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# Akv Murine Leukemia Virus Enhances Bone Tumorigenesis in hMT-c-fos-LTR Transgenic Mice

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hMt-c-fos-LTR transgenic mice (U. Rüther, D. Komitowski, F. R. Schubert, and E. F. Wagner. *Oncogene* 4, 861–865, 1989) developed bone sarcomas in 20% (3/15) of females at 448 ± 25 days and in 8% (1/12) of males at 523 days. After infection of newborns with Akv, an infectious retrovirus derived from the ecotropic provirus of the AKR mouse, 69% (20/28) of female animals and 83% (24/29) of males developed malignant fibrous-osseous tumors. The tumors in infected transgenics developed with higher frequency and a 200-days shorter mean tumor latency period. The hMt-c-fos-LTR transgene was expressed in all the fibrous-osseous tumors. They also showed newly integrated Akv proviruses, but in most tumors Akv was detected and expressed in only a small number of the tumor cells. Wild-type C3H mice infected with Akv developed benign osteomas with an incidence of 33% and a latency period of 474 days. The data indicate that Akv exerts distinct pathogenic effects on the skeleton. In hMt-c-fos-LTR transgenic mice, predisposed to bone sarcomagenesis, Akv acts synergistically with the fos transgene, resulting in the development of fibrous-osseous tumors. © 1995 Academic Press, Inc.

## INTRODUCTION

The Akv murine leukemia virus is derived from an endogenous provirus of the AKR mouse and belongs to a genus of related retroviruses (Chattopadhyay *et al.,* 1980; Coffin, 1990) which are essentially considered as nonpathogenic (Celander and Haseltine, 1984; Hays and Vredevoe, 1977; Lenz *et al.,* 1982; Lenz and Haseltine, 1983; Nishizuka and Nakakuki, 1968; Pedersen *et al.,* 1981). Other ecotropic retroviruses derived from endogenous proviruses of BALB/c and C57/BL mice have been shown to harbor pathogenic potential in susceptible mice (Pedersen *et al.,* 1990; Schmidt *et al.,* 1988). Moreover, Akv λ623 derived from the AKR mouse ecotropic provirus (Lowy *et al.,* 1980) induces lymphomas (Speth *et al.,* 1995) as well as benign bone lesions (Luz *et al.,* 1991) after prolonged latency. A retroviral etiology of certain mouse bone tumors has been shown for FBJ MSV (Finkel *et al.,* 1966) and FBR MSV (Finkel *et al.,* 1975). These v-fos carrying retroviruses isolated from spontaneous and radiation-induced osteosarcomas are highly oncogenic for skeletal cells both *in vivo* (Michiels *et al.,* 1984; Ward and Young, 1976) and in tissue cultures of mandibular condyles (Schmidt *et al.,* 1986).

A large body of data on the biological effect of c-fos (see references in Grigoriadis *et al.,* 1993) suggests that the fos protooncogene plays a pivotal role in the regulation of differentiation and proliferation (Angel and Karin, 1991; Bravo, 1990; Curran, 1988), as well as in mouse (Schön *et al.,* 1986) and human (Wu *et al.,* 1990) skeletal neoplasia. This notion is supported by osteosarcoma development in c-fos transgenic mice (Grigoriadis *et al.,* 1993; Rüther *et al.,* 1989), and chondrosarcoma development in c-fos chimaeric mice (Wang *et al.,* 1992). Given the transforming potential of the fos gene product, it might be expected that hMt-c-fos-LTR transgenic mice would be highly susceptible to osteosarcoma development, but only 18% of mice develop tumors (Rüther *et al.,* 1989). The low incidence of osteosarcomas in hMt-c-fos-LTR transgenic mice suggests that genetic factors in addition to c-fos are necessary for bone tumor development.

Previous observations on cell lines established from osteosarcomas of hMt-c-fos-LTR transgenic mice (Goralczyk *et al.,* 1990) indicated that in bone neoplasia there may be a cooperation between activated endogenous retroviruses and c-fos. We observed high expression of endogenous retroviruses in these tumor cell lines, but not in cell lines established from normal tissues of hMt-c-fos-LTR transgenic mice (unpublished data). In order to test whether activated endogenous retroviruses cooperate with the fos oncogene in skeletal neoplasia we infected newborn hMt-c-fos-LTR transgenic mice with Akv, a retrovirus derived from the ecotropic provirus of the AKR mouse (Lowy *et al.,* 1980). Compared to nonin-

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fected transgenic controls, infected hMt-c-fos-LTR transgenic mice showed significantly increased bone tumor incidence and a considerably shortened tumor latency period. Thus it seems that activated endogenous retroviruses and the fos oncogene cooperate in bone neoplasia.

## MATERIALS AND METHODS

### Mice

hMt-c-fos-LTR transgenic C3H/HeJ mice (Rüther *et al.,* 1987) were mated to C3H/He/R1/Nhg mice from the breeding colony of the GSF. Newborn offspring were infected with Akv as described (Speth *et al.,* 1995). Mice were killed, X-rayed, and autopsied when they showed illness or tumor development or at the end of the experiment (586 to 599 days in Akv-infected transgenics; 711 to 716 days in noninfected transgenics). The bone tumors were diagnosed roentgenographically and in part histologically after staining with H&E and with von Gieson stain. The definition "bone tumors" included all expansively growing neoplasms containing newly formed bone as well as bone neoplasms described by Rüther *et al.* (1989). All other tumors were diagnosed as described (Schmidt *et al.,* 1984). For statistical evaluation we used Fisher's exact test and the log rank test.

### Tumor transplantation

Transplantability of bone tumors was determined by subcutaneous implantation of primary Akv-induced tumor tissue into syngeneic newborn wild-type C3H mice. The mice were killed when the transplant tumors reached 10 mm. The tumors were diagnosed and processed as above.

### DNA blot analysis

High-molecular-weight DNA was isolated from the tumors, digested with the appropriate restriction endonucleases, and analyzed by Southern blotting. Ten micrograms of restriction enzyme-digested cellular DNA was separated on 1% agarose gels, transferred to Zeta probe-GT membranes (Bio-Rad), and probed with $^{32}$P-labeled probes. Hybridizations and washings were performed as described (Church and Gilbert, 1984). Proviral integration of Akv was detected by *Eco*RI digestion of tumor DNA and hybridization of the filters with an ecotropic virus-specific probe (Chattopadhyay *et al.,* 1980). Control tissues included spleen from uninfected hMt-c-fos-LTR transgenic C3H/HeJ mice, three liver adenomas from hMt-c-fos-LTR transgenic and normal C3H/HeJ mice, four malignant lymphomas from normal C3H/HeJ mice, and normal spleen tissues from Akv-infected hMt-c-fos-LTR transgenic, bone tumor-bearing mice. The transgene was detected by digestion with *Eco*RI, *Eco*RV, and *Kpn*I and hybridizing with a v-fos specific probe (Curran, 1988).

### PCR analysis of DNA

Figure 1 shows the strategy used to distinguish Akv sequences from the closely related C3H mouse endogenous provirus emv-1. One PCR primer in each of the two pairs (Akv-1 and Akv-4) recognizes the two 99-bp repeat junction present in the U3 region of Akv LTR. The second primer in each of the two pairs (Akv-2, Akv-3) recognizes sequences homologous to the C3H provirus. Akv-1 and Akv-2 primers amplify a 285-bp region. Akv-3 and Akv-4 primers amplify a 449-bp Akv-env fragment. The primers Akv-8 and Akv-9 amplify a 382-bp fragment in the C3H provirus and a 481-bp fragment in Akv. This primer set and a pAkv-CAT plasmid containing an Akv LTR region were used to generate an Akv LTR probe.

PCR was performed on Gen Amp PCR system 9600 (Perkin Elmer). The initial cycle of 94° for 1 min, 65° for 1 min 30 sec, 72° for 2 min was followed by 28 cycles of 94° for 24 sec, 65° for 2 min, 72° for 2 min and by an elongation step at 72° for 10 min. The amplification temperature for a control actin PCR was 60°. Ten microliters of PCR product was separated on a 2% agarose gel, visualized with ethidium bromide, blotted to Zeta probe-GT membranes (Bio-Rad) by electroblotting, and hybridized with random primer-labeled probes. Nucleotide sequencing was performed using the Automated Laser Fluorescent DNA sequencing system 373A (Applied Biosystems) protocols for ABI dye terminator cycle sequencing.

### RT PCR

Total cellular RNA was reverse transcribed into cDNA and used as template for PCR. Random hexanucleotides were used as primers for reverse transcription; 0.5 $\mu$g of total RNA was centrifuged (10,000 rpm, 30 min), lyophilized, dissolved in 20 $\mu$l water, and treated with DEPC. The RNA was heated (68°, 10 min) to denature its secondary structure and chilled; 100 $\mu$l reaction mix (1 × PCR reaction buffer, (Perkin Elmer Cetus), 100 $\mu$l each of dGDP, dCTP, dTTP, and dATP (Pharmacia), and 10 U RNasin (Promega, 40 U/$\mu$l, 5 U AMV reverse transcriptase (Boehringer, 24 U/$\mu$l), 5 $\mu$g hexanucleotides (Pharmacia) and sterile water) were added and the reaction was stopped by freezing the tubes at −20°.

Hot start PCR (D'Aquila *et al.,* 1991) was performed (Thermocycler 480, Perkin Elmer Cetus) for amplification of the transcripts from the integrated Akv and the hMt-c-fos-LTR transgene. The reaction mix contained 1 × PCR reacting buffer, 100 $\mu M$ each dNTP, 1 $\mu M$ each primer, and 2.5 U Taq polymerase (Perkin Elmer Cetus). The initial cycle of 94° for 2 min, 60° for 2 min, and 72° for 2 min was followed by 35 cycles for the transgene and 26 cycles for Akv in which the denaturation step at 94° was shortened to 1 min. The final elongation reaction was at 72° for 10 min. PCR products were electrophoresed on a 2% agarose gel and transferred to Zeta-Probe membranes (Bio-Rad). Akv transcripts were hybridized

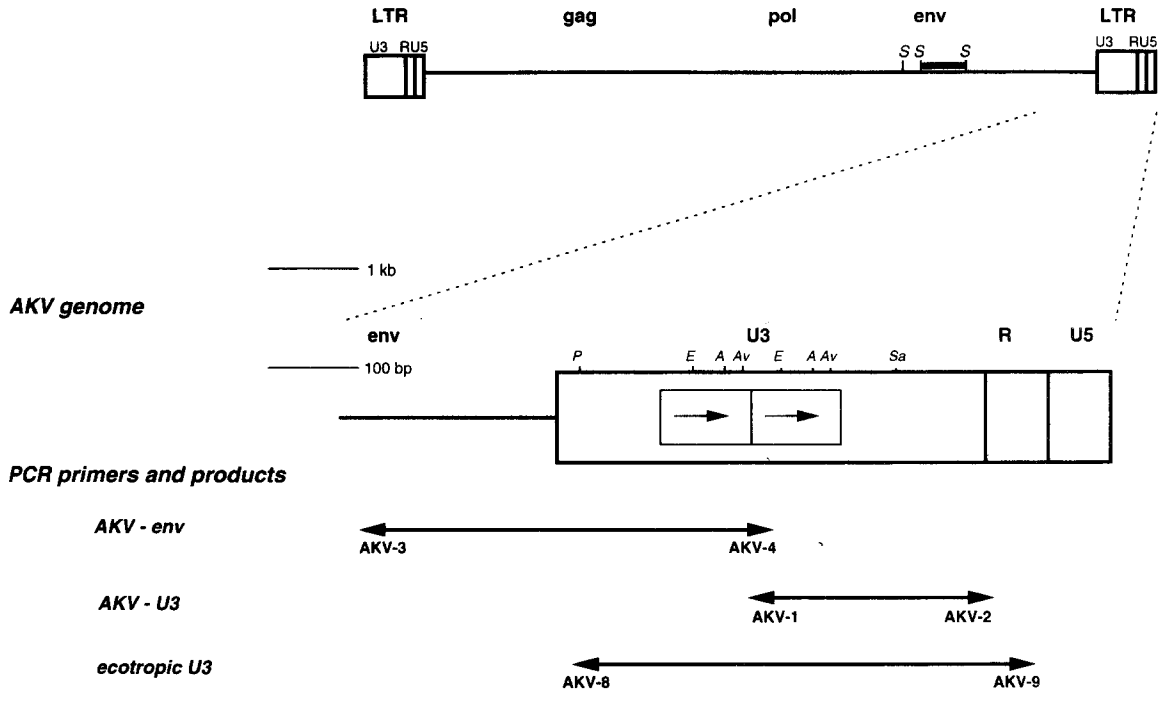


FIG. 1. Strategy for detection of Akv sequences in hMt-c-fos-LTR transgenic C3H mice carrying the emv-1 ecotropic provirus. PCR primers were targeted to the Akv-specific 99-bp repeat in the U3 region of the LTR (Etzerodt *et al.*, 1984). The location of the ecotropic-specific virus probe delimited by two *Sma*I (S) sites in the env gene of Akv (base No. 5748-6076) is indicated on the Akv genomic map. The enlarged region underneath encompasses the 3′ part of the Akv p15E gene and the 3′ LTR. Distinctive restriction sites in this region are *Pst*I (P), *Eco*RV (E), *Apa*I (A), *Ava*II (Av), and *Sau*3A (Sa). Primer pair Akv-1 and Akv-2, giving a 285-bp product, was used for amplification of the Akv-U3 region; primer pair Akv-3 and Akv-4, giving a 449-bp product, was used to amplify the 5′ portion of the U3 region and part of p15E. Primers Akv-1 and Akv-4 are homologous to the 99-bp repeat junction which is characteristic of the Akv LTR. Primer pair Akv-8 and Akv-9 are homologous to the U3 and R regions of most ecotropic retroviruses and have been used to generate hybridization probes by PCR amplification of an Akv-LTR region from a plasmid. The Akv specificity of the PCR primers was demonstrated by the absence of Akv-related sequences in PCR products from DNAs isolated from six different C3H/HeJ mice (Fig. 3, lane C3H) and the presence of a PCR product with $\beta$-actin primers (data not shown). To exclude an inhibitory effect of C3H DNAs on Akv-PCR, C3H DNA was mixed in a 1:1 ratio with DNA of tumors shown to contain Akv sequences, and the DNA mixtures were used as template in PCRs with primers Akv-1 and Akv-2 (see Fig. 3, lanes 85, 85 + C3H and C3H). Akv-U3 (285 bP) PCR primers: Akv-1 (5′) CCCCAGAAACAG-AGAGGC; Akv-2 (3′) GCGCGCCGAGTGTGG. Akv-env (449 bp) PCR primers: Akv-3 (5′) GTCCCCTTGGTTCACCAACC; Akv-4 (3′) GCCTCTCTGTTTCTG-GGGACC. Akv-ecotropic U3 (Akv: 481 bp), (C3H provirus: 381 bp) PCR primers: Akv-8 (5′) CAGCTAACTGCAGTAACGCCAT; Akv-9 (3′) CGACTCAGT-CTATCGGAGGACT.

with the PCR-amplified Akv-3/Akv-4 fragment (Fig. 1). PCR products were characterized by restriction analysis as well as by direct sequencing of PCR products of selected tumors. The fos-PCR product was confirmed by hybridization to a $^{32}$P-labeled v-fos probe.

## RESULTS

### Akv enhances bone tumor development in hMt-c-fos-LTR transgenic mice

Untreated hMT-c-fos-LTR transgenic C3H mice were observed for up to 716 days (Table 1). Twenty percent of females and 8% of males developed single bone tumors after a mean latency period of 448 and 523 days, respectively. After infection of newborn hMt-c-fos-LTR transgenic mice with Akv, a total of 76% of the mice developed fibrous-osseous tumors with a mean tumor latency of 274 days. In females tumor incidence was 69% with a mean of 3.1 tumors per tumor-bearing mouse; in males tumor incidence was 83% with a mean of 4 tumors per tumor-bearing mouse. Akv-infected wild-type C3H mice developed osteomas with an incidence of up to 33% and a mean latency of 477 days. No bone tumors were detected in 35 control C3H mice within a 702-day observation period. In other studies 199 female control C3H mice included in a 4-year genetic monitoring program developed 3 (1.5%) bone tumors after a latency period of $2\frac{1}{2}$ years.

The majority of the bone tumors observed in the Akv-infected hMt-c-fos-LTR transgenic mice were fibrous-osseous tumors. In addition to these, we also detected 2 mice with osteomas. In the Akv-infected wild-type C3H mice all bone tumors were diagnosed as osteomas, a benign, expansively growing bone tumor characterized by a smooth outline and compact bone. Tumor tissue of an Akv-induced fibrous-osseous bone tumor gave rise to a continuous line of osteogenic transplant tumors after transplantation indicating its malignancy. Four out of 54 Akv-infected transgenic mice developed lymphomas and

TABLE 1

ENHANCEMENT OF BONE TUMORIGENESIS IN hMt-c-fos-LTR TRANSGENIC C3H MICE WITH Akv

| Mice | No. of mice | Bone tumor incidence | | Mean latency period (days, f/m)[a] | Observation period (days) |
|---|---|---|---|---|---|
| | | Females | Males | | |
| Wild type | 35 | 0% (0/23) | 0% (0/12) | | 702–716 |
| Akv-infected wild type | 17 | 33% (6/18) | 13% (1/8) | 477[b] | 536–544 |
| hMT-c-fos-LTR | 27 | 20%[c] (3/15) | 8%[d] (1/12) | 448 ± 25/523 | 711–716 |
| Akv-infected hMT-c-fos-LTR | 57 | 69%[e] (20/28) | 83%[f] (24/29) | 268 ± 122/279 ± 109 | 586–592 |

*Note.* The data show tumor incidence at Day 600 of the experiment unless otherwise noted. The tumors were diagnosed by X-ray and by histological sections after staining with H&E and von Gieson.

[a] The mean latency period was calculated over the entire observation period; f, females; m, males.

[b] Mean latency period of osteomas in three mice which were detected before the end of the observation period.

[c] Two additional female mice with single tumors were observed on Days 700 and 714.

[d] Three additional male mice with a mean of 1.3 tumors per mouse were observed on Days 603, 609, and 713.

[e] $P = 0.001$, Fisher's exact test, compared to noninfected females; $P < 0.0001$, log rank test for the entire observation period compared to noninfected females.

[f] $P = 0.000003$, Fisher's exact test, compared to noninfected males; $P < 0.0001$, log rank test for the entire observation period compared to noninfected males.

2 developed liver adenomas. In conclusion, Akv infection significantly enhanced bone tumor development and shortened the tumor latency period by about 200 days in hMt-c-fos-LTR transgenic mice.

### c-fos is expressed in the majority of the bone tumors

The presence of the c-fos transgene in genomic DNA from 24 randomly selected tumors of Akv-infected hMt-c-fos-LTR transgenic mice was confirmed by *Eco*RI, *Eco*RV, and *Kpn*I digestion of tumor DNA, Southern blotting, and hybridizing with a v-fos probe. Neither the transgene nor the endogenous c-fos fragments were altered. The expression of the transgene was shown by RT-PCR from tumor RNA using primers spanning c-fos and p15E sequences of the vector. An 890-bp RT-PCR product which hybridized with the v-fos probe was detected in 21 out of 24 (87%) bone tumors. Northern blot analysis of bone tumors showed varying amounts of fos mRNA without correlation between the mRNA levels and other tumor features. The 890-bp RT-PCR product was not found in RNA isolated from the normal liver of a noninfected hMt-c-fos-LTR transgenic mouse with an osteogenic tumor, or in nontransgenic control mice.

### Proviral Akv is not present in all tumor cells

DNAs from 24 fibrous-osseous tumors, 3 lymphomas, and 3 liver tumors from infected mice were analyzed for the presence of Akv sequences. Digestion with *Eco*RI showed the endogenous ecotropic provirus (emv-1) of C3H/HeJ mice on an approximately 18-kb restriction fragment (Jenkins *et al.*, 1982). Fourteen out of 24 showed additional ecotropic provirus sequences on Southern blots. In 6 out of these 14 tumors (Nos. 67, 78, 84, 85, 91, 95) the intensity of the signals suggested the presence of a provirus in most of the tumor cells, indicating clonal growth of virus-infected cells. In 8 out of 24 tumors (Nos. 63, 70, 73, 75, 77, 79, 90, 92) new proviruses were present in a small proportion of tumor cells. No additional proviral

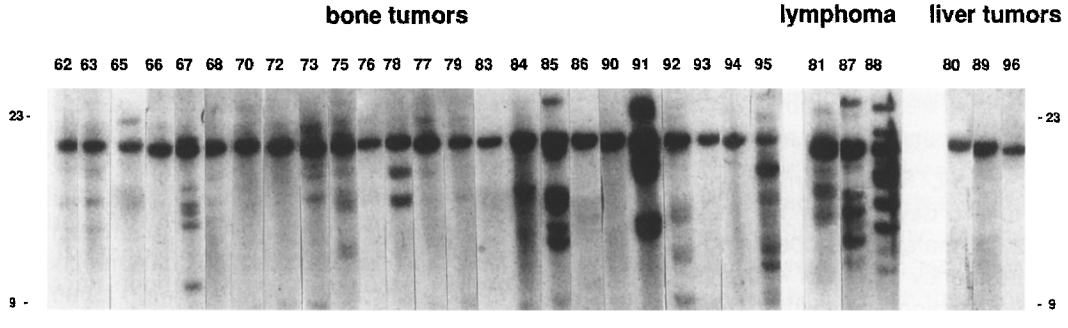


FIG. 2. Southern blot analysis of tumor DNAs from Akv-infected hMt-c-fos-LTR transgenic mice. High-molecular-weight DNAs were digested with *Eco*RI, and the restriction fragments were electrophoresed, blotted, and probed with the ecotropic retrovirus-specific probe. The DNAs are derived from fibrous-osseous (bone) tumors, lymphomas, and liver tumors. Molecular size markers are indicated in kilobases. The numbers refer to individual tumors as in Figs. 3 and 4.

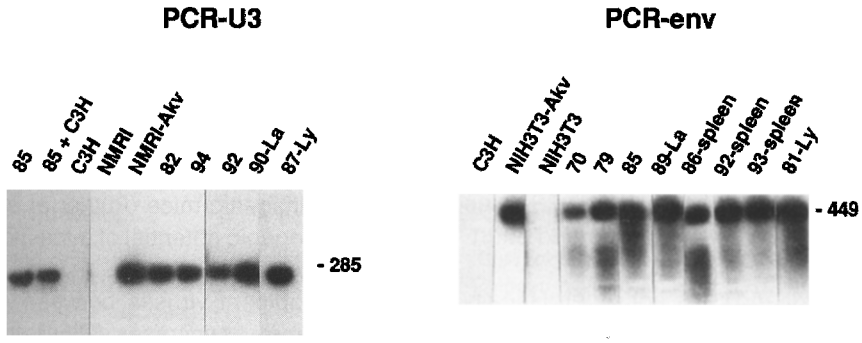


FIG. 3. PCR analysis showing the presence of Akv sequences in tumor DNAs. PCR-U3, PCR amplification of a 285-bp Akv U3 sequence with Akv-1 and Akv-2 primers in tumors and control cells from Akv-infected transgenic C3H mice. 85, fibrous-osseous tumor; 85 + C3H, C3H DNA mixed (1:1) with 85-DNA; C3H, C3H genomic DNA; NMRI and NMRI-Akv, genomic DNA of noninfected and Akv-infected NMRI mice; La, liver tumor, and Ly, lymphoma, in bone tumor bearing mice. PCR-env, PCR amplification of a 449-bp Akv env sequence with Akv-3 and Akv-4 primers in tumors and control cells from Akv-infected hMt-c-fos-LTR transgenic C3H mice. C3H, genomic DNA from a C3H mouse; NIH3T3-Akv and NIH3T3, DNA from Akv-infected NIH3T3 and noninfected NIH3T3 cells; the numbers refer to fibrous-osseous tumors; La, liver tumor; 86-, 92-, 93-spleen, spleen cells from bone tumor-bearing mice; Ly, lymphoma.

sequences were detected by Southern analysis in the other 10 tumors (Fig. 2). The lymphomas in Akv-infected transgenic mice contained several roughly equimolar signals from newly acquired ecotropic proviruses. No additional proviruses were found in liver adenomas, a tumor which also appears spontaneously in wild-type C3H mice (Fig. 2), in lymphomas from control mice, in normal spleens from bone tumor-bearing Akv-infected transgenic mice, and in spleens from noninfected transgenic controls (data not shown).

Southern blot analysis could not discriminate between newly integrated Akv proviruses and potentially activated and reintegrated endogenous ecotropic provirus from the emv-1 locus. To identify newly integrated proviruses and to check for the presence of Akv in Southern blot-negative tumors we subjected tumor DNAs to PCR analysis. Akv specific primers were used which allowed discrimination of newly integrated Akv from the endogenous ecotropic provirus emv-1 of C3H mice (Fig. 1). The primer pairs Akv-1/Akv-2 and Akv-3/Akv-4 detected Akv sequences in DNAs of all tumors from Akv-infected hMt-c-fos-LTR transgenic mice (Fig. 3). The sequence homology to Akv was confirmed by direct sequencing of HPLC-purified DNAs of three randomly selected PCR products (data not shown).

To correlate the different results obtained by Southern blot (Fig. 2) and PCR analyses (Fig. 3), we quantified the PCR analysis by varying the number of amplification cycles. DNA from tumors showing multiple Akv sequences on Southern blots (85, 95, 87), generated the characteristic Akv env band of 449 bp after 19 cycles. DNA from tumors showing either a weak (70, 79, 83) or no (89) Akv env signal on Southern blots generated a band of similar intensity only after 21–23 and 26 PCR cycles (not shown). These data indicate that the results obtained by quantitative PCR are compatible with the results from Southern blot analysis. Thus Akv was present in all tumors, but in about 40% of the fibrous-osseous tumors Akv provirus was present only in a small cell population.

### Akv is expressed in the osteogenic tumors

Akv env gene transcripts were detected by PCR amplification of reverse-transcribed total cellular RNA (RT-PCR) in all but two osteogenic tumors, in two out of three lymphomas, and in two out of the three liver tumors analyzed. Representative data are shown in Fig. 4. Integrity of the RNAs was confirmed by amplification of the PCR-negative RNAs using primers for $\beta$-actin (not shown). These results showed that the sequences spanning the Akv env and LTR/U3 (bp 7593–8042) region were expressed, although at a low level, in virtually all the fibrous-osseous tumors which developed in Akv-infected hMt-c-fos-LTR transgenic C3H mice.

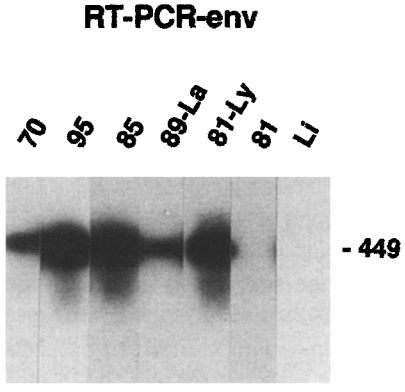


FIG. 4. RT-PCR analysis with total cellular RNA of tumors from Akv-infected hMt-c-fos-LTR transgenic and nontransgenic control mice. The 449-bp product was generated by use of the Akv-env primer pair Akv-3 and Akv-4 and probed with the PCR product of an Akv LTR-containing plasmid by use of Akv-8 and Akv-9 primers. The data show representative fibrous-osseous tumors; 89-La, liver tumor; 81-Ly, lymphoma; Li, liver DNA from an hMt-c-fos-LTR transgenic mouse.

## DISCUSSION

In this paper we describe the influence of Akv, a retrovirus derived from the endogenous ecotropic provirus of the AKR mouse (Lowy *et al.,* 1980), on c-fos-induced bone tumor development in hMt-c-fos-LTR transgenic mice. The results show that infection of hMt-c-fos-LTR mice with Akv increases the bone tumor incidence and shortens the tumor latency period.

The data suggest that Akv and c-fos act synergistically to enhance the oncogenic process in the skeleton. The effect of Akv on wild-type C3H mice differed markedly from the effect on the hMt-c-fos-LTR transgenic mice. Thirty-three percent of the infected wild-type mice developed benign osteomas, whereas 76% of transgenic mice developed malignant bone tumors exhibiting a characteristic trabecular bone pattern together with fibrous tissue. Only 4% of the infected transgenics developed benign osteomas. Fibrous-osseous tumors and osteomas could be clearly differentiated by X-ray and histological analysis (Luz *et al.,* 1991). The appearance of malignant tumors in hMt-c-fos-LTR transgenic mice after Akv infection, and the drop of osteoma incidence from 33% in Akv-infected wild-type mice to 4% in Akv-infected transgenic mice, indicates a coupling of the biological activities of Akv and c-fos (Luz *et al.,* 1991; Ruddle *et al.,* 1993; Rüther *et al.,* 1987; Schmidt *et al.,* 1988).

Earlier reports indicating a nonpathogenic nature of Akv probably resulted from the shorter observation periods used (Celander and Haseltine, 1984; Hays and Vredevoe, 1977; Lenz *et al.,* 1982; Lenz and Haseltine, 1983; Nishizuka and Nakakuki, 1986; Pedersen *et al.,* 1981). Extension of the observation period for Akv-infected mice for up to 700 days postinfection clearly shows the leukemogenic (Speth *et al.,* 1995) and bone pathogenic (Luz *et al.,* 1991) potential of this virus in different strains of mice.

In contrast to Moloney MuLV-induced lymphomagenesis (Corcoran *et al.,* 1984; Tsichlis and Lazo, 1991; van Lohuizen *et al.,* 1991, 1989), the underlying mechanism of Akv pathogenicity is not yet clear (Kung *et al.,* 1991). One out of 24 Akv-induced bone tumors contained an additional 4.8-kb fragment, as detected by hybridization of *Kpn*I-digested DNA hybridized with a bmi-1 specific probe. No alterations were found after hybridization with a fos probe or with an mlvi-1 probe (not shown). The p53 locus, although frequently altered in other tumors (Strauss *et al.,* 1992) and recently identified as a target for ETn insertion in radiation-induced osteosarcoma (Mitreiter *et al.,* 1994), was not rearranged in any of 24 bone tumors. Neither were the DNAs from liver adenomas or lymphomas rearranged in the p53 (Mowat *et al.,* 1985; Wolf and Rotter, 1984), mlvi-1 (Tsichlis and Lazo, 1991; Tsichlis *et al.,* 1983), or bmi-1 (Berns, 1991; van Lohuizen *et al.,* 1991) loci. These data do not rule out Akv integration into other relevant target sites; however, the absence of common bands on the Southern blots and the morphological characteristics of the fibrous-osseous tumors suggest a synergism between Akv and the fos oncogene in bone tumorigenesis. This synergistic activity results in the appearance of a bone tumor which looks morphologically more benign than the bone tumors in noninfected c-fos transgenic mice (Rüther *et al.,* 1989) but revealed the pathogenic potential of c-fos-induced osteosarcomas (Goralczyk *et al.,* 1990).

Recombinant viruses between Akv and endogenous nonecotropic proviruses (DiFronzo and Holland, 1993; Lung *et al.,* 1983; Stoye *et al.,* 1991) were only found in 1 out of 24 tumors by Southern blotting and hybridizing with an MCF-1 probe (Chattopadhyay *et al.,* 1982) (data not shown). Moreover, the low lymphoma incidence was only 7% in Akv-infected mice. These data indicate that MCF type viruses do not play a key role in Akv-induced bone tumors.

In the majority of the tumors Akv provirus was present in only a small number of the tumor cells. This finding is surprising and, together with the morphological appearance of the fibrous-osseous tumors, points toward a particular role for replication-competent, slow-transforming retroviruses in bone neoplasia (Pedersen *et al.,* 1990; Schmidt *et al.,* 1984, 1988). The presence of irregular bone within the well differentiated tumors is compatible with expression of Akv in a few distinct areas of the tumors, and also with the direct correlation between the steady state levels of Akv mRNA and the size of the tumors (data not shown here). Emv-1 provirus expression could be ruled out by PCR analysis and seemed not to be involved in the tumorigenic processes.

From our data we hypothesize that expression of an Akv-encoded protein and its binding to a heterologous, bone cell-specific receptor may enhance cell growth as described for SFFV gp55 and the erythropoietin receptor (Li *et al.,* 1990). Similarly, a virus-induced, locally acting factor may exert mitotic and/or differentiation-inducing activity in virus-infected cells of the osteoblastic lineage. Such a mechanism has been observed following HTLV-1 infection (Motokura *et al.,* 1988; Watanabe *et al.,* 1990) and suggested in HTLV-1 LTR-tax transgenic mice (Ruddle *et al.,* 1993). Possible effector molecules with a direct effect upon cells of the skeleton may include the proto-oncogene c-fos (Nagata *et al.,* 1989), parathyroid hormone-related peptide (PTHrP) (Motokura *et al.,* 1988; Watanabe *et al.,* 1990), transcription factors (Paul *et al.,* 1990), cytokines (Tschachler *et al.,* 1989) and their receptors (Inoue *et al.,* 1986), or both (Green *et al.,* 1989; Maruyama *et al.,* 1987; Siekewitz *et al.,* 1987).

As in c-fos-transgenic and c-fos-chimaeric mice (Rüther *et al.,* 1987, 1989; Grigoriadis *et al.,* 1993; Wang *et al.,* 1992), the time of onset of the initiating event in bone tumorigenesis seems crucial to the tumor phenotype. In C3H strain mice spontaneous provirus expression appears after adolescence with a low incidence,

resulting in a relatively small number of tumors and allowing the c-fos transgene to determine the tumor phenotype. In the experiments described here, Akv was injected at birth and followed by early viremia, exerting its biological activity at an earlier developmental stage of the skeleton. This allows cooperating events to ensue and a distinct tumor to develop.

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