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TRANSGENIC MICE, CARRYING AN EXPRESSED ANTI-HIV RIBOZYME IN THEIR GENOME, SHOW NO SIGN OF PHENOTYPIC ALTERATIONS

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Transgenic mice are suitable model animals for testing the *in vivo* functionality of custom-tailored ribozymes. Transgenic experiments can demonstrate whether a ribozyme is able to cleave any RNA transcript of the host animal or not. Most probably, this kind of cleavage activity gives rise to phenotypic alterations in mice. In the present paper we demonstrate that an anti-HIV ribozyme does not cause any detectable phenotypic effect in mice carrying and expressing it. Our transgenic mice developed well and were indistinguishable from their wild type counterparts.

Keywords: Transgenic – mice – ribozyme – HIV – *in vivo* model

INTRODUCTION

Ribozymes are a family of unique RNA molecules that are capable of catalyzing the site-specific cleavage of RNA. Several types of ribozymes exist in nature [2, 3, 8, 17]. The catalytic reactions mediated by a number of ribozymes have been well-characterized *in vitro* [1, 11, 12, 18], demonstrating that ribozymes can be used to cleave or splice targeted RNA molecules [5, 9, 10, 16, 22, 23]. Based on a shared secondary structure and a conserved set of nucleotides, the term "hammerhead" has been given to one group of ribozymes originated from certain plant virusoids and viroids [6, 7, 14]. The simplicity of the hammerhead catalytic domain has made it a popular choice in the design of *trans*-acting ribozymes. Virtually any RNA molecules can be cleaved by smartly designed *trans*-acting ribozymes and thus they have tremendous potential as gene control therapeutics.

In our experiments we used a hammerhead ribozyme targeted to the HIV-1 *gag* transcripts [4, 21]. The functionality of this ribozyme was tested in HeLa CD4+ cells [4, 21]. However, a functional test in a human cell line does not answer the question whether this catalytic molecule is capable to recognize target sequences amongst the RNA molecules of a living animal or not. In the present study we demonstrate that this RNA enzyme does not cause any alterations in the normal development and viability of the transgenic mice carrying and expressing it.

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MATERIALS AND METHODS

Generation of transgenic mice

Transgenic mice were generated by pronuclear microinjection of fertilized eggs as described [13]. C57BL/6J × CBA/J F2 fertilized eggs were used for producing transgenic mice. A plasmid vector, pCePur132 (Fig. 1), was linearized by digestion with NruI restriction endonuclease. After digestion it was phenol and phenol : chloroform (1 : 1) extracted and precipitated with absolute ethanol. The resulted DNA pellet was resuspended in 1–5 μg/ml concentration in injection buffer and filter sterilized to remove particles that can clog the injection capillary.

PCR and RT-PCR reactions

PCR and RT-PCR reactions were performed in a Perkin Elmer GeneAmp PCR System 9600 instrument. A PCR reaction contained 30 mM Tris (pH 8.5), 5 mM β-merkaptoethanol, 0.1 mg/ml gelatin, 0.05% Tween-20, 1 pmol/μl of each primers,

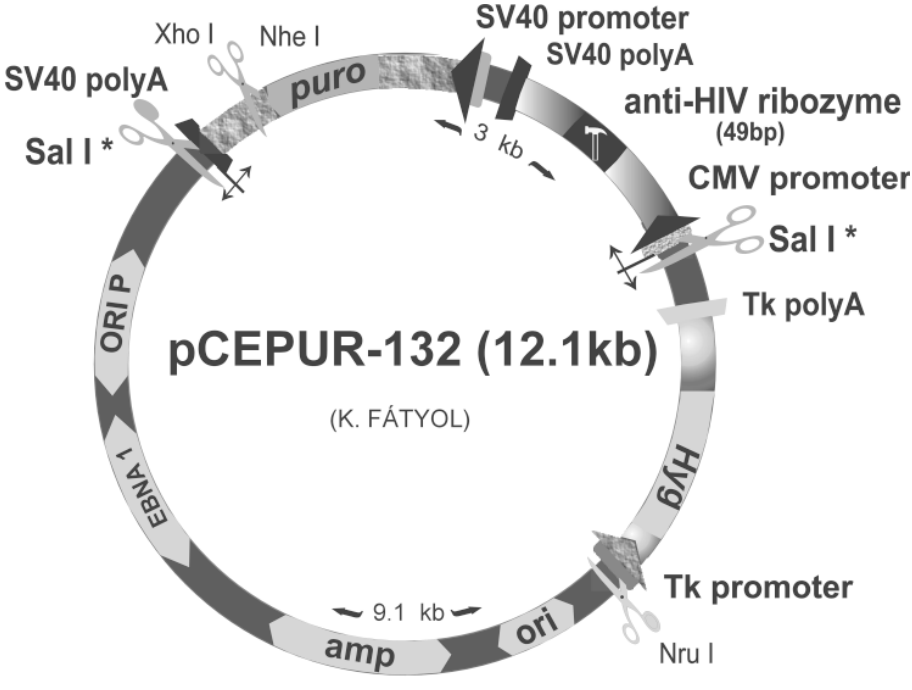

Fig. 1. The pCePur132 plasmid vector was linearized with NruI restriction enzyme. The linear plasmid DNA was dissolved in 1–5 μg/ml concentration and was injected into the male pronuclei of fertilized eggs

2 mM $MgCl_2$, 0.25 mM dNTP mix, 2.5 units of Perkin Elmer AmpliTaq and the required amount of template. The conditions of the reactions were as follows: 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 65 °C for 45 seconds and primer extension 72 °C for 1 minute. RT-PCR reactions were performed by the TITAN RT-PCR Kit (Boehringer-Ingelheim) as described. In the RT-PCR reactions approximately 2 μg of poly(A) RNAs samples were used. The sequences of the primers used in the PCR and RT-PCR reactions were as follows:

HygF primer:
5'-GAATCTCGTGCTTTCAGCTTCGAT-3'
HygR primer:
5'-GTTTCCACTATCGGCGAGTACTTC-3'
PuroF primer:
5'-ATGACCGAGTACAAGCCCACGGTGCGC-3'
PuroR primer:
5'-TCAGGCACCGGGCTTGCGGGTCATGCA-3'
PacR primer:
5'-GAGGCCTTCCATCTGTTGCTGCGC-3'
CCMV primer:
5'-CTGCAGCCCAAGCTTCGAGGGAT-3'
SV400929 primer:
5'-TGTCCAAACTCATCAATGTATC-3'

Purification of poly(A) RNA

The poly(A) RNA samples were purified by Invitrogen's Fastrack poly(A) RNA purification Kit as described.

Southern hybridization

Mouse genomic DNA samples were digested by EcoRI restriction endonuclease to completion, blotted by vacuum transfer onto Hybond-N (Amersham) nylon membrane, UV crosslinked with an UVP crosslinker to the membrane and hybridized by EcoRI-SmaI digested and radioactively labeled pCePur132 plasmid vector. The radioactive labeling was performed by the Amersham Nick translation Kit as described. Gel preparation, restriction endonuclease digestion and blotting was performed as described [20].

RESULTS

The linearized pCePur132 plasmid DNA construct (Fig. 1) was injected into the male pronuclei of fertilized mouse eggs (see Methods) and seven primary transgenic mice (E17, E20, E28, E31, E48, E49 and E68) were obtained out of 72. The injected plas-

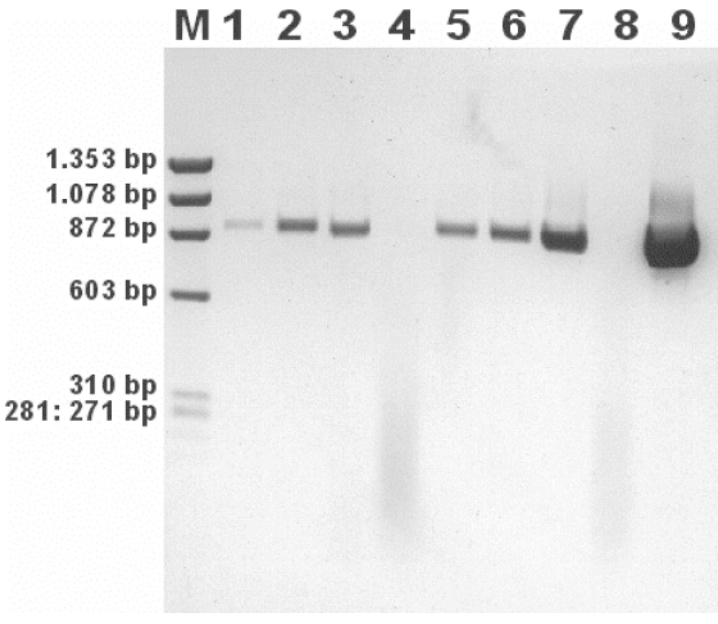

Fig. 2. RT-PCR experiments were performed on poly(A) RNA samples derived from mice of the established transgenic lines. The primers were specific to the transgene confering resistance to hygromicin B compound (Hyg-RT-PCR). M: Marker. HaeIII restriction endonuclease digested ϕX174 phage DNA. 1. Hyg-RT-PCR experiment on liver poly(A) RNA sample originated from a mouse of the E20 transgenic line. 2. Hyg-RT-PCR experiment on liver poly(A) RNA sample originated from a mouse of the E48 transgenic line. 3. Hyg-RT-PCR experiment on liver poly(A) RNA sample originated from a mouse of the E28 transgenic line. 4. Negative control: Hyg-RT-PCR experiment on liver poly(A) RNA sample originated from a non-transgenic (normal F1) mouse. 5. Hyg-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E20 transgenic line. 6. Hyg-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E48 transgenic line. 7. Hyg-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E28 transgenic line. 8. Negative control: Hyg-RT-PCR experiment on kidney poly(A) RNA sample originated from a non-transgenic (normal F1) mouse. 9. Positive control: Hyg-RT-PCR experiment on poly(A) RNA sample originated from a cell line expressing the gene confering resistance to the hygromicin B compound

mid vector carried an anti-HIV ribozyme gene and two mammalian selectable marker genes that were conferring resistance to puromycin and hygromicin-B compounds, respectively. Five out of seven primary transgenic mice could hand down the injected transgenes into their progeny (E17, E20, E28, E31, and E48). It was possible to establish three homozygous lines (E20, E28 and E48) by breeding experiments. In every case the presence of the transgenes in the genomic DNA of the mice was demonstrated by PCR reactions that were specific to the sequence of the puromycin resistance gene (Puro-PCR). The PCR results were verified by Southern hybridization. In this latter case the utilized probe was the radioactively labeled pCePur132 plasmid DNA itself.

The expression of the integrated transgenes was detected by RT-PCR experiments. The poly(A) RNA samples for these RT-PCR reactions were purified from kidney and in some cases from liver tissues of the homozygous transgenic mice. These poly(A) RNA samples were tested for genomic DNA contamination by transgene-specific PCR experiments. Only genomic DNA contamination free poly(A) RNA samples were used for further experiments to avoid false positive results. Approximately 2 μg of a poly(A) RNA sample was used in every PCR and RT-PCR experiments.

The thymidine kinase promoter from *Herpes simplex* virus has driven the expression of the hygromicin resistance gene. The RT-PCR reaction gave an 870 bp product with the used primers (HygF and HygR). Both primer sequences were from the coding sequence of the gene. The expression of the hygromycin resistance gene was successfully detected in all three transgenic lines. Higher level of expression was detected from kidney tissue than from liver tissue. In later experiments only the

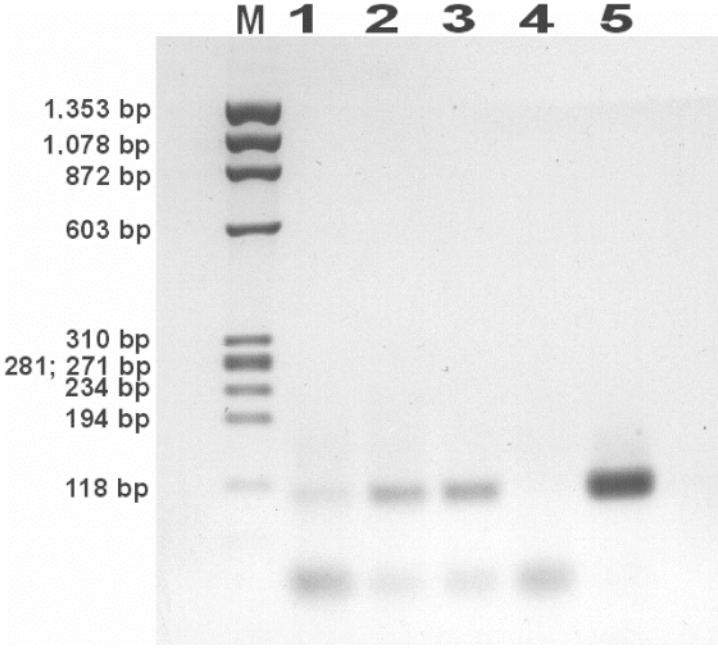

Fig. 3. RT-PCR experiments were performed on poly(A) RNA samples derived from mice of the established transgenic lines. The primers were specific to the anti-HIV ribozyme transgene (Ribo-RT-PCR). M: Marker. HaeIII restriction endonuclease digested ϕX174 phage DNA 1. Ribo-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E20 transgenic line. 2. Ribo-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E48 transgenic line. 3. Ribo-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E28 transgenic line. 4. Negative control: Ribo-RT-PCR experiment on kidney poly(A) RNA sample originated from a non-transgenic (normal F1) mouse. 5. Positive control: Ribo-RT-PCR experiment on poly(A) RNA sample originated from a cell line expressing the anti-HIV ribozyme gene

poly(A) RNA samples purified from kidney tissue were used to examine the expression of transgenes (Fig. 2).

Cytomegalovirus promoter was used to express the anti-HIV ribozyme molecule. In this case the RT-PCR reaction gave a 120 bp product. The primers were specific to the CMV promoter (CCMV primer) and to a section of the SV40 polyadenylation signal before the poly(A) addition site (SV400929 primer), respectively. The CCMV primer contained the first 4 bp of the anti-HIV ribozyme gene. The expression of this transgene was detected in all three transgenic lines (Fig. 3).

SV40 promoter was utilized to express the message of the puromycin resistance gene. Both primers sequences (PuroF and PacR) were from the coding sequence of the gene and the RT-PCR reaction resulted in a 330 bp product. This product was present in all transgenic lines, demonstrating the expression of the puromycin resistance gene (Fig. 4).

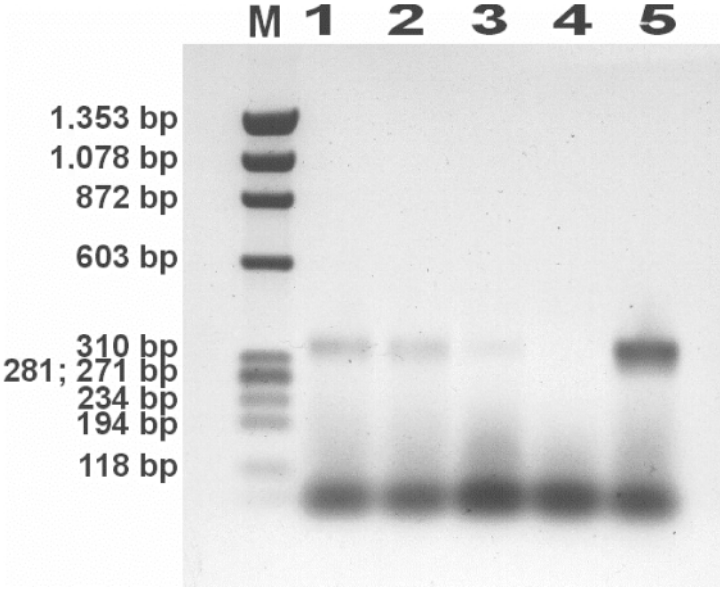

Fig. 4. RT-PCR experiments were performed on poly(A) RNA samples originated from mice of the established transgenic lines. The primers were specific to the transgene confering resistance to the puromycin compound (Puro-RT-PCR). M: Marker. HaeIII restriction endonuclease digested ϕX174 phage DNA 1. Puro-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E28 transgenic line. 2. Puro-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E48 transgenic line. 3. Puro-RT-PCR experiment on kidney poly(A) RNA sample originated from a mouse of the E20 transgenic line. 4. Negative control: Puro-RT-PCR experiment on kidney poly(A) RNA sample originated from a non-transgenic (normal F1) mouse. 5. Positive control: Puro-RT-PCR experiment on poly(A) RNA sample originated from a cell line expressing the gene confering resistance to the puromycin compound

DISCUSSION

We successfully generated homozygous transgenic lines carrying an anti-HIV ribozyme, a puromycin and a hygromycin resistance gene. The expression of these transgenes was demonstrated by RT-PCR experiments. The functionality of the ribozyme was tested elsewhere [4, 21]. Theoretically, a ribozyme is capable to recognize cleavable sites in the RNA transcripts of a living animal. The only way to observe this phenomenon in living organisms is to produce transgenic animals carrying and expressing the functional ribozyme in question. We can expect phenotypic alterations in transgenic mice, if the ribozyme was capable to cleave RNA molecules of the host animal. We did not observe any phenotype in the case of our transgenic lines. The most probable explanation of this phenomenon is that the ribozyme did not recognize any cleavable target sequences amongst the host's RNA molecules. Another possibility is that it finds and cleaves target sequences in the host organism, but this cleavage does not cause phenotypic alterations or the turnover of the reaction is not enough fast to remove an effective amount of the cleaved RNA molecules.

Locardi et al. demonstrated that mice could be infected with AIDS virus [15]. By utilizing the methods described in that paper, we will be able to further test the functionality of this ribozyme and estimate the turnover of the reaction that it catalyses.

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