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Akv Murine Leukemia Virus Enhances Lymphomagenesis in myc-kappa Transgenic and in Wild-Type Mice

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The contribution of endogenous retroviruses to the multistep process of lymphomagenesis was investigated in wild-type mice and in two different myc-κ transgenic mouse lines by infection with Akv. This retrovirus is derived from the endogenous ecotropic provirus of the AKR mouse and was previously considered to be nonlymphomagenic. The mice of the two myc-k transgenic lines are predisposed to B-cell lymphomagenesis and were therefore considered to be more susceptible to Akv. For comparison, the same mouse strains were also infected with the exogenous Moloney murine leukemia virus (MoMuLV). Both MoMuLV and Akv increased the tumor incidence and shortened the tumor latency period in wild-type mice and in the transgenic mouse lines. The differences in pathogenicity, number of provirus integrations, and level of virus expression between MoMuLV and Akv indicate different mechanisms of lymphomagenesis: while MoMuLV induced tumors apparently by insertional mutagenesis involving common integration sites similar to previous reports, the enhancement of lymphomagenesis by Akv seems to be directed by other mechanisms.

INTRODUCTION

The mouse genomic locus Akv-1 harbors the prototype (Risser and Horowitz, 1983) of endogenous ecotropic murine leukemia viruses, Akv (Chattopadhyay *et al.,* 1975). In contrast to other MuLVs, Akv is regarded as nonlymphomagenic in healthy mice (Risser and Horowitz, 1983). Molecular cloned Akv did not accelerate the onset of disease in AKR/J mice nor did it induce disease in C3H(f)/Bi mice or CBA/J mice within an observation period of 200 days after infection of newborns (Nishizuka and Nakakuki, 1968; Hays and Vredevoe, 1977; Pedersen *et al.,* 1981; Lenz *et al.,* 1982; Lenz and Haseltine, 1983; Celander and Haseltine, 1984). In contrast, Moloney murine leukemia virus (MoMuLV), an exogenous ecotropic MuLV originally isolated from a sarcoma passaged in Balb/c mice (Moloney, 1960), induces predominantly T-lymphomas within 3 months following its injection into newborn mice.

Several potential mechanisms have been discussed for lymphoma induction by murine leukemia retroviruses which lack a cellular oncogene homologue (Kung *et al.,* 1991; Tsichlis and Lazo, 1991). These include enhancement of the expression of a cellular oncogene by integration of proviruses into its vicinity (promoter insertion), alterations of the protein structure or mRNA stability of a cellular oncogene as a result of the integration event, and the *de novo* generation of recombinant viruses, as well as the biological effects of viral-encoded proteins which may also have a pathogenic potential. Whereas MoMuLV exerts its pathogenicity mainly by insertion mutagenesis, the biological effects of endogenous ecotropic retroviruses in their natural hosts are less clear. Similar to the integration of exogenous retroviruses, relocation of activated retroviral sequences within the host genome can act as insertional mutagens (Kung *et al.,* 1991; Mitreiter *et al.,* 1994).

Although previous experiments indicated that Akv is nonlymphomagenic in mice of strains AKR, CBA, and C3H, endogenous ecotropic retroviruses do have a lymphomagenic potential. For example, molecularly cloned Balb/c-derived endogenous ecotropic MuLVs have been shown to induce lymphomas after a long latency period (Pedersen *et al.,* 1990).

For a more detailed investigation of the biological effect of the molecularly cloned Akv on lymphoma development we took advantage of new transgenic NMRI mouse lines whose transgene construct mimics the genetic rearrangement found in human Burkitt's lymphoma (Lipp *et al.,* manuscript in preparation). It was hypothesized that these mice are prone to develop lymphomas after additional somatic alterations, and therefore are possibly more sensitive for lymphomagenic effects of retroviruses. Our studies show that Akv has an enhancing potential on lymphoma development in these myc-κ transgenic

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mice. Equally, by extending the observation period, Akv could be shown to induce lymphomas in wild-type NMRI mice, although at lower incidence. The lymphomagenic potential of Akv, the induced tumor type, and the integration and expression pattern of Akv are different from that of Moloney murine leukemia virus, suggesting distinct pathogenic pathways for Akv and MoMuLV-induced tumors.

MATERIALS AND METHODS

Myc-kappa transgenic and wild-type mouse lines

Three different mouse lines were used to determine the pathogenicity of Akv in comparison to MoMuLV: wild-type NMRI mice and two myc-κ transgenic NMRI mouse lines (lines 614 and 615). NMRI is originally derived from Swiss mice (Staats, 1985) and has been maintained in our animal facilities as an outbred breeding colony since 1966. The generation of myc-κ transgenic NMRI mice will be described elsewhere in detail (Lipp *et al.*, manuscript in preparation). Briefly, the transgene was a fusion construct between the human myc gene and the rearranged human kappa light chain gene; both genes were isolated from human Burkitt's lymphoma cell line BL64. The human myc gene carries point mutations in the first exon and the first intron; the functional kappa gene contains the intron enhancer between the J exon and the C exon (Hartl and Lipp, 1987). Two transgenic genotypes were used for further breeding, giving rise to the transgenic lines 614 and 615. Line 614 has approximately 6 transgene copies integrated into the cellular DNA, whereas line 615 has about 50–100 copies.

Retroviral infection

Female offspring of heterozygotes representing transgenics and nontransgenic controls were infected intraperitoneally within 36 hr after birth with 100 μl of cell-free supernatant either from an Akv-producing NIH3T3 cell line (Lowy *et al.*, 1980) or from a BC cell line producing Moloney MuLV (Weiland and Mussgay, 1976). The inoculum contained 2×10^5–2×10^6 infectious virus particles. Infected and uninfected control mice were checked on 5 days per week. Mice were killed when they showed illness or tumor development or at the end of the experiment at 520 days postinfection. A complete autopsy including X-ray analysis was performed. The tumors were diagnosed by Northern and by Southern blotting. For statistic evaluation we used the log-rank test.

DNA and RNA isolation

DNA was isolated according to standard procedures. For Southern blot analysis, 12 μg of total genomic DNA from each mouse was digested with restriction enzymes and separated on 0.8% agarose gels. DNA was transferred to a ZetaProbe nylon membran (Bio-Rad) by alkali transfer blotting according to standard procedures and crosslinked to the filters by uv light (254 nm).

RNA was extracted by the guanidinium isothiocyanate method of Chomczynski and Sacchi (1987) using the modification by Puissant (1990). For Northern blot analysis, 5 μg of total RNA was denatured with glyoxal/DMSO (McMaster and Carmichael, 1977), separated on agarose gels, and transferred to nylon membrane by neutral capillary blotting. Filters were stained with methylene blue and hybridized as described below.

Hybridization and probes

Filters were hybridized with ^{32}P-labeled hybridization probes under stringent conditions according to the method of Church and Gilbert (1984).

The following probes were used für hybridization: bmi 19.1 (van Lohuizen *et al.*, 1991), Mlvi-1/pTS26pp, Mlvi-2/pTS6, Mlvi-2/pTS10 (Wirschubsky *et al.*, 1986), pim-1/Probe A (Cuypers *et al.*, 1984), N-myc/Nb-1 (Schwab *et al.*, 1983), murine pSV c-myc (Land *et al.*, 1983), human c-myc/S421 (*Pvu*II fragment of 1st exon (Lipp *et al.*, 1987)), T-cell receptor β/RBL5 (Caccia *et al.*, 1984), and an ecotropic virus specific probe (Chattopadhyay *et al.*, 1980). As NMRI mice do not harbor endogenous ecotropic retroviruses crosshybridizing with this probe (Leib-Mösch *et al.*, 1986), the probe is specific for Akv proviruses in Akv-infected NMRI mice and for the ecotropic MoMuLV in MoMuLV-infected NMRI mice. For GAPDH we used a PCR-product derived from rat DNA with the primers 5′-CCACCACCCTGTTGCTGTAGC-3′ and 5′-TGGCCAAGGTCATCCATGACAACT-3′. The murine μ-probe was an Xba fragment from clone pSVμM5 (M. Reth, Freiburg), containing the exon C1-4 of the μ-chain.

RESULTS

Acceleration and enhancement of lymphoma development by Akv

The effect of molecularly cloned Akv on lymphoma development was tested in wild-type NMRI mice, and in two transgenic NMRI lines bearing the human myc gene fused to the rearranged human kappa light chain gene. The two transgenic mouse lines (614 and 615) differed in the number of copies of the transgene integrated into the cellular genome (Lipp *et al.*, manuscript in preparation). Lymphoma development was examined in all three mouse lines during a total observation period of 520 days (Figs. 1A–1C). Since uninfected wild-type NMRI mice developed lymphomas spontaneously after 360 days, the tumors which developed after 1 year were considered age-related rather than induced and are not discussed further.

Lymphoma were detected in 5% uninfected wild-type NMRI mice (Fig. 1A, Table 1), in 14% myc-κ transgenic mice from line 614 (Fig. 1B), and in 88% transgenic mouse

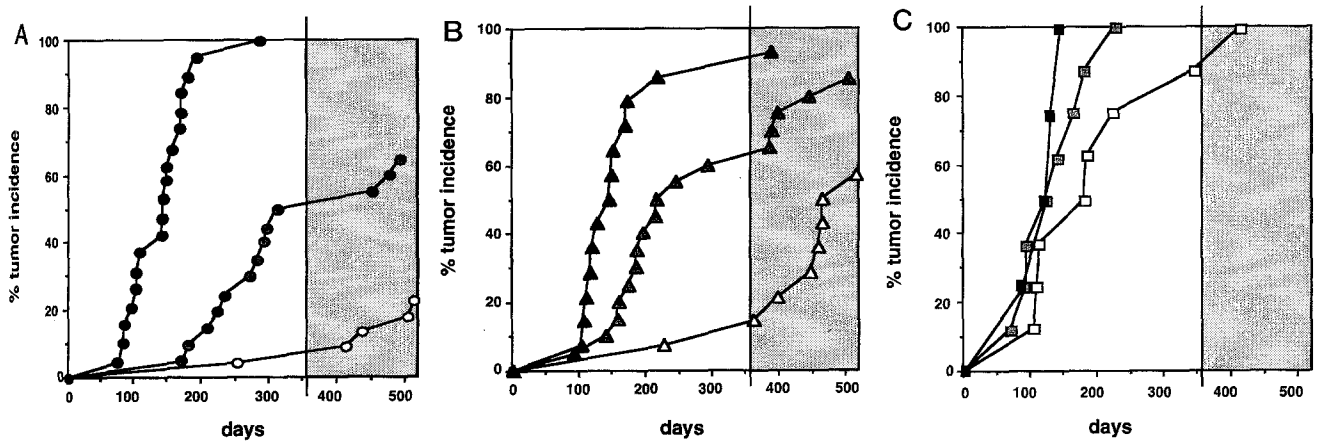

FIG. 1. Tumor incidences from uninfected (white symbols), Akv-infected (grey symbols), and MoMuLV-infected (black symbols) wild-type and myc-κ transgenic NMRI mice. Shaded area: observation time 360–520 days, the period for spontaneously occurring tumors in noninfected wild-type and myc-κ transgenic mice. (A) Cumulative tumor incidence of uninfected (n = 22), Akv-infected (n = 20), and MoMuLV-infected (n = 19) wild-type mice. (B) Cumulative tumor incidence of uninfected (n = 14), Akv-infected (n = 20), and MoMuLV-infected (n = 14) myc-κ transgenic mice of line 614. (C) Cumulative tumor incidence of uninfected (n = 8), Akv-infected (n = 8), and MoMuLV-infected (n = 4) myc-κ transgenic mice of line 615.

line 615, which harbors a high copy number of the myc-κ transgene (Fig. 1C). The only tumor in wild-type mice was detected at Day 255 (Table 1); earlier independent experiments showed tumor development in 3 of 99 wild-type NMRI mice after a mean latency period of 330 ± 21 days (Müller *et al.*, 1988). The mean latency period for line 614 was 293 ± 95 days and 180 ± 86 days for line 615. This difference is highly significant ($P < 0.0001$).

After infection of newborn mice with Akv, the tumor incidence increased in all three mouse lines. Tumors developed in 50% infected wild-type NMRI mice with a significantly ($P < 0.0001$) shorter mean latency period of 249 ± 49 days compared to uninfected controls. In transgenic mouse line 614 lymphomas developed in 60% infected mice with a mean latency period of 187 ± 51 days which was significantly ($P < 0.0001$) shorter than in uninfected animals. In the transgenic high-copy mouse line 615 lymphomas developed in 100% Akv-infected mice with a mean latency period of 137 ± 52 days. The difference in latency period compared to uninfected mice of the 615 line was not significant.

Pathogenicity of Akv was compared with that of MoMuLV. Wild-type NMRI mice and mice of the two transgenic lines were infected with MoMuLV as newborns. The tumor incidence within 360 days after infection was 100% for wild-type mice, 92% for mice of line 614, and 100% for mice of line 615. The mean latency periods for these tumors were 144 ± 49 days in the wild-type NMRI mice ($P < 0.0001$ compared to Akv-infected controls), 139 ± 34 days in line 614 ($P < 0.01$ compared to Akv-infected 614-line mice), and 117 ± 23 days in line 615 (not significant compared to Akv-infected 615-line mice). The mean tumor latency period in MoMuLV-infected line-615 mice was 22 days shorter than in line 614 mice, but the difference was not significant.

In summary, Akv infection enhanced the tumor incidence and shortened the tumor latency period both in wild-type NMRI mice and, more particularly, in mice of the myc-κ transgenic lines. MoMuLV enhanced lympho-

TABLE 1

TUMOR INCIDENCES AND MEAN LATENCY PERIODS OF TUMOR DEVELOPMENT OF WILD-TYPE AND myc-κ TRANSGENIC NMRI MOUSE LINES

	NMRI mouse line					
Virus infection	Wild-type		Myc-κ 614		Myc-κ 615	
Control	1/22 (5%)[a]	—[b,c]	2/14 (14%)	293 ± 95 days	7/8 (88%)	180 ± 86 days
AKV-infected	10/20 (50%)	249 ± 49 days	12/20 (60%)	187 ± 51 days	8/8 (100%)	137 ± 52 days
MoMuLV-infected	19/19 (100%)	144 ± 49 days	12/13 (92%)	139 ± 34 days	4/4 (100%)	117 ± 23 days

[a] Tumor incidences in number of tumor bearing animals/total number of animals. The percentage is given in parentheses. Each mouse carried only one tumor.

[b] The mean latency periods of the tumors. The mean value and standard deviation were determined from the age of the mice when the tumor was detected.

[c] Not to be determined in this experiment. A single tumor was observed after 255 days.

TABLE 2

TUMOR TYPES IN NONINFECTED, AKV-INFECTED, AND MOMULV-INFECTED WILD-TYPE AND myc-κ TRANSGENIC NMRI MOUSE LINES[a]

Virus infection	NMRI mouse line	Tumor type			
		PreB-cell	B-cell	T-cell	Other[b]
Control	wt (1)[c]	1/1 (100%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
	myc-κ 614 (2)	0/1 (0%)	1/1 (100%)	0/1 (0%)	0/1 (0%)
	myc-κ 615 (7)	0/7 (0%)	6/7 (86%)	1/7 (14%)	0/7 (0%)
Akv-infected	wt (10)	2/8 (25%)	5/8 (63%)	0/8 (0%)	1/8 (13%)
	myc-κ 614 (12)	1/10 (10%)	5/10 (50%)	2/10 (20%)	2/10 (20%)
	myc-κ 615 (8)	0/8 (0%)	7/8 (88%)	0/8 (0%)	1/8 (12%)
MoMuLV-infected	wt (19)	0/10 (0%)	1/10 (10%)	6/10 (60%)	3/10 (30%)
	myc-κ 614 (12)	1/11 (9%)	1/11 (9%)	7/11 (64%)	2/11 (18%)
	myc-κ 615 (4)	0/2 (0%)	1/2 (50%)	1/2 (50%)	0/2 (0%)

[a] Percentage of tumors of the preB-cell, B-cell, and T-cell type, which developed within the observation period of 360 days. The tumor type was determined by the presence of μ-chain, κ-chain, and TCRβ-chain mRNA.

[b] Tumors expressing both or none of the B-cell and T-cell markers.

[c] The total number of tumors which developed within 360 days is given in parentheses; 58 of 75 tumors have been characterized.

magenesis more than Akv, independent of the genetic background.

Expression of B- and T-cell differentiation markers

Lymphoid tumors which developed within 360 days were identified as preB-cell, B-cell, or T-cell tumors by determining the expression of IgM heavy chain (μ-chain) mRNA, kappa light chain (κ-chain) mRNA, and T-cell receptor β-chain (TCRβ) mRNA.

The single lymphoma in wild-type mice expressed the μ-chain, but not the κ-chain or the TCRβ (Table 2) and was classified as preB-cell lymphoma. The majority of the lymphoid tumors in noninfected transgenic mice expressed both μ-chain and κ-chain mRNA, but not TCRβ-chain mRNA. They were classified as mature B-cell tumors and included the one tumor from transgenic mouse line 614 (100%) and six of the seven (86%) tumors from transgenic mouse line 615.

In Akv-infected mice B-cell tumors were the predominant tumor type. The expression pattern of mature B-cell tumors was found in 5 of 8 (63%) tumors in Akv-infected wild-type mice, in 5/10 (50%) tumors in Akv-infected myc-κ transgenic mice of line 614, and in 7/8 (88%) of Akv-infected myc-κ transgenic mice of line 615. Only a few preB-cell and T-cell tumors were found in the Akv-infected mice (Table 2).

In MoMuLV-infected mice the majority of the tumors was classified as T-cell tumors. These tumors expressed the TCRβ mRNA, but neither μ-chain nor κ-chain mRNA. In MoMuLV-infected mice, T-cell tumors were found in 6/10 (60%) wild-type mice, in 7/11 (64%) line-614 mice, and in 1/2 line-615 mice.

In summary, noninfected wild-type NMRI and myc-κ transgenic mice predominantly develop tumors of the mature B cell type and Akv infection further enhanced the development of this type of tumor. MoMuLV induced mainly T-cell tumors in these mice, irrespective of the myc transgenic background.

Expression of Akv and MoMuLV proviruses

Akv-infected mice showed high levels of viral expression in 33/39 (85%) of the tumors (Fig. 2). In contrast, in MoMuLV-infected mice, expression of the provirus was detected in only 1 tumor of 26 (4%) in Northern blot analysis.

The steady state levels of viral mRNA did not correlate with the number of integrated proviruses found in the tumor DNA (Fig. 3). For example, tumor 177, which contained four Akv proviruses, showed abundant expression, whereas expression of viral transcripts in tumor 174, which contained seven Akv proviruses, was relatively low. Similarly, the one MoMuLV provirus in tumor

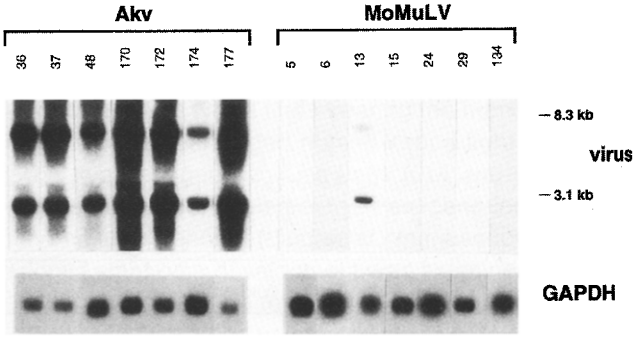

FIG. 2. Provirus expression in tumors of Akv- and MoMuLV-infected mice. Northern blots were hybridized with probes for mouse ecotropic retroviruses (Chattopadhyay *et al.*, 1980) and GAPDH. Akv and MoMuLV show both the viral genomic 8.3-kb and the spliced 3.0-kb transcripts. The numbers indicate different tumors. Tumors 4, 26, and 31 were classified as T-cell tumors, 13, 19, 54, 56, 61, 172, 174, 175, and 177 as B-cell tumors, and tumor 170 as preB-cell tumor. Tumors 30 and 134 showed neither B-cell nor T-cell markers.

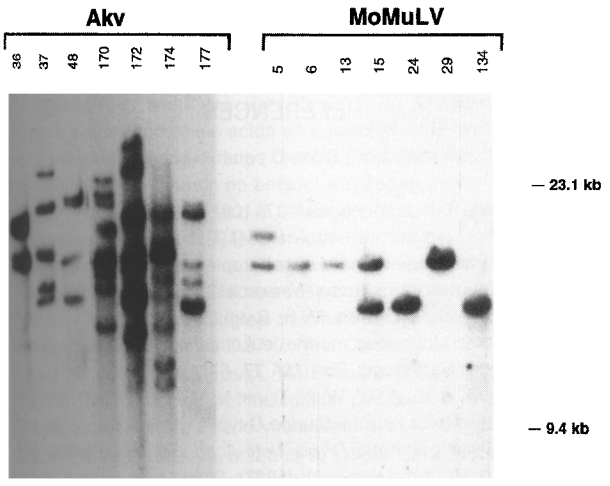

FIG. 3. Proviral integration pattern of Akv and MoMuLV. Tumor DNA from different Akv- or MoMuLV-infected mice was digested with *Eco*RI. The Southern blot was hybridized with a probe specific for mouse ecotropic retroviruses (Chattopadhyay *et al.*, 1980). The numbers indicate independent tumors as described in the legend to Fig. 2.

13 was transcribed, but no transcripts were found from the two MoMuLV proviruses in tumor 2. No correlation was observed between virus expression and the tumor type or a particular mouse line.

Proviruses in the tumor genomes and rearrangement of cellular genes

The integration patterns of Akv and MoMuLV proviruses in the DNA of the tumors were examined by Southern blot analysis (Fig. 3).

The tumors of Akv-infected mice showed 2–10 proviruses (average 5) integrated in the genome. In contrast, tumors of MoMuLV-infected mice contained 1 or 2 proviruses. DNA from 5 tumors out of 7 showed MoMuLV proviruses on a 16-kb *Eco*RI restriction fragment, 3/7 tumors on a 13-kb *Eco*RI restriction fragment when hybridized with a probe for ecotropic MuLV. One tumor had acquired proviruses on *Eco*RI restriction fragments of both sizes. Digestion of the same tumor DNAs with *Asn*I showed the same distribution of restriction fragments, indicating that these fragments represent two common integration sites of the MoMuLV provirus in myc-κ transgenic mice.

We tested several cellular genes which are known to be target genes for insertional mutagenesis by retroviruses or have been reported to be rearranged in tumors of Akv- and MoMuLV-infected mouse lines. These included pim-1 and bmi-1, common integration sites in tumors of the B-cell lineage (Selten *et al.*, 1986; Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991), c-myc, N-myc, Mlvi-1, and Mlvi-2 (Tsichlis *et al.*, 1983; Corcoran *et al.*, 1984; Li *et al.*, 1984; O'Donnell *et al.*, 1985; Tsichlis *et al.*, 1985; van Lohuizen *et al.*, 1989) as preferred integration sites for proviruses in T cell tumors, and p53, which has been found to be a target for insertion mutagenesis in retrovirus-induced leukemia (Wolf and Rotter, 1984; Mowat *et al.*, 1985; Hicks and Mowat, 1988). None of these loci were rearranged (data not shown), indicating that they were not targets for Akv or MoMuLV provirus integration in the tumors of myc-κ transgenic mice.

DISCUSSION

Akv, the endogenous ecotropic virus of the AKR mouse is commonly referred to as nonlymphomagenic (Risser and Horowitz, 1983). The results from the experiments presented here, however, show that Akv harbors significant lymphomagenic potential. In NMRI mice, Akv enhances the tumor incidence and shortens the latency period of lymphoma development.

Two factors may explain the discrepancy between our experiments and earlier reports: the extended observation period and a higher sensitivity of the NMRI mouse strain. In earlier reports Akv-infected mice of mouse strains AKR, CBA, and C3H were observed for a maximum of 230 days (Pedersen *et al.*, 1981; Lenz *et al.*, 1982; Lenz and Haseltine, 1983). The observation period of our experiment was 360 days and 50% of the infected wild-type NMRI mice developed lymphomas during this time. However, 20% of the Akv-infected NMRI mice had already developed lymphomas within 230 days, indicating that the NMRI mouse strain may also be more sensitive than the mouse strains previously tested.

The long latency period suggests that Akv alone may not be sufficient to induce lymphoma, but may rather enhance lymphoma development in a mouse which is already predisposed to lymphomagenesis by other genetic or epigenetic alterations. To investigate this possible role of Akv in tumorigenesis further, we infected myc-κ and c-fos transgenic (Schmidt *et al.*, 1995) mice, which are prone to develop tumors as a result of the transgenes' activity. The myc-κ transgenic mice used in this study carry a human myc-κ fusion gene which mimicks the rearrangement in Burkitt's lymphomas. Akv infection increased tumor incidence and reduced the mean tumor latency period by some 100 days in line 614. Akv did not significantly accelerate lymphoma development in the 615 line, most probably because of the already very efficient induction of tumors by the high copy number of the myc-κ transgene.

We observed a similar biological effect for Akv in the development of osteogenic tumors in hMt-c-fos-LTR transgenic mice (Schmidt *et al.*, 1995), suggesting that the biological effect of Akv is not restricted to lymphomagenesis.

The spontaneously arising lymphomas in noninfected NMRI wild-type mice were of mature B-cell type. The same tumor type was found in Akv-infected NMRI wild-type mice as well as in uninfected and Akv-infected myc-κ transgenic mice. In contrast, MoMuLV induced pre-

dominantly T-cell lymphomas in both infected wild-type and myc-κ transgenic mice, indicating that the pathogenic effect of MoMuLV determines tumors formation independent of the presence of a dominant acting transoncogene.

Akv and MoMuLV differed in the pattern of provirus integration. Akv-induced tumors showed several proviruses integrated into the cellular genome whereas only one to two proviruses were found in MoMuLV-induced tumors. As the integration pattern of MoMuLV on Southern blot analysis indicates two common integration sites, we tested several loci which have been identified to be preferential targets for MoMuLV integration. We found no integration of MoMuLV in genes known to be preferential targets of this virus in Eμ-myc transgenic mice developing preB lymphoma or in mice with T lymphoma.

The tumors from Akv-infected mice showed 2–10 proviruses integrated into the cellular genome. It cannot be excluded that a family of genes could have been targeted by proviral integration; however, the complex integration pattern found in these tumors could indicate a pathogenic mechanism different from insertion mutagenesis. For example, the expression of a retroviral protein may contribute directly or indirectly to cellular transformation. Strong viral RNA expression was indeed found in most tumors from Akv-infected mice. On the other hand, RNA-expression of MoMuLV was not detected in most tumors of MoMuLV-infected mice in the experiments described here.

An explanation for the different transcriptional activity in Akv- and MoMuLV-infected lymphoma cells may be found in the repressor binding site (RBS) in the LTR of both viruses. In embryonal carcinoma cells the RBS mediates the repression of MoMuLV transcription 48 hr after infection (Kempler *et al.,* 1993). It has been shown by mutation analysis that the last 3 bp of the 18-bp sequence of the RBS are necessary for the silencing effect (Kempler *et al.,* 1993). The RBS sequence is well conserved between MoMuLV and Akv, but differs in the last 2 bp in the Akv LTR. The active RBS in MoMuLV could explain why the MoMuLV expression is repressed in lymphomas, whereas the expression of Akv, containing the altered RBS, is not.

The results from the infection of both types of transgenic mice with Akv and MoMuLV suggest that Akv facilitates the development of the tumors to which the host cells are predisposed depending on their genetic background. The underlaying mechanism of Akv pathogenicity is not yet clear. Although insertion mutagenesis by Akv cannot be ruled out, the pathogenic mechanism appears to be distinct from that of MoMuLV.

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