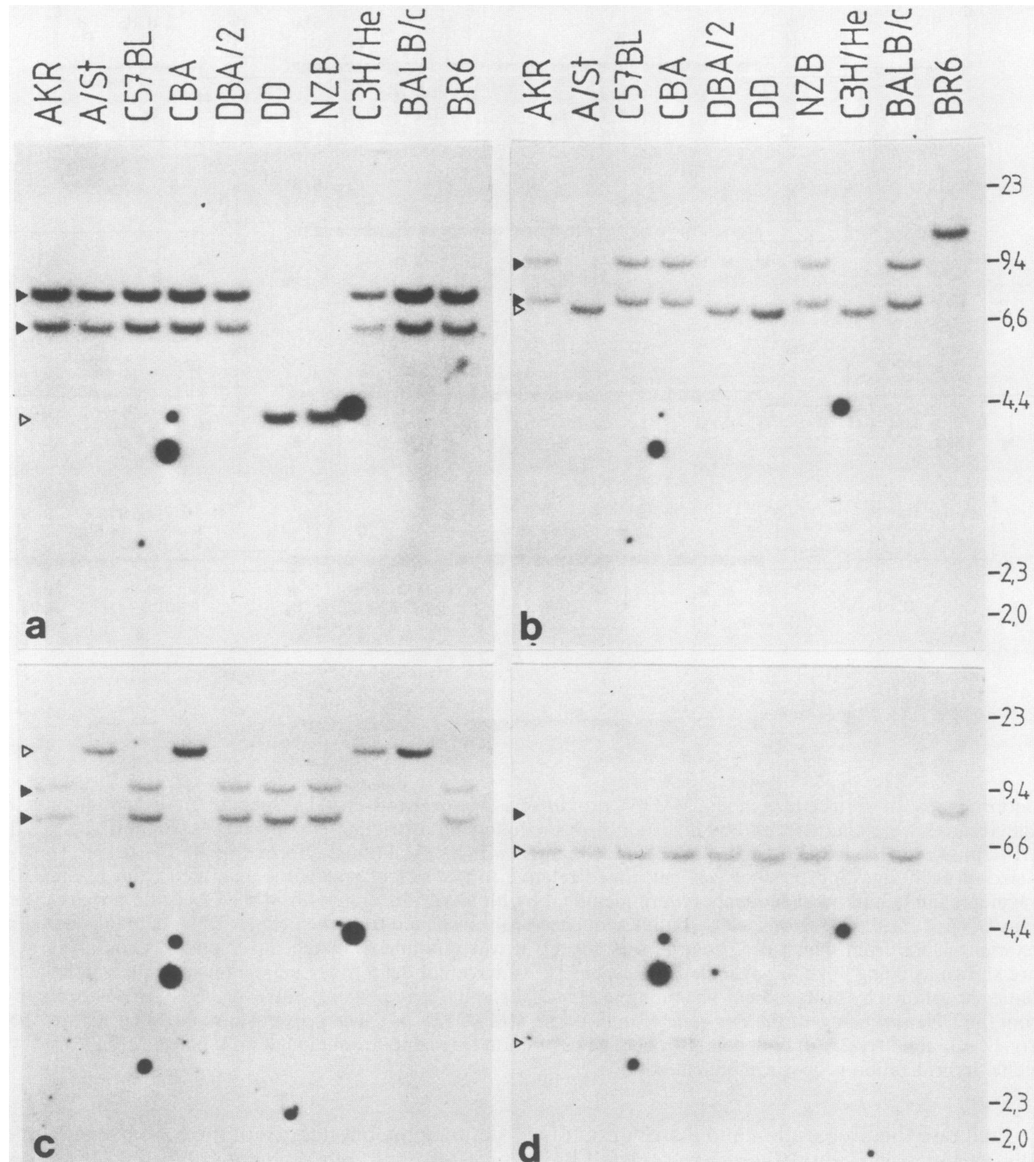

FIG. 3. Distribution of four endogenous MMTV proviruses in a panel of inbred mice. High-molecular-weight DNA extracted from the spleens of 10 different inbred strains of mice was digested with *Eco*RI and analyzed by agarose gel electrophoresis and Southern blotting. (a) The nitrocellulose filter was hybridized with a mixture of the two flanking sequence probes from the *Mtv-8* provirus, *Mtv8-5′C* and *Mtv8-3′C*, which were prepared to equivalent specific activities. These probes detected the appropriate 5′ and 3′ junctions (▶) in 8 of the 10 DNA samples and the 4.2-kb *Eco*RI fragment corresponding to the unoccupied site (▷) in the two negative mice. (b and c) The filter was then rinsed in 30 mM NaOH to remove the bound radioactivity and hybridized sequentially with the mixed 5′- and 3′-specific probes from *Mtv-9* (b) and *Mtv-17* (c). (d) Only hybridization with *Mtv21-5′C* is shown, since the corresponding 3′ probe could not be prepared free of repetitive sequences. The designation of each of these probes is presented in Fig. 2 and discussed in the text. Numbers to the right of the figure refer to the positions and relative sizes (in kilobases) of fragments of *Hin*dIII-digested lambda DNA which were used as size markers in these analyses.

probes for the flanking cellular sequences (Fig. 2). Thus, the 9.8-kb 5′ end and 8.0-kb 3′ end were shown to comprise a single proviral unit, since the probes detected the same *Eco*RI fragment in mice which were negative for this provirus (Fig. 3c). Moreover, the restriction map of the 8.0-kb 3′ end is virtually identical to that published by Peterson et al. for a provirus present in C57BL mice (40). They have tentatively designated this locus *Mtv-17* and have deduced that the 5′ end of such a provirus should yield a 10.0-kb *Eco*RI fragment (40). The data presented in Fig. 3c confirmed this interpretation and further established that the *Mtv-17* locus was common to 6 of the 10 inbred strains tested. However, the size of the *Eco*RI fragment identified as the unoccupied site in the four negative strains was around 13.5 kb, compared with a predicted size of 7.9 kb, based on the extent of cellular sequences adjacent to the 5′ and 3′ termini of the provirus. The reason for this discrepancy remains unknown, but it could reflect polymorphism in the *Eco*RI sites or, alternatively, deletion of cellular DNA sequences during or subsequent to the insertion of *Mtv-17* into the germline.

The flanking sequence probe for the remaining 8.0-kb 5′

TABLE 1. Correlation between specific mouse chromosomes and *Mtv-17* in mouse-hamster somatic cell hybrids

Mouse chromosome	No. of hybrid clones retaining *Mtv-17* or chromosome[a]				% Discordant
	Both	Neither	*Mtv-17* only	Chromosome only	
1	5	8	1	4	28
2	3	9	3	3	33
3	3	9	2	3	29
4	7	12	0	0	0
5	1	11	6	1	37
6	4	8	3	4	37
7	5	4	2	8	53
8	3	11	3	1	22
9	3	10	4	2	32
10	1	11	6	1	37
11	0	12	6	0	33
12	4	6	1	6	41
13	1	9	4	3	41
14	2	9	5	3	42
15	4	1	0	11	68
16	3	9	3	3	33
17	4	4	3	8	58
18	3	7	3	4	41
19	3	8	3	4	39
X	3	7	3	5	44

[a] 16 hybrids were karyotyped by Giemsa-trypsin banding: 3 were typed only for specific chromosome markers. All were probed for *Mtv17-5'C* and *Mtv17-3'C* sequences by Southern blot analysis.

end characteristic of BR6 mice, tentatively designated *Mtv21-5'C*, was found to hybridize to not one but two *Eco*RI fragments in BALB/c and other mouse DNAs (Fig. 3d). The size of the larger 6.2-kb fragment would be entirely consistent with the unoccupied site for a provirus comprising an 8.0-kb 5' end plus an 8.1-kb 3' end, and provisionally established the linkage between these two junctions. In an attempt to clarify the situation, both the 6.2- and 2.8-kb *Eco*RI fragments were isolated as recombinant phage clones by screening a BALB/c DNA library with the *Mtv21-5'C* probe. The eventual aim was to obtain a unique sequence probe which would specifically recognize the remaining 3' junction fragment *Mtv21-3'*, but despite repeated attempts, all eight of the fragments tested contained presumed repetitive elements which caused background problems on Southern blots. Nevertheless, the 0.4-kb *Sac*I-*Sac*I fragment depicted as *Mtv21-3'C* in Fig. 2d gave sufficiently clear results to recover a clone of the *Mtv21-3'* junction from a recombinant library of size-selected *Eco*RI fragments from BR6 spleen DNA and to confirm the linkage between the corresponding 5' and 3' ends of *Mtv-21*, with an unoccupied site of 6.2 kb. This probe did not detect the 2.8-kb *Eco*RI fragment.

Chromosome assignment of *Mtv-17* and *Mtv-21*. Since the endogenous MMTV proviruses segregate independently, they provide useful markers for tracing the outcome of genetic crosses. The chromosomal locations of *Mtv-6*, *Mtv-8* and *Mtv-9* were previously determined by forming somatic cell hybrids between BALB/c mouse and hamster cells and screening for the presence of the signature *Eco*RI fragments from these loci (2, 45). Here, we used the same approach to characterize a panel of mouse-hamster hybrids with different complements of mouse chromosomes. Southern blots were prepared of the hybrid DNAs digested with either *Eco*RI or *Pst*I and were probed in turn with each of the flanking sequence probes derived from the *Mtv-17* and *Mtv-21* proviruses (data not shown).

Table 1 correlates the presence of particular mouse chromosomes in each of the hybrid clones with positive hybridization signals by using the *Mtv17-5'C* and *Mtv17-3'C* probes. Identical results were obtained with the two probes in separate experiments, confirming the linkage between the two ends of the *Mtv-17* locus. Thus, 7 of the hybrids contained both chromosome 4 and *Mtv-17*, while 12 contained neither. In contrast, all other mouse chromosomes displayed discordant segregation with *Mtv-17*, reinforcing the assignment of this endogenous provirus to mouse chromosome 4.

With the *Mtv-21* provirus, the results were complicated by the presence of two mouse-specific DNA fragments, both of which were detected by the *Mtv21-5'C* probe. However, the bona fide unoccupied site, represented by a 6.2-kb *Eco*RI fragment, segregated consistently with chromosome 8 (Table 2). In contrast, the 2.8-kb *Eco*RI fragment mapped to the X chromosome (data not shown).

Survey of endogenous MMTV proviruses in 10 inbred mouse strains. As discussed in previous sections and presented in Fig. 3, DNAs from uninfected tissues of 10 arbitrarily chosen strains of mice were analyzed for the presence of the *Mtv-8*, *Mtv-9*, *Mtv-17*, and *Mtv-21* loci. The same or similar blots also provided an opportunity to compare the full complement of MMTV proviruses in these strains and to correlate them with loci other than the four specific examples characterized here. Hybridization was performed with either a 5'-specific (Fig. 4A) or 3'-specific (Fig. 4B) MMTV probe. As expected, the various 5' virus-cell junction fragments mapped in Fig. 2 were readily distinguishable and corresponded exactly to the bands identified by the flanking cellular sequence probes in Fig. 3; similar results were obtained for the 3' junctions. Moreover, since the size of each of these *Eco*RI fragments had been accurately determined by restriction mapping, a relatively precise estimate could be made of the sizes of other 5' and 3'

TABLE 2. Correlation between specific mouse chromosomes and *Mtv-21* in mouse-hamster somatic cell hybrids

Mouse chromosome	No. of hybrid clones retaining *Mtv-21* or chromosome[a]				% Discordant
	Both	Neither	*Mtv-21* only	Chromosome only	
1	7	10	2	6	32
2	5	13	4	3	28
3	3	12	0	4	21
4	7	13	3	3	23
5	2	14	8	1	36
6	8	12	2	3	21
7	8	6	2	10	46
8	8	16	0	0	0
9	6	15	4	1	19
10	2	15	8	1	35
11	0	16	5	0	24
12	3	8	0	6	35
13	1	12	1	3	23
14	3	14	7	2	35
15	3	2	0	13	72
16	4	12	0	3	16
17	5	8	0	8	38
18	3	12	0	3	17
19	9	12	1	4	19
X	7	11	1	5	25

[a] 17 hybrids were karyotyped by Giemsa-trypsin banding, 9 were typed for specific chromosome markers, and all were probed for *Mtv21-5'C* sequences on Southern blots.

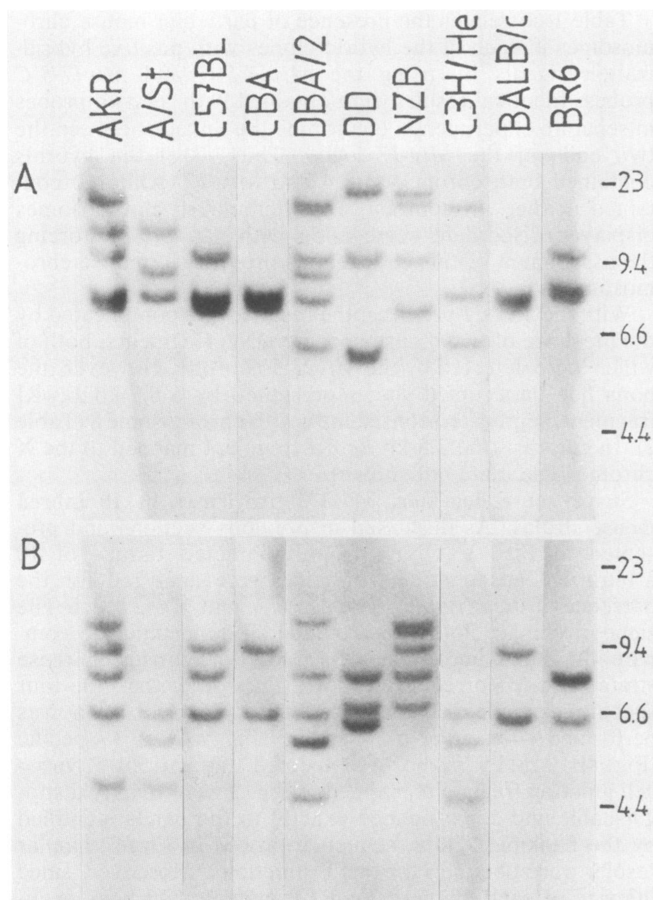

FIG. 4. Survey of endogenous MMTV proviruses in 10 inbred strains of mice. Southern blots prepared from *Eco*RI-digested DNA as described in the legend to Fig. 3 were hybridized with a probe specific for either the 5′ (A) or the 3′ (B) region of the MMTV provirus as described in Materials and Methods. The cloned restriction fragments used as probes are depicted in Fig. 2e and have been described previously (39). Numbers to the right of each panel show the positions and relative sizes (in kilobases) of fragments of *Hin*dIII-digested lambda DNA.

junction fragments detected in these analyses. A summary of these data is presented in Table 3. As discussed later, these data also allowed a tentative linkage scheme to be developed to match the 5′ and 3′ junctions (see Table 4).

DISCUSSION

This characterization of the endogenous proviruses of BALB/c and BR6 mice developed out of our interest in sites of proviral integration in mammary tumors, and in particular the *int-2* locus (8, 39). As a consequence, the study was not undertaken systematically, but, rather, it evolved as a by-product of our cloning efforts. The one exception was the 3′ end of the *Mtv-21* provirus which had proved elusive until more direct measures were adopted for its recovery.

Since the majority of the cloning involved construction of random libraries of total, *Eco*RI-digested DNA, it is interesting to reflect on the relative frequencies with which the various 5′ and 3′ junction fragments were isolated. Of approximately 1.5×10^6 recombinants generated from BALB/c DNA, we obtained 6 and 5 copies, respectively, of *Mtv8-5′* and *Mtv8-3′*. In contrast, only one *Mtv9-5′* clone was recovered compared to 10 copies of the corresponding 3′ end, *Mtv9-3′*. Since the sizes of these various fragments were equally optimal for cloning in λgtWES · λB, one might speculate that the relative paucity of *Mtv9-5′* clones reflects some inherent property of these sequences, echoing the difficulties encountered by others in propagating clones containing segments from the 5′ end of the MMTV provirus (10, 11, 18, 29, 49). In this context, it is worth noting that only a single clone of *Mtv17-5′* has been identified to date and that during transfer of this 9.8-kb *Eco*RI fragment into a plasmid vector, an insertion of approximately 800 base pairs of bacterial DNA occurred in the vicinity of the so-called poison sequences in the MMTV provirus (data not shown). A similar phenomenon has been observed in some isolates of *Mtv21-5′* and in other clones comprising the 5′ end of milk-transmitted MMTV present in BR6 mice (S. Brookes and G. Peters, unpublished observations). However, one of the *Mtv21-5′* isolates was transferred into a plasmid vector with no apparent interruption or rearrangement and yielded the restriction map presented in Fig. 2d.

Direct comparison of the restriction maps of the four MMTV proviruses depicted in Fig. 2 sheds no lights on why some of the *Eco*RI fragments should be more amenable than others to propagation as cloned DNA sequences in procaryotes. Some hallmarks attributed to endogenous proviruses, such as an *Hpa*I site in the LTR, or the lack of a 1.3-kb *Bam*HI fragment in the 3′ region (3, 4, 7, 9, 21, 28), are fulfilled in *Mtv-8*, *Mtv-9*, and *Mtv-17* but clearly do not apply to *Mtv-21*. In this regard, *Mtv-21* is much more analogous to the milk-borne MMTV characteristic of GR mice in that it is cleaved three times by the enzyme *Sac*I (7). However, none of the endogenous proviruses described here would yield the 4.0- to 4.2-kb *Pst*I fragment commonly ascribed to milk-transmitted viruses (3, 4, 18, 46, 47), and the absence of this additional *Pst*I site in the vicinity of the poison sequences may in part explain our success in cloning these 5′ junctions.

The *Mtv-8* locus. The *Mtv-8* locus is probably the best characterized of all the endogenous MMTV proviruses. It

TABLE 3. Estimated sizes of 5′ and 3′ junction fragments derived from endogenous MMTV proviruses[a]

Mouse strain	Sizes of 5′ *Eco*RI fragments (kb)	Sizes of 3′ *Eco*RI fragments (kb)
AKR	17, 11.8, **9.8**,[b] **7.7**,[c] **7.3**[d]	11.5, **9.3**,[d] **8.0**,[b] **6.3**,[c] 4.6
A/St	11.8, 8.8, **7.7**[c]	14.5,[e] **6.3**,[c] 5.5, 4.6
C57BL	**9.8**,[b] **7.7**,[c] **7.3**[d]	**9.3**,[d] **8.0**,[b] **6.3**[c]
CBA	**7.7**,[c] **7.3**[d]	14.5,[e] **9.3**,[d] **6.3**[c]
DBA/2	15.5, 15.0, **9.8**,[b] 8.8, **7.7**,[c] 6.1	14.5,[e] 11.5, **8.0**,[b] **6.3**,[c] 5.5, 5.5, 4.4
DDSio	20, **9.8**,[b] 5.9	**8.0**,[b] 6.6, 6.1
NZB	20, 17, **9.8**,[b] **7.3**[d]	11.5, 11.0, **9.3**,[d] **8.0**,[b] 6.6
C3H/He	15, **7.7**,[c] 6.1	14.5,[e] **6.3**,[c] 5.5, 4.4
BALB/c	**7.7**,[c] **7.3**[d]	14.5,[e] **9.3**,[d] **6.3**[c]
BR6	**9.8**,[b] 8.0, **7.7**[c]	8.1, **8.0**[b] **6.3**[c]

[a] Data shown were obtained from analyses of *Eco*RI-digested DNA, hybridized with either 5′- or 3′-specific MMTV probes as illustrated in Fig. 4. Numbers in boldface refer to the virus-host junctions that have been molecularly cloned and whose known sizes were used, in conjunction with *Hin*dIII-digested lambda DNA, to calibrate graphs relating fragment size in kilobases to distance migrated in gels. Note that these analyses do not include fragments which hybridize exclusively to MMTV LTR sequences.

[b] Fragment corresponding to *Mtv-17*.

[c] Fragment corresponding to *Mtv-8*.

[d] Fragment corresponding to *Mtv-9*.

[e] Fragment which hybridized only weakly to the 3′ probe.

was initially defined as endogenous unit II, on the basis of Southern blotting analysis of BALB/c DNA (3, 6), and numerous subsequent studies have verified these original findings and described analogous sequences in many different inbred strains. Until recently, only feral mice and specific colonies bred to be negative for endogenous MMTV have been shown to lack *Mtv-8*, reinforcing the notion that the majority of the inbred strains of laboratory mice were derived from a common ancestor (1, 5, 6, 13, 45). By using the specific *Mtv-8* probes generated in this study, we have serendipitously identified two strains, DD and NZB, which lack this provirus (Fig. 3a and Table 3).

While the majority of previous reports on *Mtv-8* relied on restriction enzyme digestion and Southern blotting, the *Mtv-8* locus has already been cloned, either as an intact provirus or as two separate junctions from the A/J, C3H, GR, and C57BL/6 strains of mice (9, 10, 12, 21, 40). Although there are slight discrepancies between the published restriction maps and ours, we would conclude that the *Mtv-8* provirus itself has remained unaltered since it was established in the ancestors of the strains from which it has now been cloned. Certainly the C57BL/6 and BALB/c maps are essentially identical (Fig. 2a and reference 40), and it remains arguable whether the minor variations detected between BALB/c and the GR or A/J clones result from restriction site polymorphisms or simply inaccuracies in the derived maps.

On the basis of both DNA hybridization and direct sequencing, Fanning et al. have concluded that, at least in the case of GR mice, the *Mtv-8* provirus has integrated within the *Bam*HI family of interspersed repetitive DNA (12). Since the restriction maps of flanking cellular sequences for the GR and BALB/c clones are very similar, we would expect the *Mtv8-5'C* probe derived here to contain some of these repetitive sequences. However, in the high-stringency conditions we used to wash Southern blots (0.1× SSC at 65°C), we successfully used *Mtv8-5'C* and *Mtv8-3'C* as essentially unique sequence probes (Fig. 3a).

The *Mtv-9* locus. The *Mtv-9* locus was originally designated endogenous unit III in Southern blotting analysis of BALB/c DNA (3, 6) and has been assigned to mouse chromosome 12 (2). The restriction map of *Mtv9-3'* presented in Fig. 2b is virtually identical to that derived by Peterson et al. for the analogous junction fragment cloned from C57BL/6 mice (40). We have extended their findings by providing the first detailed map of the corresponding 5' end and have derived two flanking sequence probes specific for the *Mtv-9* locus (Fig. 2b). The use of these probes to screen a panel of inbred mice (Fig. 3b) confirmed that *Mtv-9* is quite widely distributed, being present in 5 of the 10 strains tested, but, unlike *Mtv-8*, it is not ubiquitous among strains derived from the original Bagg × DBA cross (6).

The *Mtv-17* locus. In tentatively designating the 9.8-kb 5' junction and the 8.0-kb 3' junction mapped in Fig. 2c as *Mtv-17*, we are conforming to the recent precedent set by Peterson et al. (40). It is clear that what they refer to as an 8.4-kb *Eco*RI fragment cloned from C57BL/6 mice was equivalent to the 3' end isolated here from BR6 mice, one of whose parental strains was C57BL, and that their prediction of a matching 10-kb 5' end was also fulfilled. However, it should be emphasized that this designation as *Mtv-17* is somewhat arbitrary and could lead to confusion since it is likely that the 9.8-kb fragment is analogous to that described as a subgenomic unit and designated *Mtv-15* by Traina et al. (47). Moreover, a similar pair of *Eco*RI fragments in GR mouse DNA has been assigned the name *Mtv-20* (20), while two completely different fragments have previously been designated *Mtv-17* (48). The derivation of specific probes for the *Mtv-17* provirus as defined here should help to resolve this issue, as will the unambiguous assignment of this locus to mouse chromosome 4 (Table 1). In this latter context, *Mtv-17* is distinct from the other endogenous MMTV provirus assigned to chromosome 4 in the A/HeJ strain of mice (28, 35), since the specific probes failed to detect *Mtv-17* in DNA from our A/St colony (Fig. 3c).

The *Mtv-21* locus. The two cosegregating *Eco*RI fragments, each about 8 kb in size, which were isolated and characterized from BR6 mice were arbitrarily designated *Mtv-21* to avoid confusion with other previously named loci. This decision is supported by our inability to detect the provirus in nine other strains of mice (Fig. 3d) and our failure to find any reference to such a provirus in the literature. It is also the only *Mtv* locus so far assigned to mouse chromosome 8.

Since BR6 mice were derived from a cross between a C57BL female and an RIII male (15), we have assumed that the *Mtv-21* locus was inherited from the RIII parent, although it is also possible that it has become established as a novel endogenous provirus within the 40 years since the BR6 strain was developed. While there is a noticeable similarity between the viral sequences of *Mtv-21* and those of milk-borne MMTV, we have successfully isolated clones derived from the milk-transmitted virus in BR6 mice, and the restriction maps are quite distinct from that of *Mtv-21* (unpublished observations). Moreover, *Mtv-21* does not correspond to the mammary tumor-inducing locus of RIIIf mice since foster nursed BR6 mice have a very low tumor incidence (A. Lee, personal communication). Irrespective of its origins, the *Mtv-21* locus presumably became established subsequent to the dispersion of the *Mtv21-5'C*-specific sequences onto different chromosomes, since the 2.8-kb *Eco*RI fragment, mapped to the X chromosome (data not shown), was present in BR6 as well as all other mice tested (Fig. 3D).

Other endogenous MMTV proviruses. By preparing the flanking cellular sequence probes described here, it became possible to unambiguously ascribe *Eco*RI fragments detected on Southern blots to specific MMTV proviruses. There is therefore no doubt that the ICRF colony of C57BL contains three intact endogenous proviruses (*Mtv-8*, *Mtv-9*, and *Mtv-17*), as does the BR6 strain (*Mtv-8*, *Mtv-17*, and *Mtv-21*), all of which have now been cloned and characterized. Taking account of these, the remaining 5' and 3' junction fragments detected in several of the other strains examined were sufficiently well resolved to discern obvious segregation patterns. For example, the AKR mice evidently contained two proviruses in addition to *Mtv-8*, *Mtv-9*, and *Mtv-17*, yielding 5' junctions of around 17 and 11.8 kb, with 3' junctions at 11.5 and 4.6 kb. The only other strain shown to carry an 11.8-kb 5' end was A/St, suggesting that the 4.6-kb 3' end common to these two strains is likely to be linked to the 11.8-kb fragment. By a process of elimination, therefore, the 8.8-kb 5' end in A/St mice must match the 5.5-kb 3' end. Such a conclusion was supported by the fact that DBA/2 mice also contained this cosegregating pair of *Eco*RI fragments and that an analogous pair has been previously described in A strain mice by Morris and co-workers (28, 35). The latter analyses mapped this provirus to chromosome 4, and it seems likely that it corresponds to the locus designated *Mtv-13* in reference 48. By continuing this type of reasoning, it proved possible to establish a tentative linkage scheme for virtually every *Eco*RI fragment detected in these analyses, as summarized in Table 4. The only remaining ambiguities concerned an additional 11.0-kb 3'

TABLE 4. Designation of *Mtv* loci and their distribution among inbred mice[a]

Locus designation (chromosome)	Size of fragment (kb) in:		Presence in the following strain[b]:										References
	5′ junction	3′ junction	AKR	A/St	C57BL	CBA	DBA/2	DDSio	NZB	C3H/He	BALB/c	BR6	
Mtv-1 (7)	6.1	4.7	–	–	–	–	+	–	–	+	–	–	34, 47, 48, 51, 54, 55
Mtv-6 (16)[c]			–	+	–	+	+	–	–	+	+	–	2, 3, 6
Mtv-7 (1)	15.5	11.5	–	–	–	–	+	–	–	–	–	–	17, 28, 42, 48
Mtv-8 (6)	7.7	6.3	+	+	+	+	+	–	–	+	+	+	3, 6, 45
Mtv-9 (12)	7.3	9.3	+	–	+	+	–	–	+	–	+	–	2, 3, 6, 40
Mtv-11 (14)	15	5.5	–	–	–	–	+	–	–	+	–	–	17, 28, 41, 43
Mtv-13 (4)	8.8	5.5	–	+	–	–	+	–	–	–	–	–	28, 35, 47
Mtv-17 (4)	9.8	8.0	+	–	+	–	+	+	+	–	–	+	40; this paper
Mtv-21 (8)	8.0	8.1	–	–	–	–	–	–	–	–	–	+	This paper
Undefined	17	11.5	+	–	–	–	–	–	+	–	–	–	
Undefined	11.8	4.6	+	+	–	–	–	–	–	–	–	–	
Undefined	20	6.6	–	–	–	–	–	+	+	–	–	–	
Undefined	5.9	6.1	–	–	–	–	–	+	–	–	–	–	

[a] Summary of data presented in Fig. 3 and 4 and in the existing literature (see references). In particular, the various 5′ and 3′ specific junction fragments whose sizes were tabulated in Table 3 are assigned to previously defined *Mtv* loci and to specific mouse chromosomes where known.
[b] +, Present in the indicated strain; –, not present in the indicated strain.
[c] *mtv-6* is only one 14.5-kb fragment, neither 5′ nor 3′.

fragment in NZB mice, for which there was no obvious candidate 5′ end, and uncertainty as to whether DD mice contained more than diploid amounts of the 5.9- and 6.1-kb pair of fragments as suggested by the intensities of bands on the autoradiographs.

While the data indicated a number of cosegregating pairs which could not be unequivocally related to designated *Mtv* loci, others were readily recognizable from previous reports. For example, the 6.1-kb 5′ end and the 4.4-kb 3′ end common to DBA/2 and C3H/He mice were presumably derived from the *Mtv-1* locus, responsible for the mammary tumor incidence in foster-nursed animals of these strains (34, 41, 47, 48, 51, 54, 55). Similarly, the weakly hybridizing 14.5-kb *Eco*RI fragment detected with a 3′ probe was undoubtedly *Mtv-6*, a partial provirus consisting mostly of LTR sequences and originally designated unit I (3, 6). An *Eco*RI fragment of about this size has recently been cloned from C3H/He DNA, but it proved to be an authentic 5′ junction fragment (43). We have assumed that this corresponds to the 15-kb 5′ end detected in our C3H/He colony, and that the corresponding 3′ end would be the 5.5-kb fragment (see also reference 41). Such a conclusion is entirely consistent with the findings of Prakash et al., who showed that a flanking cellular sequence probe from their cloned 5′ end recognized an unoccupied site at around 11 kb, exactly as predicted from this proposed linkage (43).

Some confusion has arisen in the past from the segregation patterns of *Eco*RI fragments in crosses involving DBA/2 mice, since these mice contain two 5′ junctions at around 15 kb (28, 47). One of these is now presumed to be linked to the 5.5-kb 3′ end as described above, while the other has been shown to cosegregate with an 11.5-kb 3′ end on mouse chromosome 1 (17, 28, 42). Our data are entirely compatible with this notion (Tables 3 and 4). However, confusion has also arisen between these 5′ ends and the previously defined unit I of BALB/c mice, leading to names such as unit Ia and unit Ib in the literature. These have since been arbitrarily replaced by *Mtv* numbers, but have led to situations in which unit Ib has been designated either *Mtv-7a* or *Mtv-11* depending on the report, while unit Ia is most commonly called *Mtv-7* (17, 41–43, 48). We have taken account of these nomenclature problems in Table 4 and suggest that the 15.5- and 11.5-kb pair should be designated *Mtv-7* and the 15.0- and 5.5-kb pair should be designated *Mtv-11*.

In conclusion, we would argue that the only way to characterize these endogenous genomes unambiguously is to obtain molecular clones of the corresponding *Eco*RI fragments and to isolate specific probes for unique flanking sequences, as was achieved here for four such endogenous MMTV proviruses.

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