ENDOGENOUS MURINE LEUKEMIA VIRUSES

Germline transmission and involvement in generation of recombinant viruses

Wim Quint

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Voor Ans

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Chapter 1

ENDOGENOUS VIRUSES AND LEUKEMOGENESIS

1. Introduction

The induction of leukemia in mice by a cell-free filtrate as dicovered by Gross(1951), from the high leukemic AKR mouse strain, has been an indication for the viral etiology of the disease (29). The origin of these viruses and their role in the development of malignancies is being investigated now in many laboratories (102).

The RNA tumor viruses (Oncovirinae) are one of the three subfamilies of the Retroviridae (22). On the base of their morphology, as revealed by electron microscopy, retroviruses can also be classified into five categories, so-called A- , B- , C- , D- and E-type particles (for review: 30, 110). Their inclusion into a single family is defined by four major characteristics (22): the architecture of the virion, a diploid single-stranded RNA genome, the presence of reverse transcription in virions and the requirement for a DNA intermediate in viral replication. All these parameters are reviewed in detail elsewere (111) and only some general properties will be described below.

The genomic RNA of the virus is a dimer complex composed of two identical single stranded RNA subunits of 6-10 kilobases long. Each of these RNAs resemble an eukaryotic mRNA with at the 5'end a methylated cap structure and a polyA tract at the 3' terminus. The replication competent genome harbors three genes encoding the structural proteins: "gag" codes for the core proteins of the viral particle, "pol" codes for the reverse transcriptase, and "env" gene codes for envelope proteins. The order of these three genes is invariable in all retroviruses: 5'-gag-pol-env-3' . A number of the acute transforming retroviruses have a fourth gene ,"onc", that occurs in addition to, or instead of, some of the viral structural genes and is involved in transformation (111). After absorption of the virion to specific cell receptors (18), synthesis of linear retroviral DNA from a template of genomic RNA is the first step in the virus life cycle. An important structural feature of this linear DNA genome is the appearance of a Long Terminal Repeat (LTR) at both termini (48). They are generated

during the process of reverse transcription and comprise sequences from the 5' and 3'end of viral RNA (27).More specifically, the LTR contains base pairs from a region unique to the 3' terminus (the U3 sequence), a repeat sequence (R) present at both ends of the viral RNA, and sequences from a region unique to the 5' terminus (the U5 sequence).The lengths of U3 and U5 sequences vary among retroviruses (111). In the nucleus, the linear DNA, is closed to a circular form (97) which comprises one or two LTRs (50, 109). Integration into the host DNA occurs colinearly, without disturbing the linear order of the virus genes. The integrated proviral genome has a uniform structure in which the coding region is flanked by two identical LTRs (91). Such a provirus is integrated in a stable manner and its gene expression and replication is in a manner similar to those of cellular genes (5). When integrated in germline cells these proviruses, now called endogenous, are transmitted as Mendelian genes. This is in contrast with the exogenous viruses, which are transmitted horizontally from one animal to another and only infect specific target cells. The genomes of endogenous viruses are found in multiple DNA copies in many vertebrate species. The endogenous viruses analyzed in most detail in the past decade are those of the chicken and the mouse. Most of the laboratory mice contain at least four types of endogenous viral loci: proviruses of the C-type and B-type viruses, A-type particles (15) and VL30 RNAs (53). The latter two types have been related to viruses in various ways but have not been shown to form infectious virus particles. The presence of loci for both theC-type(Murine Leukemia Viruses (MuLVs)) and B-type(Mouse Mammary Tumor virus (MMTV)) viruses in the AKR and GR mouse strain, respectively, has been related to the development of malignancies.

Thymic lymphomagenesis in AKR mice involves several stages of progression (34). The viral factors affecting AKR lymphomagenesis have been studied intensively and is the main subject of this thesis.

2. Endogenous Viruses

Endogenous viral genes are retroviral sequences present in the chromosomes of normal uninfected cells. For the mouse estimations run

as high as 0.3% of the total genome (44). The first convincing biochemical evidence for the existence of endogenous murine leukemia viruses as inherited viral genomes was reported by Rowe and Hartley (85). They found that a new genetic element related to MuLV was activated spontaneously at low frequency in AKR mouse cells. In addition, Lowy et al. (67) reported efficient activation of MuLVs by 5-iododeoxyuridine and 5-bromodeoxyuridine in cells which gave previously no detectable expression of viral antigens . Endogenous viral genes have now been described in species as diverse as reptiles, birds and mammals (111, 43).

These viruses share a number of characteristic properties.

- They are present in all cells of an organism (somatic and germline) and are transmitted vertically as Mendelian genes. Segregation studies of endogenous proviruses in crosses have revealed that they behave like stable Mendelian genes. The endogenous viruses can be mapped for instance to specific linkage groups (39).
- Endogenous proviruses have grossly the same structure. Although frequently defective they often retain genes that are completely intact. The linear proviral DNA is flanked by direct repeats (LTRs)(99). Each of these LTRs contains a small inverted repeat (3-12bp) which in addition to three to seven adjacent nucleotides is required for integration into the host genome (80). A short direct repeat (4-6bp) of cellular DNA is generated upon integration, during which at a distinct site the viral DNA is joined to a nonhomologous site in host cellular DNA (98). The structure resembles those of transposable elements found in procaryotes, yeast and Drosophila (98). Like transposable elements, retroviruses can also act as insertion mutagens and affect expression of cellular genes. This can either lead to enhanced expression of genes under the influence of the LTR or to physical disruption of cellular genes (37, 106).
-The endogenous viruses are usually present in multiple, non-identical copies. The number and chromosomal distribution of endogenous proviruses vary greatly among species. Species barriers can be crossed by horizontal infection and subsequent integration into germline cells. For example, nucleic acid homology and immunological relatedness of viral proteins indicated that RD114, an endogenous primate virus, and a second virus related to a rat endogenous virus has infected ances-

tors of the domestic cat (3, 101).

- The wide distribution among so many different species raised the question about their possible involvement in essential physiological functions, or in development and differentiation. A support for this view was the expression of endogenous virus antigens during early development and in some tissues of the adult (44). However, Astrin et al.(2) were able to breed a fertile chicken which lacked endogenous viral sequences and still seemed to be completely healthy. Similarly, strains of mice with no endogenous MMTV or ecotropic MuLV (chapters 2 and 3) remain completely healthy relative to virus positive animals. Furthermore, the pattern of tissue-specific expression of viral gene products revealed to be mouse strain specific. It seems unlikely, therefore, that endogenous viral genes mediate a direct functional role in normal differentiation processes. However, the unusual susceptibility of chickens free of functional endogenous viruses to the infection with exogenous retroviruses may indicate a more subtle role for these viruses in conferring a protection against pathogenesis by exogenous viruses (84).

- The expression of endogenous viruses is phenotypic complex and varies greatly from that found in exogenously infected cells. Besides the spontaneous production of infectious virus in some cases only a portion of the endogenous genome (particularly gag and env gene) is expressed (36). Expression of part of the viral genome could be the result of mutation in the proviral genes or be caused by host regulatory factors. In other cases, the presence of complete proviruses could only be demonstrated by activation with a wide range of inducing agents (111). Structural alterations of the endogenous LTRs might affect the expression of these proviruses as a result of their role in modulating the synthesis and processing of viral mRNAs (55). Furthermore the transcription of proviruses is subjected to controls similar to those of other genes (44). The correlation which can be made between chromatine structure, methylation and expression of endogenous retroviral genomes is in agreement with this hypothesis (32, 96).

- The susceptibility of tissue culture cells, or spreading in the animal of the virus is an important feature affecting infectious virus production from inherited proviruses. Loci which determine this sus-

ceptibility have been described in chickens and mice (111). For instance the ability of both ecotropic MuLV and MCF-MuLV viruses to spread in mouse cells (or mice) is determined by the Fv-1 locus (42, 56, 87, 94). It has recently been shown that only the difference in two consecutive amino acids in gag p30 of the MuLVs is responsible for the different host range (20). Hartly et al. (35) found an additional gene (Rmcfr) of the mouse ,distinct from Fv-1 that inhibits exogenous infection of cells by MCF viruses but not by ecotropic viruses. Furthermore, the presence of another "resistance" gene, resembling Rmcfr in its effect, has been identified in crosses of AKR mice with C57 and SJL mice (90). In addition to effects on infectivity of cells a number of loci in the mouse are known to affect expression of endogenous viruses and several forms of spontaneous and induced viral leukemias . Some of these loci, like those linked to the major histocompatibility (H-2) locus are thought to be involved in immunologically mediated responses to virion components or to virus infected cells (111, 81). - The expression of endogenous viruses of some inbred mouse strains appears to be involved in leukemia induction, particularly in high leukemia strains, like AKR. However, most endogenous viruses are non-pathogenic and cannot be associated with a higher incidence of disease. For example chickens with RAV-0 viremia show no higher incidence of disease (74, 83).

2.1 Endogenous MuLV C-type viruses.

The genome of Mus musculus contains many copies of murine leukemia virus related DNA sequences as part of their normal genetic complement. Endogenous MuLVs can be divided into two classes with respect to their host range (95): the ecotropic MuLVs which infect and replicate in mouse cells but not in heterologous cells and the xenotropic MuLVs, which can productively infect heterologous cells. These ecotropic and xenotropic endogenous viruses can give rise to a new class of viruses which form cytopathic"foci" on mink cells (MCFs)(34, 51). These MCF viruses are dualtropic and replicate both in murine and heterologous cells (24, 34, 51, 103). Analysis of the structure of these MCF viruses suggests that they arise by a recombination event

between ecotropic and xenotropic viruses (12, 21) and are not inherited as such (23, 49, 64, 72).

2.1.1 Endogenous ecotropic viruses.

Viruses capable of infecting exclusively mouse cells, the ecotropic viruses, are present and expressed in some mouse strains, whereas in other strains these genomes are absent or repressed by host control systems (47, 66). In AKR/N mice, two loci for ecotropic inducibility have been mapped on chromosome 7 and 16 (59, 88). The BALB/c and C3H/He strains contain a single inducible ecotropic locus mapped to chromosome 5 (42, 56), while the ecotropic locus of C57Bl/10 mice has been assigned to chromosome 8 (57). Ecotropic proviruses are absent from SWR, NZB, 129 and NIH-Swiss strains (61, 81). The genomes of these and other mice strains have been analyzed by the Southern blotting technique (92) using ecotropic specific probes (chapter 3, and ref. 45, 46). The endogenous ecotropic MuLV DNA sequences present in most mouse strains, share a set of characteristic restriction endonuclease sites, indicating that they have originated from a common ancestor (9). Nearly all the ecotropic proviruses are structurally related to the AKR ecotropic provirus (AKV) (10). Examination of 22 wild-type mice for the AKV strain of ecotropic MuLVs revealed the presence of such proviruses only in Japanese mice suggesting that the AKV is of Asian origin (22, 93).

The low viremic strains possess one ecotropic provirus. Such proviruses are indistinguishable, including flanking cellular DNA sequences, from the four prototype proviruses found in BALB/c, C57BL, DBA, and NZW (chapters2 and 3, and ref. 45, 46). The distribution of ecotropic MuLV loci shared in these strains was in accordance with their genealogical relationship. Since some of these strains have been separated for nearly 40-60 years this observation illustrates the stability of the integrated proviral element and supports the hypothesis that these loci were acquired by independent germline integrations.

Within the high viremic strain AKR, an increase in AKV proviral copies can be observed (chapters 2 and 3, and ref. 89). The sequential acquisition of AKV proviruses is predominantly seen in strains with high virus titers and seems therefore related to viremia. In the substrains of the AKR mouse considerable variation is observed in number

and site of integration of AKR genomes. Only one Akv locus is present in all these sublines. This common locus could have functioned as a parental gene for the other endogenous ecotropic genomes. The chromosomal position of the AKV proviruses in the various sublines is in complete agreement with the segregation pattern of these substrains (chapter 3). The presence of this relation indicates that the newly acquired AKV proviruses once integrated, become immobilized in the host chromosomal DNA. The new germline integrations accumulate continuously, with an average of one new provirus per 30 generations. Theoretically, the germline reinsertions of retroviruses could occur either by infection of germline cells or by the transposition of a copy of the resident provirus by mechanisms similar to that used by transposable genetic elements. In agreement with the mechanism of infection of germline cells is the detection of a MCF-type recombinant in the germline of the AKR/Cnb strain. Since these recombinants are generated in the preleukemic and leukemic tissues, later in live, integration in the germline most likely occurs by infection of germcells, oocytes or early embryos by circulating virus(chapters 2 and 3). In addition Rowe and Kozak (89) showed that the acquisition of novel virus containing loci is only associated with viruspositive females. A further support for the amplification of proviral loci by infection of germ cells can be derived from the observation that in low leukemic strains, which do not show high virus titers, such amplification is very rarely found (75).

2.1.2 Endogenous xenotropic viruses.

Xenotropic MuLVs are unable to replicate in mouse cells, but they do infect a wide range of other species, such as rat, mink, human, and even quail cells (63, 65). Since mouse cells lack receptors for xenotropic MuLV, activation of such viruses in vivo is not readily detected. In mice they grow poorly and expression of xenotropic viruses is strain dependent. The NZB mouse strain produces relatively high levels of virus throughout life, whereas no expression could be detected in strains like NIHSwiss and C57L (64). In AKR mice significant xenotropic virus-related antigen production can be found in the preleukemic thymus (51). Upon induction with a number of agents infectious viruses are produced (1). In five mouse strains, including AKR and BALB/c, in-

ducible loci for xenotropic MuLV have been mapped genetically to a chromosome 1 locus, Bxv-1, (58). These results coupled with hybridization studies have indicated that a cluster of different xenotropic proviruses and xenotropic env gene related sequences is closely linked to the Bxv-1 locus and to genes involved in the immune response (6, 38). Xenotropic MuLV genomes have been found in multiple-copies (18 to 28) in DNA of all mouse strains and is therefore more widely spread than the ecotropic genomes (8, 62). Most of the xenotropic MuLV proviruses are stable components of the mouse genome and are present in DNAs of sublines separated during many years of inbreeding (38). The abundant presence of xenotropic viruses in the mouse genome, may be the result of an efficient replication and amplification of these viruses (like ecotropic viruses in the AKR mouse). By natural selection, resistance to infection of these viruses may have arisen in the murine ancestor, after a large number of proviruses became integrated. A majority of the endogenous MuLV proviruses exist as multiple families of different but closely related proviral DNAs (38). At least seven classes were detected in mouse DNA two of which are indistinguishable from proviruses associated with known infectious xenotropic MuLVs (38).

Various functions have been suggested for endogenous xenotropic sequences. Besides their relationship to the immune system (73) and differentiation specific cell-surface antigen formation (104) they are also involved in the generation of recombinant MCF viruses (chapters 4 and 5).

3. MCF viruses and thymic lymphomagenesis.

The study of endogenous murine retroviruses in relation to spontaneous leukemia was facilitated by the development of inbred strains of mice with a high incidence of spontaneous leukemia. The AKR inbred strain of laboratory mice was derived by brother-sister matings of mice derived from a commercial Pennsylvania mouse dealer in 1928 by Jacob Furth (26). The prototype strain (A), with an infrequent in-

cidence of leukemia, was further selected to derive the AKR strain with an incidence of 90% by 6-10 month of age (31).

The disease can be described as a thymic lymphoma that spread to other organs (30, 33, 71, 105). The development of thymomas is in the AKR mice accompanied by a complex phenotypic and genetic series of alterations in the type C retroviruses which are produced by these mice .

1) The endogenous N-ecotropic MuLV is spontaneously expressed from one or more Akv loci shortly before birth (86, 87): infectious virus can be detected in embryo extracts as soon as day 18 or 20 of gestation (86).

2) The number of virus producing cells within the different organs (spleen, liver, and thymus) and in precursor cells of the haemopoietic system (28) increases dramatically between day 3 and day 10 after birth (25). The virus does not infect a high proportion of thymocytes (34). Neutralization of this virus between birth and day 3 by passive immunization can prevent the onset of viraemia and supress the development of the lymphoma (25, 41).

3) The preleukemic period is characterized by alterations in the structure and expression of endogenous MuLV genomes specifically in thymus (4,51,76,78). Multiple independent rounds of genetic recombination occur between different endogenous MuLV sequences and the infectious ecotropic viruses, resulting in the full-fledged leukemogenic polytropic viruses (chapter 4, and ref.13, 82, 100). Genomic analysis of the leukemogenic polytropic viruses (MCFs) has revealed that they differ from the AKV parent in two regions. One difference is located in the gp70 gene, where the non-AKV sequences are acquired from a set of endogenous, probably non-infectious, xenotropic-like genomes (chapter 4, ref. 11, 21, 40, 54). The other difference is situated in the U3 non-coding region of the LTR and the 3' end of the p15E gene (chapters 4 and 5, ref. 40, 52, 69). The non-AKV sequences in the LTR of these thymic MCFs are obtained from an unique endogenous virus of the xenotropic class. We have designed this virus LTRdXe (LTR donating Xenotropic virus) (chapter 4). The acquisition of the nonecotropic sequences in these two regions of the MCF viruses can occur independently as is suggested by the detection of ecotropic viruses with exclusively recombinant p15E-U3 sequences (17, 100).

4) MCFs were found to infect and replicate preferentially in immature lymphocytes, only present in thymic cortex (14). The gathering of non-AKV sequences within the 3'end of p15E and or the U3 region might be necessary in order to allow viral replication in target thymocytes (19). The infection of thymocytes and spread of MCFs occurs in AKR mice in preleukemic stage (around 5 month of age), and is manifested by the expression of MCF encoded env gene products (79). Ultimately this results in the transformation of a thymocyte, which proliferates, giving rise to a monoclonal thymoma (chapters2, and refs. 7, 11, 30, 37, 107, 108).

Some MCF viruses isolated from preleukemic or leukemic AKR thymuses are able to accelerate leukemia in AKR mice (68). This may be due to the altered gp70 molecule of the new recombinants, which may interact with thymus receptors (70) or elicits the continuous recruitment of antigen-reactive T-cells thereby increasing the probability of transformation (60). Alternatively, transformation might be the result of the activation of cellular genes by proviral integration (16, 37, 77).

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Chapter 2

CHARACTERIZATION OF AKR MURINE LEUKEMIA VIRUS SEQUENCES IN AKR MOUSE SUBSTRAINS AND STRUCTURE OF INTEGRATED RECOMBINANT GENOMES IN TUMOR TISSUES.

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Characterization of AKR Murine Leukemia Virus Sequences in AKR Mouse Substrains and Structure of Integrated Recombinant Genomes in Tumor Tissues

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A specific cDNA probe of AKR murine leukemia virus (AKR-MLV) was prepared to detect AKR-MLV sequences in normal and tumor tissues in a variety of AKR mouse substrains. AKR strains contained up to six endogenous AKR-MLV genomes. All substrains tested had one AKR-MLV locus in common, and closely related substrains had several proviruses integrated in an identical site. Virus-induced tumors in the AKR/FuRdA and AKR/JS strains showed a reintegration pattern of AKR-MLV sequences unique for the individual animal, suggesting a monoclonal origin for the outgrown tumors. An analysis of tumor DNAs from the AKR/FuRdA and AKR/JS substrains with restriction enzymes cleaving within the proviral genome revealed a new *Eco*RI restriction site and *Bam*HI restriction site not present in normal tissues. The positions of these sites corresponded both with cleavage sites of *Eco*RI and *Bam*HI in integrated Moloney recombinants and with the structure of isolated AKR mink cell focus-forming viruses. All tumors analyzed to data contain nearly identical integrated recombinant genomes, suggesting a causal relationship between the formation of recombinants and the leukemogenic process.

It has been established that the endogenous AKR murine leukemia viruses (AKR-MLVs) are transmitted from parent to offspring within a variety of inbred strains of mice (1). The segregation of AKR-MLV proviral loci has been identified by the backcross of mice of the AKR strain and the virus-negative NIH/Swiss strain; these studies determined the presence of at least three AKR-MLV proviral loci (15). Two high-inducibility loci (*Akv-1* and *Akv-2*) have been mapped on chromosomes 7 and 16, respectively (24, 25) and have been characterized by restriction endonuclease mapping (27). AKR mice express high levels of endogenous AKR-MLV throughout their life-span and show a high incidence of leukemia (70 to 95%) after a latency of 7 to 9 months (25). Leukemogenesis in AKR/J mice is accompanied by an amplification of AKR-MLV sequences in tumor tissues caused by reintegrations of endogenous MLV sequences (2, 4, 27).

Recombinants between ecotropic AKR virus and endogenous xenotropic MLVs have been detected in the thymuses of leukemic and preleukemic AKR mice and in leukemic tissues of NIH/Swiss mice that inherit either the *Akv-1* or the *Akv-2* virus-inducing locus (13). RNase T_1 fingerprinting and heteroduplex analysis showed that these mink cell focus-forming (MCF)-type viruses are *env* gene recombinants with considerable heterogeneity among the various isolates (9, 14, 19, 23). In contrast, Moloney MLV (M-MLV)-induced tumors in mice all contain similar recombinant genomes, indicating the necessity for a specific type of recombination (H. van der Putten et al., Cell, in press). Therefore, we searched for common characteristics of recombinant virus genomes integrated in the DNA of AKR tumor tissues.

In the study presented here, a cDNA probe that was specific for AKR-MLV and that did not cross-hybridize with other endogenous viral sequences was used. We applied the Southern blotting technique (26) to identify and characterize the different endogenous AKR-MLV sequences in a number of AKR substrains and in a variety of normal and malignant tissues of these strains.

MATERIALS AND METHODS

Virus. AKR virus, isolated from *Akv-1* congenic mice, was propagated in monolayers of NIH/3T3 cells in roller bottles on a Smith Kozoman autoharvester (Bellco). The AKR virus was isolated as described previously (32).

Mice. BALB/c and 129 mice of inbred strains and the outbred strain S.E.(Swiss) were obtained from the Central Animal Laboratory of the University of Nijmegen, Nijmegen, Holland. AKR/FuRdA mice were obtained from Netherlands Cancer Institute,

Amsterdam, Holland. AKR/J mice were from the Weizmann Institute of Science, Rehovot, Israel. Substrain AKR/Cnb was from Centre d'Etude de l'Energie Nucleaire, Mol, Belgium. Organs from AKR/JS mice were obtained from R. Jaenisch, Heinrich Pette-Institut, Hamburg, Federal Republic of Germany. AKR/Abom Fib mice were obtained from the Breeding and Research Center, Gl. Bomholtgaard Ltd., Ry, Denmark. Organs from the *Akv-2* strain of mice were generously supplied by A. Colombatti, Istituto di Anatomia, University of Padova, Padova, Italy. AKR clone 623 was kindly provided by D. Lowy, National Cancer Institute, Bethesda, Md.

Preparation and selection of the cDNA probe. AKR viral cDNA was prepared in the endogenous polymerase reaction as described previously with minor modifications (32). For the removal of sequences from the AKR-MLV cDNA which cross-react with other endogenous viruses, AKR-MLV cDNA was selected against DNA from 129 mice exactly as described by Berns and Jaenisch (2).

Extraction of DNA, electrophoresis, and elution. DNA was isolated, purified, and cleaved with restriction endonucleases as described previously (32). Restriction endonuclease fragments were separated on the basis of size by electrophoresis in 0.5 or 0.8% neutral agarose gels as reported previously (32). DNA was eluted and concentrated from 2-mm gel slices by electrophoretic elution; preparative elution of DNA fragments from agarose gel slices was carried out for 14 h at 70 V in a buffer containing 12.5 mM Tris base and 1.5 mM EDTA, adjusted to pH 8.0 with boric acid. The eluted DNA (400-μl samples) was adjusted to 0.3 M NaCl and 0.5% sodium dodecyl sulfate, extracted twice with 1 volume of TE buffer (10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA)-saturated phenol (pH 8) and twice with 1 volume of CHCl-isoamyl alcohol (25:1), and precipitated twice with ethanol.

Blotting and hybridization. After electrophoresis, the DNA was transferred with 10× SSC (1× SSC: 0.15 M NaCl, 0.015 M trisodium citrate) to nitrocellulose filters as described previously (30). Before hybridization, the filters were incubated for 2 h at 42°C in a volume of 200 μl of pre-hybridization mix (50% formamide, 5× SSC, 5× Denhardt solution [1× Denhardt solution: 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone], 250 μg of denatured salmon sperm DNA per ml, 0.05 M sodium phosphate [pH 7.0]) per cm^2. Hybridization was performed at 42°C for 16 h in a volume of 50 $\mu l/cm^2$ with a solution containing 50% formamide, 5× SSC, 1× Denhardt solution, 0.02 M sodium phosphate, 10% dextran sulfate, 100 μg of denatured salmon sperm DNA per ml, and 0.5×10^6 to 1×10^6 cpm of cDNA per ml (33). After hybridization, the filter was washed with hybridization mix from which the probe and dextran sulfate were omitted for 2 h at 42°C, once for 15 min with 2× SSC–0.1% sodium dodecyl sulfate at 50°C, once with 0.1× SSC–0.1% sodium dodecyl sulfate at 45°C, and once with 0.1× SSC–0.1% sodium dodecyl sulfate at 67°C. The filters were dried and autoradiographed for 2 to 6 days with Kodak Royal X-Omat R film and intensifying screens (30).

RESULTS

Characterization of the AKR cDNA probe. For the detection of AKR-MLV-specific sequences in the genomes of different mouse strains, an AKR-MLV-specific ^{32}P-labeled cDNA probe was prepared and selected as described by Berns and Jaenisch (2). The unfractionated AKR-MLV cDNA contains sequences which cross-hybridize with other endogenous mouse viruses. These sequences were removed by hybridizing the unfractionated AKR cDNA to DNA from mice of the 129 strain, which lacks the AKR-type virus (2). For a determination of the specificity of AKR-MLV cDNA, the probe was hybridized to *Eco*RI restriction endonuclease DNA fragments from liver DNA of mice which lack AKR-MLV-type proviruses (129 and Swiss) (17) and mice which contain one copy of AKR-MLV per haploid genome (BALB/c and NIH/Swiss *Akv-2* congenic) (8, 22, 32). No hybridization was detected with 129 and Swiss DNAs, whereas the AKR-MLV cDNA probe hybridized to a single *Eco*RI restriction fragment of 20 kilobase pairs (kbp) in BALB/c DNA and a fragment of 26 kbp in NIH/Swiss *Akv-2* congenic DNA (Fig. 1A). It has already been suggested that this specific probe could represent 30 to 35% of the genome (2), allowing recognition over the entire genome. Indeed, the AKR-MLV-specific cDNA displayed hybridization with all four *Bam*HI restriction endonuclease fragments (27) of a cloned AKR-MLV integrated provirus (clone 623) at a concentration identical to the relative number of AKR sequences in cellular DNA samples (18). However, not all four *Bam*HI restriction endonuclease fragments of clone 623 were recognized equally (Fig. 1B). The fragment corresponding to the 5′ end of the viral RNA showed less hybridization as compared with the fragment assigned to the 3′ end of the genome.

Endogenous AKR-type sequences of AKR mice. To characterize the endogenous AKR-MLV sequences in the AKR strains, we used the Southern blotting technique (26). Restriction endonuclease *Eco*RI does not cleave in vitro-synthesized full-length AKR proviral DNA (28) and cloned AKR-MLV DNA (18). This implies that the sizes of cellular *Eco*RI DNA fragments containing AKR proviral sequences will depend on *Eco*RI recognition sites present in cellular DNA flanking the integrated provirus. Restriction endonuclease *Bam*HI cleaves AKR proviral DNA at three sites, resulting in two internal DNA fragments of 1.9 and 3.0 kbp and two viral DNA fragments linked to the adjacent cellular sequences (27).

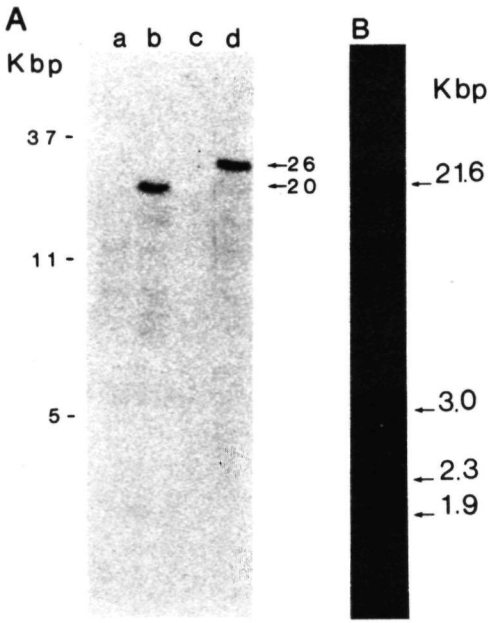

FIG. 1. *Characterization of the selected cDNA probe. (A) EcoRI-digested DNAs were analyzed by the Southern procedure (26) and hybridized with the specific cDNA probe. EcoRI does not cleave the viral DNA (28). Lane: a, liver DNA of a 129 mouse; b, BALB/c liver DNA; c, S.E.(Swiss) liver DNA; d, NIH/Swiss Akv-2 congenic liver DNA. The molecular sizes of the AKR-MLV-specific restriction endonuclease fragments are indicated at the right. Adenovirus type 2 viral DNA and phage λ DNA, digested with EcoRI, HindIII, and BamHI, were used as molecular weight markers. (B) BamHI digestion of 50 pg of AKR-MLV clone 623 (18), analyzed by the Southern procedure and hybridized with a specific cDNA probe. The positions of the internal proviral fragments (3.0 and 1.9 kbp) and the fragments containing the 3′ (21.6-kbp) and 5′ (2.3-kbp) junctions with cellular and phage λ DNAs are indicated at the right.*

Liver DNA from AKR/A, AKR/JS, and AKR/FuRdA mice showed two, four, and six *Eco*RI restriction endonuclease DNA fragments, respectively, which hybridized to the AKR virus-specific cDNA probe (Fig. 2, lanes b, d, and e). All fragments were larger than 8.8 kbp and might therefore contain a complete AKR-MLV genome. This was also suggested by nearly equal amounts of radioactivity detected in each band. The six *Eco*RI restriction endonuclease DNA fragments of the AKR/FuRdA strain were treated with restriction endonuclease *Bam*HI and further analyzed for the presence of AKR-MLV-specific sequences (27). *Eco*RI restriction endonuclease fragments from liver DNA (300 μg) of AKR/FuRdA mice were separated on the basis of size by electrophoresis on a 0.4% neutral agarose gel. DNA from 2-mm gel slices was electroeluted and digested with restriction endonuclease *Bam*HI, and DNA fragments were separated on 0.8% agarose gels. The six endogenous AKR-MLV-containing fragments appeared to contain the 3.0- and 1.9-kbp internal *Bam*HI DNA fragments, as indicated by the increased intensity in the autoradiograph in the corresponding positions. Fragments of identical size could be obtained from a cloned AKR proviral genome (clone 623). In addition to the internal proviral DNA fragments, a clearly unique distribution of additional fragments was found in all six AKR-MLV-containing *Eco*RI fractions (Fig. 3A). These additional fragments represented the overlap of the 3′ and 5′ parts of AKR-MLV genomes with the flanking cellular sequences. Each of the AKR-MLV-containing *Eco*RI fragments was characterized further with restriction endonucleases *Bam*HI plus *Bgl*I and *Pst*I. *Bgl*I reduced the 3.0-kbp *Bam*HI DNA fragment to 2.0 kbp, whereas the 1.9-kbp *Bam*HI fragment was trimmed to 1.8 kbp (data not shown), in

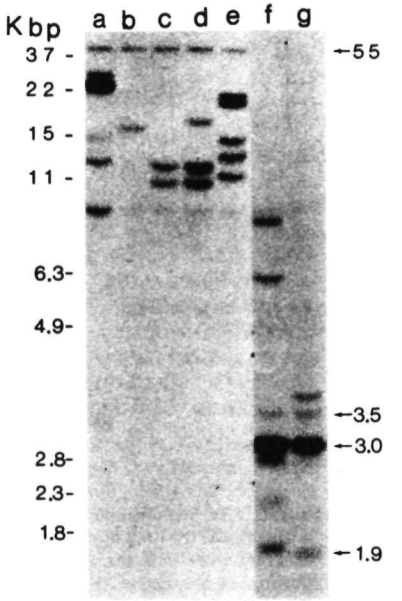

FIG. 2. *Identification of endogenous AKR-MLV sequences in substrains of AKR mice. EcoRI-digested DNAs (lanes a to e) and BamHI-digested DNAs (lanes f and g) were analyzed by the Southern procedure. The lanes represent liver DNAs from the AKR/Cnb (lane a), AKR/A (lane b), AKR/J (lane c), AKR/JS (lanes d and g), and AKR/FuRdA (lanes e and f) strains. The molecular sizes of some individual AKR-MLV-containing fragments are indicated at the right. The molecular weight markers at the left are as described in the legend to Fig. 1.*

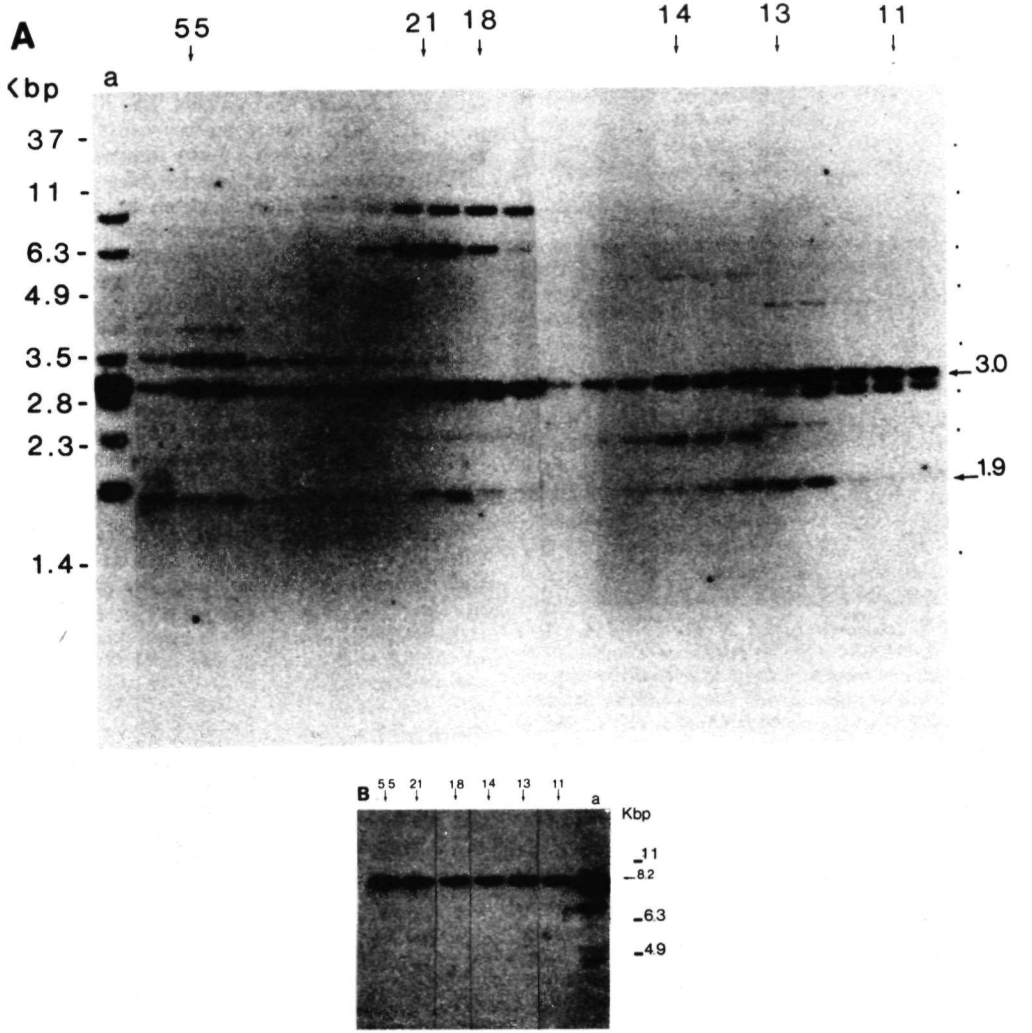

FIG. 3. *Characterization of the six endogenous AKR-MLV genomes present in the AKR/FuRdA strain. EcoRI restriction fragments from liver DNA (300 μg) of AKR/FuRdA mice were separated on the basis of size on a 0.4% agarose gel. DNA from 2-mm gel slices was electroeluted. (A) Digestion of DNA from the 2-mm gel slices with BamHI after separation of the fragments on 0.8% agarose gels and Southern analysis. Lane a represents 10 μg of AKR/FuRdA liver DNA digested with EcoRI plus BamHI. The positions of the two internal BamHI fragments (3.0 and 1.9 kbp) are indicated at the right. The molecular weight markers at the left are as described in the legend to Fig. 1. (B) Digestion of DNAs from the six AKR-MLV-containing EcoRI fragments with PstI after separation of the fragments on 0.8% agarose gels and Southern analysis. Lane a represents 10 μg of AKR/FuRdA liver DNA digested with EcoRI plus PstI. The position of an 8.2-kbp fragment is indicated at the right. The molecular weight markers at the right are as described in the legend to Fig. 1. The molecular sizes given at the top correspond to the positions of the six endogenous AKR-MLV genomes shown in Fig. 2, lane e.*

agreement with the location of the *Bgl*I sites in the molecularly cloned provirus (clone 623). Restriction endonuclease *Pst*I cleaves the unintegrated AKR-MLV genome only within the terminal redundancy, generating an 8.2-kbp fragment (28). Digestion with *Pst*I of the six AKR-MLV-containing *Eco*RI restriction endonuclease fractions yielded in all instances the 8.2-kbp internal fragment (Fig. 3B). Therefore, the AKR/FuRdA strain contains six endogenous AKR-MLV proviral genomes, integrated in different sites of the mouse genome.

Digestion of DNA from the AKR/A strain with *Eco*RI gave rise to two fragments of 55 and

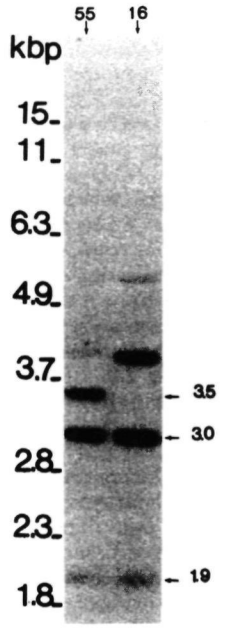

FIG. 4. *Characterization of the two endogenous AKR-MLV genomes present in the AKR/A strain. The 55- and 16-kbp EcoRI restriction fragments from liver DNA (300 μg) of AKR/A mice were isolated as described for AKR/FuRdA mice. The isolated fragments were cleaved with BamHI and analyzed by the Southern procedure. The molecular weight markers at the left are as described in the legend to Fig. 1.*

16 kbp, respectively. More detailed analyses of each fragment by digestion with *Bam*HI revealed the 3.0- and 1.9-kbp internal *Bam*HI fragments and two additional fragments representing the 3′ and 5′ overlaps with cellular sequences (Fig. 4). The 55-kbp *Eco*RI DNA fragment of the AKR/A strain contained the same flanking fragment of 3.5 and 4.0 kbp as found after digestion of the 55-kbp *Eco*RI fragment present in the AKR/FuRdA strain of DNA (compare Fig. 3A and 4). *Bam*HI digestion of the 16-kbp *Eco*RI fragment from the AKR/A strain gave rise to fragments not seen in the AKR/FuRdA strain. Therefore, one of the two AKR-MLV proviral genomes within the AKR/A strain is integrated in the same chromosome site as occupied in the AKR/FuRdA strain, whereas the other is unique for the AKR/A strain.

Similarly, one of the four endogenous proviral genomes present in a 55-kbp *Eco*RI DNA fragment in the AKR/JS strain has the same chromosomal location as found in the AKR/FuRdA and AKR/A strains. Double digestion with restriction endonucleases *Bam*HI and *Eco*RI of liver DNA of the AKR/FuRdA and AKR/JS strains showed, besides two internal fragments, clearly one other common fragment of 3.5 kbp (Fig. 2, lanes f and g). This fragment was also found after digestion with *Bam*HI of the isolated 55-kbp *Eco*RI DNA fragment from the AKR/FuRdA and AKR/A strains (Fig. 3A and 4). Double digestion with restriction endonucleases *Eco*RI and *Hin*dIII showed two fragments of 30 and 25 kbp within the AKR/FuRdA and AKR/JS strains (data not shown). *Hin*dIII cleaves the in vitro-synthesized full-length AKR proviral DNA once (28). These fragments can only belong to the AKV locus contained in the 55-kbp *Eco*RI DNA fragment. In all substrains of AKR mice, the 55-kbp *Eco*RI restriction endonuclease fragment could be detected, whereas the numbers and integration sites of the other endogenous AKR-MLVs varied (Fig. 2).

The same three AKR-MLV-containing *Eco*RI fragments present in the AKR/J genome were also detected within the AKR/JS strain of DNA (Fig. 2, lanes c and d). The AKR/JS strains had, in addition, an AKR-MLV-containing *Eco*RI fragment of 17 kbp. This indicates that germ line reinsertions of AKR-MLV occur rather frequently and that substrains which have been separated recently have more AKR-MLV-containing *Eco*RI fragments in common.

We conclude that the different AKR substrains contain various numbers of endogenous AKR-MLV genomes and that only a single AKR provirus is integrated in an identical site in all AKR strains. Closely related substrains have, in general, several proviruses in common.

Integration of AKR-MLV sequences in tumor DNA. Previously, it has been shown by liquid hybridization studies that leukemogenesis in AKR mice is accompanied by an amplification of AKR-MLV sequences in tumor tissues (2). These experiments did not provide information about specific integration sites and the structure of reintegrated viral genomes; therefore, AKR sequences were analyzed in tumor and nontumor tissues of AKR/FuRdA mice. DNA was isolated from these tissues and analyzed by restriction endonuclease digestion, blotting, and hybridization to a specific cDNA probe. Figure 5 (lanes b to i) shows the distribution pattern of AKR-MLV provirus-containing fragments from tumor DNAs of different animals, as well as from tumor DNAs obtained from different anatomical sites (thymus, spleen, and lymph nodes) of one animal.

Additional fragments were observed in tumor DNAs that were absent in normal liver DNAs. These reintegrated AKR-MLV sequences, ranging in size from 2 to 26 kbp, displayed a unique pattern for each individual animal. However, tumor DNAs from different tissues within one animal showed the same reintegration pattern.

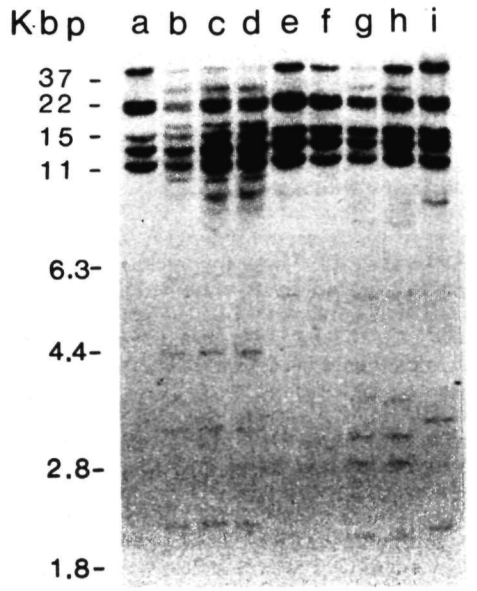

FIG. 5. *Detection of new AKR-MLV-type-containing fragments in the DNAs of leukemic tumors from AKR/FuRdA mice. Ten micrograms of tissue DNA from several tumors was digested by EcoRI and analyzed as described in the text. Lane: a, normal liver DNA; b, c, and d, DNAs from the thymus, spleen, and lymph nodes of animal 1; e and f, DNAs from the spleen and lymph nodes of animal 2; g and h, DNAs from the spleen and lymph nodes of animal 3; i, DNA from the thymus of animal 4. The molecular weight markers at the left are as described in the legend to Fig. 1.*

These observations indicate that outgrown tumors are composed largely of the clonal descendants of one or a few transformed cells, which are metastasized at different anatomical sites. Furthermore, the sizes of the different *Eco*RI restriction endonuclease DNA fragments that contain the endogenous AKR-MLV sequences remain unchanged in tumor tissues. Similar results have been obtained for genetically transmitted M-MLV (16). The reintegrated AKR-MLV sequences in tumor tissues show a reduced level of hybridization as compared with the genetically transmitted AKR-MLV copies, as expected for integrations probably occurring only once per diploid genome equivalent.

Individual *Eco*RI DNA fractions obtained from a lymphoma of an AKR/FuRdA mouse were analyzed for the presence of the characteristic internal *Bam*HI DNA fragments. Besides the characteristic fragments of 3.0 and 1.9 kbp diagnostic for the authentic AKR MLV genome, a new fragment of 2.2 kbp was detected after *Bam*HI digestion of some *Eco*RI DNA fractions comprising newly acquired AKR sequences. *Bam*HI digestion of DNA from tumor tissues of different animals gave rise to a 2.2-kpb *Bam*HI fragment, indicating that this fragment is derived from modified AKR genomes (Fig. 6, lanes 2 to 5). This fragment was localized on the physical map of the AKR genome: the 2.2-kbp *Bam*HI fragment was trimmed 0.5 kbp in size by *Xho*I digestion, generating a 1.7-kbp fragment not seen in DNAs from normal tissues (Fig. 6, lanes 6 to 8) and not present in *Xho*I-digested DNAs of tumor tissues (data not shown). This positioned the new 2.2-kbp *Bam*HI fragment from 4.1 to 6.3 kbp on the physical map (Fig. 7). Probably due to methylation, the 3-kbp internal *Bam*HI fragment was only partly digested by *Xho*I, resulting in the presence of both a 2.5-kbp fragment and a 3-kbp fragment in double digests of *Bam*HI plus *Xho*I. Similarly, digestion of DNAs from all tumor tissues isolated so far by a combination of *Eco*RI and *Xba*I revealed a fragment of 1.0 kbp (Fig. 6, lanes 9 and 10) not detectable in DNAs from normal tissues (Fig. 6, lane 11).

Figure 7 shows the localization of this new fragment on the viral genome. The position of the *Eco*RI cleavage site (position 6.8) was further confirmed by double digestion with *Eco*RI and *Pst*I. *Pst*I cleaves AKR-MLV proviral DNA in the long terminal redundancy at map positions 0.1 and 8.3 kbp. Digestion with *Pst*I and *Eco*RI should generate two fragments of 1.5 and 6.7 kbp. Figure 6 (lanes 12 to 16) shows that the 1.5-kbp fragment could only be found within DNAs from tumor tissues. Hybridization to a 6.7-kbp fragment was also seen in DNAs from normal tissues and in DNAs from mice lacking the endogenous AKR genomes (data not shown). Apparently, a large number of other endogenous viruses can give rise to a 6.7-kbp fragment after digestion with *Eco*RI and *Pst*I. The large molar excess of a fragment of this size might result in the recognition of this fragment by a small amount of nonspecific cDNA still present in our selected cDNA preparation.

The presence of an *Eco*RI cleavage site and a *Bam*HI cleavage site within reintegrated viral genomes indicates the acquisition of non-AKR-MLV sequences. In all of the tumors analyzed, the same new fragments were found, indicating a rather conserved organization of recombinant proviral DNAs in tumors. Similar results were obtained in M-MLV-induced tumors of BALB/Mo and BALB/c mice, in which *Eco*RI and *Bam*HI sites have been located at nearly identical positions in recombinant genomes (van der Putten et al., in press).

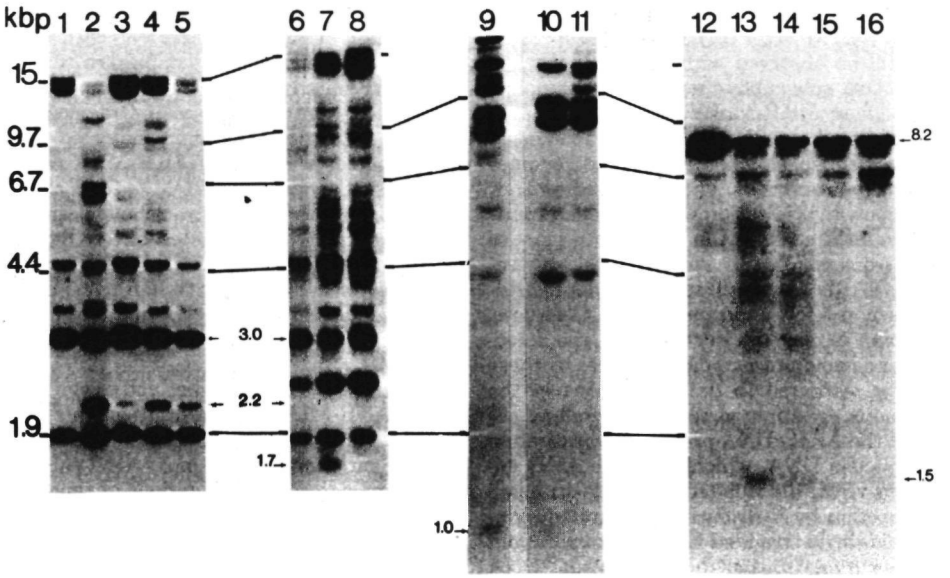

FIG. 6. *Recombinant AKR-MLV sequences in AKR/FuRdA and AKR/JS mice. Ten micrograms of DNA from tumors of different animals was cleaved with the indicated restriction endonucleases and analyzed by the Southern procedure. Lane: 1, BamHI digestion of normal AKR/FuRdA liver DNA; 2 to 5, BamHI digestion patterns of tumor DNAs from four AKR/FuRdA animals, showing the new 2.2-kbp BamHI fragment; 8, digestion with XhoI plus BamHI of normal AKR/FuRdA liver DNA; 6 and 7, AKR/FuRdA tumor DNAs, revealing the additional 1.7-kbp XhoI-plus-BamHI fragment; 11, XbaI-plus-EcoRI digestion of normal AKR/JS liver DNA; 9 and 10, XbaI-plus-EcoRI cleavage patterns of tumor DNAs from a spleen of an AKR/FuRdA animal (lane 9) and a thymus of an AKR/JS animal (lane 10) containing a 1.0-kbp fragment; 12, PstI-plus-EcoRI digestion of normal AKR/FuRdA liver DNA; 13 to 16, AKR/FuRdA tumor DNAs from four animals, digested with PstI plus EcoRI. The molecular weight markers at the left are as described in the legend to Fig. 1.*

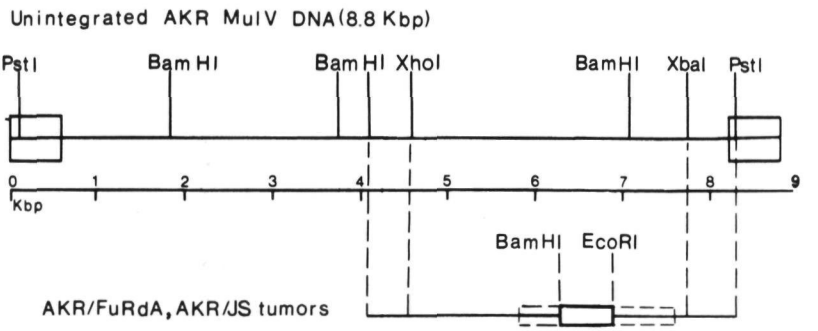

FIG. 7. *Structure of integrated recombinant proviral genomes. The upper line represents the map of restriction endonuclease cleavage sites on the unintegrated AKR-MLV genome (partly obtained from reference 28). The boxes at the ends indicate the long terminal redundancies. The scale of the map is shown in the middle. The line at the bottom represents the recombinant structure, with the positions of the EcoRI and BamI sites. The difference between the broken-line frame (representing recombinants as found in M-MLV-induced tumors) and the solid frame (minimum substituted sequences as indicated by BamHI and EcoRI digestions in this paper) reflects the most probable positions of the recombinant sites.*

DISCUSSION

Many type C viruses are part of the genome complement of inbred mice (7). The majority of these viral sequences can be detected by DNA probes complementary to most leukemia viruses. To detect more specifically the AKR endogenous virus, Steffen et al. removed most of

the AKR viral sequences which this virus has in common with M-MLV (27). Cross-hybridization could still be observed with other endogenous viruses. Also, subgenomic segments from molecularly cloned AKR-MuLV DNA (clone 623) have been used as probes to analyze and identify ecotropic sequences integrated in the genomes of several mouse strains. These probes, however, do not show hybridization with the endogenous ecotropic MLVs present in some of the mouse strains from which this virus can be induced, such as C57BL (9), or still seem to cross-hybridize with non-AKR endogenous type C viruses, such as in 129 mice (5). Furthermore, the detection of endogenous ecotropic sequences by these probes is restricted to defined regions of the endogenous ecotropic genomes. Therefore, we selected the AKR cDNA probe by hybridization to 129 mouse DNA, which lacks the AKR endogenous virus, and isolated the single-stranded DNA fraction by hydroxyapatite chromatography. This single-stranded DNA fraction did not show any hybridization to chromosomal DNA from strains which have been reported to lack the endogenous AKR virus (129 and Swiss); on the other hand, DNA from strains which have been shown to contain a single AKR endogenous virus exhibit hybridization with a single unique restriction fragment (BALB/c and NIH/Swiss *Akv-2* congenic).

The selected probe represents 30 to 35% of the viral genome (2) and recognized the four AKR-MLV-specific *Bam*HI restriction endonuclease fragments at a dilution similar to the amount of AKR proviral sequences present in cellular DNA samples used in our agarose gels. This indicates that the sequences present in the cDNA-specific probe are distributed over the entire AKR genome, although not all fragments are recognized equally; DNA fragments corresponding to the envelope region, where most dissimilarities with other mouse type C viruses are found by heteroduplex analysis (9), seem to be recognized preferentially.

Ihle and Joseph presented evidence for three AKR-MLV proviruses in AKR mice (15). We observed six endogenous AKR-MLV sequences in the AKR/FuRdA strain and two genomes in the AKR/A strain, whereas four genomes were detected in the AKR/JS strain; other substrains, such as AKR/Cnb and AKR/J, appear to contain various numbers of integrated AKR genomes (Fig. 2). The characterization of the six AKR-MLV-containing *Eco*RI fragments within the AKR/FuRdA strain by digestion with *Bam*HI, *Bgl*I, and *Pst*I suggests that they represent six complete authentic AKR-MLV genomes. This was substantiated further by backcross studies, which revealed the independent segregation of all six proviral loci, each with an identical internal restriction pattern (data not shown). Similarly, *Bam*HI digestion of the two AKR-MLV-containing *Eco*RI fragments detected in the AKR/A strain indicated that in this strain two complete AKR-MLV genomes are present. Digestion of DNAs from all AKR substrains (14 different strains have been tested so far) with *Eco*RI revealed an AKR-specific fragment of 55 kbp. The other AKR sequences are detected in differently sized fragments. A 55-kbp fragment has also been found in the AKR/N, AKR/J, and AKR/Cu mouse strains by others (9, 27).

The differences in number and size of endogenous AKR-MLV-containing fragments among several substrains demonstrated considerable genetic variability. Since most AKR substrains have segregated for long periods of time (up to 50 years), independent germ line reintegrations may have taken place, giving rise to the high variability in integrated AKR genomes in these strains. The AKR endogenous viral genome, contained in the 55-kbp *Eco*RI fragment (probably corresponding to the *Akv-1* locus [6, 27]) and present in all substrains, may have acted as a progenitor of other endogenous AKR genomes. The AKR/J and AKR/JS substrains have three proviruses integrated in the same chromosomal site, whereas the AKR/JS strain possesses one additional provirus not present in the AKR/J strain. This indicates that three proviral loci were present before separation and that the integrated structure is stable for many generations. The additional provirus within the AKR/JS strain may have arisen from a new integration event after separation, or the difference may have been due to the loss of one provirus by the AKR/J substrain. Another explanation for the high variability of the endogenous AKR genomes can be given by assuming a high excision or mutation rate for some of these integrated genomes, possibly by virtue of their transposon-like feature (29, 31). However, our recent analysis of 14 different AKR substrains, all with unique integration sites, did not give any indication of the occurrence of excision as an explanation for the variability in number and integration site of endogenous AKR genomes in this strain. Therefore, we believe that most proviruses became stably integrated in the chromosomal genome by accidental germ line integration.

The hybridization pattern of amplified AKR-MLV sequences observed in tumor tissues was identical for tumors obtained from different anatomical sites of a single animal but was different

among individual animals. This suggests a monoclonal origin of outgrown tumors which metastasized at different anatomical sites of a single animal. The number of somatically acquired AKR-MLV sequences in tumors of AKR mice differs considerably. Similar results have been obtained for tumors from BALB/Mo mice (16), for M-MLV-infected BALB/c mice, and for M-MTV-type sequences in mice with mammary carcinomas (10, 20). Within the tumors of different animals, no common reintegration site was detected (Fig. 4, lanes b to i). This suggests that a specific reintegration site of these viruses is not an etiological cause of leukemia in mice. Further characterization of viral integration sites are being performed to clarify this point. Furthermore, the genetically transmitted AKR genomes seem to be unaffected in these tumors; the AKR-MLV-containing restriction endonuclease fragments observed in nontumor tissues are present in similar amounts in tumor DNAs.

An analysis with restriction enzymes cleaving within proviral genomes revealed internal fragments in tumor DNAs not seen in normal tissues. A 2.2-kbp *Bam*HI fragment could be assigned exclusively to the newly acquired sequences in tumor DNAs. This fragment was localized on the restriction endonuclease map of the AKR genome between map positions 4 1 and 6.3 kbp. In all tumor DNAs, AKR proviruses were detected with an *Eco*RI restriction site at position 6.8 kbp. The presence of this *Eco*RI site and a *Bam*HI site within these reintegrated viral genomes, which were detected in all tumors analyzed, suggests that a recombination has taken place under strong selection pressure. The observed restriction pattern in the recombinant region is in agreement with the restriction map of integrated Moloney recombinants detected in M-MLV-induced lymphomas (van der Putten et al., in press). An *Eco*RI site at position 6.9 kbp from the 5′ end was also found within proviral DNA of the AKR MCF-247 recombinant virus (21). Therefore, it is likely that the newly acquired cellular sequences are derived from endogenous xenotropic viral sequences and represent AKR MCF proviruses. The recombinant-specific sequences (solid-line box between map positions 6.3 and 6.8 on the restriction endonuclease map of Fig. 7) represent the minimum (observed) length of the substitution. The positions are in full agreement with the MCF-specific regions found by heteroduplex analysis of AKR MCF and Moloney MCF isolates (6.05 to 6.95 kbp from the 5′ end of the RNAs) (9). The major envelope glycoprotein of M-MLV coded for by a 2.7- to 3.0-kbp-long fragment located at the 3′ end of the genome starts at position 6.4 kbp from the 5′ end of the genome (11, 12, C. Van Beveren, personal communication). Therefore, a heteroduplex analysis (3), our data, and the data obtained by an analysis of recombinant structures within DNAs of M-MLV-induced tumors (van der Putten et al., in press) strongly suggest that the amino terminus of the major glycoprotein of MCF is contributed by xenotropic viruses, whereas the carboxy terminus originates from the parental ecotropic virus. The presence of the same conserved recombinant proviral structure in DNAs of all AKR-MLV- and M-MLV-induced leukemias (we have analyzed 30 different tumors to date) strongly suggests that the formation of these recombinants is a prerequisite for leukemic transformation rather than the accidental consequence of neoplastic outgrowth.

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Chapter 3

MOBILITY OF ENDOGENOUS ECOTROPIC MURINE LEUKEMIA VIRAL GENOMES WITHIN MOUSE CHROMOSOMAL DNA AND INTEGRATION OF A MINK CELL FOCUS-FORMING VIRUS-TYPE RECOMBINANT PROVIRUS IN THE GERMLINE.

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Mobility of Endogenous Ecotropic Murine Leukemia Viral Genomes Within Mouse Chromosomal DNA and Integration of a Mink Cell Focus-Forming Virus-Type Recombinant Provirus in the Germ Line

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Characterization of endogenous ecotropic Akv proviruses in DNA of low and high leukemic mouse strains revealed the presence of one to six copies of the Akv genome per haploid genome equivalent integrated in the germ line. Low leukemic strains analyzed so far contained only one complete copy of the Akv proviral DNA. The site of integration varied among strains, although genetically related strains often carried the Akv proviral gene in the same chromosomal site. The different substrains of the AKR mouse displayed the presence of variable numbers (two to six) of Akv genomes. In all substrains one Akv genome was present in an identical chromosomal site; this locus probably comprised the progenitor genome. Closely related substrains had several Akv proviral DNAs integrated in common sites. The accumulation of Akv genomes in the germ line of the AKR/FuRdA strain is likely the result of independent integration events, since backcross studies with the Akv-negative 129 strain showed random segregation of all six proviral loci. The AKR/Cnb strain carried a recombinant provirus in the germ line. This provirus resembled in structure the AKR mink cell focus-forming viruses, which are generated by somatic recombination during leukemogenesis. Therefore, the germ-line amplification of Akv proviral DNAs occurs most likely through infection of embryonic cells by circulating virus.

Most mouse strains carry multiple copies of murine leukemia viral (MLV) genomes as genetic elements in the host chromosome (15, 17, 22). These endogenous viruses have been classified according to their host range: ecotropic viruses, which can only infect mouse cells; xenotropic viruses, which are only infectious for cells of other species; and amphotropic viruses, which can infect both types of cells (7, 15, 20). These endogenous viruses are expressed in some mouse strains, whereas in other strains these genomes are repressed by host control systems (13, 16, 28). The expression of the ecotropic Akv virus is associated with the occurrence of lymphatic leukemia in various strains; a high incidence of leukemia is associated with a high level of Akv expression like in the AKR, C58, and C3H/Fg strains, and a low incidence of leukemia is seen in strains in which only occasionally Akv virus expression is observed, like BALB/c, DBA, and C3H, whereas in strains without an increased frequency of leukemia, like 129, no Akv virus is expressed (4, 9, 10, 14, 17, 21, 26). Both liquid hybridization studies and DNA analyses with the Southern transfer technique have shown that the nonleukemic strains lack the genetic information for ecotropic virus (17, 19, 26). The endogenous ecotropic viruses present in the low and high leukemic strains share a set of restriction endonuclease sites, suggesting that they have originated from a common ancestor (26). Since all of these endogenous proviruses are part of the host genome complement and are transmitted from parent to offspring according to Mendelian expectations, we were interested to see whether the characteristic integration sites of these ecotropic viruses were conserved among related mouse strains. The transposon-like structure of RNA tumor viruses and the ability to generate infectious virus and reinfect host cells would in principle allow considerable variation in the number and sites of integrations. We chose both high and low leukemic strains to perform this analysis. The high leukemic AKR strains originate from Jacob Furth (6) who started to select mice for high incidence of leukemia. C. Lynch continued inbreeding of several sublines up to 1946. Most of the now available

sublines are derived from her AKR/M line (6). Some of these lines differ in several genetic markers (1).

We analyzed 14 different substrains of the high leukemic AKR strain and various low leukemic strains to study the mobility of the Akv viral genome within the chromosomal DNA. We addressed the following questions. At what frequency do germ-line integrations of Akv occur spontaneously? What is the stability of the integrated structure and through what mechanisms are germ-line integrations established, through infection of embryonic cells by circulating virions, as suggested by the results of Rowe and Kozak (23), or through intracellular pathways?

MATERIALS AND METHODS

Virus. The AKR virus was isolated and propagated as described previously (26).

Mice. Mice or organs from the different strains were obtained as follows: BALB/c, 129, C57B1/10ScSn, and C3H/St-Z strains from the Central Animal Laboratory of the University of Nijmegen, Nijmegen; AKR/JS*, C3H/HeJ, C57B1/6J, and A/J livers from F. Jensen, Scripps Clinic, La Jolla, Calif.; AKR/FuRdA, B10Y, ACR (or AKR/FuA), and C3H/HeA mice from Netherlands Cancer Institute, Amsterdam. NZW and DBA/2 strains from the Central Institute for the Breeding of Laboratory Animals-TNO, Zeist, The Netherlands; GRS/A strain from the Radiobiological Institute-TNO, Rijswijk, The Netherlands; AKR/Cnb and AKR/Rb-k strains from Centre d'Etude de l'Energie Nucleaire, Mol, Belgium; AKR/J and AKR/Cu strains from the Weizmann Institute of Science, Rehovot, Israel; AKR/A Bom and AKR/H Bom strains from the Breeding and Research Center, GL Bomholtgaard Ltd., Ry, Denmark; AKR/JS DNA and organs from R. Jaenisch, Heinrich Pette-Institute, Hamburg, Federal Republic of Germany; AKR/Tl strain from H. Pogosianz, All Union Cancer Research Centre, Moscow, USSR; AKR/Sn organs from Health Research, Inc., Roswall Park Memorial Institute, New York, N.Y.; AKR/NH strain from Zentral Institut für Versuchstiere, Hannover, Federal Republic of Germany; AKR/N strain from National Cancer Institute of Health, Bethesda, Md.; and AKR/Rho strain from IFFA CREDO, l'Arbresle, France.

Preparation and characterization of the cDNA probe. An AKR-MLV specific ^{32}P-labeled cDNA probe was prepared with the endogenous polymerase reaction. The AKR-MLV cDNA sequences that cross-hybridize with other mouse viruses were removed by hybridization to an excess of 129 mouse DNA followed by hydroxyapatite chromatography exactly as described by Berns and Jaenisch (2). The remaining single-stranded fraction was tested for specificity by hybridizing with DNAs of different strains (129, NIH Swiss) which do not contain the Akv provirus and by hybridization with BALB/c DNA and NIH Swiss Akv-2 congenic DNA, which contain one copy of AKR-MLV (19). All four *Bam*HI restriction endonuclease fragments of a cloned AKR-MLV integrated virus (clone 623) hybridized with this probe, indicating that this probe contains sequences complementary to the different regions of the AKR-MLV genome.

Analysis of DNA. Isolation of DNA from mouse livers, restriction endonuclease digestion gel electrophoresis, transfer to nitrocellulose filters, and hybridization were carried out as described previously (19).

RESULTS

Determination of Akv-specific sequences. To detect exclusively Akv sequences, a cDNA probe was prepared as described previously and outlined above (19). The resulting Akv cDNA probe did not cross-hybridize to non-Akv endogenous proviruses present in mouse DNA. Using this Akv-specific probe, we have analyzed the genomic organization and integration sites of Akv proviral DNA in a number of inbred mouse strains. To characterize the integration sites we used restriction endonuclease *Eco*RI, which does not cleave the Akv proviral DNA. Therefore, the size of the generated fragment is determined by the position of *Eco*RI sites in adjacent host DNA. The presence of Akv sequences in identically sized *Eco*RI fragments among different strains would therefore strongly suggest that they are integrated in the same chromosomal site. Liver DNAs from a series of laboratory mouse strains were analyzed for the presence of Akv-containing *Eco*RI DNA fragments. The results of such an analysis performed on low leukemic and nonleukemic strains is shown in Fig. 1. It appeared that different strains with low virus titers carried one copy of the ecotropic MLV genome (Fig. 1B, lanes 1 through 10), whereas in virus-negative strains no endogenous Akv sequences were detectable (Fig. 1B, lanes 11 through 13). In various strains the Akv sequences were present in *Eco*RI fragments of the same size, whereas other strains carried Akv sequences on differently sized fragments, e.g., a fragment of 20 kilobase pairs (kbp) was detected in the ACR, C3H/HeA, C3H/HeJ, A/J, and BALB/c strains of mice (Fig. 1B, lanes 1, 2, 3, 5, and 6), a 31-kbp fragment was found in the C57BL/6J, C57BL/10Sn, and B10Y strains of mice (Fig. 1B, lanes 4, 9, and 10), whereas the NZW and DBA/2 strains of mice had differently sized *Eco*RI fragments of 24 kbp (Fig. 1B, lane 7) and 16 kbp (Fig. 1B, lane 8), respectively. The differences in proviral integration sites were further substantiated by *Bam*HI digestion of DNA from these inbred strains. This analysis showed different virus-cell junction fragments (data not shown). The coincidence of the Akv-containing *Eco*RI fragments among strains is in full agreement with the genealogy of the mouse strains (Fig. 1A). The Akv genome detected in a 20-kbp fragment was apparently conserved in the segregation of BALB/c, A/J, and C3H/He strains from the Bagg strain. DNA from mice of the C3H/St-Z strain did not show hybridization with the AKR-MLV cDNA. Either the C3H

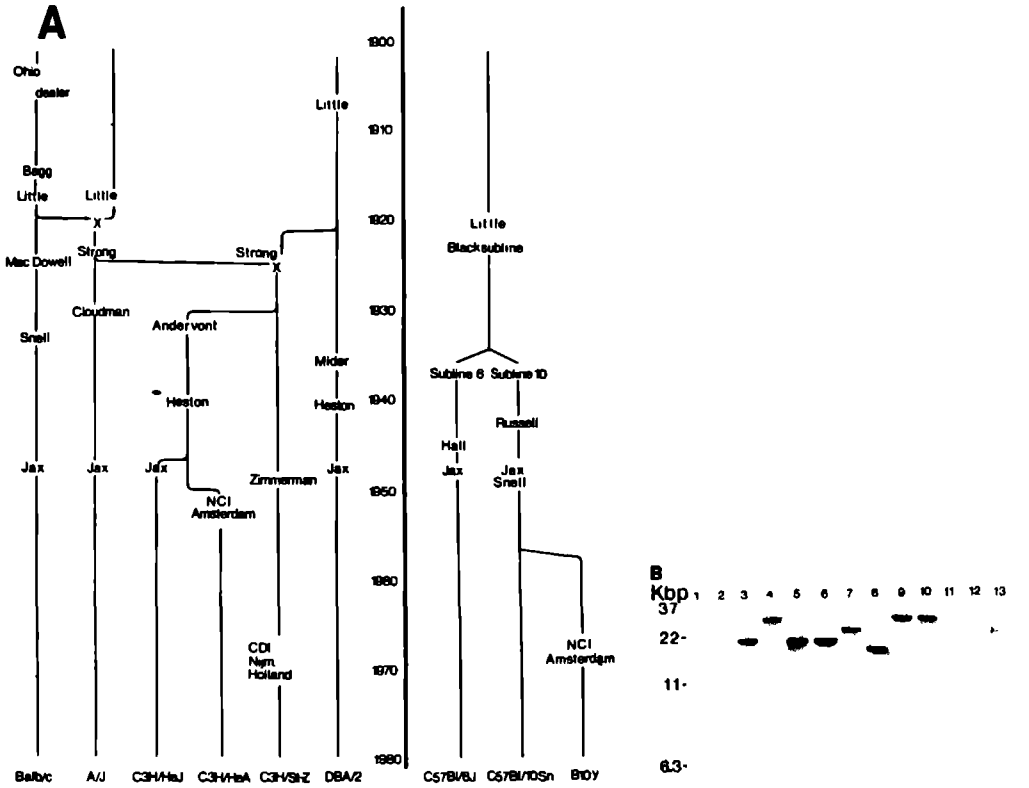

FIG 1 Segregation of endogenous Akv proviruses during inbreeding of low-virus-titer strains A, The genealogy of the mouse strains was derived from the Mouse News Letter, inbred strains of mice by J Staat, (ed), Jackson Laboratories, and are plotted against the dates for mating and segregation B, *Eco*RI-digested DNAs were analyzed by the Southern procedure (24) and hybridized with the specific cDNA probe *Eco*RI does not cleave the Akv viral DNA (25) Lanes 1 through 13 represent liver DNAs from the following strains ACR (lane 1), C3H/HeA (lane 2), C3H/HeJ (lane 3), C57B1/6J (lane 4), A/J (lane 5), BALB/c (lane 6), NZW (lane 7), DBA/2 (lane 8), C57B1/10Sn (lane 9), B10Y (lane 10), GRS (lane 11), C3H/St-Z (lane 12), and 129 (lane 13) Adenovirus type 2 viral DNA and phage λ DNA digested with *Eco*RI, *Hin*dIII, and *Bam*HI were used as molecular weight markers

strain maintained by Strong was not homozygous for this Akv locus at the time of transfer to Andervont and subsequently lost the locus during inbreeding, or the Akv locus in the C3H/St-Z has been lost. The conservation of integrated sites among strains that have been separated for nearly 60 years (BALB/c, A/J, and C3H) and 40 years (C57BL sublines) is indicative of a stable integration of the endogenous Akv genome Furthermore, it shows a very low frequency of new integration events during the process of inbreeding of low-virus-titer strains.

Akv sequences in the AKR strains. Previously it has been shown that several sublines of the high-virus-titer AKR strain contain variable numbers of endogenous Akv genomes integrated in different chromosomal sites (19) Figure 2 shows the genealogical relation and the patterns of Akv-specific *Eco*RI fragments in liver DNA from 14 AKR sublines maintained at different laboratories. All of the sublines carry an Akv genome on a 55-kbp *Eco*RI DNA fragment. Substrains derived from the Jackson group and the Law group, which separated in 1950, have a second fragment of 12 kbp in common. In addition, each of these two groups contains a third common fragment, a 29-kbp fragment in the Law group and a 10 5-kbp fragment in the Jackson group Besides these common integration sites, unique additional fragments can be detected in the Jackson sublines JS, JS*, and Sn, which are not seen in other related colonies. The NH and HB sublines were separated from the N colony in 1975 and contain one additional fragment. From the different endogenous genomes present in the AKR/Rho and AKR/FuRdA strains, three

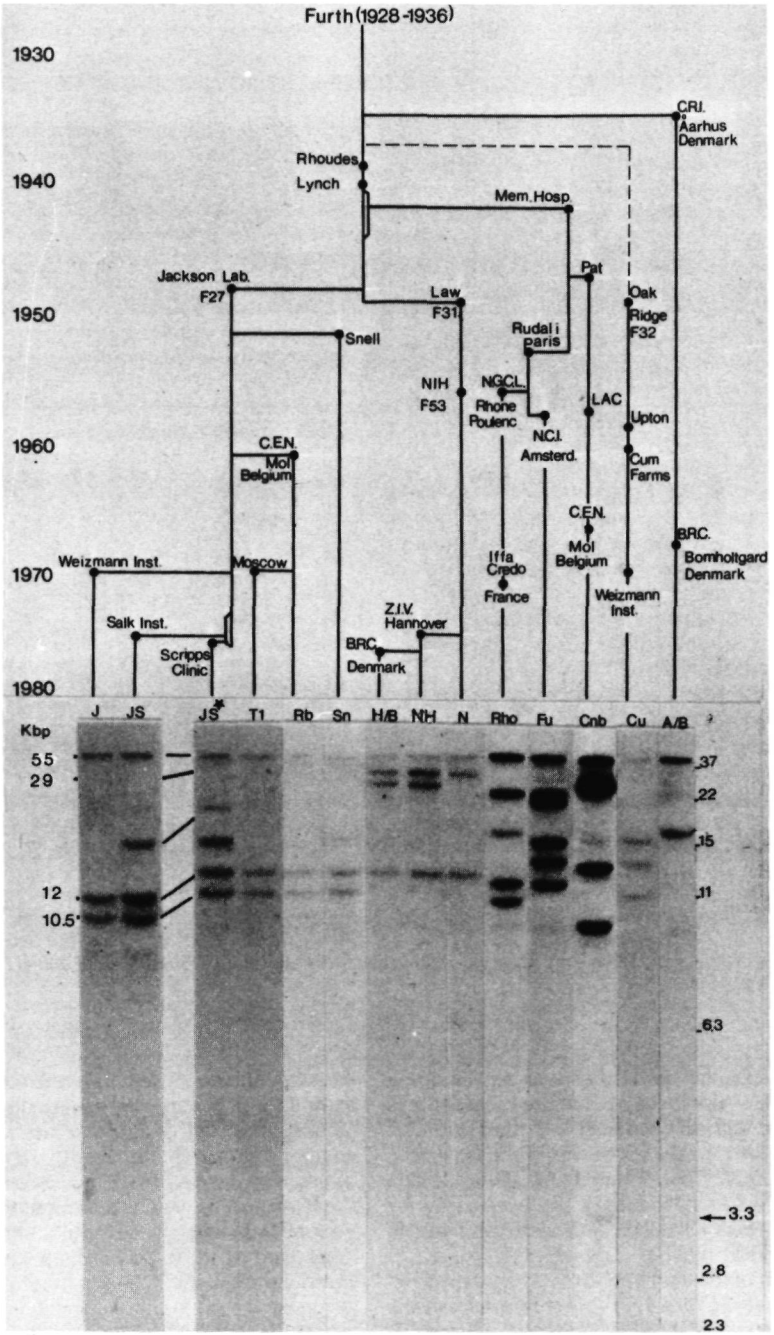

FIG. 2. Identification and segregation of endogenous Akv proviruses in 14 substrains of AKR mice. The upper part represents the genealogical tree of the AKR substrains which was obtained from the Mouse News Letter and plotted against the recorded time for segregation. The dashed line indicates the most likely historical pathway of the AKR/Cu strain. The lower part of the figure represents the Akv genotype for each substrain, which was determined by Southern blotting analysis of *Eco*RI-digested liver DNA. The molecular sizes of some individual Akv-containing fragments are indicated at the left. The molecular weight markers at the right are as described in the legend to Fig. 1.

bands are located at the same position, whereas the others are unique for each substrain. In general, the integration patterns show that strains which have been separated in the 1930's and 1940's have only the Akv genome present in the 55-kbp *Eco*RI DNA fragment in common; later-diverged strains possess several Akv genomes integrated in the same chromosomal site. The position of Akv proviruses in the different chromosomal loci is in complete agreement with the segregation pattern of the various sublines and illustrates the stability of proviral integrations in these strains.

Segregation of endogenous Akv genomes from the AKR/FuRdA strain. To study the characteristics of individual Akv loci, the AKR/FuRdA strain, which contains six endogenous genomes, was backcrossed with the ecotropic virus-negative 129 strain. We were interested to see whether the six proviral loci were genetically linked or segregated independently. Furthermore, it allows analysis of the biological activity of the individual proviral loci. From 29 mice, obtained after backcrossing 129 with (AKR/FuRdAx129) F1 males and females, liver DNA was isolated and digested with *Eco*RI. The segregation patterns of these 29 mice show random segregation of the six proviral loci (Fig. 3). Among the 29 mice analyzed, 6 mice with each one of the six loci were found. Therefore, the Akv genomes are integrated in distant unlinked positions in the AKR genome and most likely are the result of independent integration events in the germ line rather than gene duplication or tandem integrations.

Mode of germ-line integration. The accumulation of Akv proviral loci during inbreeding in AKR substrains could be the result of two different mechanisms. Either it occurs via intracellular mechanisms, e.g., reverse transcription and subsequent reintegration, or it is the result of reinfection of (primordial) germ cells or early stage embryos. The AKR/Cnb strain allowed us to obtain more information concerning the mechanism involved in amplification of proviral loci. These mice display an *Eco*RI DNA fragment of 3.3 kbp hybridizing to the Akv-specific cDNA probe (Fig. 2). Since authentic Akv proviral DNA does not carry an *Eco*RI site, this 3.3-kbp *Eco*RI DNA fragment might comprise genetic information for part of a recombinant MLV provirus. Such recombinant proviral DNAs, however, are normally restricted to tumor tissues and are generated only later in life by somatic recombination in lymphatic tissues (8, 19, 27). The presence of such a recombinant provirus in the germ line therefore suggests infection of germ-line cells.

Liver DNA (250 μg) of AKR/Cnb mice was cleaved with *Eco*RI, and the fragments were separated on the basis of size on a 0.4% agarose gel. The 3.3-kbp *Eco*RI DNA fraction was electroeluted from gel slices and further characterized by digestion with *Xba*I and *Pst*I. After cleavage with *Pst*I and *Xba*I, one (1.5-kbp) and two (1.1- and 1.7-kbp) fragments, respectively, were found which hybridized to an Akv-specific cDNA probe (Fig. 4A). Such fragments could not be generated from AKR proviral DNA. However, fragments of 1.5 and 1.1 kbp could be obtained from 3′ parts of mink cell focus-forming virus (MCF)-type recombinant proviruses detected in all leukemic tissues of AKR mice (Fig. 4B) (3, 19); therefore, the 3.3-kbp *Eco*RI fragment represents part of a AKR-MCF recombinant genome. Since recombinant proviruses are generated by somatic recombination, the presence of such a provirus in the germ line of the AKR/Cnb strain most likely indicates that introduction of this recombinant in the germ line has been caused by infection through circulating recombinant viruses produced in lymphatic tis-

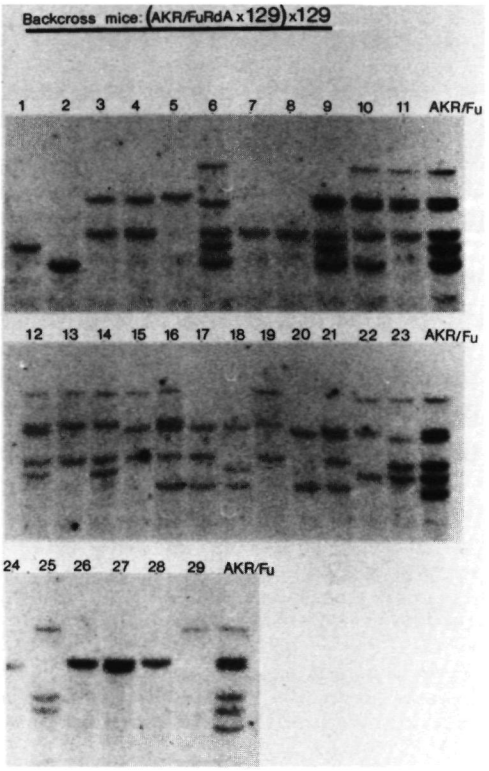

FIG. 3. Segregation of Akv proviruses in backcross of (AKR/FuRdA × 129) mice with the 129 strain. *Eco*RI-digested DNAs were analyzed by the Southern procedure. Lanes 1 through 29 represent liver DNAs from 29 mice (AKR/FuRdA × 129).

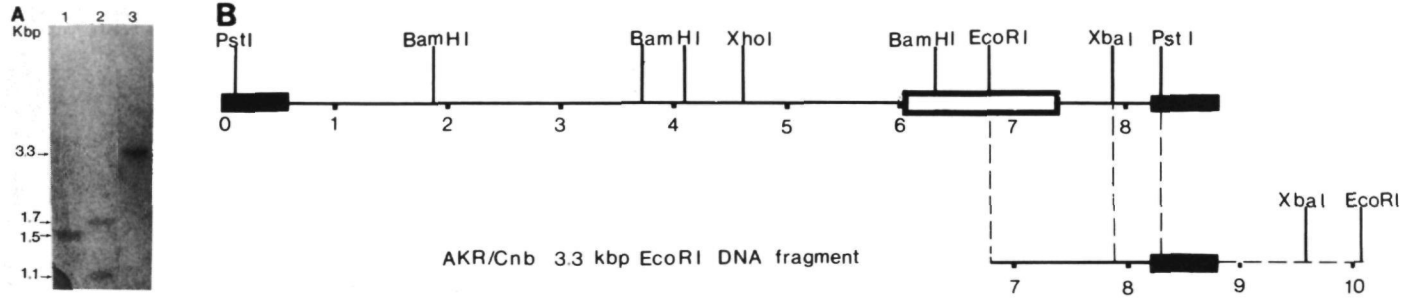

FIG. 4. Recombinant AKR-MLV sequences in AKR/Cnb mice. A, The 3.3- kbp *Eco*RI fragment from liver DNA (250 μg) of AKR/Cnb mice was isolated as described previously (19). This fragment was cleaved with *Pst*I (lane 1) and with *Xba*I (lane 2). Lane 3 represents the uncleaved 3.3-kbp *Eco*RI fragments. The molecular sizes of fragments containing the AKR-MLV sequences are indicated to the left. B, The 1.5-kbp *Pst*I fragment and the 1.1-kbp *Xba*I fragment could be mapped on the linear map of the MCF-type recombinant proviruses (19) (upper line). The positions in kbp of some restriction sites are indicated. Black boxes represent the long terminal repeat sequences. The solid frame represents the substituted sequences present in the MCF recombinant proviral genomes (19, 27). The *Eco*RI site (at 6.8) is characteristic for MCF recombinants and causes the generation of the 1.1- and 1.5-kbp fragments (19). The line at the bottom represents the structure of the 3.3-kbp *Eco*RI fragment. The solid line represents proviral sequences, and the dashed line represents the flanking cellular sequences.

sues. Whether sperm cells, oocytes, or developing embryos are the target for infection remains obscure for the moment.

DISCUSSION

We have characterized the integration sites of endogenous ecotropic genomes in DNAs from several low- and high-virus-titer strains. Previous studies have shown that cell DNA from mice with high incidence of leukemia contained multiple copies of ecotropic proviral DNA, whereas mice with low incidence of leukemia comprise in general only a single ecotropic viral genome (4). Our analyses confirm these results. The Akv genome in the low leukemic strains is found in only a few different chromosomal loci. The Akv genome in C3H/HeA, C3H/HeJ, A/J, and BALB/c strains is present in the same chromosomal site. Since these strains have been separated for nearly 60 years, this observation illustrates the stability of integrated proviral elements (Fig. 1A). The same stability is found within the C57Bl/6J and C57Bl/10Sn strains, which have been separated for 40 years and which both carry their Akv sequences in a 31-kbp *Eco*RI fragment (Fig. 1B). The presence of Akv genomes in different chromosomal loci of low leukemic strains supports the hypothesis that the endogenous Akv sequences in these strains were acquired by independent germ-line integrations (26).

Previously, we reported a difference in number and in site of integrations of Akv genomes among five substrains of the high-virus-titer AKR strain (19). Besides this variability, all of these strains contain an Akv genome in an *Eco*RI fragment of 55 kbp which is also present in nine additional AKR sublines (Fig. 2). These results clearly suggest a progenitor function of this locus as a parental gene for the other endogenous genomes. This Akv genome in a 55-kbp *Eco*RI fragment has also been found in the AKR/N, AKR/J, and AKR/Cu mouse strains by others and corresponds to the Akv-1 locus (4, 25). AKR sublines which have segragated for long periods of time (AKR/A, AKR/Cu, AKR/FuRdA, AKR/Cnb, and AKR/N) have only the Akv genome present in the 55-kbp fragment in common, whereas sublines which have segregated more recently carry several Akv genomes in identical chromosomal sites, e.g., the Jackson and Law are more related to each other (Fig. 2). This indicates that the viral genomes become immobilized in the host chromosomal DNA and behave as cellular genes.

The difference between AKR/J and AKR/N mouse strains (Fig. 2) most likely results from new integration events after the separation of these strains. However, the difference between these two sublines could also be explained by transposition of an Akv proviral genome in one of these strains after separation. Within progeny of the A/JxDBA/2 mice (Fig. 2) the DBA/2-specific locus (16-kbp *Eco*RI fragment) is not present. The absence of this Akv genome could be the result of an excision event or could be ascribed to the heterozygosity for this locus at the moment of crossing. However, the absence of this genome within the A/JxDBA/2 progeny and its presence within the DBA/2 strain could also be due to an independent germ-line integration event within the DBA/2 strain after 1925, the year in which the DBA/2 strain was crossed with the A/J strain. It is obvious that no solid evidence for an excision event can be found in any of the low leukemic strains.

The observed diversity and accumulation of Akv genomes within the sublines could be due to gene duplication, tandem integration, or reinfection of germ cells. Backcrossing the AKR/FuRdA strain with the ecotropic virus-negative 129 strain revealed independent segregation of the six loci. Therefore, the variation of Akv loci within the AKR substrains is more likely the result of independent germ-line integration events which occur on average once in 30 generations. As a consequence, integration of provirus in chromosomal DNA of germ cells has to occur with a much higher frequency, since the newly acquired genomes might be easily lost during inbreeding by the heterozygous or even mosaic character of the newly acquired locus or by being lethal in the homozygous state. The acquisition of new viral integrations is predominantly seen in strains with high virus titers and seems therefore related to the expression of the provirus. The introduction of MLV proviruses in the germ line through infection has already been reported; in vitro infection of preimplantation embryos results in the acquisition of genetically transmitted proviruses (11, 12). The observed amplification in the Akv-1 congenic mouse strain (23) and in the AKR strains most likely occurs by infection of germ-line cells. We provide evidence that, indeed, circulating virus has been involved in germ-line integration. We detected an MCF-type recombinant provirus in the germ line of the AKR/Cnb strain. Since these types of recombinants are generated only later in life in the lymphatic tissues of preleukemic and leukemic AKR mice by somatic recombination between ecotropic AKR viruses and endogenous xenotropic-like sequences (8), integration in the germ line most likely occurs by infection of primordial germ cells, oocytes, or early embryos by circulating virus. Although one could theoretically envisage the intracellular generation of MCF genomes in germ cells, this seems rather unlikely. The results of Rowe and Kozak (23), who showed that the acquisition of novel

virus-containing loci is only associated with virus-positive females, also suggest that the most likely in vivo mechanism of germ-line amplification is by the infection of oocytes or developing embryos. However, at this point we cannot exclude that postcoital infection of sperm cells occurs or that the virus is introduced during fertilization. In vitro fertilization studies in the presence of infectious virus are being performed to test the possibility of this last mode of infection.

ACKNOWLEDGMENTS

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GENERATION OF AKR-MINK CELL FOCUS FORMING VIRUSES: A CONSERVED SINGLE COPY XENOTROPIC-LIKE PROVIRUS PROVIDES RECOMBINANT LONG TERMINAL REPEAT SEQUENCES

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GENERATION OF AKR-MINK CELL FOCUS FORMING VIRUSES: A CONSERVED SINGLE COPY XENOTROPIC-LIKE PROVIRUS PROVIDES RECOMBINANT LONG TERMINAL REPEAT SEQUENCES

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ABSTRACT

AKV and AKR-MCF-specific probes from the envelope and LTR region were prepared to study the structure of recombinant proviruses in tumor tissues of AKR mice. The results show that: i. All the somatically acquired proviruses possess, besides a recombinant gp70 gene, an altered U3-LTR. In a substantial portion of the somatically acquired AKR-MCF proviruses, the LTR comprises sequences derived from the same xenotropic-like provirus. iii. This U3-LTR donating parental provirus (LTRdXe) is present only once per genome equivalent in several mouse strains. iv. In the strains which contain the LTRdXe provirus the provirus is present in the same chromosomal site. v. Restriction analysis of the LTRdXe revealed that the MCF gp70 sequences are derived from a parental provirus, different from LTRdXe. Therefore, at least two

non-ecotropic parents participate in the generation of leukemogenic AKR-MCF viruses: A xenotropic-like virus, LTRdXe, donating U3 LTR sequences and (an)other xenotropic-like virus(es) providing gp70 seqences.

INTRODUCTION

Inbred laboratory mice carry various types of murine leukemia viruses as part of their genome complement (9,21,23,32). These viruses can be classified according to their host-range. The ecotropic MuLV's which can infect and replicate only in mouse cells, and xenotropic MuLV's which only propagate in cells of other species (21,33). Endogenous ecotropic viruses are found both in high leukemic strains, like AKR, C58 and C3H/Fg as well as in low leukemic strains like BALB/c, DBA and C3H (4,18,30,31,37). The various sublines of the AKR strain each harbor, besides the Akv1 locus common to all AKR strains, several AKV genomes in unique chromosomal locations (4,30,37). The expression of these ecotropic endogenous viruses shows a causal relationship with the development of thymic leukemias later in life (39). Several stages can be discerned in the life span of these high leukemic strains: From shortly after birth these animals express high titers of the ecotropic virus (33). Prior to the onset of leukemia a marked amplification of MuLV antigen expression is observed in the thymus (19). This increased antigen production coincides with the expression of endogenous xenotropic-like sequences (19). From this time on mink cell focus-forming viruses can often be detected in thymuses of these animals (14). Unlike AKV viruses and the class II MCF's (24), most of the class I MCf's do accelerate leukemia when when injected into newborn AKR mice (10,28). It has been suggested that these MCF's are generated by multiple recombination events in which at least three endogenous viruses are involved (11,38). Comparison of the genomic structure of AKV virus with the leukemogenic MCF's shows that the differences are found predominantly in the 3' half of the genome: within the 3' portion of the p15E gene, the U3 region of the Long Terminal Repeat (LTR) and the 5' region of the gp70 gene (7,16,20,24). In this report we describe a more detailed analysis of the structure of AKR MCF proviruses integrated in leukemic tissues. We provide evi-

dence that a significant portion of the leukemogenic AKR-MCF viruses are generated by at least two recombinational events: i. Acquisition of heterologous U3 sequences, derived from a unique, single copy xenotropic-like parent and ii. Acquisition of xenotropic-like gp70 sequences from a parent, differing from the U3 donor.

MATERIAL AND METHODS

Viruses and preparation of cDNA.

AKR virus and Moloney MuLV (clone 1A) were propagated and isolated as described previously (40). Alpha-32P labeled AKR and M-MuLV cDNA was prepared by the endogenous polymerase reaction and the sequences which crossreacted with heterologous endogenous viruses, were removed as described (2,31).

Mice

AKR/JS, AKR/J, AKR/FuRdA, BALB/c, DBA, C57BL/10ScSn, and 129 mice were obtained as described (30).

DNA analysis

Isolation of DNA, restriction endonuclease digestions, gel electrophoresis, electro-elution, transfer to nitrocellulose, and hybridizations were carried out as described earlier (31,40).

Molecular cloning of AKV probes

A PvuII - EcoRI fragment from clone AKR-623 (courtesy of Dr. Lowy; see fig.1) was isolated from an agarose gel by electro-elution, and digested with restriction endonuclease Sau3A. The resulting fragments were ligated into the BamHI site of pBR322. From the Amp-resistant/Tet-sensitive colonies which reacted with AKR cDNA, but not with M-MuLV cDNA, plasmid DNA was isolated. These DNA's were nick-translated as described (39), and tested for their specificity by blot hybridization to 129, BALB/c and AKR mouse DNA.

The inserts of these plasmids were localized on the genomic map by hybridization to Southern blots, which contained a number of restriction digests of the recombinant phage AKR-623 (22).

Molecular cloning of AKR-MCF provirus from tumor DNA.

High-molecular-weight lymphoma DNA (250 #g) from the AKR/JS mouse was digested to completion with EcoRI. After gel electrophoresis in a 0.6% agarose gel, DNA was electro-eluted from 2mm gel slices (31), and

the fractions, hybridizing with the AKV-specific cDNA probe, were used for cloning. Phage cloning was performed as described (1), except that charon 28 was used as vector. The identity of plaques hybridizing with a total AKR cDNA probe was determined by restriction enzyme analysis and blot hybridization of mini-phage isolates. The insert of the AM35 recombinant phage selected by this procedure was subcloned in pBR322.

Cloning and labeling of MCF probes

Probe 1 and 2 (Fig.4) were obtained by Bal31 exonuclease digestion and cloning as described by Poncz et al.(29). The fragments were inserted into M13mp8 and M13mp9 (26). All probes were labeled by the primer extension reaction (34). For probe 4 a dideoxy nucleotide was included in the reaction.

Transfection

The pAM35 insert was ligated to a 15 Kbp EcoRI fragment, which comprises the 5' end of Moloney MCF genome. Transfection of this DNA on mink lung fibroblasts (CCL-64) was carried out as described (3,13).

RESULTS

Construction and Selection of hybridization probes, specific for Ecotropic MuLV's.

The recombinant phage AKR 623 (courtesy of Dr.Lowy, NIH), containing an infectious AKV provirus was used as source for preparing ecotropic specific hybridization probes. Various regions of the AKV genome were subcloned in pBR322 and tested for their specificity by hybridization with several mouse DNA's.

A PvuII-EcoRI fragment mapping between coordinates 5.8 and 9.8 and containing part of the polymerase gene, the total envelope gene and LTR region was digested with Sau3A and the resulting fragments subcloned in the BamHI site of pBR322 (fig. 1A). (Here, and in the remainder of the paper, viral DNA sites are referred to by their distance from the 5' end of the viral DNA.) Colonies were screened by filter hybridization using both AKV and Moloney MuLV cDNA as probe (31,39). Recombinant clones which reacted with the AKV cDNA, but not with the Moloney cDNA were further tested for their ecotropic specificity on Southern blots (35) containing EcoRI digested DNA from dif-

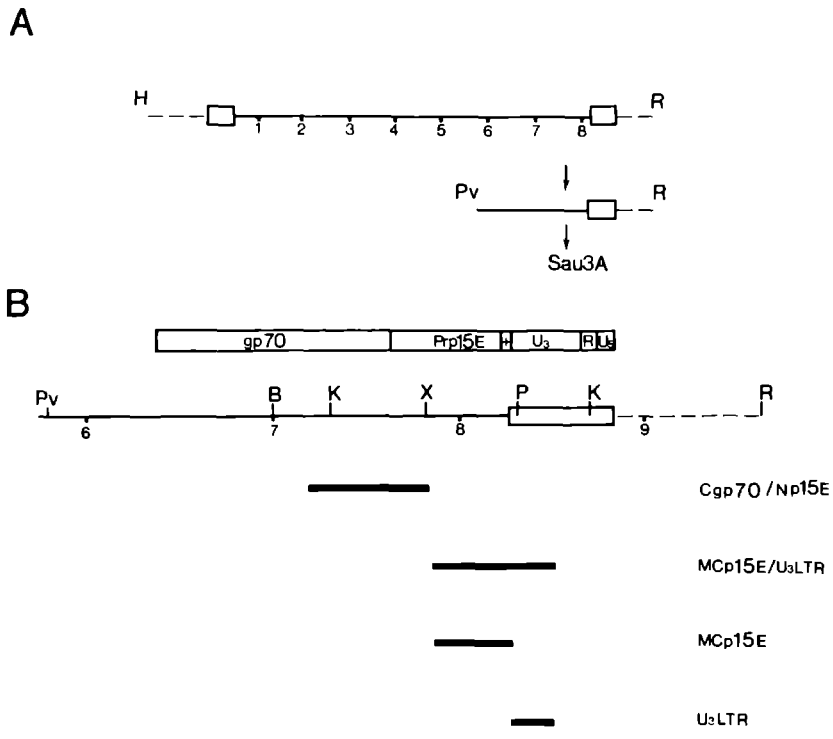

Figure 1 Structure of AKV provirus and location of the AKV-specific probes.
A. Structure of phage AKR-623 (22), and position of the PvuII-EcoRI fragment used for Sau3A digestion and cloning. The dashed line represents the flanking cellular sequence.
B. The upper box gives the location of the envelope gene and the LTR region. The positions of some restriction sites are indicated (in Kbp from the 5' end of the viral genome). The position of the AKV subclones are shown. Pv, PvuII; B, BamHI; K, KpnI; X, XbaI; P, PstI; R, EcoRI.

ferent mouse strains: The 129 strain, which does not contain an endogenous ecotropic provirus, the BALB/c strain, which carries one copy of the AKV virus in its genome, and the AKR/JS , harboring four endogenous AKV genomes (30). This analysis revealed that 2 clones and 3 probes from the envelope and LTR region were ecotropic-specific under the conditions used.

The positions of these probes on the genomic map are summarized in fig.1B. Cgp70/Np15E is located between map position 7.2 and 7.8, corresponding to the C-terminal region of gp70 and the N-terminal region of p15E. This probe overlaps the XbaI site. The MCp15E/U3LTR

clone, which is juxtaposed to the Cgp70/Np15E probe, extents from 7.8 to 8.5 on the genomic map, corresponding with the middle- and C-terminal region of p15E and the U3 region of the LTR (see fig.1B). PstI digestion of the MCp15E/U3LTR plasmid generated two fragments of 1.5 and 3.5 kbp respectively. The 3.5 kbp fragment contains besides pBR322 sequences 250 bp of DNA corresponding to a region in the U3 LTR directly to the right of the PstI site. The 1.5 Kbp fragment harbors the remaining 450 nucleotides corresponding to the middle- and C-terminal region of p15E (MCp15E) and the first 30 nucleotides of the U3 region up to the PstI site. Hybridization of the MCp15E with a Southern blot containing HindIII digested DNA from BALB/c and the AKR/J strain (with 1 and 3 Akv copies respectively) revealed one and three hybridizing fragments, corresponding to the 3' regions of the viral genomes (fig.2A). Since HindIII cleaves the AKV genome once (36), hybridization with the U3LTR probe should give rise to two and six hybridizing fragments, corresponding to both 3' and 5' portions of the proviral genomes (fig.2B). The Cgp70/Np15E probe showed similar specificity for the ecotropic proviral genomes (data not shown).

As has been shown by others for the gp70 and p15E region (8,16), our results indicate that an ecotropic specific U3LTR probe can be prepared which allows the recognition of both 3' and 5' portions of integrated MuLV genomes.

Construction of hybridization probes for MCF proviruses.

We have molecularly cloned the 3' half of an AKR-MCF provirus present in the chromosomal DNA of an AKR/JS lymphoma: The tumor DNA was digested with EcoRI, and the fragments reacting with an AKV-specific cDNA probe characterized (31). Fig.3 shows the result of a blot hybridization. Lane 1 represents liver DNA, lane 2 tumor DNA. Within the tumor DNA two additional fragments of 3.5 and 20 Kbp were detected. Since AKR-MCF's have an EcoRI recognition site at position 6.9 on the genomic map, the 3.5 kbp fragment is most likely derived from the 3'region of an AKR-MCF provirus. The 3.5 Kbp fragment was molecularly cloned in phage lambda (see material and methods). Charac-

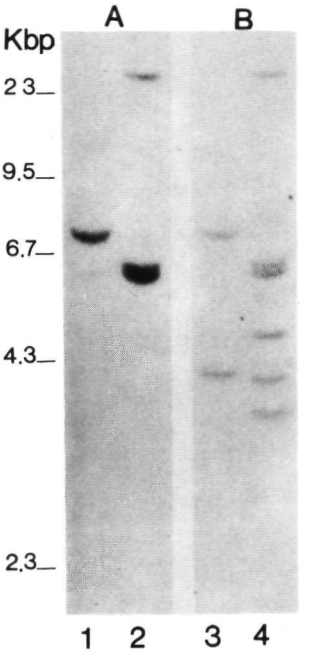

Figure 2
Characterization of the AKV-specific MCp15E and LTR probe
HindIII digested liver DNAs from the Balb/C (lane 1 and 3) and AKR/J (lane 2 and 4) mouse, hybridized with the MCp15E probe (A) and the U3LTR probe (B). HindIII cleaves the endogenous AKV proviral genome once. Phage lambda, digested with HindIII, was used as Molecular Weight marker.

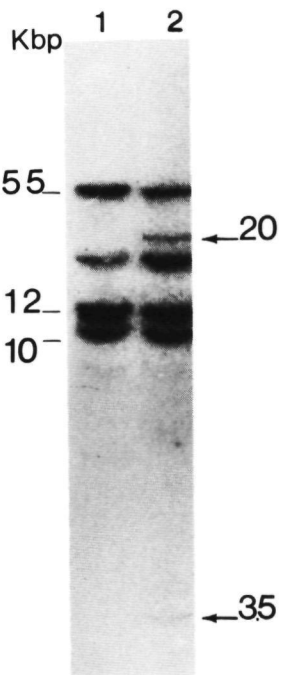

Figure 3
Detection of somatically acquired AKV sequences within a AKR/JS tumor.
EcoRI digested tumor DNA (lane 2) and normal liver DNA (lane 1) were hybridized with a AKV-specific cDNA probe (31). The Molecular Weights of the fragments which carry the additional AKV sequences are indicated at the right.

terization of this clone with various restriction endonucleases, revealed the typical structure seen in most AKR-MCF viruses (see fig.4): The ecotropic specific XbaI site at position 7.8 (6,7), the SstI site at coordinate 7.6, which is present in many MCF recombinants, but absent from the ecotropic viral genome, and an EcoRI site at position 6.9, which is found in all MCF viruses isolated so far (7,39). The biological integrety of this clone was further shown by ligation of this 3'MCF clone to the 5'part of a Moloney MCF provirus, which comprized Moloney-MCF sequences up to the EcoRI site at position 6.9. Transfection of this DNA into mink cells resulted in the production of recombinant viruses, with the expected genomic structure (data not shown).

Fig.4B shows the location of the fragments which were subcloned in phage M13 and used as hybridization probes. EcoRI digested liver DNA from the AKR/JS mouse strain was hybridized with the gp70-MCF probes 1 and 2 and the p15E-MCF probe 3. These probes reacted with about 20 different EcoRI DNA fragments (data not shown). Similar molecular weight fragments were recognized by both the gp70 and p15E probes. No hybridization was observed with the AKV proviruses, suggesting that these hybridization probes most likely annealed to the numerous xenotropic proviral sequences, detectable in many mouse strains (5,17,19).

However the LTR-MCF probe 4, which was derived from the U3 region of our AKR-MCF clone, reacted with only two EcoRI fragments of 6.7 and 9.1 Kbp in both the BALB/c and AKR/JS mouse strain (fig.5 lane 1 and 2). Hybridization of this probe with HindIII digested liver DNA from the AKR/JS strain showed exclusively annealing with a single restriction fragment of 22 Kbp (fig.5 lane 3). These results suggest that the MCF-LTR probe recognizes a proviral genome which is present only once in the genome of the BALB/c and AKR mouse. We have designated this virus LTRdXe (donating LTR Xenotropic virus).

Characterization of the LTRdXe proviral genome

Southern blots containing AKR/JS DNA cleaved with a variety of restriction endonucleases were hybridized with the MCF-LTR probe (data not shown), and a physical map of the proviral locus was constructed.

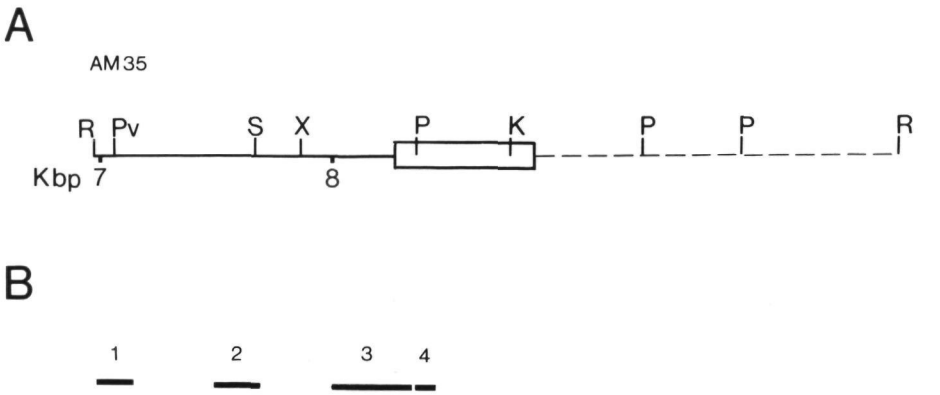

Figure 4 Structure of the 3' and of the AKR-MCF AM35 clone and location of the MCF probes
A. Physical map of the 3.5 Kbp insert of recombinant AM35. Flanking mouse sequences are indicated by the dashed line. The open box represents the LTR. The viral part of the provirus is indicated by the solid line. R, EcoRI; Pv, PvuII; S, SstI; X, XbaI, P, PstI; K, KpnI.
B. The positions of the MCF probes on the genomic map of AKR-MCF are shown. The fragments were subcloned in M13. Number 1 and 2 represent gp70 probes: probe 1 corresponds with position 6.9 to 7.05 on the genomic map of AM35, probe 2 is located between coordinate 7.4 and 7.6. The p15E probe (number 3) comprises a PstI-TaqI fragment of 313 nucleotides, positioned between 8.0 and 8.3. The MCF-LTR probe (number 4) corresponds to a PstI-TaqI fragment between map position 8.30 and 8.45.

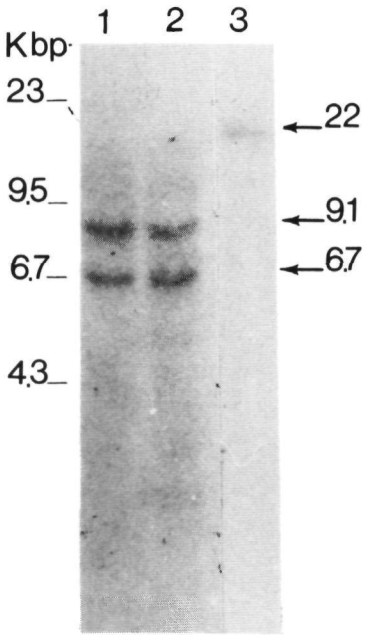

Figure 5
Characterization of the AKR MCF-LTR probe.
EcoRI (lanes 1 and 2) and HindIII (lane 3) digested DNAs were blot-hybridized with the MCF-LTR probe: lane 1, Balb/C liver DNA; lanes 2 and 3, AKR/JS liver DNA. The Molecular Weights of the hybridizing fragments are indicated at the right. Molecular Weight markers are depicted at the left.

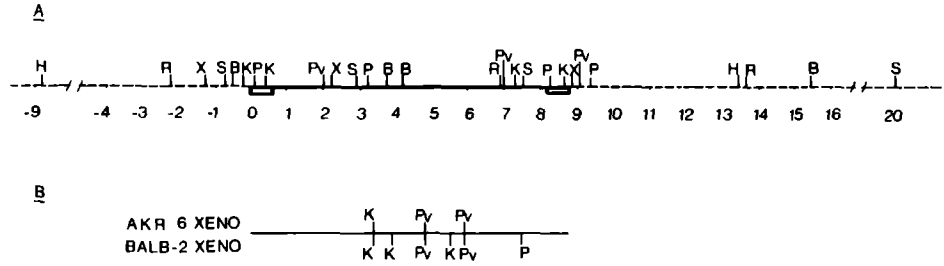

Figure 6 Physical map of the LTRdXe proviral genome with flanking cellular sequences.
The restriction endonuclease map (A) was constructed by cleaving liver DNA from the AKR/JS mouse with various restriction endonucleases, followed by blot-hybridization to the 32P labeled MCF-LTR probe. This map was indistinguishable from the map of AKR-6 and Balb-2 xenotropic virus (7), except that the position of the restriction sites depicted in (B) could not be determined using the MCF-LTR probe. Restriction endonucleases are abbriviated as described in legends to figs 1 and 4. H; HindIII.

Fig.6A shows the the restriction map of LTRdXe with adjacent cellular sequences. Comparison of this structure with the physical map of known xenotropic MuLV's (7), showed a close relationship with the AKR-6 and Balb-2 xenotropic viruses. Some restriction sites, which were shown to be present within the genomes of AKR-6 and Balb-2 xenotropic virus (7), could not be determined in the LTRdXe genome with the MCF-LTR probe (fig.6B). Because of the close resemblance with these known xenotropic viruses, it is justified to conclude that the sequences present in the MCF-LTR probe are derived from a provirus which is similar or identical to the AKR-6 or Balb-2 xenotropic virus.

In order to determine whether the same endogenous virus was present in other laboratory mouse strains as well, liver DNA from various strains was analyzed by Southern blot analysis. The AKR, BALB/c, DBA and C57BL/10 strains all contained a single proviral copy of LTRdXe, present in the same chromosomal site, as indicated by hybridization to identical sized fragments. The 129 mouse strain lacked LTRdXe sequences. The presence of the same proviral structure in the same chromosomal location in different strains indicates that the LTRdXe proviral genome was present in these strains before they diverged at least 80 years ago (27).

Structure of somatically acquired MCF proviruses

It has been shown previously that leukemogenesis in AKR mice is accompanied by amplification of AKV sequences in DNA of tumor tissues (1,9,16,31). The structure of these somatically acquired copies closely resembled the structure of MCF proviruses. Unlike the ecotropic MuLV they all contained an EcoRI site at map position 6.9 and a BamHI site at map position 6.2. We have analyzed these recombinant proviruses with the different probes described above. DNA was obtained from lymphomas of the AKR/FuRdA strain, which contains six endogenous AKV genomes (31).

The ecotropic MCp15E probe shows the presence of these six endogenous proviruses in normal liver DNA upon digestion with EcoRI (fig.7a, sample 5 and 22). Within tumor DNA from the AKR/FuRdA strain many additional fragments were recognized, representing 3' portions of recombinant proviruses (fig.7a, sample 3,4,7,8,10 and 23). The ecotropic MCp15E probe was able to recognize considerable more MCF-like proviruses than the ecotropic Cgp70/Np15E probe, suggesting a preference for ecotropic sequences in the MCp15E region of the MCF genome. This observation is in good agreement with the described structural heterogeneity among MCF viruses (16).

Analysis of the same DNA samples was also performed with the ecotropic specific LTR probe under hybridization conditions of high and low stringency. Under low stringency conditions tumor 4,8, and 23 revealed the presence of somatically acquired proviruses with this probe. In tumor 3, 7 and 10 no additional proviruses were seen (fig.7b), although they were detected by the MCp15E probe. When the stringency of hybridization was raised, the additional fragments in tumor 4,8 and 23 were no longer seen, although the AKV endogenous sequences hybridized normally (see fig.7c). The reduced efficiency of hybridization of these MCF proviruses with the ecotropic LTR probe, is best explained by the presence of only part of the AKV U3 LTR sequences in the U3 region of these MCF proviruses. The results suggest that all integrated MCF proviruses in tumor tissues have acquired non-ecotropic U3 sequences.

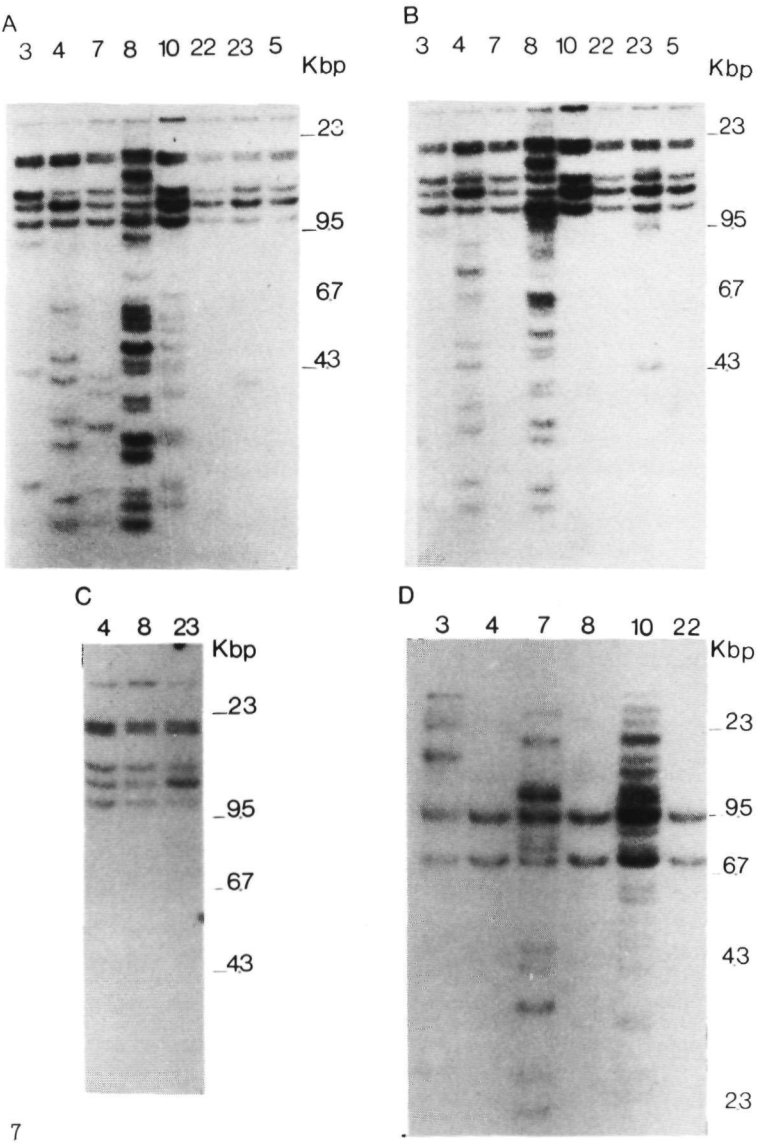

Figure 7

Detection of somatically acquired proviruses by the AKV and MCF-specific probes.

10 micrograms of DNA from AKR/FuRdA mice was digested with EcoRI, and blot-hybridized. Number 3,4,7,8,10,23 represent thymoma DNA from different mice. Number 5 and 22 represent control liver DNA. Molecular Weight markers are as described in legend to Fig. 2.

A. Hybridization to AKV-specific MCp15E probe.

B. Hybridization to AKV-specific U3LTR probe under low stringency condition (0.1xSSC + 0.1% SDS, 15 min. at 62 degrees).

C. Hybridization of thymoma DNA 4, 8 and 23 to AKV-specific U3LTR probe under high stringency condition (0.1xSSC + 0.1% SDS, 15 min. at 65 degrees).

D. Hybridization of thymoma DNA 3, 4, 7, 10 and liver DNA 22 to MCF-LTR probe.

In order to obtain more information concerning the origin of the LTR sequences in MCF proviruses, the same DNAs were analysed with the MCF-LTR probe under high stringency hybridization conditions. It can be seen from fig.7d that tumor 3,7 and 10 exhibit, besides the two endogenous EcoRI fragments of 6.8 and 9.1 Kbp, a number of additional fragments. Some of these fragments also hybridized with the ecotropic MCp15E probe (compare fig.7a) and therefore constitute 3' end of recombinant genomes. The fragments detectable with the MCF-LTR probe, but not with MCp15E, most likely represent 5' ends of MCF proviruses. The somatically acquired proviral genomes within tumor 4 and 8 which reacted with the ecotropic LTR probe under low stringency conditions were not recognized by the MCF-LTR probe.

The structure of the proviral MCF's, detected in tumor DNA, is reminiscent of the structure of the "class I" MCF viruses (24,25). The gp70 region as well as the MCp15E and U3 region of this "class I" viruses are not of ecotropic origin. The changes in the gp70 region of the somatically acquired recombinants includes the acquisition of a characteristic BamHI site at map position 6.2 (31,39). However the Xeno-dl provirus involved in the generation of the MCF proviruses in tumor 3, 7, and 10 does not contain a BamHI site at this position (data not shown). Similarly a KpnI site present in the LTRdXe provirus at map coordinate 7.3, is frequently absent from MCF proviruses, which have acquired a SstI site at position 7.6 (compare figs.4 and 6)(7,39).

Therefore the generation of the MCF viruses involves recombinant events in which often at least two non-ecotropic parents participate: One xenotropic-like virus, LTRdXe, is donating U3, and possible p15E, to many of the somatically acquired MCF proviruses, whereas (an)other xenotropic-like parent(s) provide the gp70 sequences.

DISCUSSION

Previously we have provided evidence, using a specific cDNA probe, for the presence of many somatically acquired proviruses in AKR lymphomas (31). Nearly all these proviruses exhibited MCF structures with non-ecotropic restriction sites within the gp70 gene and ecotropic-specific restriction sites near the 3'terminus (16,31,39).

Here we present a more detailed analysis of these proviruses with ecotropic- and MCF-specific probes, derived from the gp70, p15E and LTR region. In agreement with the data of others (16) , our results show the presence of two separated regions (gp70 and LTR) with non-ecotropic sequences within AKR-MCF proviruses found in tumor tissues. In earlier studies it has been shown that the ecotropic sequences comprizing the C-terminal region of p15E are not retained in the majority of the MCF proviruses (16). Analysis with the ecotropic Cgp70/Np15E and MCp15E probe showed that only a fraction of the MCF proviruses which were recognized by the MCp15E probe could be detected by the Cgp70/Np15E probe. Therefore the ecotropic sequences in the p15E zone, common to most MCF proviruses, seem to be restricted to a small region around the XbaI site at position 7.8. This area also comprises the ecotropic specific oligonucleotide 18, which is present in all the leukemogenic "class I" MCF's (24). The data suggest that the need for ecotropic sequences in this region, a requirement which has been recognized by many authors (6,16,24), is restricted to a very limited region of approximately 100-200 nucleotides near the XbaI site.

All the MCF recombinant proviruses we have analyzed gained non-ecotropic sequences within the LTR region. This was shown by the use of an ecotropic- specific LTR probe under stringent hybridization conditions. Hybridization under high stringency conditions allowed the exclusive recognition of the endogenous ecotropic proviruses (see fig.7c), indicating that all MCF proviruses recognized by the ecotropic MCp15E probe carried an altered LTR. The recombinant proviruses which could still hybridize with the ecotropic LTR probe under low stringency conditions (fig.7b), did not hybridize with the MCF-LTR probe, whereas the MCF proviruses which did react with the specific MCF-LTR probe, did not hybridize with the ecotropic-specific LTR probe (compare fig.7b and 7d). This complementary reactivity is most likely due to the different recombination positions in the MCF LTR's. Dependant on the amount of ecotropic sequences left in the U3 LTR of the MCF genome, the provirus can be recognized by the ecotropic-specific LTR probe or by the MCF- specific LTR probe. In those cases, in which the MCF proviruses are not recognized by the MCF-specific LTR probe, the origin of non-ecotropic sequences is still uncertain. However from

our hybridization analyses, and sequence comparisons of the MCF-LTR probe with the published sequence of the leukemogenic AKR-MCF-247 (20), we can conclude that a significant portion of the leukemogenic AKR-MCF proviruses have acquired LTR sequences from the LTRdXe provirus. This suggests an important role for these sequences in the leukemogenic process. The complementary reactivity between the ecotropic LTR probe and the MCF-LTR probe is not restricted to single proviral integrations, but is seen in all proviruses within one tumor (compare fig.7b and 7d). Most likely MCF viruses with an identical or at least a very similar structure, are integrated in multiple sites in the DNA of target cells. One could speculate that, once a "proper" MCF KX generated, it will proliferate and become responsible for most of the integration events detected in the outgrown tumor.

Hybridization of the MCF-LTR probe to normal mouse DNAs revealed that the MCF-LTR sequence is derived from a xenotropic-like provirus, which is present only once in the same chromosomal site within the BALB/c, C57BL/10, AKR and DBA, but absent from the 129 mouse strain. The presence of the LTRdXe virus in an identical chromosomal site in such unrelated strains like BALB/c and C57BL, indicates that this LTRdXe virus became integrated in the germ line more than 80 years ago.

Restriction analysis of the proviral MCF recombinants in tumor DNA showed that the xenotropic related gp70 in the MCF's cannot be derived from the LTRdXe virus: The MCF recombinant proviruses we analyzed (31,39) (see fig.8) all acquired a BamHI restriction site at map position 6.2, while many did not contain a KpnI site at position 7.3. In contrast, the LTRdXe provirus contains a KpnI site at position 7.3 and lacks the BamHI site at 6.2. If the gp70 region is derived from a single parent, the presence of a BamHI at position 6.2, an EcoRI site at 6.9, a SstI site at 7.6, and the absence of a KpnI site at 7.3, places this virus in a distinct subclass of xenotropic like sequences, which have not been identified in infectious isolates. Therefore the generation of a significant portion of the leukemogenic AKR-MCF viruses involves the recombination with at least two xenotropic-like proviruses: i. The LTRdXe provirus, donating part of the LTR, and ii. Other xenotropic-like sequences providing gp70 genetic information. These results confirm and extend suggestions by others, who documented

the independent expression of the non-AKV gp70 and p15E-U3 sequences (11,38).

The retention within MCF viruses of ecotropic p15E sequences and gathering of U3 sequences from a specific xenotropic locus, corroborate the notion that, besides the need for an altered gp70, the U3 region is also required for the leukemogenicity of the MCF virus. The LTRdXe provirus might provide LTR sequences which change the tropism of the virus, allowing it to replicate in the thymus. Such a role has recently be assigned to LTR sequences (12). The absence of the LTRdXe locus in the 129 strain might contribute to the high resistance of this strain to AKV induced leukemogenesis. This in contrast to the high susceptibility of this strain for Moloney MuLV induced leukemogenesis (39). In this respect it is interesting to note that the leukemogenic Moloney MCF has retained Moloney specific LTR and p15E sequences (3, our unpublished results). Apparently the LTR (and p15E) sequences in Moloney MuLV can fulfill the requirements for the leukemogenic potential of the Moloney MCF. The construction of a series of hybrid viruses will eventually establish the functional requirements for the different regions, involved in the generation of leukemogenic MCF recombinant viruses.

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GENERATION OF AKR MINK CELL FOCUS FORMING VIRUS: NUCLEOTIDE SEQUENCE OF THE 3' END OF A SOMATICALLY ACQUIRED AKR-MCF

Virology, submitted.

GENERATION OF AKR MINK CELL FOCUS FORMING VIRUS: NUCLEOTIDE SEQUENCE OF THE 3' END OF A SOMATICALLY ACQUIRED AKR-MCF

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ABSTRACT

The 3' end of an AKR-MCF provirus (MCFr35) was cloned and found to be biologically active. Comparison of the nucleotide sequence of MCFr35 with the sequence of other MuLVs reveals that the MCFr35 is most likely derived from the same xenotropic and ecotropic parents, which were involved in the generation of AKR-MCF247. Ecotropic sequences are present around the XbaI site at position 7.9 on the genomic map and in the Long Terminal Repeat. Most of the T1 oligonucleotide sequences, characteristic for the leukemogenic "class I" MCFs are also present in MCFr35, with the exception of T1 oligonucleotides 108 and 18. The MCFr35 LTR contains a duplicated enhancer sequence from a xenotropic-like provirus, which is present only once per haploid genome equivalent. The 3' end of MCFr35 consist predominantly of xenotropic-

like sequences, thereby delimiting the positions of recombination in various MCF viruses.

INTRODUCTION

High-leukemic inbred strains of mice inherit DNA copies of ecotropic type C viruses. Expression of these loci leads to a lifelong viremia, which results ultimately in leukemia (Lilly et al., 1975). These ecotropic viruses themselves appear not to be leukemogenic, but give rise, by recombination, to novel type C viruses, called Mink Cell Focus inducing viruses (MCF), some of which are able to accelerate lymphomagenesis upon inoculation into mouse strains (Cloyd et al., 1980; O'Donnell et al., 1981; Rowe et al., 1979; Hartley et al., 1977; Kawashima et al., 1976). In AKR mice these MCF's are generated by recombination between the germ-line transmitted ecotropic AKV virus and xenotropic-like viruses (Lung et al., 1983; Quint et al., 1984; Thomas and Coffin, 1982). The MCF viruses possess novel gp70s, resulting in a different host range, being able to infect both mouse cells like their ecotropic parental virus and heterologous cells like their putative xenotropic progenitors (Chien et al., 1978; Elder et al., 1977; Fischinger et al., 1978; Rommelaere et al., 1978). MCF viruses have been divided into two classes with different biological characteristics (Cloyd et al., 1980; Rowe et al., 1979). MCFs isolated from thymus (class I MCF's) accelerate leukemia when injected into AKR mice, whereas isolates from non-thymic tumors (class II MCFs) do not. These classes can also be distinguished by RNase T1 fingerprinting of genomic RNA (Lung et al., 1983). The class I MCF viruses possess besides altered gp70 genes also recombinant p15E genes, with the 5' to mid portion derived from their ecotropic parents and the extreme 3' portion derived from one of their xenotropic-like progenitors. Also the U3 portion of the LTR is from non-ecotropic origin. Recently it has been shown that at least two different xenotropic-like viruses are involved in the generation of the class I MCF's: One donating gp70 sequences, and the other donating U3 LTR and probably p15E sequences (Quint et al., 1984). The class II MCFs have gp70 and p15E genes from xenotropic origin, and the LTR from their ecotropic parent. To get more insight in the structure of somatically acquired MCF proviruses,

we have sequenced part of a DNA clone (AM35), which contains the 3' region of an integrated recombinant provirus (MCFr35). This region comprizes part of the coding sequence for the C-terminal region of gp70, Prp15E, the long terminal repeat (LTR), and the host-virus junction sequences. The nucleotide sequences, and the predicted amino acid sequences of the coding regions, are compared with the sequences of AKR-MCF247 (Holland et al., 1983; Kelly et al., 1983), Moloney-MCF (Bosselman et al., 1982), and AKV (Herr et al., 1982; Lenz et al., 1982; Van Beveren et al., 1982).

MATERIALS AND METHODS

Molecular cloning

Lymphoma DNA of an AKR/JS mouse (Quint et al., 1984) was digested with restriction endonuclease EcoRI, and separated on agarose gels (Quint et al., 1981; Van de Putten et al., 1981). The DNA fractions hybrizing with an AKV-specific cDNA probe were cloned in Charon 28 as described (Berns et al., 1980). The DNAs of AKV cDNA positive plaques were analyzed by restriction enzyme digestions and blot hybridization. The selected AM35 recombinant was subcloned in pBR322. This AM35 insert was ligated to a 15 Kbp EcoRI fragment which comprized the 5' end of a Moloney MCF genome up to the EcoRI site at position 6.9 (Bosselman et al., 1982). Transfection of this DNA into Mink lung fibroblasts was carried out as described (Bosselman et al., 1982; Graham and van der Eb, 1973).

Nucleotide Sequencing

The AM35 clone was linearized by digestion with SmaI (map position 8.7), and treated with exonuclease Bal31 (Poncz et al., 1982). At various times samples were withdrawn from the incubation mixture. The viral part of the insert was separated from the pBR322 vector by digestion with EcoRI, separated on size and inserted into the EcoRI-SmaI site of M13mp8 (Messing and Vieira, 1982). In addition, fragments obtained by digestion with HpaII, TaqI, Sau3A, and also EcoRI, PstI, and XbaI were inserted into the polylinker region of M13mp8-11. The nucleotide sequence of the inserts was determined by the dideoxy chain termination method as described by Sanger et al. (1977). The gel reading were recorded, edited, and compared using the Staden programs (Staden, 1982).

The 3' half of a somatically acquired recombinant provirus (MCFr35) was molecularly cloned from the chromosomal DNA of an AKR/JS lymphoma (Quint et al., 1984). Restriction endonuclease analysis revealed a physical map, characteristic for MCF proviruses (Quint et al., 1981; van der Putten et al., 1981; Chattopadhyay et al., 1981; Quint et al., 1984). Ligation of this clone to the 5' part of a Moloney MCF provirus, which contained Moloney-MCF sequences up to the EcoRI site at position 6.9 (Fig.1), followed by transfection into mink cells, resulted in the production of recombinant viruses with the expected genomic structure (Fig.1), as revealed by blot hybridization using MCF- and ecotropic-specific probes (data not shown). This established the biological activity of the MCFr35 clone.

The nucleotide sequence of the 3' region of gp70, p15E, LTR and virus-host junction was determined by using the M13 dideoxy chain termination method (Sanger et al., 1977). The strategy is depicted in Fig.2. The MCFr35 sequences were compared with the sequences of the corresponding regions of the ecotropic progenitor AKV (Herr et al., 1982; Lenz et al., 1982; Van Beveren et al., 1982), AKR-MCF247 (Holland et al., 1983; Kelly et al., 1983), and Moloney MCF provirus (Bosselman et al., 1982).

gp70 sequences

Comparison of the sequence of MCFr35 with the nucleotide sequence of AKV revealed that the recombination position between the parental ecotropic and xenotropic-like proviruses occurred around position 760 (Fig.3) just in front of the coding sequence for the mature p15E. The gp70 carboxy termini of murine leukemia viruses have not been determined directly so far. Since the proteolytic cleavage to mature gp70 could well be accompanied by the loss of several amino acids (Engelman et al., 1981; Jou et al., 1980), the C-terminal part of the gp70 protein, encoded by the MCFr35 provirus, might well be completely of xenotropic-like origin. From position 6.9 to 7.2 on the genomic map (nucleotide 1-300, Fig.3) MCFr35 is for 99.4% homologous to AKR-MCF247. From this position on to the 3' end of the gp70 gene (nucleo-

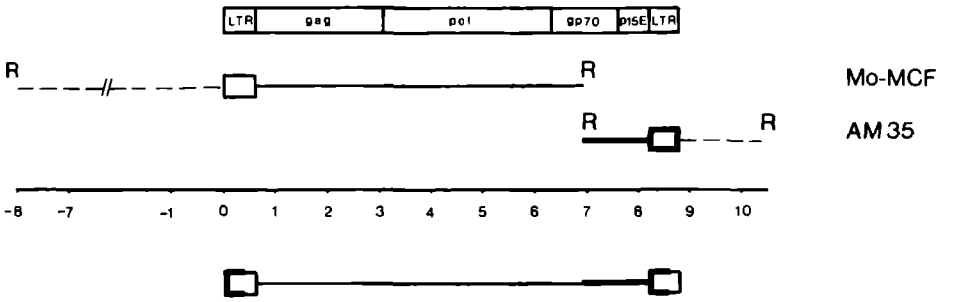

Figure 1: Characterization of the AM35 clone.
Schematic representation of the DNA fragments of the AM35 insert, containing the 3' end of MCFr35 and the 5' end of a MoMCF genome (Besselman et al., 1982). Flanking mouse and pBR322 sequences are indicated by the dashed lines. The upper box gives the location of the different viral genes and the LTR region. Bottom; the genomic structure of the virus, generated after transfection of the MoMCF 5' end and the MCFr35 3' end into mink cells. Abbreviation; R, EcoRI.

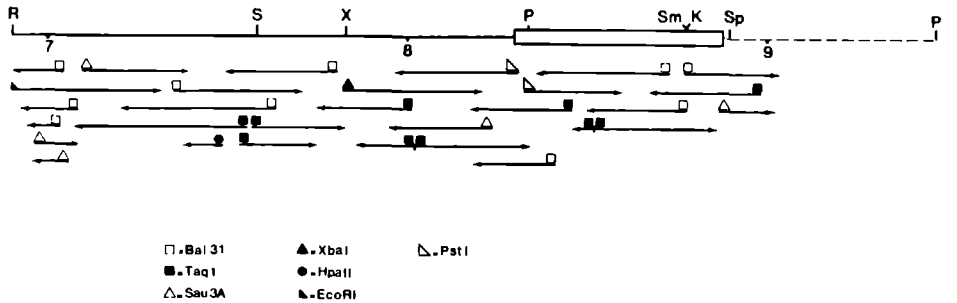

Figure 2: Strategy for sequencing the MCFr35.
The extends of individual readings are indicated in the 5'-3' direction by horizontal arrows. Squares, circles and triangles stand for the enzymes which were used in order to obtain small fragments, suitable for M13 sequencing. Abbreviations: R, EcoRI; S, SacI; X, XbaI; P, PstI; Sm, Sma; K, KpnI ; Sp, SphI.

tide 300-765), AKR-MCF247 is clearly of ecotropic origin. The MoMCF sequence (Bosselman et al., 1982) resembles the MCFr35 sequence very closely up to position 562, whereas the Friend Spleen Focus Forming Virus (SFFV) sequence shows close resemblance up to position 370 (Clark and Mak, 1983; Wolff et al., 1983). The recombination position in these different viruses is schematically represented in Fig.6. Comparison of the sequence of large T1 resistant oligonucleotides, identified previously by Rommelaere et al. (1978), and Lung et al. (1983) with the DNA sequence of MCFr35 revealed the presence of the MCF-specific T1 oligonucleotide 102, and the absence of the ecotropic-specific oligonucleotides 9, 3, 8, and 22. This pattern was also frequently observed in other MCF isolates (Lung et al., 1983; Rommelaere et al., 1978). These data indicate that the MCFr35 gp70 sequences are derived from a provirus which is closely related or identical to the provirus involved in the generation of AKR-MCF247, MoMCF, SFFV, and other MCF viruses (Lung et al., 1983).

Prp15E sequences

It is apparent from Fig.3 that the sequences coding for the N-terminal region of p15E (nucleotide 765 to approximately 1000) are derived from the ecotropic AKV parent. In this region only a single nucleotide difference was observed between MCFr35 and AKV. It also harbors the AKV-specific XbaI cleavage site (Chattopadhyay et al., 1981; Quint et al., 1981), and the ecotropic-specific T1 oligonucleotides 35 and 19. The sequence corresponding to ecotropic-specific T1 oligonucleotide 18 shows a single base-pair difference. Comparison of the sequences coding for the middle and C-terminal region of p15E (nucleotide 1000-1369) with the sequence of AKV, shows that this region is of nonecotropic origin. From nucleotide 1263 to the end of the p15E gene, MCFr35 does not show a single nucleotide difference with AKR-MCF247, suggesting strongly that the same progenitor donated xenotropic-like p15E sequences to both viruses. In this region the MCF-specific T1 oligonucleotide 101 is contained.

The presence or absence of distinct T1 oligonucleotides present in genomic MCF RNAs has been related to their biological properties (O'Donnell et al., 1979; Kelly et al., 1983; Lung et al., 1983). The

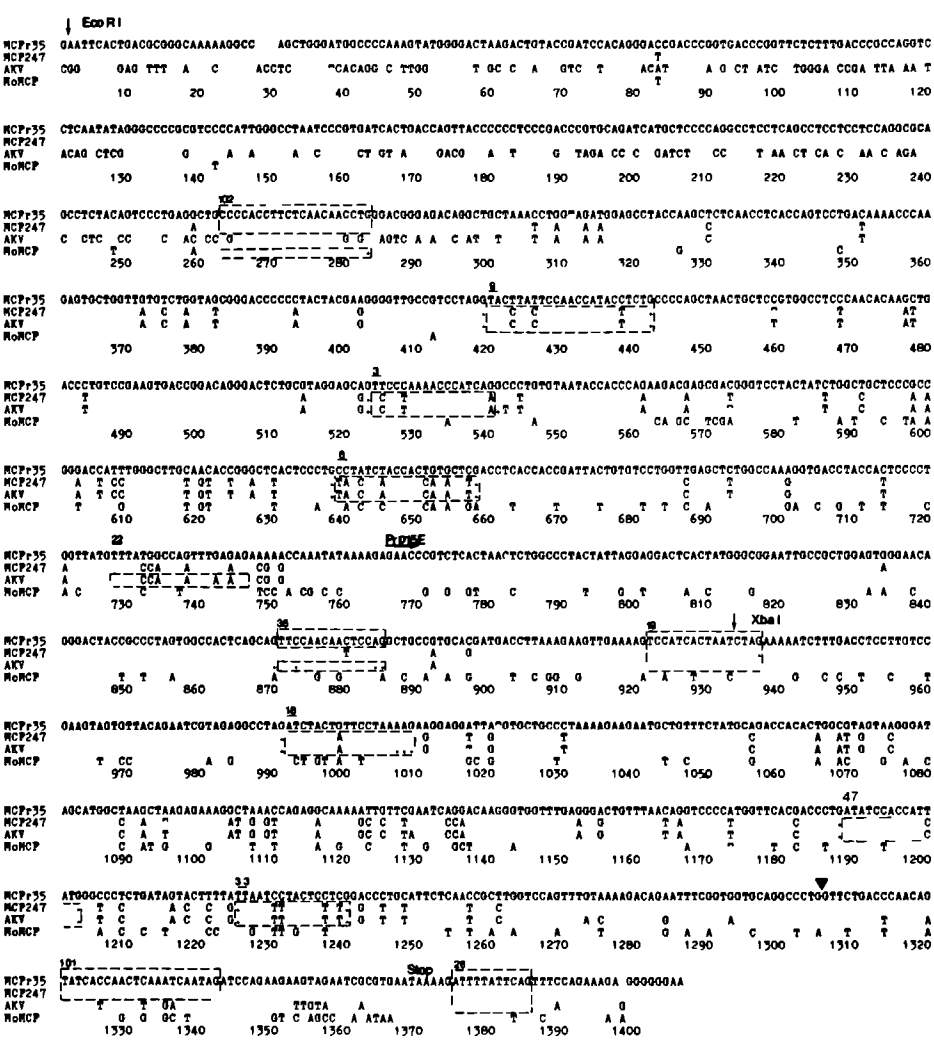

Figure 3 Nucleotide sequences of part of gp70 and p15E of MCFr35, AKR-MCF247, AKV, and MoMCF

The nucleotide sequences of the gp70 3' terminus and Prp15E of MCFr35 were determined as described in the text. The nucleotide sequence for MoMCF and AKV are those of Bosselman et al. (1982) and Lenz et al. (1983), respectively. The nucleotide sequence of the AKR-MCF247 gp70 is from Holland et al. (1983), the sequence of the AKR-MCF247 Prp15E and LTR are those of Kelley et al. (1983). The sequence of MCFr35 was used as the "standard". Dots indicate nucleotide identicalness; blank space, absence of a nucleotide. The dashed boxes correspond to the T1 oligonucleotides previously identified in fingerprints of AKR-MCF247 or AKV (Herr et al., 1982; Kelly et al., 1983, Lenz et al., 1982; Rommelaere et al., 1978).The large arrowhead above the sequence indicates the position of the proteolytic cleavage site to generate mature p15E (Green et al., 1981, Henderson et al., 1978). Search for homology and optimal alignment was done by the computer program Diagon (Staden, 1982).


MCF247 like MCFr35
MoMCF like MCFr35
MCFr35 like AKV
Prp5E
35
19
MCFr35 like AKV
18
MCFr35 like MCF247

presence of T1 oligonucleotides 19, and 35, and the absence of oligonucleotides 47, and 33 is observed frequently in the Class I MCF viruses, in which the recombination between AKV and one of the xenotropic-like parents occurred approximately in the middle of p15E. The sequence corresponding to oligonucleotide 18, which has so far been found in all leukemogenic class I MCF's, is absent from MCFr35. However, since the single nucleotide difference (position 999), which is reponsible for the absence of T1 oligonucleotide 18, as well as the other base-pair changes up to position 1068, do not affect the amino acid sequence (see Fig.4), the MCFr35 provirus almost certainly belongs to the pathogenic class I MCF's.

LTR sequences

As can be seen from Fig.5, the nucleotide sequence of the MCFr35 LTR is very similar to the AKR-MCF247 LTR sequence; Only 5 base-pair differences can be discerned. The R and U-5 sequences of MCFr35 are identical to the AKV sequences. The MCFr35 possesses in the U-3 region an internal duplication of 58 nucleotides, and a spacer of 5 nucleotides, which are absent from AKR-MCF247. The direct repeat present in AKR-614 (Van Beveren et al., 1982) is longer (arrows at position 109 and 280, Fig.5) and covers the region in which the duplication in MCFr35 occurred (position 127-185). The variability in many LTR's is caused by the duplication of U-3 enhancer regions. Sequence

Figure 4: Comparison of the predicted amino acid sequences of the gp70 C terminus and the Prp15Es of MCFr35, MCF247, MoMCF and AKV viruses. The predicted amino acid sequences from MCF247 were obtained from Kelly et al.(1983) and Holland et al.(1983). The amino acid sequence from MoMCF and AKV are those from Bosselman (1982) and Lenz (1982), respectively. Amino acids shared with the MCFr35 are indicated by dashes; blank space indicate the absence of an amino acid. Maximum homology was obtained as indicated in legend to figure 3. The open boxes enclose T1 oligonucleotides analyzed previously in AKV and MCF247 fingerprints. The large arrowhead indicates the cleavage site for generating mature p15E. These sites were derived by comparing our sequence with the data of Moloney MuLV (Henderson et al., 1978; Shinnick et al., 1981).

analyses of proviral LTR's, present in lymphomas of BALB/Mo mice, revealed duplications of 74-136 nucleotides (Berns et al., 1983). In the direct vicinity of the boundary of the duplication the sequence TGAA or TTCA was often found. Besides the presence of this sequence in the MCFr35 LTR at the termini and at position 60 and 324, it is also found at the beginning of the 58 nucleotide long duplication in MCFr35. The presence of this sequence at the termini of the LTR, its frequent presence near proviral integration sites in host sequences, and its occurrence near the boundaries of duplicated LTR regions, suggests that the enzyme system which is involved in the integration event, might also play a crucial role in the generation of duplications.

The core sequence which has been recognized in papova virus enhancers (Moreau et al., 1981; Tyndall et al., 1981), and which is found in the MoMuLV LTR (Berns et al., 1983; Jolly et al., 1983), is also present in the MCFr35 LTR. The underlined sequence (Fig.5) at positions 163 and 226 within the direct repeats, exhibits a stretch of 10 nucleotides, 8 of which are identical to the sequence found in the SV40 enhancer (Moreau et al., 1981). At position 303, outside the duplicated region, another stretch of 11 nucleotides is present, which is identical to the core sequence of SV40. This latter sequence is absent from the AKV LTR. None of the Class I MCF's, isolated sofar, contain oligonucleotide 14. The absence of this T1 oligonucleotide from all thymotropic MCF's could reflect the requirement of gathering non-ecotropic enhancer sequences in order to gain a different target-cell specificity as has recently been suggested by DesGroseillers et al. (1983). Nearly all class I MCF's contain the MCF-specific oligonucleotides 106 and 108. The MCFr35 contains the sequence corresponding to T1 oligonucleotide 106. In the region of T1 oligonucleotide 108 a single base-pair difference is found.

Recently we have shown that the parental provirus which donated the U-3 region to MCFr35, is present only once per haploid genome equivalent (Quint et al., 1984). Both the hybridization data with a MCF-specific LTR probe (Quint et al., 1984) as well as the strong sequence homology between the U-3 region of MCFr35 and AKR-MCF247 (only 5 base-pair difference) indicates that the U-3 region of AKR-MCF247 was donated by the same xenotropic-like parent.

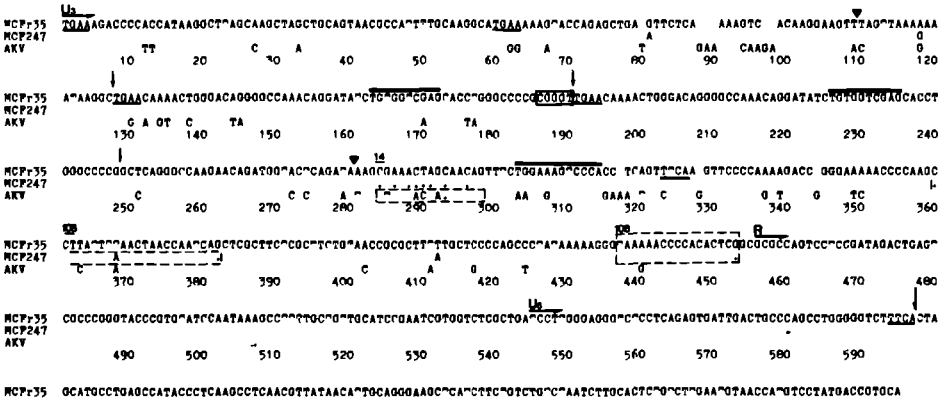

Figure 5: Nucleotide sequence of the LTRs of MCFr35, MCF247, and AKV. The positions of the U-3, R, and U-5 were determined as described (Van Beveren et al., 1982). The positions of the large RNase T1 oligonucleotides in this region are indicated with dashed boxes. The small arrows indicate the boundaries of the direct repeat in MCFr35. The solid box represent a spacer sequence between the direct repeats. The arrowheads indicate the position of the direct repeat (represented only once in this figure) as determined in AKV (Van Beveren et al., 1982). The heavy black lines above the MCFr35 sequence show the position of a sequence shared with Papova virus enhancers (Moreau et al., 1981; Tyndall et al., 1981). The adjacent 3' host DNA sequences of the integration site are indicated to the right of the large arrow.

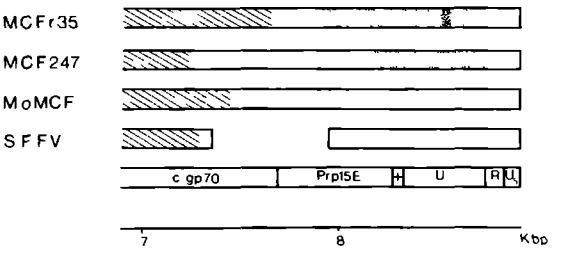

Figure 6: Schematic representation of the 3' ends of various recombinant viruses.
Open boxes: derived from ecotropic parent. Dotted boxes: Xenotropic LTR donating virus. Dashed boxes. Xenotropic gp70 donating virus. The positions of recombination were derived from the DNA sequence as described in the text. Heavy shaded area in MCFr35: duplicated enhancer region.

This analysis, and the work of others (Datta et al., 1983; Lung et al., 1983; Thomas and Coffin, 1982), suggest that at least two and possible three regions in the MCF genome are essential for their leukemogenic potential; i. gp70 sequences. The requirement for xenotropic-like gp70 sequences could best be explained by assuming that infection of a different target cell, which is refractory for viruses with an ecotropic envelope, is an essential step in lymphomagenesis. ii. LTR sequences. In order to allow efficient replication in the "new target cell" another more "tissue adapted" enhancer might be required. Whether this new enhancer is needed for efficient replication of the recombinant virus or for the activation of distinct host genes is a matter of speculation. In this respect, it is interesting to note that during MoMuLV induced lymphomagenesis no new LTR sequences are acquired by the MoMCF genomes. Although the formation of leukemogenic MoMCF's is frequently accompanied by the duplication of the enhancer region, the enhancer encoded in the MoMuLV is apparently able to work efficiently also in the MoMCF. iii. The apparent requirement for ecotropic p15E sequences (T1 oligonucleotide 18 (Lung et al., 1983) and the presence of the XbaI site (Chattopadhyay et al., 1981; Quint et al., 1981)) can be explained in two ways; Either an ecotropic N-terminal p15E region is essential, or the presence of this ecotropic region is in some way coupled to the acquisition of gp70 sequences and LTR sequences from different xenotropic-like viruses. One could imagine that recombination with the LTR donating virus, LTRdXe (Quint et al., 1984), probably the first recombination event (Datta et al., 1983; Thomas and Coffin, 1982), can only be followed by a recombination in the gp70 gene, if the recombination with the LTRdXe virus is restricted to the C-terminal region of p15E and the U-3 LTR. The in vitro generation of a series of recombinant proviruses will probably resolve the role of the different subgenomic regions for the leukemogenic potential of the MCF viruses.

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Summary

Mice of the AKR inbred strains develop after a latency of 7-12 months a spontaneous leukemia. The development of the disease is accompanied by the expression of endogenous murine leukemia viruses which are present as normal genes in the mouse genome. Some of these endogenous viral loci are activated early in life (ecotropic MuLVs) or during the preleukemic period (xenotropic MuLVs). An important step in the pathway leading to the disease forms the generation of novel-type MuLVs which are recombinants between the endogenous ecotropic and different xenotropic viruses. Recombinant-viruses, isolated from the thymus (class I MCFs) in contrast to the ecotropic and xenotropic parental viruses, are able to accelerate leukemia upon injection into AKR mice.

Chapter 1 summarizes various molecular biological aspects of RNA-tumor viruses. In addition, a number of characteristic properties of endogenous viruses and the involvement of MCF viruses in leukemogenesis are described.

The genome organization of endogenous ecotropic proviruses present within the low viremic mouse strains is indistinguishable from high viremic strains (chapter 2, first part). Within the high virus titer substrain of AKR mice, a considerable variation in number and site of integration of AKVs in the germline can be observed, except for one progenitor locus which is present in all the substrains. The position of AKV proviruses in the different chromosomal loci is in complete agreement with the segregation pattern of sublines (chapter 3). The new germline integrations accumalate continuously, with an average of one new provirus per 30 generations. The presence of a provirus with a MCF structure, which is generated later in life, in one of the AKR substrains indicates that germline integrations are the result of infection of oocytes or developing embryos by circulating viruses.

In the second part of chapter 2 and in the chapters 4 and 5 the structure of recombinant proviruses, as found integrated in tumor tissues of the AKR mouse, is described.

DNA obtained from tumors in different anatomical sites (thymus, spleen, and lymph-nodes) within one animal showed the same reintegra-

tion pattern (chapter 2). Furthermore, as identified by cDNA probes, the structures of these reintegrated recombinants were similar in all the tumors investigated and resembled closely those of described MCFs. A more detailed analysis was performed with molecular clones containing AKV and AKR-MCF specific probes, which recognize different regions of the viral genome (chapter 4). The results showed that, besides the ecotropic AKV-genome, at least two non-ecotropic parents participate in the generation of leukemogenic AKR-MCF viruses: A xenotropic-like virus, LTRdXe, donating U3 LTR and p15E sequences and (an)other xenotropic-like virus(es) providing gp70 sequences. The LTRdXe genome is unique in the mouse genome and is involved in the recombination of the majority of the somatically acquired AKR-MCF proviruses.

In chapter 5 the molecular cloning and the sequence of the 3' end of a reintegrated AKR-MCF provirus is presented. The insert of the clone is 3.5 kilobases and comprises 1.9 kbp of viral sequences (MCFr35). Comparison of the nucleotide sequence of MCFr35 with that of a pathogenic AKR-MCF (MCF247) shows that the same xenotropic parents were involved in the generation of both MCF viruses. In addition, nucleotide sequence comparisons revealed the positions of the recombination within several MCFs.

Muizen van de AKR-inteeltstam ontwikkelen spontaan een lymfatische leukemie na een latentie-periode van 7-12 maanden. Bij deze leukemie ontwikkeling zijn retrovirussen betrokken, die als normale stabiele genetische elementen aanwezig zijn in het muizengenoom (endogeen virale genen) of hieruit, de novo, via recombinatie gevormd worden. Enkele van deze virale genomen welke coderen voor ecotrope en xenotrope murine leukemie virussen (MuLVs) komen vlak na de geboorte en in de preleukemische fase in de AKR-muis tot expressie. Een belangrijke stap in het ontstaan van de ziekte is de vorming van een nieuw type MuLV, dat een recombinant is tussen de ecotrope en verschillende xenotrope virussen. Deze recombinant-virussen (MCFs) zijn veelal in staat, na injectie in pasgeboren AKR-muizen, de ontwikkeling van leukemie aanzienlijk te versnellen, dit in tegenstelling tot de ecotrope ouder en de verschillende typen xenotrope virussen.

Het in dit proefschrift beschreven onderzoek geeft een moleculair-biologische analyse van de verschillende virussen welke betrokken zijn bij de leukemie ontwikkeling.

In hoodstuk 1 wordt een korte inleiding gegeven t.a.v. een aantal moleculair-biologische aspecten van RNA-tumor-virussen. Tevens wordt aandacht besteed aan de recente literatuurgegevens, welke betrekking hebben op de eigenschappen van endogene virussen en de betrokkenheid van MCF-virussen in de leukemieontwikkeling bij AKR-muizen.

Het eerste gedeelte van hoofdstuk 2 laat zien dat de structuur van de ecotrope endogene provirussen bij verschillende AKR-stammen gelijk is, terwijl het aantal provirussen per stam varieert. Bij alle stammen is een provirus op een identieke plaats geintegreerd. De onderlinge stamboomrelatie tussen de verschillende hoog-leukemische AKR-stammen en de laag-leukemische stammen enerzijds en de aanwezigheid van ecotrope endogene virussen anderzijds, wordt in hoodstuk 3 verder uitgewerkt. Deze studie geeft aan dat in de hoog-leukemische AKR-stam een continue accumulatie van provirussen in de kiembaan wordt waargenomen met een frequentie van een nieuw provirus per 30 generaties. De aanwezigheid in een van de stammen van een provirus met een MCF-structuur,welke pas gedurende leukemogenese gemaakt wordt, geeft aan

dat kiembaanintegraties mogelijk plaats vinden door infectie van kiembaancellen.

In het tweede gedeelte van hoofdstuk 2 en de hoofdstukken 4 en 5 worden structuuranalyses beschreven van geintegreerde recombinantprovirussen in tumorweefsel van de AKR-muis.

DNA van uitgegroeide tumoren, welke afkomstig zijn van diverse anatomische plaatsen uit een muis, vertonen eenzelfde herintegratiepatroon van recombinantgenomen (hoofdstuk 2). De structuur van de geherintegreerde recombinanten was in alle tumoren dezelfde en leek op het geisoleerde MCF-type. Bij deze structuurbepaling is gebruik gemaakt van verschillende cDNA-probes. Een meer gedetailleerd structuuronderzoek is gedaan met moleculair gekloneerde AKV- en AKR-MCF-specifieke probes, welke verschillende gedeelten van het genoom herkennen (hoofdstuk 4). De resultaten laten zien dat naast het ecotrope AKV-genoom tenminste twee niet-ecotrope ouders deelnemen aan de vorming van MCF-virussen: Een ouder met een xenotrope genoomstructuur (LTRdXe) levert de U3LTR-sequenties, terwijl een ander endogeen virus de gp70-sequenties doneert. Het LTRdXe-genoom, welke uniek is in het muizengenoom, is in de meeste MCF-provirussen betrokken bij de recombinatie.

Hoofdstuk 5 beschrijft de klonering van het 3'-uiteinde van een geherintegreerd AKR-MCF-provirus, dat biologische actief is. De kloon is 3,5 kilobaseparen groot en bevat 1,9 kb aan virale sequenties (MCFr35). Vergelijking van de nucleotide sequentie van dit virale deel met die van een pathogene AKR-MCF (MCF247), laat zien dat, zowel voor de recombinatie in het gp70-genoom als voor de recombinatie in het c-terminale deel van p15E en de LTR, bij beide virussen dezelfde xenotrope ouders gebruikt zijn. Voorts kon uit vergelijking van de nucleotidesequentie van MCFr35 met andere retrovirussen de recombinatieposities in een aantal MCFs worden vastgesteld.

♦ Ecotrope virussen zijn in staat muizencellen te infecteren, dit in tegenstelling tot de xenotrope virussen welke niet goed groeien in muizencellen maar wel in cellen van heterologe oorsprong (b.v. hamster, rat, mens.).

Wim Quint werd geboren te Heerlen op 23 april 1951. Na het behalen van de eindexamens HAVO in 1970 en Atheneum in 1972 aan het Rombouts College te Brunssum, begon hij de studie Biologie aan de Katholieke Universiteit te Nijmegen.

Het candidaatsexamen (B4) werd in januari 1976 afgelegd. In april 1979 werd het docteraalexamen behaald met als hoofdrichting Chemische Cytologie (Prof. Dr. Ch. Kuyper) en de bijvakken Moleculaire Biologie (Prof. Dr. J. Schoenmakers) en Biochemie (Prof. Dr. H. Bloemendal).

Vanaf april 1979 tot en met december 1982 was hij, als wetenschappelijk medewerker in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO), werkzaam op het laboratorium voor Biochemie van de K.U. Nijmegen. In deze periode heeft hij onderzoek verricht aan integratie in de kiembaan en de vorming van recombinant Murine Leukemie Virussen onder leiding van Dr. A. Berns.

Tijdens de promotieperiode werd in october 1979 deelgenomen aan een EMBO-cursus "RNA Tumor Viruses" in Berlijn. In september 1980 werd deelgenomen aan een EMBO-cursus "Cellulaire en moleculaire aspecten van de vroege zoogdier embryogenese" op Kreta. In mei 1981 werd de RNA-tumor-virus-vergadering te Cold Spring Harbor (New York) bijgewoond.

Vanaf januari 1983 is hij, in dienst van het Centraal Diergeneeskundig Instituut (CDI), werkzaam op de afdeling Biochemie van de K.U. Nijmegen om onderzoek te verrichten aan ADV-mutanten.

Stellingen

1

Het ecotrope specifieke oligonucleotide 18 in recombinant MCF virussen mag niet, zoals Kelly et al. beweren, beschouwd worden als een genetische marker voor leukemogene klasse I MCF virussen.
Kelly, M., C. Holland, M. Lung, S. Chattopadhyay, D. Lowy en N. Hopkins. (1983). J. Virol. 45:291-298.

2

De conclusie van Yoshimura en Levine, dat MCF virussen integreren in identieke plaatsen bij verschillende AKR thymus lymphoma DNAs, is, op grond van de vertoonde gegevens, niet gerechtvaardigd.
Yoshimura, F., en K. Levine. (1983) J. Virol. 45:576-584.

3

De afwezigheid van AKR ecotrope provirus amplificatie in AKR leukemische thymussen, zoals geconcludeerd door Yoshimura en Breda en Chattopadhyay et al., geldt niet algemeen en is gebaseerd op onderzoek aan een te beperkt aantal tumoren.
Yoshimura, F., en M. Breda. (1981) J. Virol. 39:808-815.
Chattopadhyay, S., M. Cloyd, D. Linemeyer, M. Lander, E. Rands en D. Lowy. (1982) Nature 295:25-31.

4

De positionering van het Bam H1 15 fragment door Ihara et al. op het Pseudorabies virale genoom moet betwijfeld worden.
Ihara, S., L. Feldman, S. Watanabe, en T. Ben-Porat. (1983) Virology 131:437-454.

5

De afwezigheid van virale transcripten, gecodeerd op het Bam H1 7 fragment , 0-2 uur na infectie van konijnenier cellen met pseudorabies virus, zoals vermeld door Feldman et al. in de figuren 4 en 5, is niet in overeenstemming met de getoonde filterhybridisatie experimenten (figuur 2).

Feldman, L., F. Rixon, Jong-Hojean, T. Ben-Porat en A. Kaplan. (1979) Virology 97:316-327.

6

Het onderscheiden van homozygote en heterozygote muizen op basis van band intensiteiten in Southern blots, zoals bepaald door Wagner et al. is vanuit moleculair biologische standpunt bekeken een slechte methode.

Wagner, E., L. Covarrubias, T. Steward, en B. Mintz. (1983) Cell 35:647-655.

7

Het gebruik van vaccinia virus vectoren voor de productie van polyvalente levende vaccins tegen andere humane en veterinaire ziekteprocessen vraagt om veilige en langdurige testprogramma's.

Smith, G., M. Mackett en B. Moss. (1983) Nature 302:490-495.
Panicali, D., S. Davis, R. Weinberg en E. Paoletti. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:5364-5368.

8

Angst voor computers wordt soms weggewuifd met het argument dat een computer slechts doet wat hem wordt opgedragen. Ten onrechte, immers datgene wat men een computer opdraagt is in de meeste gevallen iets anders dan men beoogde.

9

Om het jachtluipaard voor uitsterven te behoeden zal een goed doordachte kruisingsstrategie nodig zijn.
O'Brien, S., D. Wildt, D. Goldman, C. Merril en M. Bush. (1983) Science 221:459-461.

10

Het gebruik van Viditel als publiek gegevensbestand zou aanzienlijk verbeterd en goedkoper kunnen worden door invoering van trefwoorden als selectiesysteem voor informatie.

11

De opwaardering van huishoudelijk werk zal er toe leiden dat het kiezen voor een levensinhoud zowel minder mannen als vrouwen zal frustreren.

Nijmegen, 14 februari 1984
W. Quint.