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Infection of Central Nervous System Cells by Ecotropic Murine Leukemia Virus in C58 and AKR Mice and in In Utero-Infected CE/J Mice Predisposes Mice to Paralytic Infection by Lactate Dehydrogenase-Elevating Virus

GRANT W. ANDERSON, GENE A. PALMER, RAYMOND R. R. ROWLAND, CHEN EVEN, AND PETER G. W. PLAGEMANN*

Department of Microbiology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455-0312

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Certain mouse strains, such as AKR and C58, which possess N-tropic, ecotropic murine leukemia virus (MuLV) proviruses and are homozygous at the *Fv-1^n* locus are specifically susceptible to paralytic infection (age-dependent poliomyelitis [ADPM]) by lactate dehydrogenase-elevating virus (LDV). Our results provide an explanation for this genetic linkage and directly prove that ecotropic MuLV infection of spinal cord cells is responsible for rendering anterior horn neurons susceptible to cytocidal LDV infection, which is the cause of the paralytic disease. Northern (RNA) blot hybridization of total tissue RNA and in situ hybridization of tissue sections demonstrated that only mice harboring central nervous system (CNS) cells that expressed ecotropic MuLV were susceptible to ADPM. Our evidence indicates that the ecotropic MuLV RNA is transcribed in CNS cells from ecotropic MuLV proviruses that have been acquired by infection with exogenous ecotropic MuLV, probably during embryogenesis, the time when germ line proviruses in AKR and C58 mice first become activated. In young mice, MuLV RNA-containing cells were found exclusively in white-matter tracts and therefore were glial cells. An increase in the ADPM susceptibility of the mice with advancing age correlated with the presence of an increased number of ecotropic MuLV RNA-containing cells in the spinal cords which, in turn, correlated with an increase in the number of unmethylated proviruses in the DNA extracted from spinal cords. Studies with AKXD recombinant inbred strains showed that possession of a single replication-competent ecotropic MuLV provirus (emv-11) by *Fv-1$^{n/n}$* mice was sufficient to result in ecotropic MuLV infection of CNS cells and ADPM susceptibility. In contrast, no ecotropic MuLV RNA-positive cells were present in the CNSs of mice carrying defective ecotropic MuLV proviruses (emv-3 or emv-13) or in which ecotropic MuLV replication was blocked by the *Fv-1$^{n/b}$* or *Fv-1$^{b/b}$* phenotype. Such mice were resistant to paralytic LDV infection. In utero infection of CE/J mice, which are devoid of any endogenous ecotropic MuLVs, with the infectious clone of emv-11 (AKR-623) resulted in the infection of CNS cells, and the mice became ADPM susceptible, whereas littermates that had not become infected with ecotropic MuLV remained ADPM resistant.

Age-dependent poliomyelitis (ADPM) is a unique paralytic disease of mice which is linked to an interaction between two unrelated, normally harmless viruses: an endogenous, N-tropic, ecotropic murine leukemia virus (MuLV) and lactate dehydrogenase-elevating virus (LDV). The disease is induced in old mice (12 months old and older) of highly leukemic mouse strains, such as C58, AKR, PL/J, and C3H/Fg, by infection with neuropathogenic strains of LDV (for reviews, see references 40 and 42). Paralysis develops between 2 and 3 weeks postinfection (p.i.) and results from a cytocidal infection of motor neurons by LDV (3, 7, 9). LDV belongs to a new, not yet named family of enveloped positive-strand RNA viruses (40, 42). Normally, LDV productively infects only macrophages in all strains of mice and infection does not result in overt disease (40, 42).

The susceptibility of certain mouse strains to paralytic LDV infection has been genetically linked to possession of multiple copies of endogenous N-tropic, ecotropic MuLV proviruses and homozygosity at the *Fv-1^n* locus (35, 36, 38, 39), which permits the efficient replication of N-tropic, ecotropic MuLVs (5, 25). Previous experiments using Northern (RNA) and in situ hybridization analyses indicated that the susceptibility of spinal cord motor neurons to LDV infection correlated with the expression of ecotropic MuLV in these cells (8, 9). Mice younger than 10 to 12 months of age were generally found not to develop paralytic disease after LDV infection but could be rendered ADPM susceptible by a single treatment with X-irradiation or cyclophosphamide when they were at least 5 months of age (9, 35, 36). This effect is attributable to an inhibition of the generation of anti-LDV immune responses that protect motor neurons, but not macrophages, from cytocidal LDV infection (17, 42). Although the results suggested that expression of ecotropic MuLV in anterior horn neurons specifically renders these cells susceptible to cytocidal infection by LDV (8, 9), a number of uncertainties and questions concerning this model persisted. First, there was a discrepancy between the finding of relatively low concentrations of ecotropic MuLV RNA in the spinal cords of C58 mice by Northern hybridization analyses, whereas in situ hybridization suggested that practically all motor neurons in the spinal cords

* Corresponding author. Mailing address: Department of Microbiology, Medical School, University of Minnesota, Box 196 UMHC, 420 Delaware St. SE, Minneapolis, MN 55455-0312. Phone: (612) 624-3187. Fax: (612) 626-0623. Electronic mail address: micro@lenti.med.umn.edu.

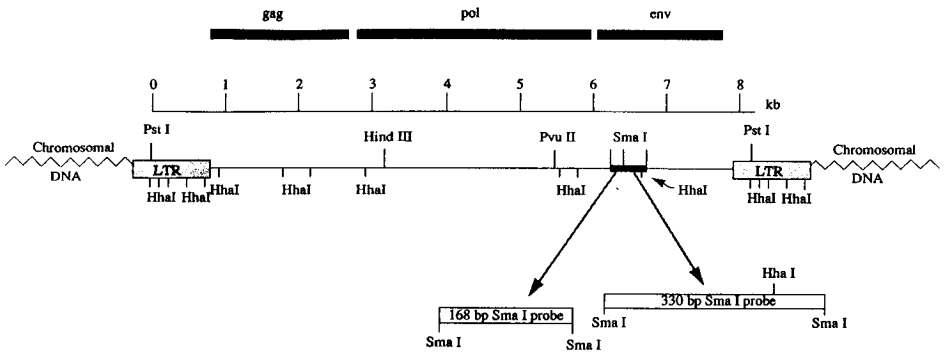


FIG. 1. Organization and partial restriction map of the infectious clone AKR-623 of ecotropic MuLV provirus emv-11 and locations of 168- and 330-bp *Sma*I restriction fragments specific for ecotropic MuLVs (4). The restriction sites are based on the sequence of the AKR-623 clone (18). LTR, long terminal repeat.

of 6-month-old C58 mice contained ecotropic MuLV RNA. Second, the genetic linkage between ADPM susceptibility and homozygosity at the *Fv-1*^{n} locus was unexplained, and the question of whether specific ecotropic MuLV proviruses were linked to ADPM susceptibility was unanswered. Third, there was no direct proof for a causal linkage between MuLV expression in motor neurons and the susceptibility of the mice to paralytic LDV infection. We now have answers to all of the questions described above, and our results provide direct proof for a causal linkage between ecotropic MuLV expression in central nervous system (CNS) cells, most likely glial cells, and susceptibility of the motor neurons to cytocidal infection by neurovirulent LDV. A prime question that remains is how ecotropic MuLV expression in glial cells mediates LDV permissiveness of anterior horn neurons.

MATERIALS AND METHODS

Mice and LDV. C58/M, AKXD-16, CE/J, and C58/M F_1 hybrid mice were bred in the animal facility of the Department of Microbiology. Outbred Swiss mice were purchased from Biolabs, Inc. (St. Paul, Minn.), and were used for LDV titration by an endpoint dilution assay (41). All other mice were obtained from Jackson Laboratories (Bar Harbor, Maine).

For ADPM experiments, mice were injected intraperitoneally (i.p.) with about 10^6 50% infectious doses (ID_{50}) of LDV-v. When indicated, the mice were also injected i.p. with 200 mg of cyclophosphamide per kg of body weight 1 day before and at weekly intervals after LDV-v infection to continuously suppress the formation of motor neuron-protective anti-LDV antibodies (42). The mice were subsequently monitored for paralytic symptoms until at least 5 weeks p.i. LDV-v is a neuropathogenic strain of LDV which was isolated from the spinal cord of a paralyzed C58/M mouse that had been injected with Ib-LDV obtained from W. Murphy (35, 36). Stock LDV-v was prepared by injecting groups of Swiss mice with LDV-v and harvesting their plasma 1 day p.i. The plasma contained 10^9 to 10^{10} ID_{50} per ml.

MuLV and infection of midgestation embryos. *Mus dunni* cells infected with ecotropic, polytropic, and xenotropic MuLV were kindly provided by Jonathan Stoye and John Coffin (51) and propagated in RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum. The *M. dunni* cells had been transfected with molecularly cloned, infectious proviral DNA of the individual prototype MuLVs, namely, clone AKR-623 (33) in the case of ecotropic MuLV. Ecotropic AKR-623 MuLV was harvested from the culture fluid of persistently infected *M. dunni* cells and concentrated by centrifugation through a 0.5 M sucrose layer in an SW27 rotor at 21,000 rpm for 4 h at 4°C in a Beckman ultracentrifuge. The pelleted MuLV was resuspended in ice-cold phosphate-buffered saline (PBS) and stored at −70°C until use. MuLV was titrated by a focal immunoassay (48) using rat anti-gp70 monoclonal antibody 83A25 (12) kindly provided by John Portis. Embryos at 8 to 10 days of gestation were injected with 0.2 μl of concentrated ecotropic AKR-623 MuLV (about 400 focus-forming units) as described by Jaenisch (22). The pregnant mothers were anesthesized by injection of sodium pentobarbitol before the operation.

Northern hybridization analysis. Total RNA was extracted from tissues by the acid guanidium thiocyanate method, glyoxylated, and analyzed by Northern hybridization as described previously (29, 30). Blots were hybridized with LDV-specific, ecotropic MuLV-specific, or actin-specific probes (see below).

Genomic DNA extraction, Southern analysis, and digestion with restriction enzymes. Mice were perfused with 12 ml of ice-cold PBS, and their tissues were removed and quick-frozen on dry ice. Genomic DNA was then extracted from these tissues or pieces of tail and was analyzed by Southern hybridization using ecotropic MuLV-specific probes as described by Sambrook et al. (45). When indicated, samples of 20 to 50 μg of genomic DNA were digested overnight with *Pvu*II, *Pst*I, or *Hha*I restriction endonuclease (New England Biolabs, Beverly, Mass.) before Southern analysis.

In situ hybridization. Mice were perfused under anesthesia. Tissues were removed, fixed in neutral formalin for 1 to 2 h (spinal cords) or 4 h (brain), and then placed in ethanol at 4°C for at least 4 h. The tissues were embedded in Amerffin (American Scientific Products, Minneapolis, Minn.). Sections of 8 μm each were cut and floated on a drop of 3% (vol/vol) Elmer's white glue on a slide pretreated with Denhardt's medium and then acetylated. The slides were dried, deparaffinized, pretreated, and hybridized with DNA probes as described by Blum et al. (2).

All hybridizations included negative controls, i.e., sections of spinal cords from CE/J mice which are devoid of endogenous ecotropic MuLVs, RNase-treated sections, and sections hybridized with a nonspecific probe. The RNase controls were dehydrated before acetylation, incubated with 0.25 μg of RNase A and 840 U of RNase T_1 per ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min at 37°C, washed in distilled H_2O, acetylated, and redehydrated.

After hybridization, the slides were extensively washed (2), autoradiographed, stained with Mayer's hematoxylin and 0.5% (vol/vol) eosin Y in ethanol (Sigma, St. Louis, Mo.), and examined under a Leitz microscope. Stated magnifications pertain to those of the microscope. Photographic enlargements varied between three- and fivefold but were the same for all frames in a single figure.

Hybridization probes and radiolabeling. The LDV-specific probe was a 437-bp cDNA (4-55) which represents the 3′ end of the genome (29). The ecotropic MuLV-specific probes were 168- and 330-bp *Sma*I restriction fragments which represent the *env* gene (Fig. 1). They were prepared by *Sma*I digestion of the infectious ecotropic MuLV proviral clone AKR-623, which was provided by Doug Lowy (33), separation of the fragments by gel electrophoresis, and subcloning into pBSIIKS(+) (Stratagene, LaJolla, Calif.). The probes are specific for ecotropic MuLVs (4). This result has been confirmed; the probes hybridized to RNA extracted from ecotropic MuLV-infected *M. dunni* cells but not to RNA extracted from *M. dunni* cells infected with polytropic or xenotropic MuLVs (8) (data not shown).

The cDNAs were radiolabeled by random priming using the Random Priming Labeling kit from Boehringer Mannheim (Indianapolis, Ind.) according to the procedure recommended by the manufacturer with [α-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.) for Northern and Southern hybridization analyses or ^{35}S-dATP and ^{35}S-dCTP (Amersham) for in situ hybridizations. All probes were phenol-chloroform-isoamyl alcohol extracted before use in hybridizations.

In one experiment, Northern blots were hybridized with an ecotropic MuLV *env*-specific oligonucleotide as described previously (8, 9). This oligonucleotide and a mouse actin-specific oligonucleotide (7) were labeled at the 3′ end using terminal deoxynucleotidyl transferase and [α-^{32}P]dATP as described by Collins and Hunsaker (6).

RESULTS

Expression of ecotropic MuLV in the CNSs of C58 mice as a function of age. Previous Northern blot hybridization analyses indicated the presence of ecotropic MuLV RNA in the spinal cords of 6- to 7-month-old C58 mice, but little, if any, ecotropic MuLV RNA was detected in the spinal cords of 2-month-old mice (8, 9). A more detailed time course analysis of ecotropic MuLV RNA in the spinal cords of C58 mice is

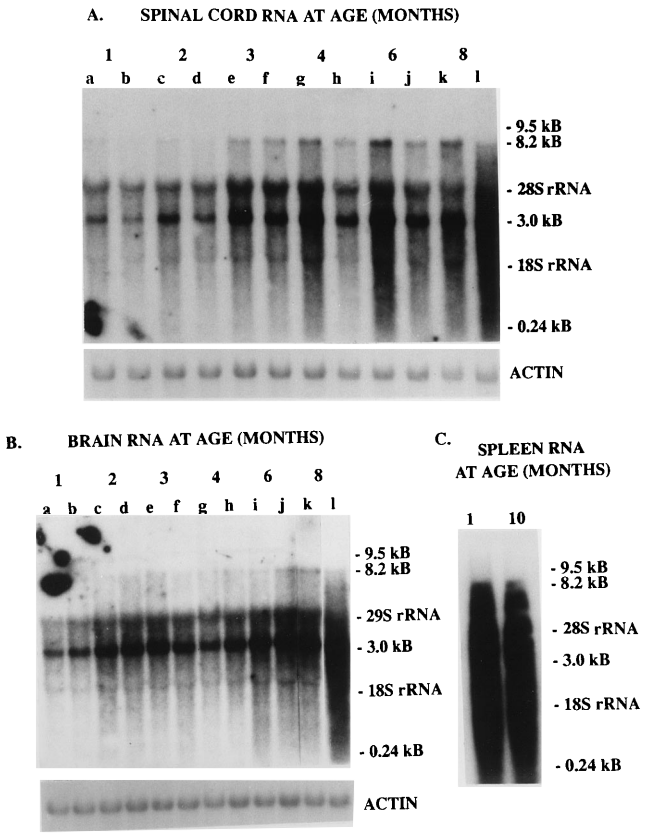


FIG. 2. Age-dependent expression of ecotropic MuLV in the spinal cords (A), brains (B), and spleens (C) of C58/M mice. Total RNA was extracted from tissues of two individual mice of each age and was analyzed by Northern hybridizations using the ecotropic MuLV-specific 168-bp *Sma*I fragment as a probe. The blots were then stripped and hybridized with a mouse actin-specific oligonucleotide (8).

shown in Fig. 2A, which also examines the variability of ecotropic MuLV RNA levels between individual mice of the same age. Using a 168-bp cDNA specific for the *env* gene of ecotropic MuLV rather than an oligonucleotide (8, 9) as a probe, we detected both full-length 8.2- and 3-kb *env* mRNAs in the spinal cords of C58 mice as young as 1 month of age (Fig. 2A). The levels of ecotropic MuLV RNA in the spinal cords increased progressively with the ages of the mice. The RNA levels differed relatively little among companion mice of the same age, except for the two 8-month-old mice. The spinal cord, as well as the brain (see below), of one of the latter contained unusually high levels of ecotropic MuLV. Some nonspecific binding of the probe to 28S and 18S rRNAs was observed (Fig. 2A) because of the relatively low stringency of the washes necessary for visualization of the signal.

In contrast to a previous study, which failed to detect ecotropic MuLV RNA in the brains of C58 mice (8, 9), we observed considerable concentrations in the brain which increased with the ages of the mice (Fig. 2B). The concentrations of ecotropic MuLV RNA in the spleen were much higher than those in the CNS and were about the same for young and old mice (Fig. 2C).

Results similar to those shown in Fig. 2 were obtained with ADPM-susceptible AKXD-16 mice (data not shown). The increase in ecotropic MuLV RNA in the spinal cords of both C58 and AKXD-16 mice with advancing age correlated with their increased ADPM susceptibility with increasing age (35, 36, 42). In contrast to the ADPM-susceptible mice, no ecotropic MuLV RNA was detected in the CNSs of old mice of non-ADPM-susceptible mouse strains that possessed defective ecotropic MuLV proviruses (DBA/2J, C3H/HeJ, and C57BL/10J mice) or did not possess any ecotropic MuLV provirus (C57L/J and CE/J mice; see below). The causal relationship between expression of the ecotropic MuLV in the CNS and susceptibility of the mice to paralytic LDV infection was proven by in utero infection of non-ADPM-susceptible CE/J mice with ecotropic MuLV (see below).

The age-dependent increase in ecotropic MuLV RNA in the CNSs of C58 mice was confirmed by in situ hybridization analyses. The results also yielded information on the identity of the ecotropic MuLV-RNA-containing cells and their distribution in the CNS, especially in the spinal cord. Figure 3 shows representative spinal cord sections illustrating the increase in the number of ecotropic MuLV RNA-containing cells with increasing ages of the mice. Only very few positive cells were detectable in the spinal cords of very young C58 mice, and most were found in foci containing at least several positive cells. Figure 3B shows such a focus of positive cells in the white matter of the spinal cord of a 1-month-old C58 mouse. These were the only positive cells found in five sections of the spinal cord of this mouse. In all other sections, no positive cells were detected in either the white or the grey matter (Fig. 3A). In contrast, practically all sections of the spinal cord of a 12-month-old C58 mouse contained a large number of ecotropic MuLV RNA-containing cells (Fig. 3D). An intermediate number of positive cells were present in the spinal cord of a 4-month-old C58 mouse (Fig. 3C). However, the grain density over positive cells indicated that the amounts of ecotropic MuLV RNA per cell were comparable in young and old mice (Fig. 3).

All ecotropic MuLV RNA-containing cells in the spinal cords of 1- to 2-month-old mice were located in the white matter. Since the white matter is composed exclusively of glial cells (27), the ecotropic MuLV RNA-containing cells were glial cells. In older C58 mice, a considerable number of positive cells were also present in the grey matter (Fig. 3D); however, contrary to earlier results (8, 9), these cells were primarily not anterior horn neurons but smaller cells, most likely also glial cells. Figure 3E shows a representative section of the spinal cord of an 8-month-old C58 mouse with several small MuLV RNA-containing cells in the grey matter and three much larger, easily recognizable, motor neurons which did not contain ecotropic MuLV RNA. Only occasionally did we find an anterior horn neuron in older mice that contained ecotropic MuLV RNA. An example is provided in Fig. 3F, which shows one positive motor neuron in the spinal cord of an 8-month-old C58 mouse and five other motor neurons that did not contain ecotropic MuLV RNA.

In agreement with the Northern hybridization analyses (see above), no positive cells were observed in sections of spinal cords from CE/J mice, which are free of any endogenous ecotropic MuLV provirus (included as a negative control in each in situ hybridization analysis; see Fig. 7F), or spinal cord sections of DBA/2J and C57BL/10J mice, which possess only replication-defective ecotropic MuLV proviruses (emv-1 and emv-2, respectively [5, 10; see below]). Furthermore, positive signals observed in sections of spinal cords from ADPM-susceptible C58 (AKR or AKXD-16) mice were consistently eliminated in sequential sections by treatment with RNases A and T_1 (data not shown).

The conclusion that ecotropic MuLV is expressed in the spinal cord in cells other than the anterior horn neurons that become infected with LDV is further supported by an analysis

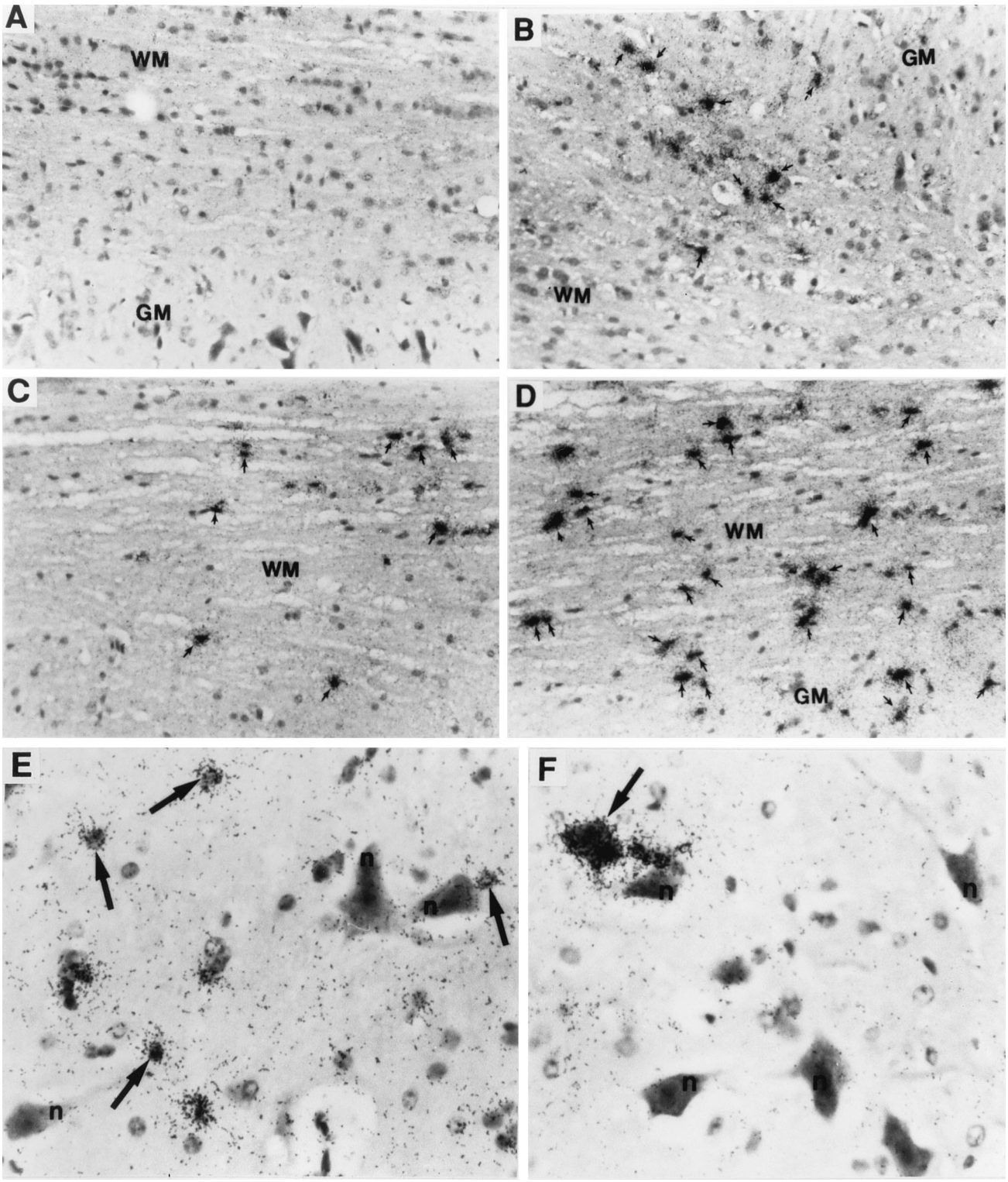
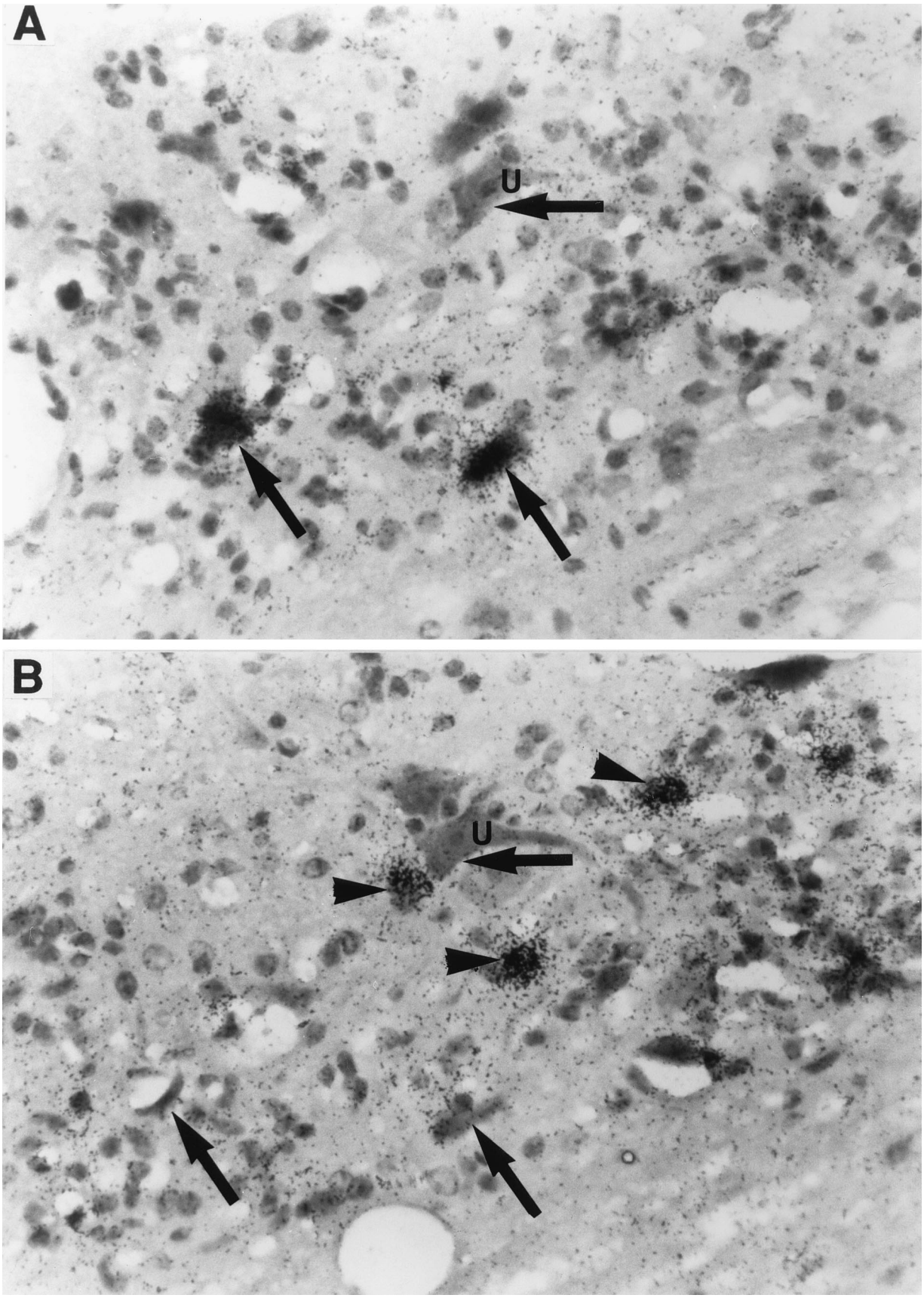


FIG. 3. Age-dependent increase in the number of ecotropic MuLV RNA-containing cells in the spinal cords of C58/M mice. Sections were prepared from spinal cords of 1 (A and B)-, 4 (C)-, 12 (D)-, and 8 (E and F)-month-old C58/M mice and were hybridized with the ecotropic MuLV-specific 168-bp *Sma*I fragment as described in Materials and Methods. Sections from at least three C58/M mice and one CE/J mouse were analyzed on the same slide. One slide from each tissue was treated with RNases A and T_1 prior to hybridization. No labeled cells were detected in RNase-treated tissue sections or tissue sections from CE/J mice (data not shown). Arrows denote ecotropic MuLV RNA-positive cells. WM, white matter; GM, grey matter; n, motor neurons. Microscopic magnifications: ×160 for panels A to D; ×400 for panels E and F.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of sequential sections of the anterior horn of a paralyzed 9-month-old C58 mouse in which one section was hybridized with an LDV-specific probe (Fig. 4A), whereas the next section was hybridized with the ecotropic MuLV-specific probe (Fig. 4B). The section in Fig. 4A shows two LDV-infected and several uninfected motor neurons (arrows). Neither the LDV-infected nor the LDV-uninfected motor neurons contained ecotropic MuLV RNA (arrows in Fig. 4B), whereas at least three smaller cells did contain ecotropic MuLV RNA (arrowheads in Fig. 4B).

In situ hybridization analyses showed that ecotropic MuLV RNA-containing cells in the brains of C58 mice were also primarily located in white-matter tracts. For example, in the brains of 8-month-old C58 mice, numerous ecotropic MuLV-positive cells were present in the commissura anterior, the pars anterior, the corpus callosum, and the fimbria hippocampi (data not shown). Only few ecotropic MuLV RNA-containing cells were detected in the grey matter, even when it was adjacent to a white-matter tract containing numerous positive cells. Single positive cells were found in the corpus striatum, the gyrus dentatus in the hippocampus, and the folia of the cerebellum; however, generally no positive cells were detectable in the deep grey matter of the neocortex or in the meninges.

Potential mechanism that could account for the increase with advancing age in the number of ecotropic MuLV RNA-containing cells in the CNS. Two mechanisms could explain the progressive increase in the number of ecotropic MuLV RNA-containing cells in the CNSs of C58 or AKR mice with advancing age: either more cells become continuously infected with exogenous ecotropic MuLV, or ecotropic MuLV proviruses already present become transcriptionally activated in a progressively increasing number of individual cells. Transcriptional activation could involve germ line proviruses or proviruses that have been newly acquired by infection at an earlier time, most likely during embryogenesis (see below), and rendered transcriptionally silent after integration. In either case, transcriptional activation may be due to provirus demethylation, since methylation of proviral DNA has been implicated in transcriptional control of retroviruses (5, 10, 16, 20, 52). For example, germ line MuLV proviruses that are not expressed are highly methylated, whereas only hypomethylated MuLV DNA is infectious (10, 11, 20, 52).

Therefore, we assessed the extent of methylation of the provirus(es) in the spinal cord of AKXD-16 mice as a function of age. AKXD-16 mice were selected in this experiment since they possess only a single germ line ecotropic provirus (emv-11 [23, 24]), contain levels of ecotropic MuLV RNA in their spinal cords similar to those of AKR and C58 mice, which increase with advancing age, and are ADPM susceptible (see Table 2). High-molecular-weight DNAs isolated from the spinal cords of AKXD-16 mice of various ages were first digested with the restriction enzyme *Pst*I, which cleaves the provirus at a single site in each long terminal repeat and thus releases the full-length 8.2-kb provirus from flanking host DNA (Fig. 1). The AKR-623 clone shown in Fig. 1 has been derived from the emv-11 provirus (33) present in AKXD-16 mice, and, therefore, both possess the same restriction sites. The DNA was then restricted with *Hha*I, which recognizes the sequence 5′-GCGC-3′ but does not cleave it when the internal C is methylated. The restricted DNA was then analyzed by Southern hybridization using both the 168- and 330-kb *Sma*I restriction fragments specific for the *env* gene of ecotropic MuLV as probes (Fig. 1).

The results in Fig. 5A show that regardless of the ages of the AKXD-16 mice, the majority of the proviruses in the spinal cords were released intact as 8.2-kb fragments (lanes 1 to 6), whereas clone AKR-623 DNA was completely restricted under the same experimental conditions (data not shown). Thus, most proviruses in the spinal cord were methylated at all or most of the 17 *Hha*I sites present within the ecotropic MuLV provirus (Fig. 1). However, with advancing ages of the mice, increasing amounts of a 900-bp fragment appeared, indicating the increasing presence of proviruses in which the *Hha*I site in the *env* gene, the next site upstream, and probably others were unmethylated. A second expected downstream *Hha*I product of 1,700 bp was not observed (Fig. 5A), probably because the levels were too low for detection, since only a small number of bases (70 bp) of the expected fragment overlapped with the 330-bp *Sma*I probe (Fig. 1). In contrast to the spinal cord proviruses, considerable as well as similar numbers of proviruses were unmethylated in the spleens of 1- and 10-month-old AKXD-16 mice, as evidenced by the presence of both the 900- and 1,700-bp *Hha*I restriction fragments (Fig. 5A, lanes 7 and 8). The presence of an additional 2.6-kb fragment indicates that some proviruses were unmethylated in the *Hha*I sites flanking the *env* gene but methylated in the *Hha*I site in the middle of the *env* gene (Fig. 1).

For comparison, the ecotropic MuLV proviruses of DBA/2J mice isolated from both spinal cords and spleens were analyzed in the same manner (Fig. 5A, lanes 9 to 12). The emv-3 provirus of DBA/2J mice differs from emv-11, which is carried by AKXD-16 mice, by only a few point mutations, one of which lies within the p15 *gag* gene and results in lack of myristylation of the Pr65 *gag* gene product, thereby rendering the provirus replication defective (10, 11). In the spleens of young DBA/2J mice, the emv-3 provirus was highly methylated; however, unmethylated proviruses had appeared in 10-month-old mice, which agrees with the results described by Copeland et al. (10). However, no unmethylated ecotropic MuLV appeared in the CNSs of DBA/2J mice (Fig. 5A, lane 10). Comparable results were obtained with 1- and 10-month-old AKXD-16 and DBA/2J mice in three additional independent experiments (data not shown).

The presence of unmethylated proviruses correlated with the synthesis of ecotropic MuLV RNA in both AKXD-16 and DBA/2J mice (cf. Fig. 5A and B). In AKXD-16 mice, ecotropic MuLV RNA was found in the spinal cords of only old mice but in the spleens of both young and old mice (Fig. 5B, lanes 1 to 4). In DBA/2J mice, unmethylated proviruses and ecotropic MuLV RNA were found only in the spleens of old mice. The synthesis of ecotropic MuLV RNA in old DBA/2J mice has been reported previously (11). It is explained by the eventual generation in a rare cell of infectious MuLV, which is derived from the defective emv-3 by recombination with other endogenous MuLVs or back mutation. As shown in Fig. 5A and B, the recombinant efficiently replicated in lymphoidal tissues but did not spread to the CNS.

The lack of expression of the defective emv-3 provirus in the CNSs of even old DBA/2J mice suggests that the germ line

FIG. 4. Lack of coincidence between LDV-v-infected and ecotropic MuLV RNA-positive cells in the grey matter of the spinal cord of a paralyzed C58/M mouse. Sequential sections of a spinal cord of a paralyzed 9-month-old C58/M mouse 16 days p.i. with LDV-v were hybridized with a ^{35}S-labeled LDV-specific cDNA (A) or the ^{35}S-labeled ecotropic MuLV-specific 168-bp *Sma*I fragment as a probe (B). Arrows denote LDV-infected and uninfected motor neurons, and arrowheads denote ecotropic MuLV RNA-positive cells. U, motor neuron not infected with LDV. Microscopic magnification, ×400.

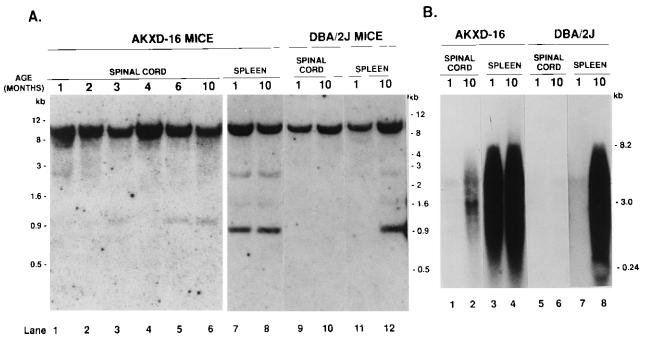

FIG. 5. Correlation between age-dependent demethylation of ecotropic MuLV proviruses (A) and synthesis of ecotropic MuLV RNA (B) in the spinal cords and spleens of AKXD-16 and DBA/2J mice. (A) Samples of 50 μg of genomic DNAs extracted from the spinal cords and spleens of individual mice of the indicated ages were digested with the restriction nucleases *Pst*I and *Hha*I, and the restriction segments were analyzed by Southern hybridization. The blots were hybridized simultaneously with the 168- and 330-bp *Sma*I DNA fragments of the AKR-623 clone. (B) Total RNAs extracted from the spinal cords of the mice were analyzed by Northern hybridization using the AKR-623 168-bp *Sma*I fragment as a probe.

TABLE 1. Correlation between the presence of ecotropic MuLV RNA in the CNSs of various mouse strains and C58/M F_1 hybrid mice and their susceptibilities to paralytic LDV-v infection

Mouse type	*Fv-1*	Expression of ecotropic MuLV in CNS[a]	Incidence of paralysis[b]
C58/M	*n/n*	+	130/140[c]
CE/J	*n/n*	−	0/20
FVB	*b/b*	−	0/6
C57BL/10J	*b/b*	−	0/6
F_1 hybrids			
C58/M × CE/J	*n/n*	+	4/4
C58/M × FVB	*n/b*	−	0/6
C58/M × C57BL/10J	*n/b*	−	0/6

[a] RNA was extracted from the spinal cords of approximately 6-month-old mice and was analyzed by Northern hybridization for the presence of ecotropic MuLV RNA (Fig. 2), and/or spinal cord sections were examined for ecotropic MuLV RNA-containing cells by in situ hybridization (Fig. 3).

[b] Other mice were infected with about 10^6 ID_{50} of LDV-v and were injected with 200 mg of cyclophosphamide per kg 1 day before infection and at weekly intervals p.i. The mice were monitored for the development of paralysis for at least 40 days p.i.

[c] Values are from reference 42.

provirus was not expressed in the CNS and that expression of the closely related emv-11 provirus in the CNSs of AKXD-16 mice, therefore, probably involves infection by an exogenous ecotropic MuLV derived from germ line emv-11. It follows that the ecotropic MuLV RNA in the CNSs of these mice is transcribed from proviruses newly acquired as a result of active infection rather than the germ line provirus present in all cells. The same must apply to the ecotropic MuLV RNAs produced in AKR and C58 mice and other mice that carry endogenous replication-competent ecotropic MuLVs. Expression of ecotropic MuLV in the CNS and ADPM susceptibility seem strictly linked to the possession of replication-competent MuLVs that become activated during embryogenesis (44) and then spread in $Fv\text{-}1^{n/n}$ mice to other tissues, including the CNS (see below).

This scenario is further supported by the following breeding experiment. ADPM-susceptible C58/M ($Fv\text{-}1^{n/n}$) mice were bred with CE/J, FVB, and C57BL/10J mice, which are resistant to paralytic LDV infection (Table 1). CE/J mice are $Fv\text{-}1^{n/n}$ but do not possess any ecotropic MuLV proviruses (5) (Fig. 6). C57BL/10J mice are $Fv\text{-}1^{b/b}$ and carry only the replication-defective provirus emv-2 (5). FVB mice are also $Fv\text{-}1^{b/b}$ and carry an undetermined number of ecotropic MuLV proviruses. The $Fv\text{-}1^{b}$ allele strongly restricts the replication of N-tropic, ecotropic MuLVs by blocking viral DNA integration into host chromosomes (5, 25). No ecotropic MuLV RNA was expressed in the CNSs of mice of the last three strains, and they were not ADPM susceptible (Table 1). *Fv-1* restriction is dominant; thus, in $Fv\text{-}1^{n/b}$ heterozygotes, the replication of both N-tropic and B-tropic ecotropic MuLVs is inhibited. The (C58/M × CE/J)F_1 hybrids carried the C58/M endogenous ecotropic MuLV proviruses, were $Fv\text{-}1^{n/n}$, and expressed ecotropic MuLV in the CNS, and all developed paralytic disease after treatment with cyclophosphamide and injection with LDV-v. In contrast, the (C58/M × FVB)F_1 or (C58/M × C57BL/10J)F_1 hybrids were $Fv\text{-}1^{n/b}$, no ecotropic MuLV expression was detected in their CNSs, and the mice were not ADPM susceptible, even though they also possessed the C58/M endogenous ecotropic MuLV proviruses (Table 1). Thus, expression of ecotropic MuLV RNA in the CNS requires efficient replication of the virus, and the MuLV RNA in the CNS is probably transcribed from proviruses acquired by exogenous infection.

A single replication-competent endogenous ecotropic MuLV provirus endows ADPM susceptibility. Earlier studies concluded that multiple endogenous ecotropic proviruses are required for ADPM susceptibility, since DBA/2J, C57BL/10J, and C3H/HeJ mice that carry only one ecotropic MuLV provirus (emv-3, emv-2, and emv-1, respectively [5, 10, 24]) were resistant, even though they are homozygous at the $Fv\text{-}1^{n}$ locus, whereas AKR, C58 PL/J, and C3H/Fg mice carrying many proviruses (5, 24, 53) were susceptible (35, 36, 38, 39). We investigated whether the susceptibility of mice to paralytic LDV infection was limited to possession of specific endogenous ecotropic MuLVs by comparing the proviral contents and ADPM susceptibilities of AKXD recombinant inbred (RI) strains. These RI strains have been derived by crossing AKR/J and DBA/2J mice. The strains possess different combinations of parental emvs but are all $Fv\text{-}1^{n/n}$ (23, 24). A Southern analysis of *Pvu*II-digested DNAs (Fig. 1) of six AKXD RI strains and their progenitor strains confirmed the proviral profiles of emv-3, -11, -13, and -14 of these strains reported previously (10, 11, 19, 23, 24, 50) but showed that AKR/J,

TABLE 2. Proviral contents and ADPM susceptibilities of AKR/J, DBA/2J, and AKXD RI strains of mice

Mouse strain	Provirus(es)[a]	No. of replication-competent proviruses	No. paralyzed/total no. of mice[b]
AKR/J	emv-11, -13, -14 + x_2	≥2	7/8
AKXD-16	emv-11	1	18/19
AKXD-24	emv-11, -13, -14 + x_1	≥2	2/3
AKXD-26	emv-3, -14	1	2/4
AKXD-11	emv-3, -13 + x_3	0	0/2
AKXD-28	emv-13	0	0/5
AKXD-20	None	0	0/15
DBA2/J	emv-3	0	0/3

[a] Proviral profiles of the strains were derived from Southern analyses of *Pvu*II restriction nuclease-digested genomic DNA by using the AKR-623 168-bp DNA fragment as a probe (data not shown). Proviruses emv-3, -11, -13, and -14 were identified on the basis of previously published data (5, 23, 24, 50). Previously, uncharacterized proviruses were designated emv-x_1, -x_2, and -x_3.

[b] All mice were 4 to 6 months of age and were injected with approximately $10^{6.0}$ ID_{50} of neurovirulent LDV-v and with 200 mg of cyclophosphamide per kg 1 day before infection and at weekly intervals p.i. with LDV. The mice were observed for development of paralysis for 40 days p.i.

AKXD-11, and AKXD-24 mice possessed additional proviruses (emv-x_2, emv-x_3, and emv-x_1, respectively) which have not been previously characterized (data not shown; Table 2).

Half of the six RI strains examined were ADPM susceptible, just like the AKR/J parent, whereas the other half were not susceptible (Table 2). One susceptible RI strain was AKXD-16, which possesses only the replication-competent emv-11 provirus. The other two susceptible RI strains possessed either emv-11 or another replication-competent provirus, emv-14 (5, 23, 54). In contrast, the strains that possessed only the replication-defective proviruses emv-3 or emv-13 (5, 10, 11) were not susceptible. Thus, ADPM susceptibility was supplied by the presence of a single replication-competent provirus but not by the presence of replication-defective proviruses. A further implication of the data is that the previously uncharacterized emv-x_3 provirus is also replication defective. It is also of interest that the presence of replication-defective proviruses had no effect on the ADPM susceptibilities of the AKR/J, AKXD-24, and AKXD-26 mice which carried replication-competent proviruses.

Generation of ADPM-susceptible mice by in utero infection of fetuses with ecotropic MuLV. Direct proof for the linkage between ecotropic MuLV infection of CNS cells and ADPM susceptibility is provided by an experiment in which endogenous ecotropic MuLV provirus-lacking CE/J mice (*Fv-1*$^{n/n}$) were infected with ecotropic MuLV in utero. The rationale for this experiment is provided by the finding that in AKR mice, ecotropic MuLV replication commences sometime during fetal development and that at birth MuLV is present in most tissues of these mice (44). This invasion of various tissues by ecotropic MuLV has been duplicated by infection of midgestation embryos of BALB/c mice with Moloney (Mo) MuLV (22). On the other hand, after infection of BALB/c mice at birth, MoMuLV replication was largely restricted to lymphoidal tissues, indicating that ecotropic MuLV infection of various tissue cells, including those in the CNS, occurs most efficiently when the cells are actively multiplying during embryogenesis (22). For example, between 7 and 8 days of gestation, the total number of cells per embryo increases approximately 20-fold (49). Characteristic of this period of development are complex patterns of cell migration and the beginning of organogenesis, including the formation of neuroectoderm. Why infection of the CNS by MuLV is largely limited to the period of fetal development is not entirely clear. In part, it may be related to the finding that MuLV provirus integration and thus productive infection occur only in dividing cells (32, 55) and that at birth most of the cells in the CNS are postmitotic. It is also possible that barriers that restrict the spread of MuLV to the CNS are formed during embryonic development.

For our experiment, CE/J mice were selected because they are devoid of endogenous ecotropic MuLVs, and genetic studies showed that (C58/M × CE/J)F_1 hybrid mice express ecotropic MuLV in the CNS and are ADPM susceptible (Table 1). Thus, there is nothing in the genetic background of CE/J mice that prevents ecotropic MuLV expression in the CNS or cytocidal LDV infection of anterior horn neurons. Midgestation embryos of CE/J mice were infected with the infectious MuLV clone AKR-623. As discussed already, AKR-623 is a full-length clone of the replication-competent provirus emv-11 (18, 33) which segregates with ADPM susceptibility in AKXD-16 mice (Table 2).

To determine whether the embryos had become infected with ecotropic MuLV, genomic DNAs were isolated from the tails of surviving pups at 2 to 3 weeks after birth, digested with *Pst*I endonuclease to release full-length proviruses, Southern blotted, and probed with the ecotropic MuLV-specific probe.

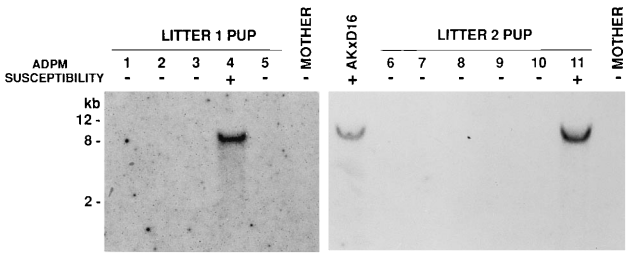

FIG. 6. CE/J mice successfully infected in utero with ecotropic MuLV are susceptible to paralytic infection by LDV-v. Genomic DNA was extracted from the tails of weanling CE/J mice of two litters (1 and 2) which had been infected in utero with ecotropic MuLV (clone AKR-623) as well as from the tails of their mothers. A sample of DNA (10 μg) was digested with restriction endonuclease *Pst*I, and the fragments were analyzed by Southern hybridization using the AKR-623 168-bp *Sma*I fragment as a probe. DNA from an AKXD-16 mouse was included as a positive control (lane 7). At approximately 7 months of age, all mice were injected with $10^{6.0}$ ID_{50} of LDV-v and with 200 mg of cyclophosphamide per kg 1 day before infection and at weekly intervals p.i. The mice were monitored for paralysis (+ or −) for 40 days, and the results are indicated above each lane. All mice were coded, and the code was not broken until paralysis was observed.

Since infectious ecotropic MuLV is present in high titers in the tails of AKR mice (44), it seemed likely that in ecotropic MuLV-infected CE/J mice as well, cells in the tail would be actively infected and would possess newly acquired proviruses. This was the case. Two pups, one from each of two litters, were found to have become infected with AKR-623, and their tail DNAs contained ecotropic MuLV proviruses (Fig. 6, pups 4 and 11). The rest of the littermates and the respective mothers were uninfected.

All mice were aged for about 7 months and were then analyzed for ADPM susceptibility, and their CNSs were analyzed for ecotropic MuLV RNA and for LDV RNA by in situ hybridization or Northern hybridization. Only the two ecotropic MuLV-infected mice (mice 4 and 11) developed paralytic disease after cyclophosphamide treatment and infection with LDV-v (14 and 11 days p.i., respectively; Fig. 6). For in situ hybridization analysis, all mice from litter 2 were sacrificed 2 days after mouse 11 developed paralysis. Extensive motor neuron infection by LDV was apparent in the spinal cord of paralyzed mouse 11 (Fig. 7A and B) but not in the spinal cords of any of the littermates that had not become infected with MuLV (e.g., Fig. 7C). Mouse 11 was paralyzed in all four limbs, and large numbers of LDV RNA-positive cells were found throughout its spinal cord.

Many ecotropic MuLV RNA-positive cells were present throughout the white and grey matter of the spinal cord (Fig. 7D and E, respectively) and of the brain (data not shown) of the paralyzed mouse 11. In fact, the density of ecotropic MuLV-positive cells in the CNS of mouse 11 was considerably higher than that observed in the CNSs of AKR or C58 mice of comparable age that become infected because of activation of a germ line provirus during embryogenesis, although the overall distributions of the positive cells were very similar. Furthermore, as observed for AKR and C58 mice, ecotropic MuLV-positive cells in the spinal cord of mouse 11 were primarily located in the white matter and those in the grey matter were not anterior horn neurons (Fig. 7E), although the latter became infected with LDV (Fig. 7A and B). As expected, no ecotropic MuLV RNA-positive cells were present in the CNSs of any of the littermates that had not become infected with AKR-623 (e.g., Fig. 7F). Similarly, Northern blot hybridization demonstrated the presence of ecotropic MuLV RNA in the spinal cord of mouse 4 of litter 1 but not in those of any of

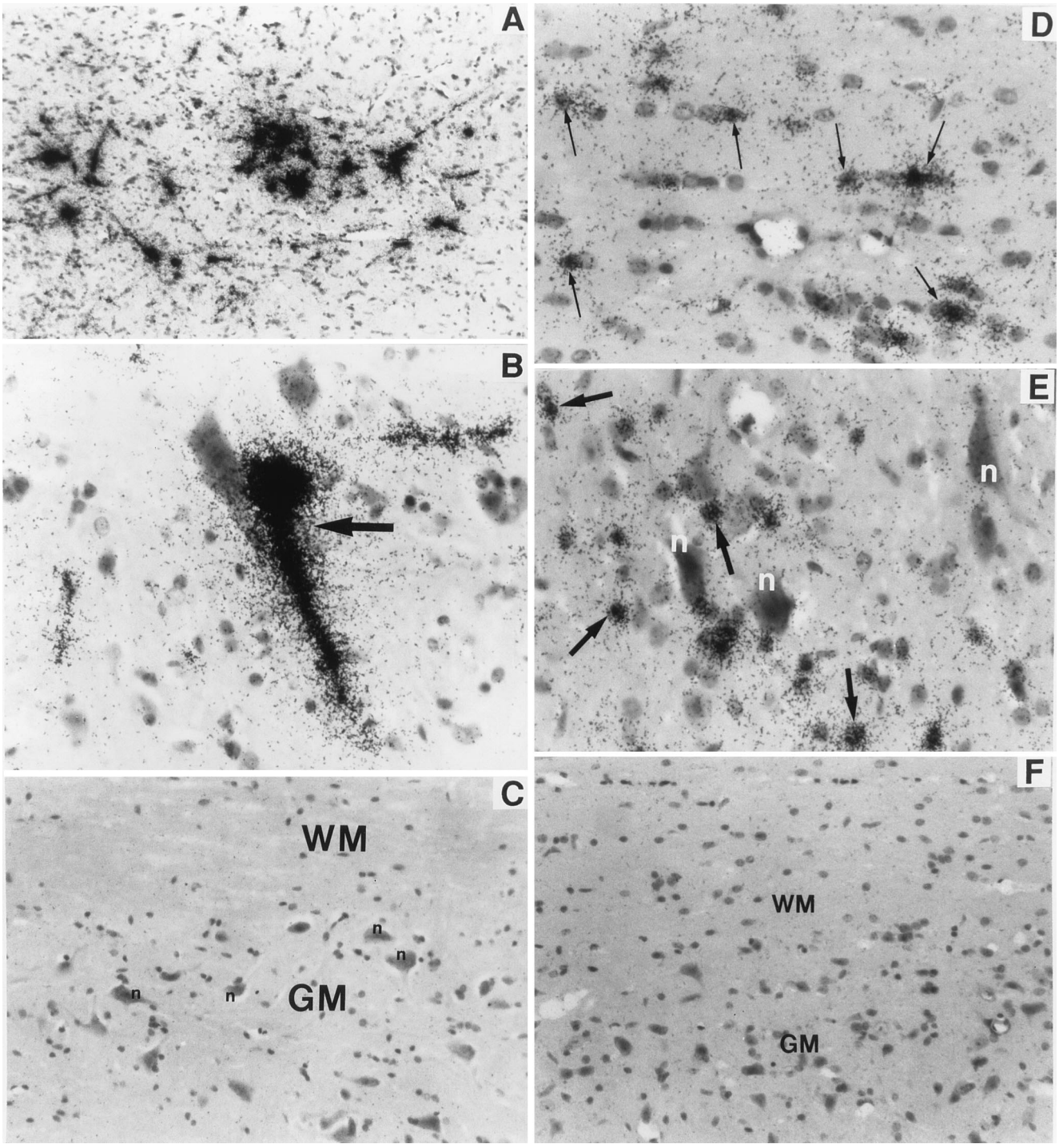

FIG. 7. LDV-infected cells (A and B) and ecotropic MuLV RNA-containing cells (D and E) in the spinal cord of a paralyzed CE/J mouse that had been infected with ecotropic MuLV in utero and with LDV-v at 7 months of age and absence of infected cells in the spinal cords of mice that did not become ecotropic MuLV infected (C and F). Sections of the spinal cords of paralyzed mouse 11 (A, B, D, and E) and of mouse 8 (C and F) not infected with ecotropic MuLV (Fig. 6) were hybridized with either a ^{35}S-labeled LDV-specific probe (cDNA 4-55 [A to C]) or a ^{35}S-labeled ecotropic MuLV probe (168-bp *Sma*I DNA fragment of clone AKR-623 [D to F]). Panels A, B, and E show areas of grey matter (GM), panel D shows areas of white matter (WM), and panels C and F show areas of both white and grey matter. n, anterior horn neurons. Microscopic magnifications: ×100 for panels A, C, and F; ×400 for panels B, D, and E. No positive cells were detected in sections of the spinal cords of any of the other mice of litter 2 (see Fig. 6) or in one uninfected CE/J mouse, all of which were analyzed on the same slide as that used for mouse 11. Similarly, no positive signal was detected in sections of replicate slides that had been treated with RNases A and T_1 before hybridization.

the other mice of this litter. Therefore, these analyses together confirmed the ecotropic MuLV infection of mice 4 and 11 indicated by the earlier Southern analyses of tail DNAs of these mice.

We also injected i.p. or intracranially 20 newly born CE/J mice (from three litters) and 9 newly born AKXD-20 mice (Table 2) with AKR-623 MuLV. When they were injected with cyclophosphamide and LDV-v at the age of 5 to 10 months, none developed paralytic symptoms. In addition, no ecotropic MuLV RNA was detected in the spinal cords or brains of the infected CE/J mice, although it was present in their spleens, indicating that the mice had become infected (data not shown).

DISCUSSION

Our results present unequivocal evidence for the conclusion that infection and subsequent expression of ecotropic MuLV in cells of the spinal cord are a prerequisite for the cytocidal infection of anterior horn neurons by the neurovirulent LDV, LDV-v. No ecotropic MuLV is expressed in the CNSs of mice in which ecotropic MuLV provirus activation or replication is blocked by a defect in the provirus (e.g., emv-1, -2, -3, and -13) or by the *Fv-1*^{b} allele or in mice devoid of endogenous ecotropic MuLVs. In all of these mice, LDV-v does not infect anterior horn neurons and thus does not cause paralytic disease. These results, along with the finding that the ecotropic MuLV RNA found in the CNS is transcribed from proviruses that have been newly acquired by active infection rather than from germ line proviruses, also explain the previously noted genetic requirements for ADPM susceptibility, namely, the presence of multiple copies of ecotropic MuLV proviruses and homozygosity at the *Fv-1*^{n} locus (35, 36, 39). The mice with multiple provirus copies that were found to be ADPM susceptible all contained at least one replication-competent ecotropic MuLV provirus, and our studies demonstrate that a replication-competent ecotropic MuLV is required to initiate infection of the CNS. In fact, our data prove that the possession of a single replication-competent provirus is sufficient to make *Fv-1*$^{n/n}$ mice ADPM susceptible. The lack of ADPM susceptibility reported for certain *Fv-1*$^{n/n}$ mouse strains that possess a single ecotropic MuLV provirus is now explained by the finding that the proviruses of these mice are replication defective (e.g., emv-2 and emv-3 in C57BL/10J and DBA/2J mice, respectively). Homozygosity at the *Fv-1*$^{n/n}$ locus is required because it permits the replication of the N-tropic, ecotropic MuLV that leads to the infection of CNS cells.

Infection of the CNS by endogenous ecotropic MuLVs seems to occur primarily or only during embryogenesis when a germ line provirus first becomes activated, probably in a rare cell (44). Direct proof for the requirement of an active infection of CNS cells by ecotropic MuLV and the expression of the virus in the spinal cord for generating the susceptibility of anterior horn neurons to cytocidal infection by LDV-v is provided by our experiment in which provirus-lacking, *Fv-1*$^{n/n}$ CE/J mice were infected in utero with ecotropic MuLV. Only the mice that became MuLV infected expressed ecotropic MuLV in their CNSs and became susceptible to paralytic LDV-v infection. All littermates that did not become infected with ecotropic MuLV remained ADPM resistant. An active infection of CNS cells by most ecotropic MuLVs seems restricted to the time of embryogenesis (22, 47); however, as discussed already, the reasons for this restriction are not entirely clear.

The main question remaining is how expression of ecotropic MuLV in the spinal cord renders anterior horn neurons susceptible to cytocidal infection by LDV-v. This effect seems highly specific for anterior horn neurons, since ecotropic MuLV expression in the brain (and probably other tissues) does not seem to render other types of cells LDV susceptible. No LDV RNA or LDV-infected cells have been detected in the brains of any of the paralyzed C58/M mice that have been examined, in spite of many ecotropic MuLV RNA-containing cells in the brain and numerous LDV-infected motor neurons in the spinal cord. Furthermore, no disease develops after LDV infection in mice in which ecotropic MuLV is expressed only in lymphoidal tissues, such as old DBA/2J mice. In these mice, as in mice in general, LDV productively infects primarily or only a subpopulation of macrophages, and a lifelong asymptomatic infection is maintained by replication of LDV in permissive macrophages that become continuously regenerated in mice at a low rate (40, 42).

An unexpected but well-documented finding was that ecotropic MuLV RNA synthesis is associated not with motor neurons in the spinal cord that become LDV permissive as suggested by earlier studies (8, 9) but rather with other CNS cells, most likely glial cells, both in the spinal cord and in the brain. The earlier conclusion that ecotropic MuLV RNA is primarily associated with motor neurons in the spinal cord (8, 9) is probably attributable to an experimental artifact, namely, a nonspecific binding of the 8.2-kb AKR-623 cDNA, which was used as the in situ hybridization probe in this study, to cellular RNAs, since most cells in spinal cord sections appeared to be positive.

In young mice, the ecotropic MuLV RNA-containing cells are exclusively located in white-matter tracts which contain only glial cells (27). In older mice, a considerable number are also present in the grey matter; however, very few anterior horn neurons were found to contain ecotropic MuLV RNA. Since very young C58 and AKR mice are susceptible to paralytic LDV infection when the generation of motor neuron-protective anti-LDV antibodies is blocked (42), we conclude that ecotropic MuLV-infected glial cells render anterior horn motor neurons susceptible to LDV infection apparently by some indirect mechanism. This effect could be mediated by direct contact between the glial cells and anterior horn neurons or via cytokines or MuLV gene products produced and released by the ecotropic MuLV-infected glial cells. The effect could be mediated by interaction with dendrites of the motor neurons at sites far removed from the cell body. In situ hybridization has clearly demonstrated the presence of large amounts of LDV RNA in dendrites of infected anterior horn neurons, and many foci of grains that seem to be associated with cells much smaller than the motor neurons might actually represent dendrites that have been cut horizontally or at an angle (Fig. 7A and B).

The apparent indirect effect of ecotropic MuLV-infected glial cells in rendering spinal cord motor neurons LDV permissive resembles the situation observed with a number of neuropathogenic ecotropic MuLVs, such as CasBrE, tsl Mo MuLV, and the Friend MuLV isolate PVC-211. When inoculated into either mouse fetuses or newborn mice, these viruses cause a noninflammatory spongiform encephalopathy later in life, resulting in progressive paralysis (13, 21, 26, 43, 56). The paralysis is caused by destruction of motor neurons in the spinal cord and brain stem. The mechanism of motor neuron destruction has not been resolved, except that it does not seem to involve a direct infection of the motor neurons. CasBrE MuLV RNA is found only in glial cells throughout the CNS but not in motor neurons (14). The distribution of the neuropathogenic ecotropic MuLV-infected glial cells in the CNS (14) is strikingly similar to that of the ecotropic MuLV-infected cells documented in the present study. Thus, as in the

case of the susceptibility of spinal cord motor neurons to LDV-v infection, the effects of the neuropathogenic MuLVs on motor neurons are induced indirectly by infection of glial cells.

Oligodendrocytes, astrocytes, and microglial cells all produce a variety of both inhibitory and stimulatory neuronal factors (15, 28, 31, 37, 46), which could either exert a cytotoxic effect on motor neurons in the case of the neuropathogenic MuLVs (14, 26) or induce LDV permissiveness in the motor neurons. Alternatively, an ecotropic MuLV gene product (perhaps the envelope glycoprotein gp70) released from the infected glial cells might play a role. It is possible that infection of glial cells by the neuropathogenic ecotropic MuLVs also induces LDV permissiveness to cytocidal infection by LDV-v. The identity of the ecotropic MuLV RNA-containing cells in the spinal cords and brains of C58 or AKR mice has not been elucidated. The morphology and location of the ecotropic MuLV RNA-positive cells in the brain and spinal cord white-matter tracts suggest that most are oligodendrocytes and astrocytes, but further studies are clearly required for their identification.

The progressive increase in the number of cells in the CNS that synthesize ecotropic MuLV RNA with advancing ages of C58 or AKR mice involves the activation of proviruses that have been acquired by infection. These proviruses are probably acquired during embryogenesis, rendered transcriptionally silent by methylation, and later activated by demethylation. Our results indicating a progressive increase in the number of unmethylated ecotropic MuLV proviruses in the CNS, which correlates with viral RNA synthesis, are consistent with this view but do not prove it. Nevertheless, CpG DNA methylation-demethylation has been implicated in the regulation of gene expression of murine retrovirus genes in other studies (5, 16, 20, 52).

On the other hand, the increase in the number of ecotropic MuLV RNA-containing cells in the CNSs of C58 and AKR mice could result from continuous horizontal spread of the infection among glial cells in the CNS. The finding of many of the positive cells in foci might support this conclusion. However, whether such horizontal transmission can occur in the CNS is in doubt. As pointed out already, MuLV provirus integration occurs only in dividing cells, and CNS cells in adult mice are thought generally to be mitotically inactive, except after neuronal damage, when replication of oligodendrocytes in the white matter and of certain astrocytes has been observed (1, 57). Furthermore, we have not been able to detect any MuLV-like particles in the spinal cords of old C58 mice by electron microscopy or isolation of infectious virus by propagation in 3T3 cells (unpublished data). A recent study with a recombinant CasBrE virus suggests that infection of microglial cells results in production of largely noninfectious particles (34). Our finding of MuLV RNA-containing cells in foci could also be due to the production by ecotropic MuLV-infected glial cells of factors that induce the activation in nearby cells of transcriptionally silent proviruses that have been acquired during embryogenesis. Regardless of the origin of the increasing number of ecotropic MuLV RNA-containing cells in the spinal cord with advancing age, this increase in the number of positive cells not unexpectedly correlates with an increase in the susceptibility of C58 and AKR mice to paralytic infection with LDV. A second factor that plays a role in the age-dependent increase in ADPM susceptibility of C58 mice is a decrease in the ability of the mice to mount a motor neuron-protective anti-LDV immune response (42).

A cooperation between two normally harmless viruses, such as between ecotropic MuLV and LDV in ADPM, could be involved in the etiology of severe motor neuron diseases in humans and other animals. Such interaction, especially if it is modulated by antiviral immune responses as with ADPM, may have thus far escaped detection.

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