EXPRESSION OF LEUKEMOGENIC RECOMBINANT VIRUSES ASSOCIATED WITH A RECESSIVE GENE IN HRS/J MICE*

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Recent work has suggested that recombination between retroviruses of the mouse may produce the agents responsible for leukemias and lymphomas in this species. The basis for this concept rests on several lines of evidence. In the AKR mouse, a strain in which leukemia develops spontaneously in greater than 90% of female animals over 6 mo of age (1, 2), high titers of ecotropic virus are present from birth (3). During the preleukemic period, there is increased expression of xenotropic virus, after which a new class of dual tropic viruses emerges (4, 5). These dual tropic viruses were first identified by their broad host range and cytopathic effect on mink cells. The latter property accounts for the term mink cell focus-inducing (MCF)[1] viruses. Thus far, MCF viruses have been isolated only from leukemic and preleukemic tissues, which is circumstantial evidence that they are associated with oncogenicity. More direct evidence of their tumor-causing potential has come from experiments in which injection of MCF viruses accelerated the appearance of leukemia in young AKR mice (6). The dual host range, interference, and neutralization characteristics of these MCF viruses suggested that they arose by recombination in the viral gene (*env*) coding for the envelope glycoprotein gp70. This interpretation was substantiated by tryptic peptide analysis, which showed the MCF gp70 to have components of both ecotropic and xenotropic viral gp70 (7). Oligonucleotide fingerprinting (8) and heteroduplex mapping studies (9) contributed further evidence for MCF *env* gene recombination. Viral structural proteins other than gp70 that have been analyzed all appear identical with proteins of AKR ecotropic parental virus. This suggested that alterations involving gp70 alone may be necessary to endow a nononcogenic virus with oncogenic potential.

The recently described polytropic viruses (PTV) of HRS/J mice may represent another instance in which *env* gene recombination leads to the acquisition of oncogenic potential. HRS/J inbred mice carry a mutant autosomal recessive gene (*hr*), which predisposes *hr/hr* homozygotes to thymic leukemia (10, 11). Additionally, *hr/hr* mice develop total alopecia around the age of 3–4 wk; this hairless phenotype (not to be

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[1] *Abbreviations used in this paper* ETV, ecotropic virus(es), FFU, focus-forming unit(s), MCF, mink cell focus-inducing, PFU, plaque-forming unit(s), PTV, polytropic virus(es) XTV, xenotropic virus(es)

confused with nude, an athymic mutant) readily identifies the homozygote. Another important aspect of HRS/J mice is that both homozygotes and heterozygotes express high titers of ecotropic (*N*-tropic) retroviruses from an early age. The number of ecotropic virus-producing infectious centers in lymphoid tissues of HRS/J mice, regardless of phenotype, are similar to those found in leukemia-prone AKR mice (11), yet the cumulative incidence of thymic leukemia in *hr/+* mice by 15 mo of age is less than 10%, in contrast with 50% in the homozygote. This finding clearly demonstrates that the ecotropic retrovirus of HRS/J mice cannot be the sole reason for the susceptibility of the homozygote to thymic leukemia. Further virological analysis of this model of leukemogenesis (11) disclosed that during the preleukemic period there is a marked and specific increase in the expression of xenotropic virus by thymocytes of the homozygote, an event that fails to occur in age-matched heterozygotes. During this time, and also during the leukemic phase, a third kind of retrovirus can be isolated from *hr/hr* thymocytes and thymomas. This virus differs from ecotropic and xenotropic viruses by its ability to replicate efficiently in either mouse or a variety of xenogeneic cells. We, therefore, named this class of agent polytropic virus (11).

The virological events in *hr/hr* mice thus resemble those in AKR mice. In the HRS/J strain, however, the action of a single recessive gene seems to provide conditions that favor the emergence of leukemogenic viruses. In the present experiments we show that the HRS/J PTV are *env* gene recombinants with unique oligonucleotide structures. These PTV not only accelerate leukemogenesis in HRS/J mice, but also induce thymic leukemia in the low leukemia strain CBA/J By contrast, cloned HRS/J ecotropic and xenotropic viruses have no leukemogenic action.

Materials and Methods

Mice. Breeding pairs of all strains used in these experiments were obtained from The Jackson Laboratory, Bar Harbor, Maine. The HRS/J mice referred to in Fig 1 have been maintained in our laboratory by brother-sister matings since 1977.

Virus Isolation. Ecotropic, xenotropic, and polytropic viruses were isolated from HRS/J mice as previously described (11) and cloned by limiting dilution In order to eliminate pseudotypes from PTV stocks, three cycles of limiting dilution on mink lung epithelium (CCL64) were followed by three cycles on mouse fibroblasts (NIH 3T3 or SC-1), then an additional three cycles on CCL64. All lines were grown in Eagle's minimum essential medium with 10% fetal bovine serum.

ETV-1 our prototype HRS/J ecotropic virus (ETV), was isolated from a 12-mo-old *hr/hr* mouse with thymic leukemia XTV-1, our xenotropic virus (XTV) prototype, was isolated from a nonleukemic 8-mo-old *hr/hr* mouse Two PTV prototypes were analyzed; PTV-1 was isolated from an *hr/hr* thymoma, and PTV-2 was isolated from a normal-appearing 15-mo-old *hr/+* mouse. MCF-247 was obtained from Dr. Janet Hartley.

Virus Production and Assay. Cloned virus stocks were seeded onto fresh cells and supernates harvested at hourly intervals for four to six cycles. Supernates were pooled, distributed into vials, and frozen in liquid nitrogen Virus release was measured by assaying reverse transcriptase activity (12, 13), or viral infectivity Infectious center assays of lymphoid cell suspensions were carried out as described previously (11) Immunofluorescent assays for XTV were done on CCL64 cells, and XC-positive *N*-tropic viruses were identified by an infectious center UV-XC test (14) using NIH 3T3 indicator cells. PTV were defined as XC-negative viruses that were able to infect both CCL64 and NIH 3T3 cells In the latter instance, an immunofluorescent focus-forming assay (4) was used. All titers are expressed as the number of fluorescent focus-forming units (FFU) or XC plaque-forming units (PFU)/10^7 cells (15) Where polytropic FFU are indicated, the number corresponds with the result of an assay on CCL64 cells

Virus Neutralization In vitro virus neutralization by serum was tested as described by Kane

et al. (16). Neutralization of PTV and XTV was measured by reduction of focus formation in cultured CCL64 cells; neutralization of ETV was measured by a reduction of XC plaque formation in NIH 3T3 cells. Controls included cells incubated with tissue culture medium alone

Leukemogenicity Assay. Neonatal mice (< 24-h-old) were injected with 8×10^3–5×10^4 FFU or PFU of test virus and returned to their mothers. They were then observed regularly for 6–12 mo, and killed when clinical examination revealed signs of tumor A complete autopsy (except central nervous system) was done on all mice, and grossly involved tissues were processed for histological sections by routine methods

Thy-1 2 Assay. Anti-Thy 1.2 serum (Searle Diagnostics Inc , Subsid. of G D. Searle & Co., Des Plaines, Ill.) was used in a standard trypan blue exclusion cytotoxicity assay.

gp70 Tryptic and Chymotryptic Peptide Analyses Gp70 were isolated from culture supernates by immunoaffinity chromatography, radiolabeled in solution, and immune precipitated before separation by electrophoresis in 5–17% sodium dodecyl sulfate-polyacrylamide gels (17). Bands corresponding to gp70 were sliced from the gel, digested, and analyzed by the method of Elder et al. (18, 19) Briefly, individual ^{125}I-labeled protein bands were cut from gels and washed with 10% acetic acid and then with 10% methanol until background in the wash solution was approximately 5% of the total incorporation. The proteins were then digested from the gel slice by the addition of 100 μl trypsin (1 mg/ml) or chymotrypsin (1 mg/ml) in 0.05 M NH_4HCO_3 buffer (pH 8.0) containing 5 mM dithiothreitol The slices were incubated overnight at 37°C, after which the solution was removed and lyophilized. The tryptic digests were then analyzed on two-dimensional peptide maps (18, 19). The samples were dissolved in 20 μl buffer 1 (acetic acid formic acid.water, 15 5 80), and 2–5 μl was spotted on to 10- × 10-cm cellulose-coated thin-layer chromatography plates The samples were then electrophoresed at 1 kV for ~30 min, dried, and chromatographed in a second plane using buffer II (butanol:pyridine·acetic acid·water 32 5 25 5 20) The plates were then dried and analyzed by autoradiography using Kodak RP-X-ray film

Oligonucleotide Maps. Mink lung fibroblasts infected with XTV or PTV or NIH 3T3 cells infected with ETV were labeled with 5 mCi of ^{32}P/100-mm culture dish for 4 d according to our usual protocol (20). ^{32}P-labeled 70S RNA was isolated from pelleted virus as described (20). A second cycle of density gradient centrifugation was used to remove a high background of low molecular weight RNA from the mink cell cultures. The labeled RNA was digested with RNase T_1, and the resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis (21). Isolated oligonucleotides were further digested with RNase A, and the digestion products were separated by ionophoresis in DEAE-paper (22). Oligonucleotides whose mobility and pattern of RNase A digestion products were identical with those of previous publications, were assigned the same numbers. The results of Rommelaere et al (8) were used to construct partial maps of these oligonucleotides. Other oligonucleotides were assigned new numbers, with the prefix H for those found in ETV, P for those in PTV but not ETV, and X for those unique to XTV

Results

Biological Properties of Cloned Virus Isolates from HRS/J Mice

INCIDENCE OF SPONTANEOUS THYMIC LEUKEMIA IN HRS/J MICE (FIG. 1). Neoplasms were not found in any HRS/J mouse <8 mo of age (0/36). Thereafter, the incidence of spontaneous thymic leukemia in *hr/hr* mice increased, and by the age of 15 mo, it was 50%. In contrast, less than 10% of *hr/+* heterozygotes had thymic leukemia by that age A relatively small proportion of extrathymic lymphomas, usually reticulum cell sarcomas of either spleen or mesenteric lymph node, was found in *hr/+* mice aged 18–24 mo (10/65).

IN VIVO TROPISM OF PTV. In order to determine organ distribution after infection, PTV-1 was injected intraperitoneally into newborn HRS/J mice at a dose of 8×10^3 FFU/mouse. Groups of animals were killed 5, 10, and 12 wk later, and their thymus,

FIG. 1 Incidence of spontaneous thymic lymphomas in HRS/J mice ☐, *hr/+*, ■, *hr/hr*

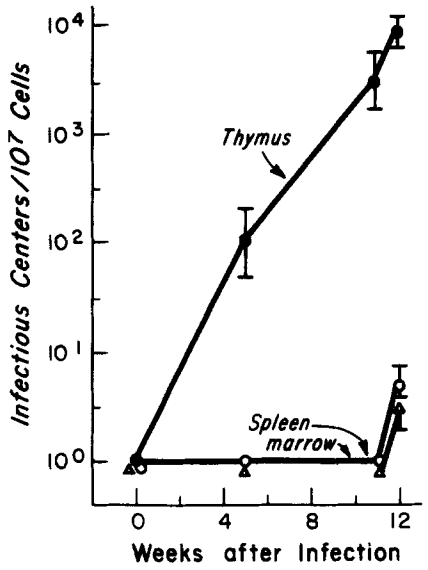

FIG 2 Organ distribution of mink-tropic virus after infection of newborn HRS/J mice with PTV-1 Vertical bars, ± SE

spleen, and bone marrow cells were assayed for infectious virus on mink lung fibroblasts. Mink-tropic virus was undetectable in the organs of age-matched control HRS/J mice that were injected with tissue culture medium. In the experimental mice, infectious mink-tropic virus was confined almost exclusively to the thymus (Fig. 2) The numbers of infectious centers in thymuses of *hr/hr* and *hr/+* mice were not significantly different ($P > 0.3$), and the values in Fig. 2 are pooled results from both phenotypes. 12 wk after infection, small amounts of virus were found in spleen and bone marrow cells of both *hr/hr* and *hr/+* mice, perhaps because of spread of virus or virus-infected thymocytes from the thymus.

The in vivo tropism of PTV-1 was compared with that of ETV-1 in CBA/J mice, a strain which does not spontaneously express ETV. 12 CBA mice injected at birth with tissue culture medium had no detectable mouse-tropic or mink-tropic viruses in thymus, spleen, or bone marrow when tested 1 mo later. 4 wk after neonatal infection

with ETV-1, XC-positive mouse-tropic virus was found in substantial amounts in thymus, spleen, and marrow cells (Fig. 3). By contrast, neonatal infection with PTV-1 resulted in preferential infection of thymocytes by mink-tropic virus. Comparable numbers of infectious centers of mink-tropic virus were found in thymuses of CBA/J and HRS/J mice 4 or 5 wk after neonatal infection ($10^{2.5}$ and $10^{3.0}$ FFU/10^7 thymocytes, respectively).

EFFECT OF NORMAL MOUSE SERUM ON THE INFECTIVITY OF PTV, ETV, AND XTV. Normal mouse serum contains a lipoprotein that neutralizes XTV, but not ETV (16). In order to determine if normal mouse serum affects PTV, five cloned isolates were tested for their susceptibility to neutralization by HRS/J serum. Heat-inactivated pooled serum from 6-wk-old HRS/J mice neutralized 50% of the in vitro infectivity of PTV-1, XTV-1, and MCF-247 at dilutions of $10^{4.4}$, $10^{4.3}$, and $10^{3.3}$, respectively. By contrast, no neutralization of either ETV-1 or an AKR ETV was found (Fig. 4). Thus, PTV-1 and PTV-2 contain an envelope determinant similar to that of XTV that is sensitive to normal mouse serum. The HRS/J serum we tested did not contain antiviral antibodies that could be detected by a double-antibody radioimmunoprecipitation assay (data not shown). Similar neutralizing activity was found in samples of AKR, C57BL/6, SWR, and NZB serum; in each case, PTV-1, XTV-1, and MCF-247 were readily distinguishable from the two ecotropic isolates (data not shown). The 50% neutralizing titer against PTV-1 in serum of 6-wk-old *hr/hr* mice was virtually identical with that found in serum of 6-wk-old *hr/+* mice ($10^{4.4}$ and $10^{4.6}$, respectively); the titers in 8-mo-old *hr/+* and *hr/hr* mice were $10^{4.2}$ and $10^{4.5}$, respectively. The susceptibility of the homozygote to leukemia, therefore, cannot be explained by a deficiency of a serum factor that neutralizes XTV and PTV.

ACCELERATION OF LEUKEMOGENESIS IN HRS/J MICE BY PTV. PTV-1, PTV-2, ETV-1, or XTV-1 were inoculated intraperitoneally into newborn HRS/J mice in a dose of 8×10^3–5×10^4 FFU or PFU. The mice were sacrificed either when external examination disclosed evidence of a tumor or by the age of 6 mo. PTV-1 is a highly active agent, and within 6 mo 60% of the mice inoculated with this virus had histologically confirmed leukemia or lymphoma (Fig. 5). HRS/J homozygotes and heterozygotes were equally susceptible; 13/22 of the former and 22/29 of the latter

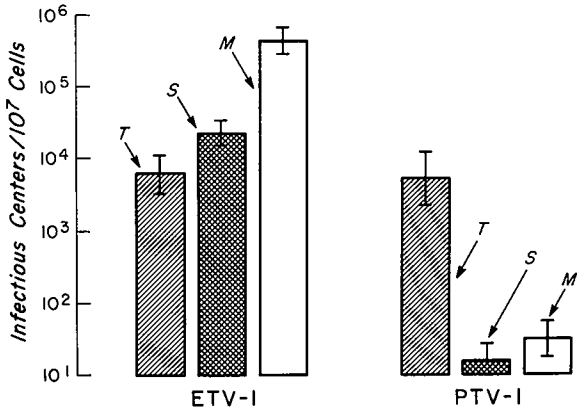

FIG 3 Organ distribution of virus 4 wk after infection of newborn CBA/J mice with ETV-1 or PTV-1. T, thymus; S, spleen; M, marrow. Vertical bars, ± SE.

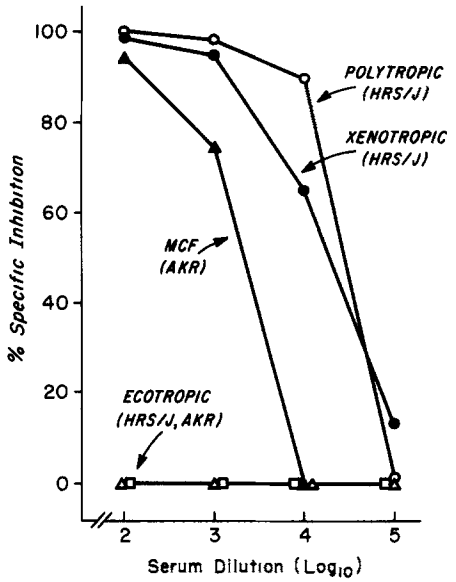

FIG 4 Neutralization of different viruses by normal mouse serum

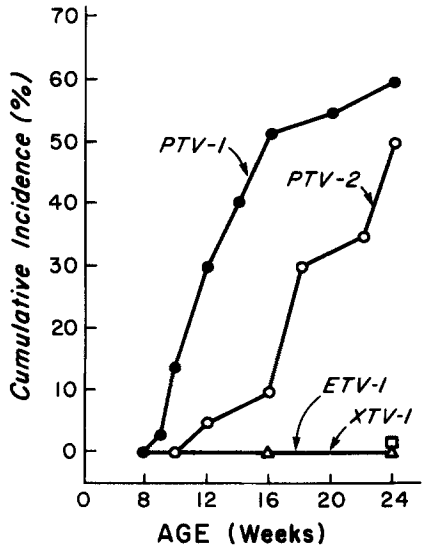

FIG 5 Development of thymic leukemia in HRS/J mice after neonatal injection of either PTV-1, PTV-2, ETV-1, or XTV-1

developed leukemia within 6 mo ($\chi^2 = 1.63$, $P > 0.1$). In this group of 51 mice, females were more susceptible than males (25/29 vs. 10/22; $\chi^2 = 9.65$, $P < 0.01$). Tumors involved the thymus grossly in two-thirds of the mice, and in most of these instances the spleen and lymph nodes were also involved. Histologically, the neoplasms consisted of sheets of lymphoblasts. 11 neoplasms were tested for the presence of the Thy-1.2 antigen, and all were positive. Of 10 HRS/J mice injected with PTV-1 when 4 wk

old, only 2 developed leukemia. The difference between this group and those injected with PTV-1 at birth is highly significant ($\chi^2 = 8.28$, $P < 0.01$).

PTV-2 was also effective in accelerating the appearance of thymic leukemia; 17/34 HRS/J mice injected at birth developed leukemia (4/17 had the *hr/hr* phenotype, and 13/17 were heterozygotes). All the tumors involved the thymus, either grossly or histologically; enormous enlargement of a tumor-filled spleen was the rule.

24 of the tumors (15 PTV-1 and 9 PTV-2) were tested virologically, and in each case high titers (range, 10^4–10^6 FFU/10^7 cells) of a mink-tropic virus were detected. The mink cell assay does not discriminate among PTV, XTV, and phenotypically mixed viruses capable of infecting mink cells; therefore, representative supernate from seven virus-positive mink cell cultures were purified by three cycles of limiting dilutions on mink cells followed by three cycles on mouse cells (Materials and Methods). Typical PTV were isolated by this means from each of the seven supernate. The RNA from an isolate obtained from a PTV-1-induced tumor was examined after co-cultivation of the tumor with mink cells but without subsequent purification. By this test at least two viruses were detected. One of these could be identified as a xenotropic-like virus, but the second, and major, virus species was identical with PTV-1. Thus, the originally injected virus could be recovered from the tumor, albeit as a mixture with a lesser amount of an endogenous XTV.

By contrast with the foregoing, ETV-1 failed to cause the appearance of any neoplasms in 15 neonatally injected HRS/J mice. Likewise, 0/11 HRS/J mice injected at birth with XTV-1 developed a tumor within 6 mo

INDUCTION OF LEUKEMIA IN CBA/J MICE BY PTV. CBA/J mice are relatively long-lived (mean life-span ~20–25 mo), and the incidence of lymphoma in this strain is low (6% in males, 15% in females) and late appearing (23). We have never observed a thymic lymphoma in our own colony of these animals. Therefore, it is noteworthy that 8/25 (32%) CBA/J mice inoculated at birth with PTV-1 and 3/19 (16%) injected with PTV-2 developed thymic leukemia within 8 mo (Table I). The tumor involved the thymus, and in all cases there was extrathymic dissemination, with huge, tumor-loaded spleens and lymph nodes. Microscopically, the CBA/J neoplasms were identical with those found in PTV-inoculated HRS/J mice. Virus with a polytropic host range was identified in and recovered from all eight tumors. In none of these instances was it possible to detect an XC-positive ETV.

TABLE I

*Incidence of Leukemia after Neonatal Injection of Cloned Viruses into Different Strains of Mice and Rats**

Strain	PTV-1	PTV-2	ETV-1	XTV-1
HRS/J	35/51	17/34	0/15	0/11
CBA/J	8/25	3/19	0/31	0/11
SWR/J	0/5	—	0/24	—
NIH/Swiss	0/32	0/17	0/15	0/10
CDF_1	0/37	—	—	—
W/Fu	0/18	0/16	—	—

HRS/J mice were observed for up to 6 mo, the other strains were held for 12 mo Numerator, number of leukemic mice, denominator, total number of mice injected, —, not tested

* CDF_1 and W/Fu

By contrast with the results in CBA/J mice, NIH/Swiss and SWR/J mice, which also had low spontaneous leukemia incidence, failed to develop neoplasms after neonatal injection with PTV-1 or PTV-2 (Table I). In addition, none of the neonatally infected CDF_1 and W/Fu rats developed tumors (Table I). ETV-1 and XTV-1 were not leukemogenic in any of the aforementioned mouse strains (observation was for up to 12 mo after neonatal inoculation). Autopsy of 1 of the 29 SWR/J mice revealed a uterine neoplasm.

Peptide Analyses of gp70 of Cloned Virus Isolates from HRS/J Mice. The tryptic and chymotryptic gp70 maps of ETV-1, XTV-1, PTV-1, and PTV-2 are shown in Fig. 6. ETV-1 possessed a gp70 typical of endogenous ecotropic murine retroviruses (18). The maps of XTV-1 gp70 are characteristic of other endogenous xenotropic murine retroviruses, as exemplified by the AKR xenotrope (18). gp70 isolated from four different HRS/J XTV were analyzed, and all had the pattern of XTV-1 (data not shown).

Analyses of PTV-1 and PTV-2 revealed that the gp70 of these viruses contained some, but not all, of the peptides characteristic of both the ecotropic and xenotropic HRS/J isolates. Furthermore, the gp70 of these two PTV were distinctly different. Specifically, the tryptic peptide maps of both PTV gp70 revealed the presence of the xenotropic spot (Fig. 6 B and C, brackets), a peptide that is the hallmark of xenotropic murine retroviruses (Fig. 6 D, brackets), and that is never seen in maps of ETV gp70. The chymotryptic map of PTV-1 gp70 contained a formation distinctive of the HRS/J- and AKV-2-type ETV (Fig. 6 E, F, arrows); these peptides differed considerably from the analogous xenotropic peptides. In contrast, the chymotryptic map of PTV-2 gp70 (Fig. 6 G) closely resembled that of XTV and did not contain the five-spot ecotropic formation of PTV-1. Tryptic and chymotryptic maps of both PTV-1 and PTV-2 also contained some peptides shared by ecotropic and xenotropic gp70. Other spots characteristic of ecotropic and xenotropic gp70 were not present, substan-

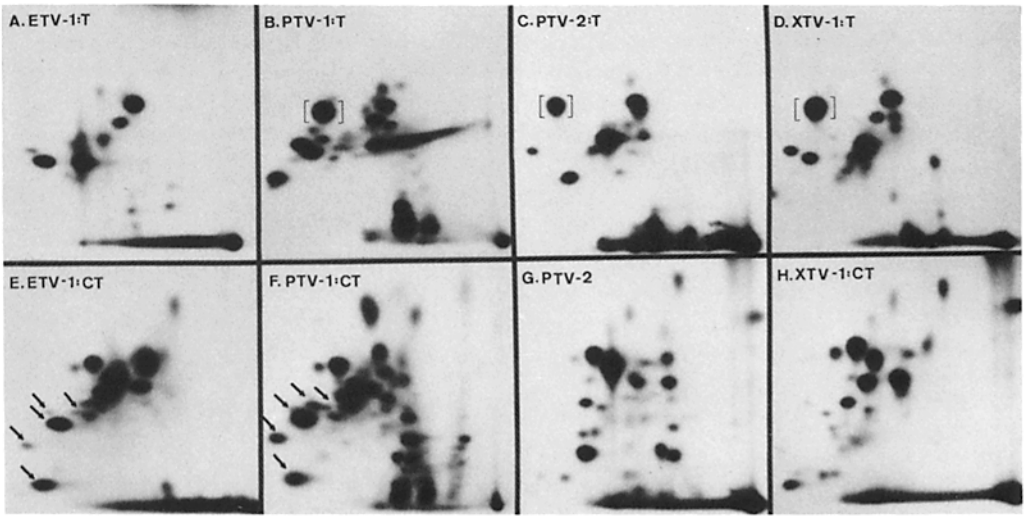

FIG 6. Peptide analyses of ^{125}I-labeled gp70 isolated from viruses of HRS/J mice A–D, tryptic peptide maps of gp70 from ETV-1, PTV-1, PTV-2, and XTV-1, respectively E–H, chymotryptic peptide maps of gp70 from ETV-1, PTV-1, PTV-2, and XTV-1, respectively

tiating that the PTV isolates were recombinants rather than mixtures of ETV and XTV. Unique PTV peptides not found in either ecotropic or xenotropic HRS/J viruses were evident, particularly in the lower right quadrant of the PTV-1 and PTV-2 chymotrypsin maps.

Genomes of Cloned Viruses Isolated from HRS/J Mice. High-molecular-weight RNA of the viruses isolated from HRS/J mice was analyzed by T_1-oligonucleotide fingerprinting. Fingerprints of the genomes of one ecotropic (ETV-1), two polytropic (PTV-1 and PTV-2), and three xenotropic viruses (XTV-1, XTV-2, XTV-3) are shown in Fig. 7. Using previously published fingerprints and oligonucleotide maps as guides, partial oligonucleotide maps from the six viruses were constructed as shown in Fig. 8. Unmapped oligonucleotides are tabulated in the lower portion of Fig. 8. Oligonucleotides are numbered according to the convention of Rommelaere et al. (8), and the assignments were confirmed by RNase A digestion of each oligonucleotide.

The genome of ETV-1 closely resembled those of ecotropic AKR viruses (AKV-1 and AKV-2) and *N*-tropic BALB/c isolates (24, 25). Like the BALB/c virus genome, it lacked the AKV oligonucleotides 2 and 12 and contained the 3′-proximal oligonucleotide 106 not found in AKV-1 and -2. The ETV-1 genome contained four unmapped oligonucleotides (119, 123, H1, and H3) absent from both AKR and BALB/c viruses. Oligonucleotides 106, 119, and 123 have previously been identified in AKR PTV. Oligonucleotides H1 and H3 appear to be unique to the HRS/J viruses.

The two PTV (PTV-1 and PTV-2) were distinct from the ETV as well as from each other. Some differences were found in all portions of the genome. The only feature common to both PTV isolates was the replacement of a set of ecotropic oligonucleotides presumably within *env* (26). Five oligonucleotides (102, 104, 113, B1, and 111) were found in this region in the PTV. Three of these (102, 104, and 113) were shared by both viruses. It is probable that this shared region accounts for the novel host range. This substitution pattern, beginning at oligonucleotide position 20 ± 1, is similar to previously mapped AKR and HIX PTV (8, 26). In particular, oligonucleotide 104 is present in all published polytropic viral fingerprints. Seven more unmapped and unshared oligonucleotides (123a, 128, 129, P1, P7, P20, and P36) were present in the genome of the two PTV. Of the 12 PTV-specific oligonucleotides, 6 were also found in one or more of the XTV genomes. The other six, including three (102, 111, and 113) mapping within the proposed *env* gene, appear to be unique to PTV-1 and PTV-2.

Examination of the fingerprints of the three XTV showed that they were closely related, yet unique. XTV-1 and XTV-2 contained several oligonucleotides present in less than molar yield indicating that they were mixtures of more than one species. The three XTV shared many oligonucleotides with the ETV particularly in the 5′-half of the genome. Interestingly, oligonucleotide 39, which is specifically associated with *N*-tropism in several strains of virus (27), was present in the XTV isolates, suggesting that these XTV carried the *N*-tropic allele. The right half of the genomes of XTV-1, -2, and -3, including the entire *env* region, was almost completely different from the ETV genome, with the exception of oligonucleotide 9 in XTV-1 and 106 in XTV-3.

Of the envelope-related oligonucleotides, number 104 was present in all three viruses (and in all PTV as noted above), whereas B-1 (also found in the BALB/c *B-*

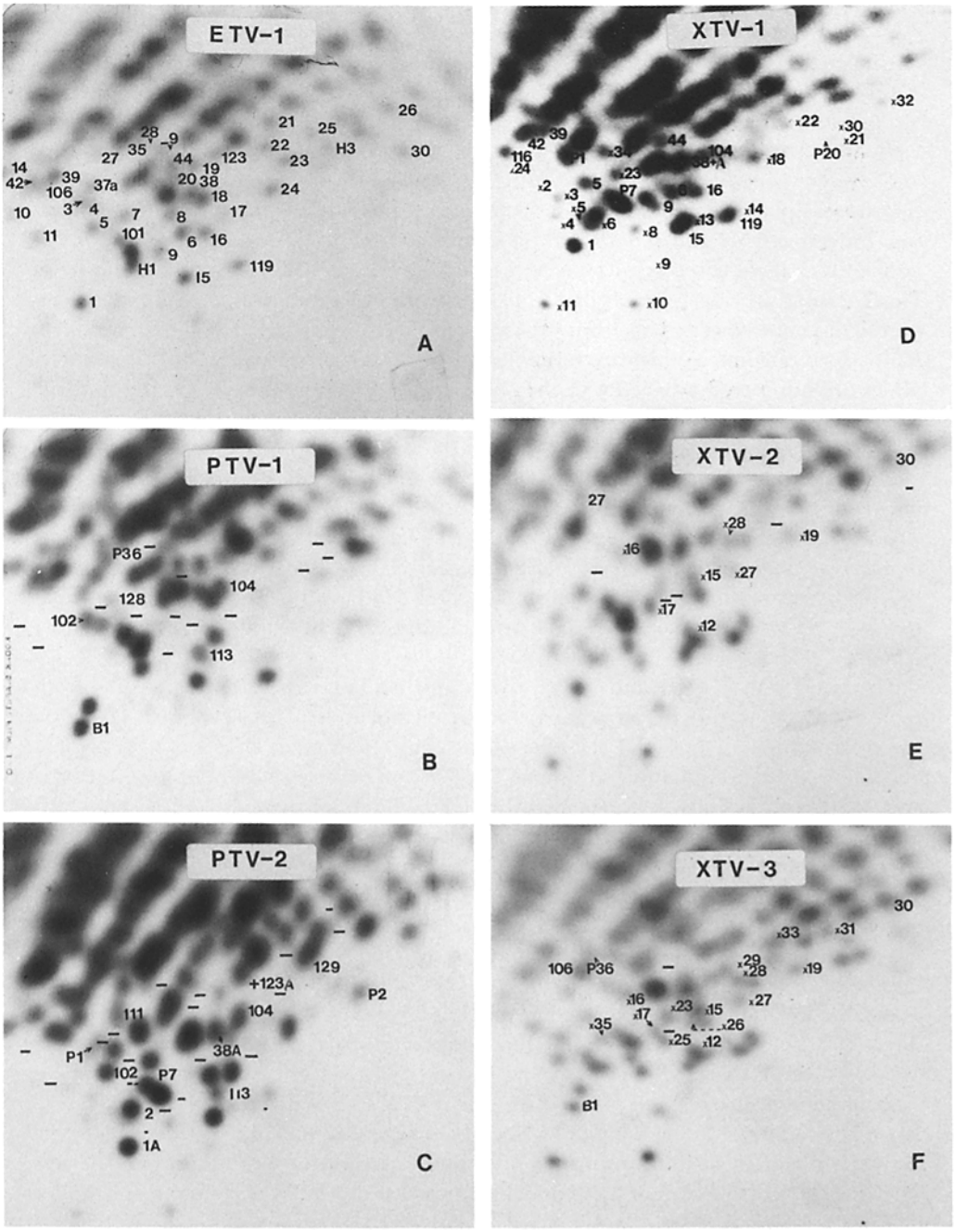

FIG 7 T_1 oligonucleotide fingerprints of HRS/J virus genomes All viruses were grown on mink cells except for ETV-1, which was grown on NIH 3T3 cells 70S RNA was labeled with ^{32}P, digested with RNase T_1, and analyzed by two-dimensional gel electrophoresis (A) ETV-1 Each oligonucleotide is numbered immediately to its right (B) and (C) PTV-1 and PTV-2 Only oligonucleotides not present in the ETV-1 genome are numbered The absence of ETV-1-specific oligonucleotides is indicated by (–) (D) XTV-1 (E) and (F) XTV-2 and XTV-3, numbered by comparison with XTV-1, as on (B) and (C) The contaminating XTV oligonucleotides seen in PTV-2 RNA were absent from fingerprints prepared after additional passages of the virus on NIH 3T3 cells (data not shown)

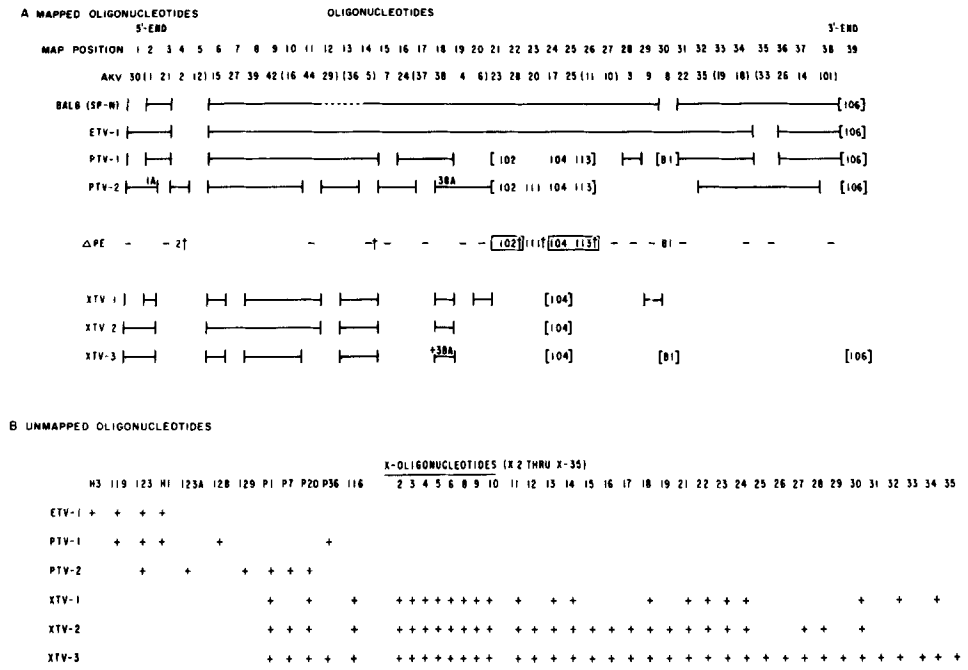

FIG 8 (A) Oligonucleotide maps of HRS/J virus genomes The viral RNA is represented in linear form with the 3'-end to the right Oligonucleotide numbers and map locations were assigned by comparison with AKR and BALB/c viruses, according to Rommelaere et al (8) as explained in Materials and Methods. The presence of an oligonucleotide in each virus, also found in AKV, is indicated by an uninterrupted solid line directly below the corresponding AKV position number Dotted lines show probable but unconfirmed shared oligonucleotides Absence of lines between vertical bars represents substitution or deletion of the appropriate oligonucleotide Bracketed numbers identify the approximate map location of substituting oligonucleotides ΔPE is a partial genomic map of a hypothetical parent that upon recombination with ETV-1 could yield the PTV In this map, dashes (−) represent the absence of the corresponding oligonucleotide Boxed numbers are common to PTV-1 and PTV-2, and those symbols marked with (†) identify genomic characteristics unique to the PTV and the hypothetical parent (B) Unmapped oligonucleotides of the HRS/J viruses. The presence in each virus of an oligonucleotide is shown by a (+) We have assigned numbers to oligonucleotides 128, 129, and those prefixed with P or X P-oligonucleotides were found in PTV and XTV genomes, but not in the ETV X-oligonucleotides were unique to the XTV genomes.

tropic isolate LP-B [25]) was present only in XTV-3. In addition, many unmapped oligonucleotides were found to belong to one, two, or three of the viruses. Some of these (P-1, P-7, P-20, and P-36) were also present in PTV-1 or PTV-2.

Discussion

A striking aspect of spontaneous thymic leukemia in the mouse is the variety of retroviruses that can be isolated from preleukemic and leukemic cells. Included among these agents are XTV (4), and PTV (11), as well as XC-positive (3), XC-negative (28), and replication-defective (29) ETV. Some of these viruses, such as certain isolates of AKR XC-negative virus and PTV, are highly leukemogenic (6, 30), whereas others, including AKR XC-positive ETV, are not (6, 30). Exactly how these numerous variants relate to each other in the pathogenesis of leukemia is far from settled. Nevertheless, the idea that leukemogenic recombinants originate from nonleukemo-

genic precursors is an appealing one and permits the formulation of testable hypotheses about the pathogenesis of leukemia in the mouse.

The HRS/J strain is a particularly attractive system for studies of the generation of viral diversity and its role in leukemogenesis because a single recessive allele influences both the appearance of PTV and the development of leukemia. Moreover, there is increasing evidence that links this mutant allele to defective differentiation of thymocytes during the preleukemic period (31). A major goal of our studies is to determine whether the abnormality in thymocyte differentiation is in turn linked to the generation of retroviral diversity. With this in mind, we have analyzed the properties of the three major types of retroviruses isolated from HRS/J mice.

The close similarities between the genomes and gp70 of HRS/J, AKR, and BALB/c ETV indicate that the ETV loci are limited in number and that genetic drift has been slight between strains. Recently we examined another ETV from an HRS/J mouse, and compared with ETV-1, the oligonucleotide fingerprint of the genome of this virus is even more similar to that of the AKR ETV (data not shown). Much of the variation among ETV may therefore arise during their growth in vivo. By contrast, the three XTV shown here are genetically distinct. This finding, as well as the presence of viral mixtures in XTV-1 and XTV-2, suggests that these viruses are the products of multiple genetic loci. The great variability in tryptic and chymotryptic peptide analyses of xenotropic envelope proteins from various mouse strains (18) supports this conclusion, as do the results of DNA blotting experiments which showed the presence of large numbers of endogenous virus-related sequences in cellular DNA (32).

Presumably representative clones of both ETV and XTV HRS/J did not induce or accelerate the appearance of leukemia after injection into CBA/J or HRS/J mice. By contrast, both polytropic isolates were highly leukemogenic. These latter viruses, moreover, had the biological and biochemical attributes of recombinants derived from ecotropic and xenotropic precursors. Taken together, these results indicate that genetic recombination between nonleukemogenic viruses can give rise to a virus with newly acquired leukemogenic properties. This interpretation rests on certain inferences, however, because the experimental system does not permit unequivocal identification of the parents that generated a particular recombinant retrovirus.

Indirect evidence that PTV-1 and PTV-2 are recombinants is their host range as well as the presence of nonecotropic functional envelope determinants. In vitro infectivity of the PTV was neutralized by normal serum, an attribute of XTV that is not shared with ETV. The normal serum neutralizer is a lipoprotein (16) that presumably inhibits infection by interaction with a structure on the virus envelope This hypothetical structure, lacking on ETV, is probably shared by XTV and PTV

The tryptic and chymotryptic peptide maps presented here provide direct structural evidence that the PTV of HRS/J mice are *env* gene recombinants. The gp70 of PTV-1 consists of peptides characteristic of both HRS/J ETV and XTV, with an overall pattern similar to that of the AKR MCF viruses (7). As in the MCF viruses, recombinant-specific peptides not present in either the ecotropic or xenotropic parental viruses are evident in PTV-1 and PTV-2 These distinctive spots indicate unique structural markers, but the relationship, if any, that they may have with functional properties of the viruses is unknown. Two additional polytropic isolates that possess a gp70 structure similar to PTV-1 have been studied (data not shown). All three of

these isolates were obtained from *hr/hr* preleukemic or leukemic tissue, which suggests the existence of a class of HRS/J PTV that is analogous, both in terms of source (preleukemic or leukemia tissue only) and gp70 structure, to the family of AKR MCF viruses.

A second class of HRS/J PTV may be exemplified by our PTV-2 isolate. Its ecotropic gp70 component is not prominent and appears to be confined to spots which are shared by both ETV and XTV. This finding is confirmed by the genomic maps, which demonstrate a greater substitution of *env*-related ecotropic oligonucleotides in PTV-2 as compared with PTV-1. PTV-2 also has a different history from PTV-1 family in that it was isolated from normal (*hr/+*) tissue. Nevertheless, both viruses are leukemogenic. Why was the animal that harbored PTV-2 not leukemic? One possibility is that this mouse was one of the exceptional preleukemic *hr/+* animals.

The genomes of PTV-1 and PTV-2 differed substantially from ETV-1 and from one another. Although both genomes contain ecotropic- and xenotropic-related oligonucleotides, they share only one distinctive feature, namely, replacement of the set of ecotropic oligonucleotides at position 21–27, presumably lying within the *env* gene. Genomic maps of AKR MCF and HIX viruses show a virtually identical area of alteration as found in PTV-1 and PTV-2. Thus, this region is implicated in the altered host range of these viruses.

12 new nonecotropic oligonucleotides were found in the PTV isolates, but only 6 could be identified in the XTV genomes. Moreover, of the five oligonucleotides previously mapped within the *env* gene, only two could be identified in the XTV isolates. One of them (104) was present in all the PTV we and others (8, 26, 33) have examined, as well as in all three of our xenotropic isolates. Our inability to account for all the PTV oligonucleotides and peptides in the genomes and gp70 of the XTV and ETV suggests the existence of still other endogenous viral genomes that contain the unaccounted for polytropic-specific oligonucleotides and peptides. Therefore, there is no formal evidence that the nonecotropic parent has a xenotropic host range, and we may consider the possibility that one of the parental viruses is a defective PTV. This hypothetical virus, however, may be closely related to the XTV, as indicated by the presence of oligonucleotide 104 and some xenotropic envelope-specific tryptic and chymotryptic peptides.

PTV from AKR and HRS/J mice are not the only replication competent retroviruses in which *env* gene recombination has been associated with oncogenicity. HIX virus, an oncogenic recombinant found in many Moloney leukemia stocks (34–36), radiation-induced leukemia virus of C57BL/Ka mice (37), and an MCF virus isolated from a BALB/c myeloma have all been shown to possess recombinant gp70 with both ecotropic and xenotropic characteristics (J. H. Elder. Manuscript in preparation.). As in the AKR agent, other proteins of these viruses appear identical with normal ETV components. Devare et al. reported similar findings in a study of structural changes associated with the acquisition of oncogenicity by retroviruses of the C3H mouse (38). Using a competition radioimmunoassay, they showed that oncogenic viruses generated by in vivo or in vitro passage of an iododeoxyuridine-induced C3H ETV were all characterized by the expression of gp70 determinants not present in the parental nononcogenic ETV. In contrast, internal structural proteins (p12 and p15) of these oncogenic viruses were identical with those of the nononcogenic parent.

Several aspects of the biological properties of PTV-1 and PTV-2 are noteworthy.

Both viruses faithfully reproduced the spontaneous Thy-1-positive leukemia of HRS/J mice, and viruses with a polytropic host range could be recovered from these tumors. In one case a PTV with an oligonucleotide map identical with the injected virus was recovered Both PTV-1 and PTV-2 were equally leukemogenic in *hr/+* and *hr/hr* HRS/J mice. This result means that the leukemia-resistant heterozygote has the target cells required for expression of the leukemogenic properties of these viruses. The difference in the incidence of spontaneous leukemia between heterozygotes and homozygotes must therefore hinge on other factors. Although PTV-1 induced leukemia in CBA/J mice, it has no such effect in SWR or NIH/Swiss mice. We have investigated these differences and found that the latter two strains are highly resistant to in vivo infection by PTV-1, whereas CBA/J mice are highly susceptible. The susceptibility to infection has been traced to a single autosomal dominant gene (R. S. Schwartz and R. H. Khiroya. Manuscript in preparation.). Another aspect of the results in CBA/J mice is that leukemia did not appear for at least 8 mo after neonatal injection of the virus; by contrast, leukemia began to develop in HRS/J mice within 2 mo. Both strains are $H\text{-}2^k$, so an *H-2*-related immune mechanism cannot account for the results. One outstanding difference between CBA/J and HRS/J mice is the high level of ETV expression in the later, but not in the former. Experiments to test the hypothesis that infection with nonleukemogenic ETV accelerates the induction of leukemia by PTV are in progress.

The two HRS/J PTV we studied were highly thymotropic in both HRS/J and CBA/J mice; two additional isolates behaved similarly (R. S. Schwartz and R. H. Khiroya. Unpublished results.). This suggests that thymotropism may be a common feature of this type of virus By contrast, ETV-1, when tested in virus-free CBA/N mice, infected thymus, spleen, and bone marrow to about the same extent. The only consistent difference between the genomes of the two leukemogenic PTV and ETV-1 is in the *env* gene. This difference probably accounts for the difference in tropism between the two viruses, and especially for the highly selective thymotropism of PTV. Conceivably, the distinctive thymotropism of these agents permits their dissemination among a relatively large number of susceptible target cells, thereby increasing the probability of malignant transformation of thymocytes.

Summary

HRS/J inbred mice carry a mutant autosomal recessive gene (*hr*), which in homozygotes coincides with susceptibility to spontaneous thymic leukemia. Unlike their heterozygote (*hr/+*) littermates, *hr/hr* homozygotes express high levels of xenotropic virus during the preleukemic period, and viruses with a broadened host range (termed polytropic viruses) can be isolated from their preleukemic and leukemic tissues. Because *hr/hr* and *hr/+* mice are otherwise genetically identical, the virological differences between them support the role of polytropic viruses in the generation of thymic leukemia. In the present report we show that the HRS/J polytropic viruses are *env* gene recombinants with unique oligonucleotide and peptide maps. These polytropic viruses appear to arise by recombination between ecotropic virus and an unidentified genome related, but not identical to, the endogenous xenotropic viruses. Moreover, polytropic viruses not only accelerate leukemogenesis in HRS/J mice, but also induce thymic leukemia in the low leukemia strain CBA/J. By contrast, cloned ecotropic and xenotropic viruses have no leukemogenic action

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