An Increase in Disease Latency Is Associated with a Host-Dependent Selection for Recombinant Murine Leukemia Viruses with Substitutions in the p15E (TM) Gene

CHRISTOPHER Y. THOMAS,* MICHAEL A. COPPOLA,† J. DEAN NUCKOLS,
SUSAN C. LAWRENZ-SMITH, AND ANN C. MASSEY

Department of Internal Medicine, Box 513, and Department of Microbiology,
University of Virginia Health Sciences Center, Charlottesville, Virginia 22908*

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The genomes of recombinant murine leukemia viruses recovered from HRS/J (type I *env* recombinants) and CWD (type II *env* recombinants) mice have distinct envelope gene structures. To better understand the biologic significance of these differences, we examined the differences in the responses of HRS/J and CWD mice to inoculation with an oncogenic type II *env* recombinant. The CWD recombinant accelerated the onset of lymphoma in both strains, but the disease latency in the HRS/J mice was about 2 months longer. Analysis of the recombinant viruses in the HRS/J tumors revealed that the injected type II *env* recombinant had recombined in vivo with the endogenous ecotropic viruses to generate secondary recombinants with type I envelope genes. In another set of experiments, comparison of complete or partial DNA sequences of the envelope genes from six recombinant proviruses confirmed that the origins of the sequences that encode an amino-terminal region of the TM envelope protein, p15E, distinguish type I envelope genes from type II. Taken together with the results of previous studies, these observations suggest that the differences in the responses of HRS/J and CWD mice to the oncogenic type II *env* recombinant resulted from an interaction between the viral TM protein and a host factor expressed in HRS/J mice.

Recombinant murine leukemia viruses (MuLVs) are associated with the development of tumors in strains of inbred mice that are highly susceptible to leukemia (high leukemia strains) and in mice injected with certain exogenous ecotropic MuLVs (for a review, see reference 7). The ecotropic viruses recombine with 1 of the 20 or so endogenous polytropic virus sequences to yield recombinants with substitutions within the envelope genes (1, 5, 7, 8, 18, 30–32). The envelope genes, *env*, encode a gp85 precursor polyprotein that is cleaved by cellular and viral proteases to yield the mature major envelope protein, gp70 or SU (surface protein), and minor envelope protein, p15E or TM (transmembrane protein), (16). Although there is some variation in the pattern of substitutions by polytropic sequences within the envelope genes of different recombinant viruses, polytropic sequences are always found in the 5' portion of *env*, which encodes the receptor-binding domain of gp70 (1, 7, 8, 12, 22, 28, 31, 32, 35). The receptor specificity of the gp70 confers an expanded in vitro host range to the recombinant viruses, a phenotype that has been termed mink cell focus-forming (MCF), dual-tropic or polytropic (7–9, 25).

Hopkins and coworkers originally divided recombinant MuLVs into class I and class II viruses on the basis of differences in the composition of the envelope gene and the ability to accelerate leukemogenesis in AKR mice (22, 28). This classification was later modified to type I *env* and type II *env* to specifically denote the differences in the envelope genes (32, 36) (the previous terms class I *env* and class II *env* have been changed to type I *env* and type II *env* to avoid confusion with the class I and II histocompatibility antigens). The classification is based on the observation that

type I *env* recombinants retain ecotropic virus sequences in the 5' portion of the p15E gene whereas the type II *env* recombinants do not (4, 22, 31, 32, 36). Type I *env* recombinant viruses are found in high leukemia strains HRS/J, AKR, and C58/J, whereas strain CWD, which is also highly susceptible to leukemia, and the NFS.akv strains produce type II *env* recombinants (8, 11, 22, 28, 32, 35).

The biologic significance of the type I and type II *env* designations is not well understood. The phenotypes of the two viruses are similar in that they utilize the same cellular receptor and may be either strongly or weakly oncogenic (3, 8, 27, 32, 34). However, recent studies suggest that the type I and type II *env* recombinants may have distinct biologic properties in certain mouse strains. HRS/J mice that were injected with the ecotropic AKR MuLV, SL3-3, developed tumors that contained type I *env* recombinants, whereas SL3-3-induced tumors of CWD mice contained type II *env* viruses (31). The association of type I *env* recombinants with HRS/J tumors is regulated by a dominant host gene that is located on chromosome 17 (4), but the mechanism by which this gene promotes the production of the type I *env* recombinants is not known. The studies reported here show differences in the responses of HRS/J and CWD mice to inoculation with the CWD type II *env* recombinant. The results support the hypothesis that HRS/J mice express a host factor that interacts with a determinant located between amino acids 34 and 58 of the p15E protein of recombinant viruses.

MATERIALS AND METHODS

Mice and recombinant viruses. CWD/Le and HRS/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, and maintained as described previously (32, 35). The spontaneous HRS/J type I *env* recombinant, PTV-1, and the

* Corresponding author.

† Present address: Department of Microbiology, Dartmouth Medical School, Hanover, NH 03756.

CWD type II *env* recombinants CWM-T-15 and CWM-S-101 were first isolated by cocultivation of tumor cells with mink lung fibroblasts CCL 64 (8, 32). PTV-1 was purified by six rounds of alternating end point dilutions on mink and NIH 3T3 fibroblasts (8). CWN-T-25 was obtained by cocultivation of NIH 3T3 fibroblasts with tumor cells from a CWD mouse injected with the AKR ecotropic virus, SL3-3 (31, 34) (see below). The provirus of the type II *env* recombinant CHF-2-30 was cloned directly from the DNA of a tumor from a (CWD $\times$ HRS/J) F_2 mouse that died of an SL3-3-induced lymphoma (4).

Leukemogenicity assays. The AKR ecotropic SL3-3 virus was derived from an infectious provirus clone that was a generous gift of Jack Lenz (20). The isolation of the CWD type II *env* recombinant CWN-T-25 is described above. Supernatants from chronically infected cells were collected, passed through 0.22- μ m nitrocellulose filters, and stored as 1-ml aliquots at -70°C . The CWN-T-25 virus stocks were free of the parental SL3-3 ecotropic virus, as determined by fingerprint analysis of viral RNA (31) and failure of the CWN-T-25 stocks to induce leukemia in NIH Swiss mice that are susceptible to the SL3-3 virus but not to polytropic viruses (data not shown). The virus-containing culture medium (0.05 to 0.1 ml) was injected into the peritoneal cavities of mice that were less than 48 h old. Ten HRS/J and 14 CWD mice were monitored until death or were sacrificed because of advanced disease. The presence of lymphoma was confirmed by necropsy and histologic examination of tumor tissues (33).

Isolation of HRN-T-57 and analysis of viral RNA by T_1 RNase fingerprint. The HRN-T-57 virus was recovered from the leukemic thymus of an HRS/J mouse that had been injected with CWN-T-25. The tumor cells were isolated and cocultivated with NIH 3T3 cells in the presence of 2 μ g of Polybrene per ml as previously described (31, 35). For RNA fingerprint analysis, the 70S viral RNA in the virions produced by the NIH 3T3 cells was labeled with $^{32}\text{P}_i$ and purified as reported previously (31, 35). The T_1 RNase-resistant oligonucleotides were separated by two-dimensional electrophoresis through polyacrylamide gels and detected by autoradiography (35). Oligonucleotides were numbered and aligned into maps of the viral genome according to the previously described conventions of Lung et al. (22), Pederson et al. (26), and Thomas (31).

Southern blot hybridization probes. The pAKV5 and pAKV6 probes that contain a 5' or 3' fragment of the p15E gene of the AKR ecotropic virus Akv623, respectively, were gifts of Winship Herr (11). The T25PB probe represented the 162-bp *Pst*I-*Bgl*II p15E gene fragment that contains polytropic sequences from the CWN-T-25 provirus (4). The p15Egen probe was the 500-bp *Xba*I-*Pst*I *env* long terminal repeat (LTR) fragment isolated from a clone of the Akv623 provirus; the latter was a gift of D. Lowy (21). The DNA probes were excised from plasmid DNAs by the appropriate restriction endonucleases and purified by electrophoresis through low-melting-point agarose gels. The fragments were labeled with [^{32}P]dATP by the random primer extension method under conditions recommended by the supplier of the Klenow fragment of DNA polymerase I (Boehringer Mannheim, Indianapolis, Ind.).

Southern blot and hybridization. The isolation of genomic DNAs and Southern blot analysis followed previously described procedures (4). The same procedure was used for Southern blot analysis of the polymerase chain reaction (PCR) products, except that the gels were not treated with

acid prior to denaturation and transfer of the DNAs in sodium hydroxide.

Amplification of recombinant proviruses by the PCR technique. The 5' oligonucleotide primer, poly5env, contained polytropic virus-specific envelope gene sequences located in the central portion of the gp70 gene (positions 798 to 817 from the mutated ATG start codon of the HRS/J endogenous polytropic provirus MX27 [30] [also see Fig. 4]), with the addition of a 5' extension that contained an *Eco*RI site. The 3' primer, LTReco3', corresponded to the ecotropic virus-specific sequences located at positions 318 to 341 of the U3 region of the SL3-3 virus or residues 220 to 238 of the endogenous ecotropic viruses (14, 19) and also contained a 5' extension. The oligonucleotide primers were synthesized at the University of Virginia Nucleic Acid and Protein Sequence Center with the sequences (virus sequences underlined) 5'-GCGATTCTCTATAGTCCCTGAGACTG (poly5env) and 5'-TTCCCGGGTCTCTGAAACTGTGTTG (LTReco3'). The primers for control PCRs included LTRgen3' (5'-TACAGAAGCGAGAAGCGAGC, which hybridizes to the U3 regions of most MuLVs) and PXenv5' (5'-CTCAAATCAATAGATCCAGA, which corresponds to the 3' *env* sequences of xenotropic and polytropic proviruses) (17, 22a, 23, 30).

For amplification of recombinant proviruses, the reaction tubes contained 8 μ g of genomic DNA or 0.04 μ g of control plasmid DNA per ml with 0.4 μ M each poly5env and LTReco3' primers. The reaction solution included buffer, MgCl_2 , nucleotides, and *Taq*I DNA polymerase in the concentrations recommended by the supplier of the enzyme (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl_2 , 0.001% gelatin, 200 μ M each dATP, dCTP, dGTP, and dTTP, and 20 U of *Taq*I DNA polymerase per ml [Perkin-Elmer, Norwalk, Conn.]). To prevent nonspecific priming of sequences, the nucleotides were not added until the tubes had been incubated at 80°C for 2 min. The denaturation, annealing, and extension steps each lasted 30 s at 94, 50, and 72°C , respectively, for a total of 28 cycles. Restriction enzyme digestion of the PCR products was performed under conditions recommended by the supplier (GIBCO-BRL, Bethesda, Md.). The control DNAs were the AKR ecotropic MuLV Akv623 (21), the endogenous HRS/J polytropic provirus MX27 (gift of J. Stoye and J. Coffin [30]), and the xenotropic provirus *Bxv*-1 (23).

Molecular cloning and DNA sequencing. The cellular DNAs were isolated from mink or *Mus dunni* cell lines that were chronically infected with the appropriate virus or from the CHF-2-30 tumor cells. DNA of the bacteriophage lambda EMBL4 was digested with *Eco*RI and *Bam*HI and precipitated in ethanol. After resuspension in solution, the bacteriophage DNA fragments were ligated to *Eco*RI-digested cellular DNAs as described previously (34). Briefly, the ligated DNAs were mixed with Packagene extracts (Promega, Madison, Wis.), and the reaction products were incubated with *Escherichia coli* P2-392. The infected bacteria were mixed with molten-top agarose and poured onto 150-mm agar plates so as to yield plaque densities of 10,000 to 20,000 per dish. The DNAs in the phage plaques were transferred to nylon membranes as described previously (4, 34), and the filters were hybridized to the appropriate ^{32}P -labeled probes. Because of the conserved *Eco*RI site within the gp70 gene of recombinant viruses, the 5' and 3' ends of the proviruses were cloned separately. One or more clones of the 3' *env* sequences were available for all six recombinants, and 5' *env* sequences were available for three of the viruses. The 3' clone of the HRS/J type I polytropic

virus PTV-1 was detected by hybridization of the plaque DNAs to the pAKV5 probe described above. Clones that contained the 3' end of CWM-S-101 or the 3' end of CWM-T-15 were selected by hybridization to the polytropic and ecotropic virus sequences located on the *EcoRI* *env* LTR fragment of the MCF 247 provirus (gift of C. Holland and N. Hopkins [12]). Clones of the 3' portions of the SL3-3-induced type II recombinants CWN-T-25 and CHF-2-30 were detected by hybridization to the pAKV6 probe. Bacteriophages that contained the 5' end of each of the proviruses were detected by hybridization to the *Bam*HI-*EcoRI* *pol-env* fragment of MCF 247. Positive plaques were subsequently purified, and the bacteriophage DNA was analyzed by Southern blot to confirm the structures of the provirus sequences. The 3' end of the proviral DNA of HRN-T-57 recombinant was cloned by ligation of the PCR products to the TA cloning vector (Invitrogen, San Diego, Calif.).

Restriction enzyme fragments of the different provirus clones were subcloned as required into plasmid vector pUC13 or pUC19 or bacteriophage M13mp vectors for DNA sequence analysis. The nucleotide sequences of double- and single-stranded DNA templates were determined by the dideoxynucleotide chain termination method (Sequenase system; United States Biochemical, Cleveland, Ohio).

RESULTS

Leukemogenicity of the CWD type II *env* recombinant virus in HRS/J mice. As discussed above, the generation or selection of type I *env* recombinants in HRS/J mice is a dominant trait with respect to the selection and generation of type II *env* recombinants in CWD mice (4). To determine whether there is a selective block of the replication or biologic effects of type II *env* viruses in HRS/J mice, we inoculated neonatal HRS/J mice with the type II *env* recombinant CWN-T-25. In Fig. 1, the incidence and latency of leukemia observed in the HRS/J animals are compared with those of CWD mice injected with the same virus and with those of mice that had been injected with the SL3-3 virus in previous studies (31, 34). CWD mice that were injected with the CWN-T-25 recombinant and HRS/J and CWD mice that were inoculated with SL3-3 MuLV developed leukemia at about the same time, with most of the animals dead or diseased by 16 weeks of age. CWN-T-25 also accelerated leukemogenesis in HRS/J mice, but the tumors developed about 2 months later than in the other groups. Thus, HRS/J mice were susceptible to infection and the induction of leukemia by CWN-T-25, but there was a relative delay in the appearance of disease.

The acquired proviruses within the HRS/J and CWD tumor DNAs were first characterized by Southern blot assays. The tumor DNAs were digested with *EcoRI* and *Bss*HII restriction enzymes to generate proviral fragments that contained the 3' *env* and U3 region of the recombinant viruses. Although the ecotropic and recombinant proviruses share the *Bss*HII recognition sites in the LTRs, only the recombinant viruses contain the *EcoRI* site within the gp70 gene (1). A Southern blot of the DNA fragments was then hybridized sequentially to three different probes. As shown in Fig. 2A, all five of the CWD tumor DNAs that were examined contained 1.7- to 1.9-kb DNA fragments that hybridized to the type II *env*-specific probe, T25PB. The variation in the sizes of the fragments most likely resulted from variation in the number of enhancer elements within the U3 region. In contrast, no band was seen in three of the

FIG. 1. Time course of development of leukemia in MuLV-injected CWD and HRS/J mice. •, CWD mice injected with the leukemogenic ecotropic virus, SL3-3; □, CWD mice injected with the CWD type II *env* recombinant, CWN-T-25; +, HRS/J mice injected with SL3-3; *, HRS/J mice injected with CWN-T-25. The disease latency for animals injected with SL3-3 was previously reported, but the incidence curve is shown here for comparison with those of the CWN-T-25-injected mice (31). By 40 weeks of age, less than 10% of the animals would be expected to develop spontaneous leukemia.

four HRS/J tumor DNAs and a faint band was seen in the fourth (tumor HR-K1) (Fig. 2A, lane k). When the same Southern blot was rehybridized to the type I *env*-specific probe (pAKV5), bands were detected in each of the four HRS/J tumor DNAs but not in the CWD tumor DNAs (Fig. 2B). All of the recombinant proviruses hybridized to the third probe (pAKV6), which contains sequences from the

FIG. 2. Southern blot analysis of acquired proviruses in tumor DNAs. Southern blots of control and tumor DNAs digested with *EcoRI* and *Bss*HII and hybridized to the three different probes. Lanes: a, normal CWD embryo; b to f, CWN-T-25-induced CWD tumors; g, SL3-3-induced tumor CWN-T-25; h, *M. dunni* cells infected with CWN-T-25; i to l, CWN-T-25-induced HRS/J tumors; m, HRS/J liver. (A) T25PB probe, which detects type II *env* recombinant and endogenous nonecotropic proviruses; (B) pAKV5 probe, which detects type I *env* and ecotropic proviruses; (C) pAKV6 probe, which detects SL3-3-induced recombinant viruses that retain ecotropic virus sequences in the 3' end of the p15E gene. The migration point of the predicted 1.8-kb fragment of the CWN-T-25 provirus is shown on the left of each panel.

FIG. 3. Southern blot of 3' fragments of MuLV proviruses amplified by PCR. A nonspecific *env* probe (p15Egen), a type I *env* probe, pAKV5, and a type II *env* probe, T25PB, were used. Lanes 1 to 7, PCR products with the primers poly5env and LTReco3' that amplify only recombinant proviruses with ecotropic U3 region sequences; lanes 8 to 11, PCR products obtained to verify that control DNAs can be amplified with appropriate nonspecific primers. Lanes: 1, endogenous polytropic provirus MX27 (30); 2, endogenous xenotropic provirus *Bxv-1* (23); 3, endogenous ecotropic provirus Akv623 (21); 4, mixture of endogenous polytropic and ecotropic proviruses; 5, type I *env* recombinant PTV-1; 6, type II *env* recombinant CWN-T-15; 7, type II *env* recombinant CWN-T-25; 8, endogenous polytropic provirus MX27 amplified by poly5env and LTRgen3' primers; 9, endogenous xenotropic provirus amplified by PXenv5' and LTRgen3' primers; 10, mixture of endogenous polytropic and ecotropic proviruses amplified with poly5env and LTRgen3' primers; 11, same mixture of proviruses amplified with U3-specific primers only. n, nonspecific signal; x, a smaller xenotropic *env* fragment that hybridizes only to the p15Egen probe.

extreme 3' end of the ecotropic virus p15E gene (Fig. 2C). Taken together, the results indicated that each of the CWD tumors had acquired CWN-T-25-like recombinant proviruses with type II envelope genes but that the HRS/J tumors contained only or mostly recombinants with type I envelope genes.

To confirm the results of the Southern blot assays and to determine whether the HRS/J tumors might harbor small numbers of CWN-T-25 proviruses, we developed a new method to amplify the 3' end of the recombinant proviruses by the PCR technique. The primers were chosen in an attempt to specifically amplify only the acquired recombinant proviruses and not the endogenous viruses. The 5' primer oligonucleotide contained a 20-bp sequence that is unique to the envelope gene of polytropic viruses (30), while the 3' oligonucleotide primer was homologous to ecotropic virus-specific sequences within the U3 region in the LTR (see Materials and Methods and schematic in Fig. 4A). The primers can anneal to either endogenous polytropic or ecotropic proviruses but not to both. Thus, a PCR product is generated only when the two sequences recognized by the primers are juxtaposed to one another, as within recombinant proviruses. To test the specificities of the primers and PCR, a series of control experiments with cloned endogenous and recombinant proviral DNAs were performed. Figure 3 shows a Southern blot of the control PCR products that were hybridized to different probes for MuLV *env* sequences. The amplified DNAs hybridized to the nonspe-

cific *env* probe (p15Egen) and the appropriate type I or II *env*-specific probes. Importantly, bands were not detected in reaction products that contained the proviral DNAs or mixtures of ecotropic and polytropic proviruses and the specific 5' polytropic *env* and 3' ecotropic U3 primers. However, the control DNAs were amplified by PCR when other primers were substituted for the specific primers.

The proviruses from control and CWN-T-25-induced tumor tissues were then amplified by PCR that employed the 5' polytropic *env* and 3' ecotropic U3 primers. The intact PCR products or those that were incubated with restriction enzyme *Pst*I or *Xba*I were analyzed by Southern blot. As shown in Fig. 4A, the *Xba*I restriction site is found only in type I *env* p15E gene sequences. A *Pst*I site is located in the polytropic p15E gene sequences of the type II *env* recombinants and only rarely in type I envelope genes (4; also, unpublished observations). The Southern blot of the proviral fragments was hybridized sequentially to probes that were specific for ecotropic (type I *env*) or polytropic (type II *env*) p15E gene sequences. As shown in Fig. 4B, the PCR products from the CWD tumors that were induced by CWN-T-25 contained a *Pst*I site in the p15E gene and hybridized to the type II *env* probe as predicted whereas the products from CWD mice injected with the HRS/J type I *env* recombinant, PTV-1 (8), contained only a single *Pst*I site in the U3 region. Faint bands indicative of a type II *env* recombinant were seen in only one HRS/J tumor, HR-K1. Hybridization of the blot to the type I *env* probe showed that each of the PTV-1-induced CWD tumors and the CWN-T-25-induced HRS/J tumors contained proviruses with type I envelope genes with the characteristic *Xba*I site in the p15E gene. The PCR products from the two PTV-1-induced CWD tumors that were digested with *Xba*I contained two bands. Most likely, this resulted from incomplete cleavage by *Xba*I, although it is formally possible that the larger product was derived from type I *env*-like recombinants that lacked the *Xba*I site but hybridized to the type I *env*-specific probe. In summary, the PCR studies of the envelope genes of the recombinant proviruses yielded the same results as the Southern blot assays. However, the PCR assay did reveal that one HRS/J tumor, HR-K1, contained a mixture of type I and type II *env* recombinants.

The HRS/J type I *env* recombinant HRN-T-57 was derived from the injected type II *env* recombinant. The genome of a recombinant virus in one of the HRS/J tumors, HR-57, was studied in more detail. NIH 3T3 cells that had been cocultivated with HR-57 tumor cells produced a mixture of viruses referred to as HRN-T-57. The components of this mixture were determined by T_1 oligonucleotide fingerprinting of the viral RNAs. This procedure allows direct comparison of the relative yields of allelic oligonucleotides in the different viral genomes. As shown in Fig. 5, the majority of genomes in HRN-T-57 contained oligonucleotides 208B and 379, which are markers of the CWN-T-25 genome and were originally derived from the SL3-3 virus (32, 34). These oligonucleotides are not present in HRS/J endogenous ecotropic or recombinant polytropic viruses (8, 35). The W2 and W4 oligonucleotides are also found in the genome of CWN-T-25, but their locations within the genome are not known. The major viral species of HRN-T-57 also contained the ecotropic p15E gene oligonucleotides 18, 19, and 35. The latter oligonucleotides are nearly always present in type I *env* recombinants but are absent in type II *env* viruses such as CWN-T-25 (8, 22, 32, 35, 36). The fingerprint indicates that the major component of HRN-T-57 was a virus that carried genetic markers of CWN-T-25 and type I envelope gene

FIG. 4. Southern blot of amplified proviral fragments from tumor and control DNAs. (A) The locations of restriction enzyme sites and the origins of sequences in the *env*-U3 regions of type I and type II *env* recombinant viruses are shown. The relative locations of the poly5env and LTReco3' primers, the expected PCR product of 1.57 kb, and the type I and type II *env*-specific probes are also indicated. Note that the amplified envelope gene fragment incorporates the type I or II *env*-specific sequences and the *Pst*I or *Xba*I restriction site. (B) DNAs were loaded intact (U) or after digestion with the restriction endonucleases *Pst*I (P) or *Xba*I (X). The Southern blot was then hybridized to the pAKV5 type I *env* probe (11) or to the type II *env* probe T25PB (4). Faint bands in the HR-K1 tumor lanes are indicated by dashes. Control, DNA from normal mouse liver (negative control); CW-142, CW-143, and CW-144, CWD tumors induced by the type II *env* recombinant CWN-T-25; CW-27 and CW-28, tumors induced by the HRS/J type I *env* recombinant, PTV-1 (36); HR-57, HR-59, HR-65, and HR-K1, tumors from HRS/J mice injected with CWN-T-25. The DNAs from the CWD tumor CW-25 and the HRS/J tumor HR-7 recovered from animals injected with the ecotropic MuLV SL3-3 served as positive controls for type II and type I *env* recombinants, respectively (31).

sequences, a finding that is consistent with the results of the Southern blot and PCR studies. Most likely, the HRN-T-57 type I *env* recombinant was generated in vivo by recombination between CWN-T-25 and one of the endogenous ecotropic viruses found in HRS/J mice, *Emv-1* or *Emv-3*, both of which contain oligonucleotides 18, 19, and 35 (13, 35). This conclusion is supported by DNA sequence analysis of the HRN-T-57 envelope gene (see below).

Comparison of envelope gene sequences of type I and II recombinant MuLVs. To better characterize the type I and type II envelope genes, we determined complete or partial

env sequences of six recombinant viruses that included CWN-T-25 and HRN-T-57. The other viruses represented a spontaneous HRS/J type I *env* recombinant and three type II *env* viruses that were recovered from spontaneous or SL3-3-induced tumors of CWD mice and a (CWD $\times$ HRS/J) F_2 mouse (4, 32). The results of the comparison of the envelope gene sequences are summarized in Fig. 6. The origin of the *env* sequences in each virus was determined by the degree of homology with the ecotropic or polytropic virus parent. For those regions in which the ecotropic and polytropic sequences were homologous, the origin was assigned to the same parent that had donated the immediate flanking sequences. As expected, the envelope genes of the type I *env* recombinants contained polytropic virus sequences in the 5' portion of the *gp70* gene and ecotropic virus sequences in the 5' end of the *p15E* gene. In contrast, the envelope genes of the two spontaneous type II *env* recombinants, CWN-T-15 and CWN-S-101, were composed entirely of polytropic virus sequences. The envelope genes of the two SL3-3-induced type II *env* recombinants also contained large substitutions by polytropic sequences; however, ecotropic virus sequences were retained in the extreme 3' end of the *p15E* gene.

Figure 7 compares the envelope gene sequences of the six recombinants with those of the endogenous polytropic and ecotropic viruses and the AKR type I *env* recombinant, MCF 247 (12, 17). Although the polytropic sequences in all of the recombinants were closely related to each other, no two sets of sequences were identical. This suggests that each recombinant virus had acquired polytropic *env* sequences from a different endogenous donor or donors. The unique base substitutions in the polytropic sequences of each virus may represent acquired point mutations, but most of the differences were probably due to polymorphisms in the endogenous *env* sequences. For example, the apparently unique substitutions at positions 638 and 803 of the PTV-1 and CWN-T-25 *env* sequences, respectively, were identical to those found in the envelope gene of MCF 247. Similarly, the unique residues at positions 951, 1203, and 1213 of the polytropic *env* sequences of CHF-2-30 are found in the analogous positions in the sequence of the envelope gene of the HRS/J endogenous modified polytropic virus MX33 (30). Finally, the finding that some base substitutions were shared by two of the proviruses implies that these substitutions represent polymorphisms in the polytropic *env* sequences rather than acquired mutations.

Comparisons of the *env* sequences confirmed that the origins of the 5' *p15E* gene sequences distinguished type I from type II envelope genes. The type I *env* recombinants studied here retained ecotropic virus sequences in the 3' end of the *gp70* gene and the 5' region of the *p15E* gene, whereas the allelic regions in the type II envelope genes contained polytropic virus sequences. Of note, the type I envelope gene of HRN-T-57, which was derived from the type II envelope gene of CWN-T-25, contained a substitution of approximately 300 bp of endogenous ecotropic virus sequences. These sequences started in the 3' end of the *gp70* gene and extended to just beyond the *Bgl*III site in the 5' half of the *p15E* gene. About 100 bp of the polytropic virus sequences remained between the acquired endogenous ecotropic virus sequences and the beginning of the ecotropic 3' *p15E* gene sequences that were inherited from CWN-T-25. This suggests that the type I *env* phenotype is encoded by ecotropic *p15E* gene sequences that are located 5' of the *Bgl*III restriction site. In addition, this observation taken

FIG. 5. T_1 oligonucleotide map and fingerprint of HRN-T-57 viral RNAs. The oligonucleotide maps of HRN-T-57 and CWN-T-25 genomes are compared at the top. For simplicity, only selected oligonucleotides in the fingerprint are numbered. Oligonucleotides 379, 208B, W4, and W2 are found in the genome of CWN-T-25. Oligonucleotides 18, 19, and 35 are markers of ecotropic virus p15E gene sequences found in type I *env* recombinants and are not present in the CWN-T-25 genome (8, 22, 32, 35, 36).

together with the fact that only the SL3-3-induced recombinants retained ecotropic virus sequences in the 3' end of the p15E gene implied that the origin of these sequences was influenced by characteristics of the ecotropic virus parent. Thus, different processes are responsible for the selection of

the 3' p15E gene sequences and the type I or type II *env* sequences in the 5' half of the gene.

Figure 8 shows the differences in the predicted amino acid sequences of the p15E molecules of the type I and type II *env* recombinants. The type I and type II p15E amino acid sequences were highly homologous, because many of the base differences in the DNA sequences did not alter the specificity of the corresponding amino acid codon. However, two conspicuous regions of divergence were located between residues 34 and 59 and at the carboxy terminus. The first region is encoded by the p15E gene sequences that distinguished type I envelope genes from type II. The other cluster of nonhomology, at the carboxy terminus, resulted from differences in the amino acid sequences encoded by the ecotropic and polytropic virus 3' *env* sequences. This observation provides additional support to the hypothesis that the selection for type I and type II *env* recombinants reflects a selection for a determinant in the amino-terminal half of the p15E protein rather than a selection for a specific nucleic acid sequence (4).

DISCUSSION

The leukemia acceleration assays reported here show that the type II *env* recombinant MuLV, CWN-T-25, accelerated leukemogenesis in HRS/J as well as in CWD mice but that disease developed about 2 months later in the HRS/J mice. The delay in the onset of disease was not related to a

FIG. 6. Schematic comparison of the envelope gene DNA sequences of type I and type II *env* recombinant MuLVs. CWN-S-101 and CWN-T-15, CWD spontaneous type II *env* recombinants; CHF-2-30, (CWD $\times$ HRS/J) F_2 type II *env* recombinant induced by the ecotropic virus SL3-3; CWN-T-25, CWD type II *env* recombinant induced by SL3-3; HRN-T-57, HRS/J type I *env* recombinant from a mouse injected with CWN-T-25; PTV-1, spontaneous HRS/J type I *env* recombinant. ■, ecotropic virus sequences (*Env*-1 and -3 or SL3-3 MuLV); ■■■, polytropic virus sequences; —, sequence not determined; s, sequence marker of SL3-3 MuLV. Locations of sequences that distinguish type I *env* viruses from type II are boxed. *, endogenous ecotropic proviruses *Env*-1 and *Env*-3.

FIG. 7. Comparison of the 3' envelope gene sequences of recombinant and endogenous polytropic and ecotropic proviruses. The recombinant provirus sequences are compared with those of the endogenous polytropic provirus MX27 (POLY), the endogenous ecotropic AKR virus Akv623 (ECO), and the AKR type I env recombinant MCF 247 (10, 12, 17, 30). The sequences begin at the mutated ATG start codon at

Start ecotropic sequences -->
in MCF 247; PTV-1

841 960

POLY ACCGGAGACAGGCTGCTAAACCTGGTAGATGGAGCCTACCAAGCTCTCAACCTCACCAGTCTGACAAAACCCAAGAGTGCTGGTTGTGTCTGGTAGCGGGACCCCCCTACTACGAAGGG

CWM S101

CWM T15

CHF230

CWM T25

HRN T57

PTV-1

MCF 247

ECO

961 1080

POLY GTTCCGCTCCTAGGTACTTATTCCAACCACTACCTCTGCCCCAGCTAACTGCTCCGTGGCTCCCAACACAGCTGACCCGTCCGAAGTGACCGGACAGGGACTCTGCGTAGGAGCAGTT

CWM S101

CWM T15

CHF2-30

CWM T25

HRN T57

PTV-1

MCF 247

ECO

1081 1200

POLY CCCAAACCCATCAGGCCCTGTGTAATACCACCCAGAAGACGAGCAACGGGTCTACTATCTGGCTGCTCCGCCGGGACCATTTGGGCTTGCAACACCGGGCTCACTCCCTGCCTATCT

CWM S101

CWM T15

CHF2-30

CWM T25

HRN T57

PTV-1

MCF 247

ECO

1201 1320

POLY ACCACTGTACTCGACCTCACCACCGATTACTGTGCTCTGGTGTAGCTCTGGCCAAAGGTGACCTACCACCTCCCTGGTTATGTTTATGGCCAGTTTGAGAGAAAAACCAGATATAAAGA

CWM S101

CWM T15

CHF2-30

CWM T25

HRN T57

PTV-1

MCF 247

ECO

1321 1440

p15E --> Pst I (poly); T25PB type II env probe underlined in CWM T25 sequence

POLY GAGCCGGTGTCTTAATCTGGCCCTGCTGTTGGGAGGACTTACTATGGCGGCATAGCTGAGGAGTAGGAACCGGGACTACAGCCCTAGTGGCCACCAAAATTCGAGCAGCTCCAG

CWM S101

CWM T15

CHF2-30

CWM T25

HRN T57

PTV-1

MCF 247

ECO

1441 1560

Bgl II

POLY GCAGCCATACATACAGACCTTGGGGCTTTAAAAAGTCAGTCAGTGCCTTAAAAAGTCTCTGACCTCGTTGTCTGAGGTGGTCTACAGAACCGGAGGGGATTAGATCTACTGTTCTTA

CWM S101

CWM T15

CHF2-30

CWM T25

HRN T57

PTV-1

MCF 247

ECO

Xba I (ecotropic)

FIG. 7—Continued.

generalized decreased susceptibility of HRS/J mice to MuLVs, since HRS/J and CWD mice are equally sensitive to the oncogenic effects of the AKR ecotropic virus SL3-3 and the HRS/J recombinant virus PTV-1 (31, 36). The presence or absence of endogenous ecotropic proviruses in the host animal is known to influence the pathogenicities of some recombinant MuLVs (3), but HRS/J and CWD mice carry the same two endogenous ecotropic proviruses, *Emv-1* and *Emv-3* (15). On the other hand, the two strains differ at the H-2 locus which contains genes that influence the susceptibility of C57BL congenic strains to a recombinant virus (37).

One possible explanation for the delay in the appearance of the HRS/J tumors came from the analyses of the proviruses in the tumor DNAs. The HRS/J tumors contained type I *env* recombinants rather than the expected type II *env* proviruses of the injected virus, CWN-T-25. When studied in detail, the genome of one of the HRS/J type I *env* recombinants was found to contain genetic markers of CWN-T-25 as well as the endogenous ecotropic viruses. This confirmed that CWN-T-25 had infected the HRS/J mice but had recombined with the endogenous ecotropic viruses to generate viruses with type I envelope genes. This reproducible substitution by type I *env*-specific sequences in the recombinants from each HRS/J mouse suggested that the allelic type II *env* p15E gene sequences impaired the replication or

oncogenicity of CWN-T-25. Thus, the time required for the generation, selection, and dissemination of the secondary type I *env* recombinants may explain the delay in the onset of leukemia.

It is tempting to speculate that the selection of the secondary type I *env* recombinants in the HRS/J mice was regulated by the same HRS/J gene on chromosome 17 that is responsible for the selection of type I *env* recombinants in animals injected with the SL3-3 MuLV. If so, the gene that selects for type I *env* recombinants acts at a late stage in leukemogenesis and need not influence the process that generates recombinant viruses. This hypothesis is supported by evidence that the type of recombinant produced by a given strain is not determined by the presence or absence of a specific endogenous provirus. For instance, the DNA sequence studies revealed that the noncancerous *env* sequences in the recombinant viruses were probably inherited from closely related but not identical endogenous polytropic proviruses. Thus, many of the endogenous polytropic and, perhaps, modified polytropic proviruses may have the potential to donate sequences to recombinant viruses. Also, the results indicated that the type II *env* viruses can serve as intermediates in the generation of type I *env* recombinants, a concept first proposed by Evans (6). Finally, the HRS/J gene that regulates the selection of the type I *env* recombinants is

FIG. 8. Comparison of predicted amino acid sequences for the p15E (TM) minor envelope proteins. The p15E proteins of the recombinant viruses are compared with those of the endogenous polytropic provirus MX27 (Poly) and endogenous ecotropic provirus Akv623 (Eco) (10, 30). The amino acids are represented by single letters. The dots in the sequences denote homology with the corresponding amino acid residues located directly above in the Poly sequence. Amino acid substitutions relative to the Poly sequence are indicated by the insertion of the letter symbol in the appropriate position. The absence of an amino acid in a sequence is indicated by a dash. Amino acids encoded by the envelope gene sequences that distinguish type I *env* recombinant viruses from type II are underlined. The locations of selected restriction endonuclease sites in the corresponding DNA sequence are also shown.

A novel aspect of these studies was the use of the PCR technique to amplify proviral fragments of the recombinant MuLVs. A major concern was the specificity of the oligonu-

1. **Chattopadhyay, S. K., M. W. Cloyd, D. L. Linemeyer, M. R. Lander, E. Rands, and D. R. Lowy.** 1982. Cellular origin and role of mink cell focus-forming viruses in murine thymic lymphomas. *Nature (London)* **295**:25-31.
2. **Chattopadhyay, S. K., M. R. Lander, E. Rands, and D. R. Lowy.** 1980. Structure of endogenous murine leukemia virus DNA in mouse genomes. *Proc. Natl. Acad. Sci. USA* **77**:5774-5778.
3. **Cloyd, M. W., J. W. Hartley, and W. P. Rowe.** 1980. Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. *J. Exp. Med.* **151**:542-552.
4. **Copolla, M. A., and C. Y. Thomas.** 1990. A host gene regulates

- the structure of the transmembrane envelope protein of murine leukemia viruses. *J. Exp. Med.* **171**:1739–1752.
5. Elder, J., J. Gautsch, F. Jensen, R. Lerner, J. Hartley, and W. Rowe. 1977. Biochemical evidence that MCF murine leukemia viruses are envelope (*env*) gene recombinants. *Proc. Natl. Acad. Sci. USA* **74**:4676–4680.
 6. Evans, L. H. 1986. Characterization of polytropic MuLVs from three-week old AKR/J mice. *Virology* **153**:122–136.
 7. Famulari, N. G. 1983. Murine leukemia viruses with recombinant *env* genes: a discussion of their role in leukemogenesis. *Curr. Top. Microbiol. Immunol.* **103**:76–96.
 - 7a. Frankel, W., and J. Coffin. Personal communication.
 8. Green, N., H. Hiroshi, J. H. Elder, R. S. Schwartz, R. H. Khirya, C. Y. Thomas, P. N. Tschlis, and J. M. Coffin. 1980. Expression of leukemogenic recombinant virus associated with a recessive gene in HRS/J mice. *J. Exp. Med.* **152**:249–264.
 9. Hartley, J., N. Wolford, L. Old, and W. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. USA* **74**:789–792.
 10. Herr, W. 1984. Nucleotide sequence of AKV murine leukemia virus. *J. Virol.* **49**:471–478.
 11. Herr, W., and W. Gilbert. 1983. Somatically acquired recombinant murine leukemia proviruses in thymic leukemias of AKR/J mice. *J. Virol.* **46**:70–82.
 12. Holland, C. A., J. Wozney, and N. Hopkins. 1983. Nucleotide sequence of the gp70 gene of murine retrovirus MCF 247. *J. Virol.* **47**:413–420.
 13. Horowitz, J. M., and R. Risser. 1985. Molecular and biological characterization of the endogenous ecotropic provirus of BALB/c mice. *J. Virol.* **56**:798–806.
 14. Hutchison, K. W., N. G. Copeland, and N. A. Jenkins. 1984. Dilute-coat-color locus of mice: nucleotide sequence analysis of the d^{+2J} and d^{+Ha} revertant alleles. *Mol. Cell. Biol.* **4**:2899–2904.
 15. Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee. 1981. Dilute (d) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. *Nature (London)* **293**:370–374.
 16. Karshin, W. L., L. J. Arcement, R. B. Naso, and R. B. Arlinghaus. 1977. Common precursor for Rauscher leukemia virus gp69/71, p15(E), and p12(E). *J. Virol.* **23**:787–798.
 17. Kelly, M., C. A. Holland, M. L. Lung, S. K. Chattopadhyay, D. R. Lowy, and N. H. Hopkins. 1983. Nucleotide sequence of the 3' end of MCF 247 murine leukemia virus. *J. Virol.* **45**:291–298.
 18. Khan, A. S. 1984. Nucleotide sequence analysis establishes the role of endogenous MuLV DNA segments in the formation of recombinant mink cell focus-forming murine leukemia viruses. *J. Virol.* **50**:864–871.
 19. Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine. 1984. Determination of the leukemogenicity of murine retrovirus by sequences within the long terminal repeat. *Nature (London)* **308**:467.
 20. Lenz, J., and W. A. Haseltine. 1983. Localization of the leukemogenic determinants of SL3-3, an ecotropic, XC-positive murine leukemia virus of AKR mouse origin. *J. Virol.* **47**:317–328.
 21. Lowy, D. R., E. Rands, S. K. Chattopadhyay, C. F. Garon, and G. Hager. 1980. Molecular cloning of infectious integrated murine leukemia virus DNA from infected mouse cells. *Proc. Natl. Acad. Sci. USA* **77**:614–618.
 22. Lung, M. L., J. W. Hartley, W. P. Rowe, and N. H. Hopkins. 1983. Large RNase T₁-resistant oligonucleotides encoding p15E and the U3 region of the long terminal repeat distinguish two biological classes of mink cell focus-forming type C viruses of inbred mice. *J. Virol.* **45**:274–290.
 - 22a. Massey, A. C. et al. Unpublished data.
 23. Massey, A. C., M. A. Coppola, and C. Y. Thomas. 1990. Origin of pathogenic determinants of recombinant murine leukemia viruses: analysis of *Bxv-1*-related xenotropic viruses from CWD mice. *J. Virol.* **64**:5491–5499.
 - 23a. Massey, A. C., and C. Y. Thomas. Unpublished data.
 24. O'Donnell, P. V., E. Stockert, Y. Obata, and L. J. Old. 1982. Leukemogenic properties of AKR dual tropic (MCF) viruses: amplification of murine leukemia virus-related antigens on thymocytes and acceleration of leukemia development in AKR mice. *Virology* **112**:548–563.
 25. Ott, D., and A. Rein. 1992. Basis for receptor specificity of nonectropic murine leukemia virus surface glycoprotein gp70^{su}. *J. Virol.* **66**:4632–4638.
 26. Pederson, F. S., R. L. Crowther, E. F. Hays, R. C. Nowinski, and W. A. Haseltine. 1982. Structure of retroviral RNAs produced by cell lines derived from spontaneous lymphomas of AKR mice. *J. Virol.* **41**:18–29.
 27. Rein, A., and A. Schultz. 1984. Different recombinant murine leukemia viruses use different cell surface receptors. *Virology* **136**:144–152.
 28. Rommelaere, J., D. V. Faller, and N. Hopkins. 1978. Characterization and mapping of RNase T₁-resistant oligonucleotides derived from the genomes of Akv and MCF murine leukemia viruses. *Proc. Natl. Acad. Sci. USA* **75**:495–499.
 29. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 30. Stoye, J. P., and J. M. Coffin. 1987. The four classes of endogenous murine leukemia virus: structural relationships and potential for recombination. *J. Virol.* **61**:2659–2669.
 31. Thomas, C. Y. 1986. AKR ecotropic murine leukemia virus SL3-3 forms envelope gene recombinants in vivo. *J. Virol.* **59**:23–30.
 32. Thomas, C. Y., B. J. Boykin, N. G. Famulari, and M. A. Coppola. 1986. Association of recombinant murine leukemia viruses of the class II genotype with spontaneous lymphomas in CWD mice. *J. Virol.* **58**:314–323.
 33. Thomas, C. Y., V. K. Buxton, J. S. Roberts, B. J. Boykin, and D. J. Innes. 1989. Phenotypic heterogeneity of spontaneous lymphomas of CWD mice. *Blood* **73**:240–247.
 34. Thomas, C. Y., M. A. Coppola, C. A. Holland, and A. C. Massey. 1990. Oncogenicity and U3 region sequences of class II recombinant MuLVs of CWD mice. *Virology* **176**:166–177.
 35. Thomas, C. Y., R. H. Khirya, R. S. Schwartz, and J. M. Coffin. 1984. The role of recombinant ecotropic and polytropic viruses in the development of spontaneous thymic lymphomas in HRS/J mice. *J. Virol.* **50**:397–407.
 36. Thomas, C. Y., J. S. Roberts, and V. K. Buxton. 1988. Mechanism of selection of class II recombinant murine leukemia viruses in the highly leukemic strain CWD. *J. Virol.* **62**:1158–1166.
 37. Vasmel, W. L. E., M. Zijlstra, T. Radaszkiewicz, C. J. M. Leupers, R. E. Y. de Goede, and C. J. M. Melief. 1988. Major histocompatibility complex class II-regulated immunity to murine leukemia virus protects against early T- but not late B-cell lymphomas. *J. Virol.* **62**:3156–3166.