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Oncogenic Retrovirus from Spontaneous Murine Osteomas

II. Molecular Cloning and Genomic Characterization

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An N-ecotropic murine leukemia virus (OA MuLV), originally isolated from spontaneous osteomas of strain 101 mice, was molecularly cloned. The virus induces osteomas, osteopetrosis, and malignant lymphomas in NMRI mice. The cloned virus was analyzed by heteroduplex analysis, restriction enzyme mapping, and oligonucleotide mapping. The data show a very close relationship to the endogenous Akv prototype virus with some differences in the *gag* and the *env* region. The nucleotide sequence of the U_3 region of OA MuLV LTR revealed a structure within the presumable enhancer region very similar to the U_3 sequences of the FBJ murine sarcoma virus and its associated helper virus. The significance of these specific structures for the oncogenicity of the virus and the development of the typical disease pattern is discussed.

INTRODUCTION

A C-type retrovirus (OA MuLV) was isolated from spontaneous osteomas of strain 101 mice, a strain with a high incidence of benign bone tumors (Schmidt *et al.*, 1984). The virus induces osteomas, osteopetrosis, and lymphomas in newborn NMRI mice after a latent period of 12–15 months. Fv-1 host range restriction assays classified the virus as N-ecotropic and XC-positive. RNase T_1 fingerprint analysis and tryptic p30 peptide mapping indicated that the OA MuLV isolate is a heterogenous virus complex, closely related to the endogenous ecotropic provirus of the AKR mouse (Akv). Serological analysis of the envelope proteins, however, revealed differences between OA MuLV and Akv in some epitopes of the gp70 and p15E proteins. For further characterization the virus was cloned molecularly and analyzed for genomic alterations possibly relating to the effect of OA MuLV on bone tissue differentiation and bone tumor development.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T_4 DNA ligase and DNA polymerase I were purchased from Boehringer-Mannheim or New England Biolabs, radiolabeled nucleotides from New England Nuclear or Amersham Corporation. The plasmid pAKR-59 containing the *Pst*I fragment with the entire Akv genome was provided by J. Lenz (Lenz *et al.*, 1982b). The plasmid pBW-50 derived from pAKR-59 by subcloning the eco-specific 0.4-kb *Sma*I fragment of Akv in pNF-1872 was obtained from B. M. Willumsen, Copenhagen.

Mice and cell lines. Strain 101 mice and NMRI mice were obtained from the breeding colony of the Institut für Biologie, GSF. NIH 3T3 cells were a gift from R. C. Gallo, NCI. 208 F cells were obtained from T. Curran, London. The cell lines were grown in Dulbecco's modified Eagle's medium. Infection and isolation of virus has been

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described previously (Schmidt *et al.*, 1984). OA MuLV infected 208 F cells were biologically cloned.

Isolation of unintegrated circular viral DNA. Unintegrated OA MuLV DNA was isolated from NIH 3T3 cells 48 hr after infection by a modification of the Hirt procedure (Hirt, 1967) as described by DeLorbe *et al.* (1980).

Nucleic acid hybridization. DNA cleaved by restriction enzyme digestion was separated in 0.7% agarose gels and transferred to nitrocellulose membranes by the procedure of Southern (1975). DNA used as hybridization probe was excised from the plasmid and separated from the vector by preparative gel electrophoresis. ^{32}P-labeled probes were made by nick translation (Rigby *et al.*, 1977). Nitrocellulose filters were prehybridized for at least 1 hr at 68° in 3× SSC (1× SSC = 0.15 *M* NaCL, 0.015 *M* sodium citrate), 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% ficoll, 0.05% SDS (sodium dodecyl sulfate), 250 μg/ml denatured salmon sperm DNA, and then hybridized for at least 20 hr at 68° in an identical solution containing 1-2 × 10^6 cpm/ml of denatured ^{32}P-labeled DNA (sp act 3-5 × 10^8 cpm/μg). After hybridization the filters were washed six times for 20 min at room temperature in 2× SSC, 0.5% SDS and twice for 15 min at 68° in 0.1× SSC, 0.1% SDS. Bands were visualized by autoradiography at −70° for 1-7 days using Kodak XAR-5 film and an intensifying screen.

Construction of recombinants. DNA isolated from OA MuLV infected biologically cloned rat cells was digested to completion with *Eco*RI. *Eco*RI digested Charon 4A λ-arms were prepared by electroelution from preparative agarose gels. Molar equivalents of the two DNAs were ligated with T_4 DNA ligase and packaged *in vitro* according to Hohn (1979). Phages were plated on *Escherichia coli* DP50 supF. Plaques were screened by the procedure of Benton and Davis (1977) using ^{32}P-labeled Akv DNA as hybridization probe. Hybridization conditions were as described for Southern blots. DNA from λ-clones of OA $MulV_R$ was prepared by the method of Cameron *et al.* (1977).

Heteroduplex analysis. Heteroduplex analysis was carried out by M. Sullivan, Genex Corporation, Rockville, Maryland, as described by Leder *et al.* (1978).

Transfection assay. Transfections were carried out in 3 × 10^5 NIH 3T3 cells according to Graham and van der Eb (1973). Twenty-four hours after the addition of 100 ng DNA from OA $MuLV_R$ λ-clones and 10 μg salmon sperm DNA as carrier per dish the transfected cells were split in a ratio of 1:3. Cultures were refed every 3 days. Eight days after transfection the cells were assayed for the production of MuLV group specific antigen (p30) applying the indirect immunoperoxidase method (Nexø, 1977). Virus producing cultures were cultivated with 4 μg polybrene/ml medium and virus was isolated from the supernatants.

RNase T_1 fingerprinting. The T_1 fingerprint analysis was carried out by two-dimensional gel electrophoresis of the oligonucleotides as described previously (Pedersen and Haseltine, 1980; Pedersen *et al.*, 1980, 1981).

Nucleotide sequencing. The *Pst*I-*Bssh*II restriction enzyme fragment of the 5′ LTR of OA MuLV type I containing the nucleotide sequences from the *Pst*I site in the U_3 region to the cap site was exchanged with the corresponding nucleotide sequences of a M13mp9 clone containing most of U_3, R, and part of U_5 from the Akv virus in a *Pst*I-*Sma*I fragment using standard DNA recombinant techniques. The nucleotide sequence of OA MuLV type I *Pst*I-*Bssh*II fragment was determined by the dideoxynucleotide method as described by Sanger *et al.* (1977). As primers for the sequencing reactions we used three oligonucleotides 5′ - GTACCCGGGCGACTC - 3′, 5′ - ATCTGGGGAACCTTG - 3′, and 5′ - TTCCTTGTTCTTGTT-3′. The oligonucleotides were synthesized by the phosphotriester method on a kieselguhr-polyamide support as described by Gait *et al.* (1982).

Oncogenicity assay. Newborn female NMRI mice were injected intraperitoneally with 100 μl cell-free supernatant from OA MuLV producing NIH 3T3 cells which contained 5 × 10^3 infectious particles per milliliter. The mice were observed 6 days a week. Moribund mice were killed and ex-

amined by X-ray. Diagnosis was confirmed by histological examination.

RESULTS

Analysis of Integrated and Unintegrated OA MuLV DNA

Previous data suggest a strong relationship of ecotropic OA MuLVs from the strain 101 mouse to the endogenous ecotropic virus of the AKR mouse (Schmidt *et al.*, 1984). Therefore the large *Pst*I fragment containing the entire Akv genome and a 0.4-kb ecospecific *Sma*I fragment from plasmid pAKR-59 (Lenz *et al.*, 1982b) were used as hybridization probe to analyze the integrated and unintegrated forms of OA MuLV DNA. Hybridization of *Eco*RI digested 101 DNA with the ecospecific fragment revealed a single band of 11.0 kb, indicating one ecotropic locus in this mouse strain. Digestion with *Pst*I, which is known to cleave most endogenous murine retroviruses within the LTR (Chattopadhyay *et al.*, 1980, 1982), yielded one single fragment with 8.4 kb. No difference was observed between young and old 101 mice (Fig. 1).

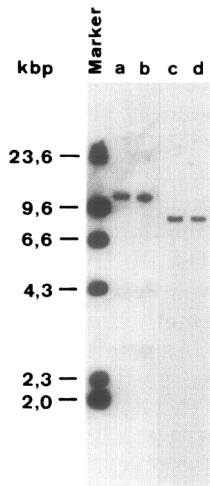

FIG. 1. Southern blot analysis of genomic DNA from strain 101 mice. Cellular DNA cleaved by restriction endonuclease digestion was subjected to electrophoresis in 0.7% agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled pBW-50 DNA containing a 0.4-kb fragment specific for ecotropic MuLV as described under Methods. (a) *Eco*RI digested embryonic DNA; (b) *Eco*RI digested DNA from a 1-year-old mouse; (c) *Pst*I digested embryonic DNA; (d) *Pst*I digested DNA from a 1-year-old mouse.

For analysis of the unintegrated circular OA MuLV DNA, OA MuLV was isolated from C3H fibroblasts, infected previously with a cell free osteoma extract from strain 101 mice. This virus isolate was used to infect NIH 3T3 cells and 48 hr later Hirt supernatant was prepared. Digestion of circular OA MuLV DNA with *Pst*I generated one fragment of 8 to 9 kb, one smaller fragment of about 5 to 6 kb, and a few weaker bands (data not shown). This demonstrates the heterogeneity of OA MuLV virus stock shown previously by RNase T_1 fingerprinting (Schmidt *et al.*, 1984).

Cloning Strategy

To facilitate the analysis of the integrated viral DNA, rat cells (208 F) were infected with OA MuLV. This strategy was chosen to avoid interference by related endogenous murine proviruses. The infected 208 F rat cells were biologically cloned. One clone, which produced a high amount of infectious virus, was used for further investigation. The virus produced by this clone was named OA $MuLV_R$. For molecular cloning, genomic DNA from OA $MuLV_R$-infected 208 F rat cells was digested with *Eco*RI, which does not cleave most endogenous ecotropic MuLV DNAs (Chattopadhyay, 1982). The *Eco*RI digested cellular DNA was ligated to the *Eco*RI arms to Charon 4A λDNA, packaged *in vitro*, and plated on *E. coli* DP50 supF. From approximately 2.5×10^5 phages eight recombinant clones were isolated, which hybridized strongly with ^{32}P-labeled Akv DNA.

Characterization of the Cloned Viruses by Restriction Enzyme Mapping

From the isolated clones three different types of viral genomes could be distinguished by their restriction enzyme cleavage sites (Fig. 2). Type I was isolated twice, each clone with the same integration site. Type II was isolated four times with different flanking cellular sequences. Both types appear to contain complete viruses of about 8.8 kb in length. Type III has a

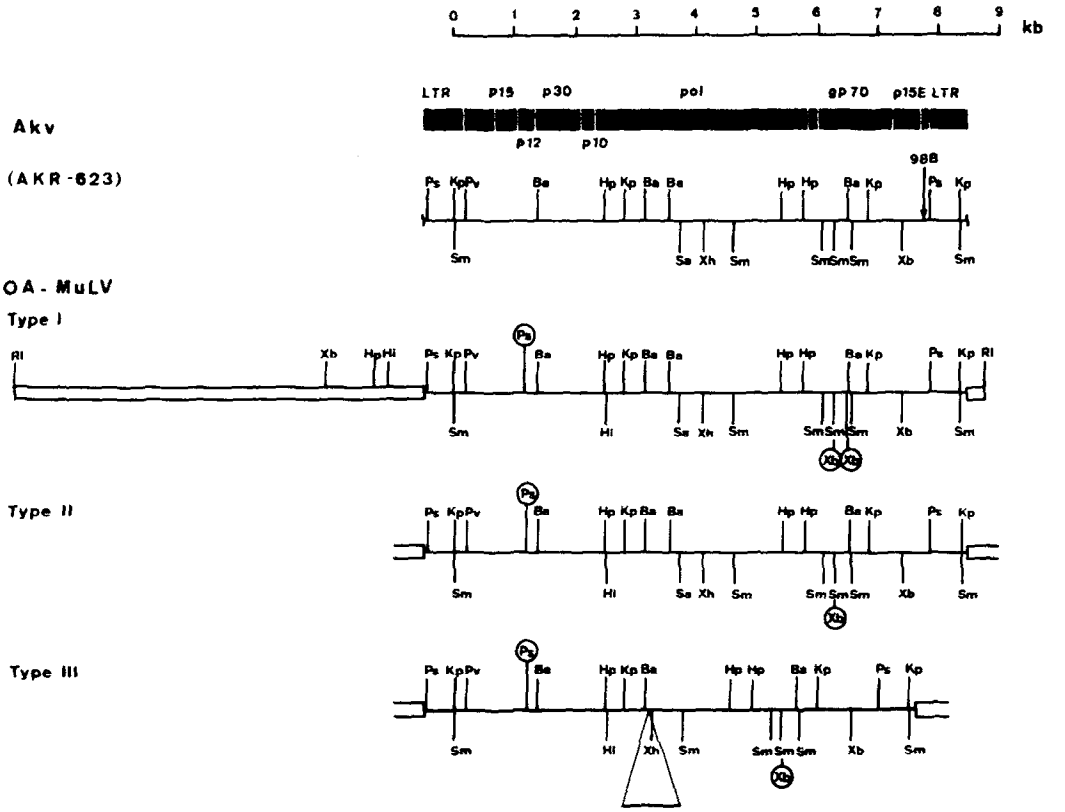

FIG. 2. Comparison of the restriction enzyme maps of cloned OA MuLV$_R$ DNAs with the Akv genome. The positions of the restriction enzyme cleavage sites in the Akv genome are based on the nucleotide sequence of the molecular clone AKR 623 (Etzerodt *et al.*, 1984; Herr, 1984). Restriction sites unique to OA MuLV$_R$ are encircled. Abbreviations: Ba (*Bam*HI), Ha (*Hpa*I), Hi (*Hind*III), Kp (*Kpn*I), Ps (*Pst*I), Pv (*Pvu*I), RI (*Eco*RI), Sa (*Sal*I), Sm (*Sma*I), Xb (*Xba*I), Xh (*Xho*I).

shortened genome of about 7.8 kb. This type was isolated twice; both clones had different flanking sequences. The restriction enzyme patterns of the three types of OA MuLV$_R$ proviral DNA are strongly related to that of the Akv genome. The differences between OA MuLV$_R$ and Akv are scattered throughout the whole viral genome. The *gag* region contains an additional *Pst*I site that was not found in the original endogenous virus of strain 101 mice (Fig. 1). The *Hind*III site in the *pol* gene of OA MuLV$_R$ is not present in the molecular clone AKR-623, but has been described for some other types of Akv (Steffen *et al.*, 1980; Chattopadhyay *et al.*, 1982). Furthermore there are two additional *Xba*I sites in the gp70 coding region of OA MuLV$_R$ type I compared with the Akv genome. Only one additional *Xba*I site is found in OA MuLV$_R$ types II and III. The deletion in the genome of OA MuLV$_R$ type III is located within the *pol* gene between the second *Bam*HI site and the *Xho*I site and extends over approximately 1 kb.

Heteroduplex Analysis

To search for possible insertions, deletions, or rearrangements heteroduplexes were formed between the three types of OA MuLV$_R$ and Akv DNA. Therefore pAKR-59 DNA, containing the 8.3-kb *Pst*I fragment of the Akv genome flanked by pBR, was allowed to anneal to the λ-clones of OA MuLV$_R$, consisting of the proviral genome flanked on each site by rat cellular sequences and the λ-vector arms. The OA

$MuLV_R$ type I DNA was homologous to the pAKR-59 DNA over a range of approximately 8.5 kb (Figs. 3a and b). The same result was found for the λ-clones of OA $MuLV_R$ type II (data not shown). If any insertions or deletions are present in the type I and type II genomes, they must be smaller than 50 bp and cannot be detected under these conditions.

OA $MuLV_R$ type III DNA and pAKR-59 DNA are annealed in one region of about 3.7 kb at the 5′ end of OA $MuLV_R$ and one region of about 4.2 kb at the 3′ end (Figs. 3c and d). The loop formed by Akv DNA comprises approximately 1 kb. The presence of this loop confirms the deletion within the *pol* gene found by restriction enzyme mapping. No evidence for a substitution in the OA $MuLV_R$ genome could be detected.

RNase T_1 Fingerprint Analysis

The biological activity of the isolated clones was tested by transfection of NIH 3T3 cells. Although the restriction enzyme maps of OA $MuLV_R$ type I and type II suggested that both contained a complete viral genome, only DNA from OA MuLV type I was infectious in a transfection assay. The RNA of OA $MuLV_R$ type I derived from transfected cells was investigated by RNase T_1 fingerprint analysis (Fig. 4a). A schematic comparison with the oligonucle-

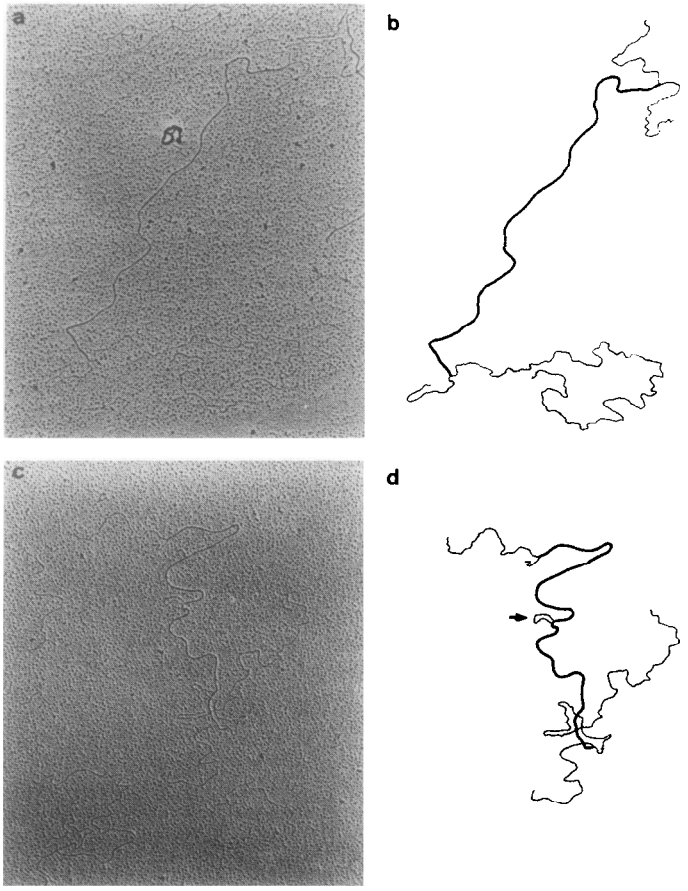

FIG. 3. Heteroduplex analysis of cloned OA $MuLV_R$ DNAs. (a, b) Heteroduplex between clone OA $MuLV_R$ type I and plasmid pAKR-59; (c, d) heteroduplex between clone OA $MuLV_R$ type III and plasmid pAKR-59. The arrow marks a loop formed by single-stranded Akv DNA.

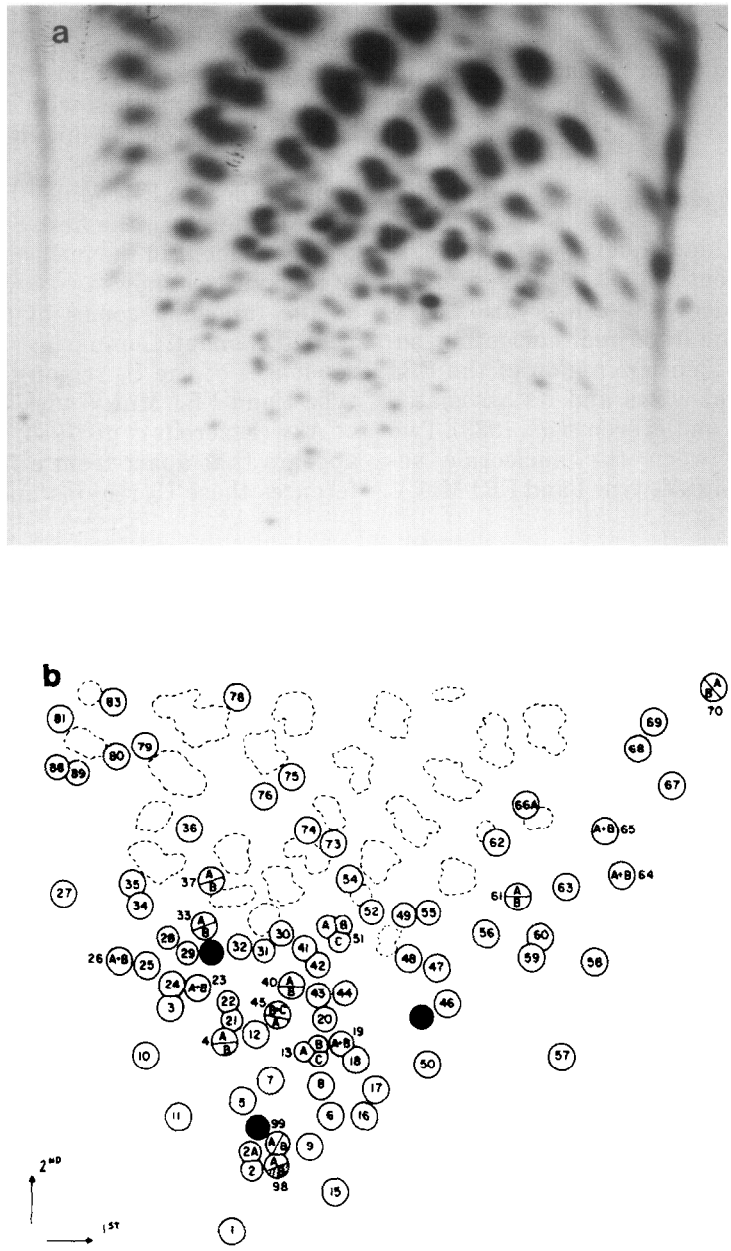

FIG. 4. RNase T_1 fingerprint analysis of OA $MuLV_R$ type I RNA. (a) OA $MuLV_R$ type I, (b) schematic comparison of OA $MuLV_R$ type I and Akv using the numbering system of Pedersen and Haseltine (1980). Open circles represent oligonucleotides shared by Akv and OA $MuLV_R$, closed circles represent oligonucleotides unique to OA $MuLV_R$, and hatched circles represent oligonucleotides unique to Akv.

otide map of AKV shows that the two viruses are closely related (Fig. 4b). The numbering system for oligonucleotides used here was brought in agreement to the Akv nucleotide sequence by Etzerodt *et al.* (1984). OA $MuLV_R$ RNA shows three oligonucleotides not detected in the Akv RNA. The oligonucleotide 98B missing in the OA $MuLV_R$ RNA is located in the p15E region of the Akv genome at position 7736–7759

(Fig. 2). The absence of this oligonucleotide and the presence of the one unique for OA $MuLV_R$ near number 99 is characteristic for some oncogenic MCF type viruses (Kelly *et al.*, 1983).

Nucleotide Sequencing

The nucleotide sequences of the *Pst*I-*Bssh*II fragment of OA $MuLV_R$ type I shows a high degree of conservation compared with the reported nucleotide sequence of the identical region of the FBJ murine sarcoma virus and its associated helper virus (Van Beveren *et al.*, 1983). Two differences between the nucleotide sequence of OA $MuLV_R$ type I and FBJ MuLV are found (Fig. 5a). The first being a point difference at position 45 where a C in OA $MuLV_R$ type I is a T in FBJ MuLV. The second difference lies within the number of appearances of a 46-bp repeated element. OA $MuLV_R$ type I has two copies of the repeat whereas FBJ MuLV has three copies. Furthermore the first copy of the repeated element in the nucleotide sequences of both viruses shows two point differences to the following copies of the 46-bp repeated element. Comparing the nucleotide sequences of the U_3 region of OA $MuLV_R$ type I and FBJ MuLV with the U_3 region of Akv (Etzerodt *et al.*, 1984; Herr, 1984) it appears that apart from a few point differences these U_3 regions mainly differ in

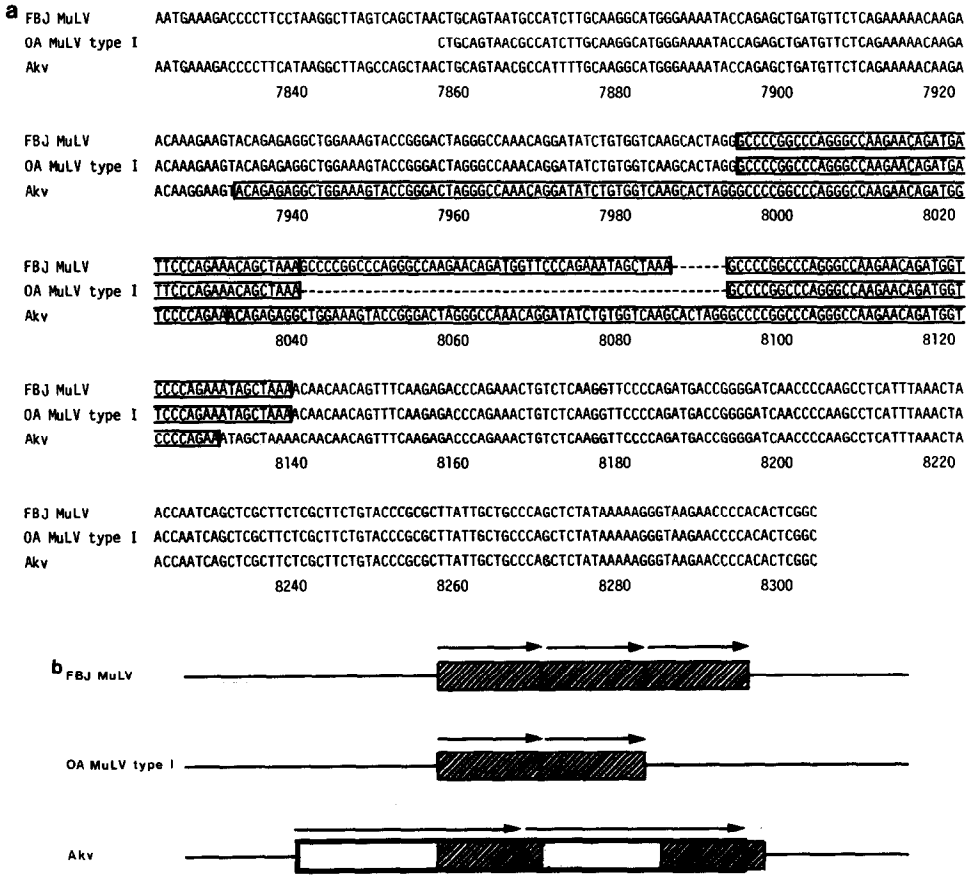

FIG. 5. (a) Nucleotide sequences of the U_3 region of FBJ MuLV (Van Beveren *et al.*, 1983), OA $MuLV_R$ type I, and Akv (Etzerodt *et al.*, 1984; Herr, 1984). The boxes show the repeated elements of the enhancer region. (b) Organization of the direct repeat regions of FBJ MuLV, OA $MuLV_R$ type I, and Akv. Hatched boxes represent the 46-bp repeated elements of FBJ MuLV and OA $MuLV_R$ type I. Thick-lined boxes represent the 99-bp repeated elements of Akv.

the way the repeated elements are organized within the nucleotide sequence (Fig. 5b).

Oncogenicity of the Molecularly Cloned Virus

The virus produced by NIH 3T3 cells, which had been transfected with the molecular clone OA MuLV$_R$ type I, was injected into newborn NMRI mice. After a latency period of 8–18 months the mice developed lymphomas, osteomas, and osteopetrosis (Table 1). DNA isolated from OA MuLV$_R$ type I-induced lymphomas was analyzed by hybridization with the ecospecific *env* fragment of Akv DNA. Digestion with *Pst*I revealed the 6.6-kb fragment characteristic for OA MuLV$_R$ (Figs. 6a–c). In DNA from uninfected NMRI mice endogenous ecotropic viruses could not be detected (Fig. 6d).

DISCUSSION

We have cloned the OA MuLV genome for molecular analysis of the regions possibly responsible for the oncogenicity of the virus and the pathological changes in bone tissues. This led to isolation of three different types of OA MuLV$_R$ recombinant DNAs, one of them infectious in a transfection assay. With the exception of the noninfectious clone OA MuLV$_R$ type III, which has a deletion in the *pol* gene, heteroduplex analysis demonstrates a strong relationship of OA MuLV$_R$ to the Akv genome. Restriction enzyme mapping and RNase T_1 fingerprint analysis indicate a homology between the Akv and the OA MuLV$_R$ genome of more than 95%. OA MuLV$_R$ type I has a U_3 structure very similar to FBJ MuLV, a virus derived from a spontaneous osteosarcoma of a CF-1 mouse. The same 46-bp element is repeated in the two viruses. However, only two copies of this element are found in the OA MuLV genome, whereas FBJ MuLV contains three copies (Van Beveren *et al.*, 1983).

The virus OA MuLV$_R$ used for molecular cloning is one component of the heterogenous virus complex which had been isolated from murine osteomas (Schmidt *et al.*, 1984). OA MuLV$_R$ was separated from the OA MuLV complex by passage on 208 F rat cells. OA MuLV$_R$ differs slightly from OA MuLV$_N$, another main component of the OA MuLV stock, which had been isolated by endpoint dilution (Schmidt *et al.*, 1984). OA MuLV$_N$ corresponds to the 9-kb *Pst*I fragment of the Hirt supernatant and lacks the additional *Pst*I site in the *gag* region, which is characteristic for OA MuLV$_R$. Nevertheless both types of OA MuLV induce the same pattern of diseases in newborn NMRI mice, and their proviral DNA could be demonstrated in lymphomas from infected NMRI mice. Therefore we conclude that the changes within the *gag* gene indicated by the additional *Pst*I site are not essential for the pathogenic capacity of the virus. But we cannot exclude that there may be some more structural changes not detected with our methods. Since major differences are found within the envelope gene and the LTR of OA MuLV, we assume that the oncogenicity and the tissue specificity of OA MuLV are mainly determined by structures in this regions. This is supported by recent studies on the highly leukemogenic murine retroviruses Gross pas-

TABLE 1

DISEASE PATTERN INDUCED BY OA MuLV IN NMRI MICE

	Osteoma		Osteopetrosis		Malignant lymphoma	
Inoculum	Animals	Mean age (days)	Animals	Mean age (days)	Animals	Mean age (days)
OA MuLV complex	18% (4/22)	389 ± 33	64% (14/22)	393 ± 75	86% (19/22)	347 ± 77
OA MuLV$_R$ type I	20% (2/10)	532 ± 65	30% (3/10)	464 ± 197	70% (7/10)	545 ± 56
Control	0% (0/50)	—	0% (0/50)	—	8% (4/50)	365 ± 157

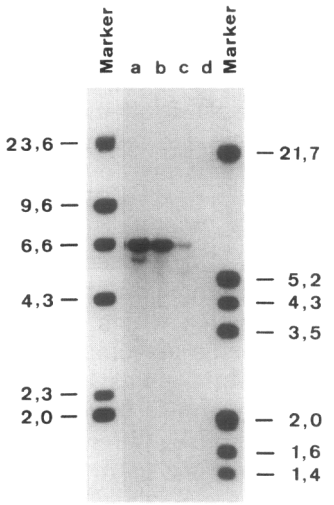

FIG. 6. Southern blot analysis of genomic DNA from lymphomas induced in NMRI mice by cloned OA $MuLV_R$ type I. Cellular DNA was digested with *PstI* and hybridized to the ecospecific 0.4-kb *env* fragment (pBW-50). (a) and (b) lymphomas; (c) spleen; (d) DNA from uninfected NMRI mouse.

sage A (DesGroseillers *et al.*, 1983b; DesGroseillers and Jolicoeur, 1984) and SL3-3 (Lenz and Haseltine, 1983; Lenz *et al.*, 1984; Celander and Haseltine, 1984). The highly leukemogenic SL3-3 virus differs by only a few base changes from the nonleukemogenic Akv with the exception of the enhancer region (Pedersen *et al.*, 1981; Lenz *et al.*, 1982a). In the experiments reported by Lenz *et al.* (1984) and Celander and Haseltine (1984) the major determinant of leukemogenicity of SL3-3 was localized within the LTR by isogeneic constructions between Akv and SL3-3. In some cases disease specificity could be transferred from one virus to another by exchanging only the LTR sequences (DesGroseillers *et al.*, 1983a; Chatis *et al.*, 1983, 1984). The role of viral enhancers in the induction of murine diseases is also supported by the detection of common viral integration sites in tumors induced by murine leukemia viruses, which in some cases are located adjacent to known oncogenes (Tsichlis *et al.*, 1983; Corcoran *et al.*, 1984; Steffen, 1984). Our findings of similar repeated elements of the enhancer region in OA $MuLV_R$ type I and in FBJ MuLV raises the possibility that elements of this structure may relate to the tissue tropism of the viruses. Our unpublished results indicate that FBJ MuLV induces the same pattern of diseases in NMRI mice as OA MuLV, whereas no osteomas are observed after injection of Akv virus. The possible role of the repeated elements for virus tropism and pathogenicity remains a subject for further study.

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