Carcinogen-induced Lymphomagenesis in *pim-1* Transgenic Mice: Dose Dependence and Involvement of *myc* and *ras*  

Marco Breuer, Ellen Wientjens, Sjef Verbeek, Robert Slebos, and Anton Berns

**ABSTRACT**

Transgenic mice overexpressing the *pim-1* oncogene in their lymphoid compartments are predisposed to T-cell lymphomagenesis but only to the extent that approximately 10% of the transgenic mice develop lymphomas within 34 weeks after birth. Recently, we have shown that lymphomagenesis in *pim-1* transgenic mice can be accelerated by infecting *pim-1* transgenic mice with murine leukemia viruses or by treating the mice with a relatively low dose of 60 mg of the carcinogen N-ethyl-N-nitrosourea (ENU) per kg of body weight. Here we describe the incidence of tumors as a function of the dose of ENU. Either 200, 15, 4, 1, or 0.1 mg/kg ENU was injected into transgenic and control mice and the tumor incidence was monitored. T-cell lymphomas developed in 100 and 70% of the *pim-1* transgenic mice treated with 200 and 15 mg/kg ENU, respectively. Approximately 20% of the *Eß-pim-1* transgenic mice developed lymphomas after treatment with either 4, 1, or 0.1 mg/kg ENU. The nontransgenic mice developed lymphomas only after injection with 200 mg/kg (45%). The data show that *Eß-pim-1* transgenic mice are approximately 25-fold more susceptible to ENU-induced lymphomagenesis than control mice.

In most tumors the expression of *c-myc* was strongly elevated, probably as a direct or indirect effect of ENU. Analysis of the lymphomas for ras mutations revealed that ~10% of the lymphomas bear a ras mutation. The data indicate that at least some of these mutations are not the direct result of alkylation by ENU but rather represent spontaneous mutations that occurred later in the tumorigenic process.

**INTRODUCTION**

The *pim-1* gene has been implicated in T-cell lymphomagenesis because of its frequent activation by proviral insertion in T-cell lymphomas induced by MuLV (1-4). The oncogenic capacity of *pim-1* became evident from the observation that approximately 10% of *Eß-pim-1* transgenic mice spontaneously develop T-cell lymphomas within 34 weeks (5). Predisposition of *Eß-pim-1* transgenic mice to lymphomagenesis became even more apparent upon infection of *pim-1* transgenic mice with MuLV. MuLV induced T-cell lymphomas in *Eß-pim-1* transgenic mice after a mean latency period of only 7-8 weeks, whereas tumor induction in nontransgenic mice required an average of 22 weeks (5). In almost all of the MuLV-induced lymphomas in *Eß-pim-1* transgenic mice either *c-myc* or *N-myc* is activated by proviral insertion, suggesting a synergistic interaction between *pim-1* and *myc* in lymphomagenesis (5, 6). Recently, we determined whether this system has a more general applicability by injecting *Eß-pim-1* transgenic mice with the alkylation agent ENU. All *Eß-pim-1* transgenic mice given a single i.p. dose of 60 mg/kg of body weight developed T-cell lymphomas, whereas only 20% of control mice did (7). This indicates that *Eß-pim-1* transgenic mice are tumor prone and that both retroviral infection and carcinogen treatment can accelerate lymphomagenesis. All ENU-induced lymphomas showed high levels of *c-myc* mRNA, supporting the notion that *pim-1* and *c-myc* cooperate in lymphomagenesis. Furthermore, ras mutations were found in 50% of the lymphomas induced in nontransgenic mice, whereas only 10% of the ENU-induced lymphomas of the *pim-1* transgenic mice carried mutations in ras. To study the dose response with respect to the increased susceptibility of *Eß-pim-1* transgenic mice for ENU-induced lymphomagenesis and to gain further insight into the synergism of the *pim-1* transgene with the *c-myc* and *ras* oncogenes, *Eß-pim-1* transgenic mice were treated with six different doses of ENU. Here we report that *Eß-pim-1* transgenic mice are approximately 25 times more susceptible to ENU-induced lymphomagenesis than nontransgenic mice. Furthermore, we provide evidence that not all mutations detected in the ras genes are directly induced by ENU but rather represent mutations occurring later in the tumorigenic process.

**MATERIALS AND METHODS**

DNA and RNA Analysis. For Southern blot analysis 10 µg of lymphoma DNA was digested with restriction endonucleases, separated on 0.6% agarose gels, and transferred to Nytran. Filters were hybridized to 32P-labeled probes and washed as described previously (1) except that 1% sodium dodecyl sulfate was added to the hybridization solutions. Final washing was with 0.1 x standard saline citrate (0.15 M sodium chloride and 15 mM sodium citrate, pH 7) at 42°C.

For Northern blot analysis 25 µg of total RNA, prepared by the LiCl-urea method, was separated on 1% agarose-formaldehyde gels (8) and transferred to Nytran. Filters were hybridized and washed as described above. The final washing was done with 0.1 x SSC at 60°C; for the MuLV hybridization the last washing was at 42°C.

Probes used for DNA or RNA analysis were (a) U3LTR probe (1), which detects the *pim-1* transgenic transcripts because of the presence of an LTR in the 3' part of the *pim-1* transgene; (b) *pim-1*, probe A (1); (c) 3' *pim-1*, probe, inserted in M13, extends from genomic map coordinate 6619 (HindIII) to 6939 (BglII) (3) and specifically detects the endogenous *pim-1* transcripts; (d) *c-myc* (1); (e) *N-myc* (5); (f) actin (9); and (g) a complete MuLV proviral clone was used as a probe for MuLV (10).

**Transgenic Mice and Lymphoma Induction.** The generation of the *Eß-pim-1* transgenic mice has been described (5). Heterozygous *Eß-pim-1* transgenic mice and C57Bl/Lia mice were crossed. At day 15 after birth, offspring from crosses between *Eß-pim-1* and C57Bl/Lia were given 200, 15, 4, 1, or 0.1 mg ENU/kg of body weight by i.p. injection. ENU was freshly dissolved in phosphate-buffered saline acidified with acetic acid to pH 6. Mice were examined every other day for lymphoma development and killed when moribund; the lymphomas were then collected for analysis.

Detection of ras Mutations. Mutations in codons 12, 13, and 61 of *K*- and *N-ras* and codons 12 and 61 of *H-ras* were detected by selective oligonucleotide hybridization on *in vitro* amplified DNA sequences (11-13). A 50-µl amplification reaction contained 250 ng of genomic DNA and 100 ng of total RNA from each lymphoma.

References
DNA, 50 pmol of each of the oligonucleotide primers listed below, 200 μM of each of the four nucleotides, and 1 unit of Taq polymerase (Cetus Corp.) in a buffer as recommended by the manufacturer. DNA fragments for codons 12 and 13 and for codon 61 of N- or K-ras were amplified in a single polymerase chain reaction. The following primers were used: K-ras codons 12 and 13, GGCGCTGGGAAAAATGACTGA and TGGTCTGAATAGCTGTAT; K-ras codon 61, CACAAAGAACCCCTCCCA and GGAGAAACCTGGTCTCCGG; N-ras codons 12 and 13, CTCTATGGGAGCATATT and GACTGAGTACAAACCTGGT; N-ras codon 61, CTCTATGGGGTCCTGCTGTA and GGTGAGACCTGCCTGGA; H-ras codon 12, GGAGGCTACTCGTCCACCA and GACAGAATACACGCTGTG; H-ras codon 61, CGCATGTAATCTGCGCCCAT and CTCTACCCGGAAAAACGTTGG. Approximately 10 ng of amplified DNA was dotted onto Nytran membranes and hybridized with tetramethyl ammonium chloride. Filters were hybridized to either the wild-type ras sequence or a mixture of oligonucleotides containing all possible activating mutations at positions 12, 13, or 61. These probes were identical to those described for the detection of ras mutations in human DNA (13), except for codon 61 of N-ras (TACTCCCTCCTCGT), codon 12 of H-ras (TGGGCGCTGGAGGCTGTA) and codon 61 of H-ras (ACAGCAGTCAAGAAGACTTATCC). Further support for a synergistic interaction came from studies in which lymphomagenesis in Eμ-pim-1 mice was accelerated by MuLV infection (5). In almost all tumors in Eμ-pim-1 mice either c-myc or N-myc was found to be provirally activated. When tumors were induced by ENU, strongly enhanced c-myc expression, but no N-myc expression, was found in nearly all tumors of both Eμ-pim-1 and control mice (Fig. 2, panels 3 and 4). High levels of c-myc, comparable to those found in tumors with a provirally activated c-myc gene, were found in the vast majority of the mice treated with 200 mg/kg ENU. Lower, but still significantly elevated, levels were found in lymphomas of mice 1, 8, 29, and 34. In the lymphomas of mice treated with 15, 4, 1, and 0.1 mg/kg ENU the c-myc mRNA levels were more variable, although even the level in the lowest expressing tumor was still significantly higher than that in a spontaneous lymphoma of a pim-1 transgenic mouse with a c-myc expression comparable to that of normal spleen (Fig. 1, panel 4; compare lanes a, b, c, and d with the other lanes). We checked whether the high levels of c-myc mRNA were associated with either amplification or rearrangements of the c-myc gene (data not shown).

c-myc Is Highly Expressed in ENU-induced Lymphomas. We have shown that, in MuLV-induced T-cell lymphomas in nontransgenic mice, proviral activation of pim-1 and c-myc or N-myc are the most predominant events. Occasionally, proviral activation of pim-1 and c-myc was found within the same cell (15-17), suggesting that c-myc and pim-1 synergize or that activation of one of these genes increases the statistical feasibility of activation of the other by increasing the number of target cells. Further support for a synergistic interaction came from studies in which lymphomagenesis in Eμ-pim-1 mice was accelerated by MuLV infection (5). In almost all tumors in Eμ-pim-1 mice either c-myc or N-myc was found to be provirally activated. When tumors were induced by ENU, strongly enhanced c-myc expression, but no N-myc expression, was found in nearly all tumors of both Eμ-pim-1 and control mice (Fig. 2, panels 3 and 4). High levels of c-myc, comparable to those found in tumors with a provirally activated c-myc gene, were found in the vast majority of the mice treated with 200 mg/kg ENU. Lower, but still significantly elevated, levels were found in lymphomas of mice 1, 8, 29, and 34. In the lymphomas of mice treated with 15, 4, 1, and 0.1 mg/kg ENU the c-myc mRNA levels were more variable, although even the level in the lowest expressing tumor was still significantly higher than that in a spontaneous lymphoma of a pim-1 transgenic mouse with a c-myc expression comparable to that of normal spleen (Fig. 2, panel 4; compare lanes a, b, c, and d with the other lanes). We checked whether the high levels of c-myc mRNA were associated with either amplification or rearrangements of the c-myc gene. Southern blot analysis of Kpnl- or £coRV-digested tumor DNA revealed no gross rearrangements or amplifications in the c-myc gene. When tumors were induced by ENU, strongly enhanced c-myc expression, but no N-myc expression, was found in nearly all tumors of both Eμ-pim-1 and control mice (Fig. 2, panels 3 and 4). High levels of c-myc, comparable to those found in tumors with a provirally activated c-myc gene, were found in the vast majority of the mice treated with 200 mg/kg ENU. Lower, but still significantly elevated, levels were found in lymphomas of mice 1, 8, 29, and 34. In the lymphomas of mice treated with 15, 4, 1, and 0.1 mg/kg ENU the c-myc mRNA levels were more variable, although even the level in the lowest expressing tumor was still significantly higher than that in a spontaneous lymphoma of a pim-1 transgenic mouse with a c-myc expression comparable to that of normal spleen (Fig. 2, panel 4; compare lanes a, b, c, and d with the other lanes). We checked whether the high levels of c-myc mRNA were associated with either amplification or rearrangements of the c-myc gene. Southern blot analysis of Kpnl- or EcoRV-digested lymphoma DNA revealed no gross rearrangements or amplifications of the c-myc gene (data not shown).

pim-1 Expression. High expression of the pim-1 transgene is found in all lymphomas of Eμ-pim-1 transgenic mice except mice 8, 26 (200 mg/kg ENU), 7, and 19 (15 mg/kg), which expressed the transgene at lower levels (Fig. 2, panel 1). Remarkably, there was a highly variable expression of the endogenous pim-1 gene in the ENU-induced lymphomas of both the Eμ-pim-1 and nontransgenic mice (Fig. 2, panel 2).
Fig. 2. Expression of c-myc, N-myc, pim-1, and endogenous viruses in ENU-induced lymphomas. Lanes A–E, positive and negative controls; lanes A–C, RNA from spontaneous lymphomas in E\(^{\beta}-\)pim-1 transgenic mice [lane A, with a high c-myc mRNA level comparable to the levels found in lymphomas with a provirally activated c-myc gene, lane B, with a c-myc mRNA level comparable to that of a normal spleen (data not shown), and lane C, with intermediate c-myc messenger RNA levels compared to those of normal spleen]; lane D, RNA from a MuLV-induced lymphoma of a nontransgenic mouse. In this lymphoma c-myc is highly overexpressed due to a proviral integration near c-myc; lane E, RNA isolated from a MuLV-induced lymphoma of an E\(^{\beta}-\)pim-1 mouse. This lymphoma bears a proviral integration in the 3'-untranslated region of the N-myc gene, resulting in high expression of a slightly shorter transcript (6). Panel A, RNA from lymphomas induced with 200 mg ENU/kg of body weight in E\(^{\beta}-\)pim-1 transgenic mice (lanes headed with a T) or in nontransgenic littermates (lanes denoted with a horizontal bar). Panel B, RNA from lymphomas of E\(^{\beta}-\)pim-1 transgenic mice treated with 15, 4, 1, or 0.1 mg ENU/kg of body weight. Tumor RNA (25 \(\mu\)g/slot) was separated on 1% agarose gels and transferred to Nytran. The same Northern blots were sequentially hybridized with the transgene-specific U3LTR probe, a 3'-pim-1 probe specific for the endogenous pim-1 transcript, c-myc, N-myc, MuLV, and actin as a control for the amount of RNA loaded. Left ordinate, ribosomal markers.
Endogenous Retrovirus Activation. Endogenous retroviruses can be induced by carcinogen treatment resulting in a viremia that in turn can activate protooncogenes by proviral insertion. To determine whether endogenous retrovirus replication had been induced in ENU-treated mice, we hybridized Northern blots of lymphoma RNAs with an MuLV probe representative for the complete MuLV genome. This probe also hybridizes with the transgene-encoded 2.8-kilobase pim-1 mRNA which contains long terminal repeat sequences at its 3' end. Additional hybridizing viral RNAs could be detected in lymphomas of mice 25 (200 mg/kg ENU), 8 (15 mg/kg ENU), and 3 (4 mg/kg ENU). However, the expression level was extremely low (Fig. 2, compare lanes D and E of MoMuLV-induced lymphoma RNAs with the others). Southern blot analysis revealed that none of the ENU-induced lymphomas had a proviral insertion near either the c-myc gene or the N-myc gene (data not shown), whereas this is found in all virus-induced lymphomas in Eμ-pim-1 transgenic mice (6). Therefore, activation of endogenous ecotropic or amphotropic viruses apparently does not play an important role in ENU-induced lymphomagenesis in mice of this genetic background, which is in agreement with experiments performed by Mayer and Dorsch-Hasler (19) and Chinsky et al. (20).

Mutations in K-, N-, and H-ras. Previous reports have shown the involvement of ras-mutations in both MNU- and ENU-induced lymphomagenesis (7, 21, 22). Screening of the ENU-induced lymphomas for the presence of point mutations in codons 12, 13, and 61 of K- and N-ras and codons 12 and 61 of H-ras (including those found in an earlier experiment in which 60 mg/kg ENU was used (7)) revealed 16 lymphomas with point mutations in 170 tumors analyzed, 9 in codon 12 of K-ras, 2 in codon 61 of K-ras, and 3 in codon 61 of N-ras (Table 1). In tumors of Eμ-pim-1 transgenic mice 5 mutations were found in codon 12 of K-ras, 2 in codon 61 of K-ras, and 3 in codon 61 of N-ras. In 25 tumors of control mice 4 mutations were found in codon 12 of K-ras and 2 in codon 61 of K-ras. Remarkably, all the mutations in nontransgenic mice were found in the group treated with a dose of 60 mg/kg ENU, whereas none were found in the group treated with 200 mg/kg (Table 1). In contrast, the frequency of ras mutations in lymphomas induced in Eμ-pim-1 transgenic mice was similar at the different doses of ENU. Five of 16 lymphomas with a ras mutation lacked the normal ras allele (Table 1). In some of the tumors with a ras mutation, the intensity of hybridization of the mutant ras allele was much lower than that of the germ line allele, suggesting that these lymphomas were subclonal for the mutation, although the same tumors were clonal with respect to T-cell receptor rearrangements. To determine whether the mutations in ras occur later in tumorigenesis, we tested tumors from different anatomical sites (spleen, thymus, mesenteric lymph nodes, peripheral lymph nodes) for the presence of ras mutations and T-cell receptor rearrangements. In 10 of 12 mice, tumors from different sites showed the same specific ras mutation and T-cell receptor rearrangements. However, two exceptions were noted. In one case, 3 of the 4 affected tissues consisted of at least 2 subclones, as determined by T-cell receptor rearrangement, of which one carried a specific ras mutation. This mutation was not detected in the other affected tissue, which was clonal with respect to T-cell receptor rearrangements. In the other case, the different tumor tissues carried the same T-cell receptor rearrangement but in only one tumor tissue was a ras mutation found.

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<th>Table 1 ras mutations in ENU-induced tumors of Eμ-pim-1 transgenic and control mice</th>
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<td>The mutations observed are in the first position of K-ras codon 12 (K12P1), position 2 of K-ras codon 12 (K12P2), position 2 of K-ras codon 61 (K61P2), position 3 of K-ras codon 61 (K61P3), position 1 of N-ras codon 61 (N61P1), and position 2 of N-ras codon 61.</td>
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<td><strong>ENU dose (mg/kg)</strong></td>
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<td><strong>Tumors</strong></td>
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<td><strong>Additional hybridizing viral RNAs could be detected in lymphomas of mice 25 (200 mg/kg ENU), 8 (15 mg/kg ENU), and 3 (4 mg/kg ENU).</strong></td>
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**DISCUSSION**

Twenty-five-fold Enhanced Susceptibility of Eμ-pim-1 Transgenic Mice for ENU. Previously, we have demonstrated that Eμ-pim-1 transgenic mice are predisposed to both MuLV- and ENU-induced lymphomagenesis (5, 7). Here we have analyzed in more detail the susceptibility of pim-1 transgenic mice for ENU-induced lymphomas (Fig. 1A). The percentage of lymphomas induced by 4, 1, and 0.1 mg/kg ENU in Eμ-pim-1 transgenic mice is essentially the same, reaching nearly 20% at 34 weeks (Table 2). This probably represents the basal level of lymphoma incidence in Eμ-pim-1 transgenic mice, which is somewhat higher than the 10% incidence we observed previously. The discrepancy is probably due to the low number of mice analyzed in the experiments presented here.

The kinetics of the dose response could be described in the Weibull formula:

\[-\ln(1 - P250) = (kd)^x\]

where \(P250\) is the lymphoma incidence up to 250 days, \(k\) is a constant characteristic for the carcinogen, \(d\) is the dose, \(n\) is the number of critical events ("hits") (14, 23). The theoretical \(P250\) can be extracted from graphs where the \(-\ln(1 - P)\) versus the latency is plotted. Fig. 1B shows the dose-response curves of ENU in pim-1 transgenic and control mice. The slopes represent the number of critical hits. For the Eμ-pim-1 transgenic
mice 0.8, and for the control mice 1.4, hits are required for tumor induction, indicating that En-pim-1 transgenic mice demand fewer hits than control mice. To get an impression of the susceptibility of the En-pim-1 we estimated the dose of ENU that is required to induce lymphomas in 50% of the treated nontransgenic mice. To compensate for the spontaneous background of the transgenic mice the dose at which 70% of the mice developed lymphomas was estimated. Comparison of these data revealed that pim-1 transgenic mice are approximately 25-fold more susceptible to ENU-induced lymphomagenesis than nontransgenic littermates.

c-myc and pim-1 Expression. Most lymphomas induced by 200 and 60 mg/kg ENU show high levels of c-myc mRNA. The levels are comparable to those seen in tumors in which c-myc is activated by proviral integration. The c-myc mRNA levels of tumors occurring in mice treated with 15, 4, 1, or 0.1 mg/kg ENU are more variable. A possible explanation is that in these cases a significant proportion of lymphomas is not induced by ENU but the result of "spontaneous" lymphomagenesis in the pim-1 transgenic mice. As reported previously, lymphomas induced with ENU are phenotypically indistinguishable from T-cell lymphomas occurring spontaneously in pim-1 transgenic mice or induced by MuLV. In a proportion of the spontaneously occurring tumors in pim-1 transgenic mice no overexpression of c-myc or N-myc was found (e.g., Fig. 2, lane B), indicating that an increased c-myc expression is not an intrinsic property of these cells (5). This is also apparent from the rather variable, although always elevated, expression of c-myc observed in the lymphomas of mice treated with 15, 4, 1, or 0.1 mg/kg ENU (Fig. 2B) and in spontaneous lymphomas of pim-1 transgenic mice (6). Therefore, it is unlikely that the high levels of c-myc mRNA in ENU-induced lymphomas are a reflection of the differentiation state or growth properties of the lymphoma cells. Rather, ENU might cause either directly or indirectly the increased c-myc mRNA levels. We have found no evidence for gross rearrangements or amplifications of c-myc in the present study, although such alterations have been detected in hepatocellular carcinomas induced by MNU (24). (Point) mutations in the c-myc gene itself or in unlinked regulatory genes might have been induced by ENU, leading to the constitutive activation of the c-myc gene.

To our surprise, a highly variable level of endogenous pim-1 expression in lymphomas of both pim-1 transgenic and nontransgenic mice was found. It is unlikely that this was caused by a selective advantage mediated by the overexpression of the endogenous pim-1 allele, because it is hard to envisage how overexpression of the endogenous pim-1 allele could confer a strong selective advantage in the presence of the highly expressed pim-1 transgene. Probably, the enhanced expression of the pim-1 germ line allele is a secondary effect of the (in)activation by ENU of other genes, as has also been found in carcinogen treated cells in vitro (25, 26).

Are ras Mutations Directly Caused by ENU? Mutations in ras have frequently been found in carcinogen-induced lymphomas. In these studies, up to 50% of the lymphomas were found to carry mutations in codons 12 in K-ras and in codons 12, 13, and 61 in N-ras. We detected point mutations in ras in 16 of 170 lymphomas that developed after treatment with ENU in both pim-1 transgenic or control mice (Table 1). We have no explanation for the variability in the frequency of ras mutations at the different concentrations of ENU in nontransgenic mice. However, if we combine the data obtained with tumors induced with 60 and 200 mg/kg ENU (the concentrations at which tumors were induced in control mice), ras mutations are found in 25% of the tumors in nontransgenic mice and in 9% of the tumors in pim-1 transgenic animals. If we calculate the percentage of ras mutations on the basis of the number of animals treated with carcinogen rather than on the number of lymphomas induced, the percentages are 6 and 9% for nontransgenic and pim-1 transgenic mice, respectively.

Interestingly, one of the tumors appeared not to be clonal for the ras mutation, although it was clonal with respect to T-cell receptor rearrangements. Therefore, either a ras mutation occurred after tumor growth had been initiated and, therefore, could not have been induced directly by ENU or, during tumor growth, selection against cells carrying the mutant ras allele caused outgrowth of a subclone which had lost the mutant allele. Although we cannot formally exclude the latter possibility, we consider this unlikely in view of the fact that in other tumors a strong selection was observed for the mutant ras allele, resulting in the concomitant loss of the germ line allele. Probably, a substantial fraction of the mutations in ras occurred later in the tumorigenic process and, consequently, were not caused directly by ENU. This notion is supported by two other observations: (a) by the occurrence of a ras mutation in 1 of 12 spontaneously occurring tumors in En-pim-1 transgenic mice and (b) by the heterogeneity in the spectrum of the ras mutations 5 K12P1, 4 K12P2, 3 K61P2, 1 K61P3, 1 N61P1, and 2 N61P2 (Table 1). This spectrum observed with ENU is at variance with MNU-induced tumors in which preferentially position 2 in codon 12 of either K- or H-ras is found to be mutated (22, 27–29). Although there are differences in the mutational spectrum of MNU and ENU, it is unlikely that this could explain the heterogeneity found in this experiment (30–33).

In conclusion, we estimate that En-pim-1 transgenic mice are 25-fold more sensitive than nontransgenic mice for the chemical carcinogen ENU. This, coupled with the low incidence of spontaneous tumors in the En-pim-1 transgenic mice, further substantiates that these mice might be very suitable for testing the carcinogenic effects of other chemical compounds.

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