Identification of new tumor suppressor genes based on in vivo functional inactivation of a candidate gene

Jingfeng Li[a], Alexei I. Protopopov[a,b], Rinat Z. Gizatullin[a,c,e], Csaba Kiss[a], Vladimir I. Kashuba[a,d], Gösta Winberg[a,c], George Klein[a], Eugene R. Zabarovsky[a,c,e,*]

[a] *Microbiology and Tumor Biology Center, Karolinska Institute, 171 77 Stockholm, Sweden*
[b] *Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Lavrentiev Ave. 10, Novosibirsk 630090, Russia*
[c] *Center for Genomics Research, Karolinska Institute, 171 77 Stockholm, Sweden*
[d] *Institute of Molecular Biology and Genetics, Ukrainian Academy of Sciences, Zabolotnogo Str. 150, Kiev 252627, Ukraine*
[e] *Engelhardt Institute of Molecular Biology, 32. Vavilov str., Moscow 117984, Russia*

Received 20 April 1999

Abstract As a step towards developing a new functional test for the identification of tumor suppressor genes, human wild type and mutant *RB* genes were expressed in the mouse A9 fibrosarcoma cell line under the transcriptional regulation of the tetracycline repressor using two new vectors: pLNCtTA and pETI. Following passage of the transfectants in immunodeficient SCID mice, the wild type *RB* gene was deleted or functionally inactivated already after the first passage in all 20 tumors tested. In contrast, a non-functional mutant *RB* gene was maintained in all 10 tumors studied. These results suggest that tests for the identification of tumor suppressor genes may be based on their functional inactivation in vivo, rather than on growth suppression.

Key words: Tumor suppressor gene; *RB* gene; Functional assay; Tetracycline; Gene expression regulation

1. Introduction

Tumor suppressor genes (TSGs) play a broad role as negative regulators in the development of human malignancies. The important classical features of TSGs include: loss of heterozygosity (LOH) accompanied by loss-of-function mutations, mutations in inherited syndromes that predispose to cancer and the ability to inhibit the growth of transformed cells in vitro [1]. Based on these modern molecular concepts of TSGs, several strategies for isolation of new tumor suppressor genes have been used (see [2,3]).

The most widely used approach to designate a candidate gene as a TSG is the unequivocal demonstration of inactivating mutations of this gene in tumor biopsies. Even such a simple requirement for mutational inactivation can be complicated in cases with dominant negative mutations, LOH or heterozygous mutations in genes whose dosage is critical and genes in which one allele is imprinted (see [4,5]). In such cases only functional approaches that demonstrate tumor suppression activity for one of the candidate genes can help solve the problem. Evidence of the ability to suppress cellular proliferation can ot always be used for identification of tumor suppressors. In many tumors which carry multiple genetic alterations, reconstituting a TSG is not sufficient to completely revert their malignant phenotype. For example, in the study by Zhou et al. [6], it was demonstrated that *Rb*-reconstituted osteosarcoma Saos 2 cells were still tumorigenic in nude mice. It must be mentioned that reconstitution experiments designed to demonstrate direct growth inhibition of tumors are difficult to perform and analyze. For instance, growth inhibition of tumors in severe combined immunodeficient (SCID) or nude mice can result from different reasons completely unlinked to tumor suppressor activity, e.g. tumor cells are infected by mycoplasma. On the other hand, a gene can easily mutate and lose its tumor suppressor activity.

Our group has previously shown [7] that a 1.6 cM region in 3p21.3 is regularly deleted during the passage of human chromosome 3 microcell/mouse A9 fibrosarcoma hybrids. We have taken this to indicate that tumor growth antagonizing or suppressor gene(s) were located in the deleted region. The present study addresses the question by asking whether a known suppressor gene, *RB*, would show a corresponding behavior. Wild type and mutated *RB* were built into appropriate constructs that permitted the expression of the gene in the absence but not presence of tetracycline. To achieve this aim a new retroviral vector, pLNCtTA, constitutively expressing tetracycline activator protein (tTA), was constructed and an A9 cell line expressing tTA was created. A new vector, pETI, that provided hygromycin resistance in the transfected cells and allowed tetracycline regulated expression of the inserted gene was also made.

Transfected A9 mouse fibrosarcoma cells were grown in SCID mice in the presence or absence of tetracycline. Wild type *RB* but not deleted *RB* was inactivated during tumor growth.

On the basis of these experiments we suggest a new functional test for TSG identification. This test is based on the functional inactivation of the analyzed genes in contrast to existing tests based on growth suppression [8–10]

2. Materials and methods

2.1. Construction of vector pETI

pETI vector (Fig. 1A) was constructed from vector pUHD10-3 [11] and pCEP4 (Invitrogen). In order to have more cloning sites we inserted oligonucleotide 5′-AATTGTGAGATCTCAACCGGTTTA-GGCGGCCGCTTCTGCTAGCTTTA-3′, 3′- CACTCTAGAGTTG-GCCAAATCCGCCGGCGAAGACGATCGAAATTTAA-5′ in the *Eco*RI site of the polylinker of pUHD10-3. Then the hygromycin resistance gene from pCEP4 was added and the CMV promoter was finally removed by digestion with *Hin*dIII and *Sal*I.

*Corresponding author. Fax: (46) (8) 31 94 70.
E-mail: eugzab@ki.se

PII: S0014-5793(99)00598-0

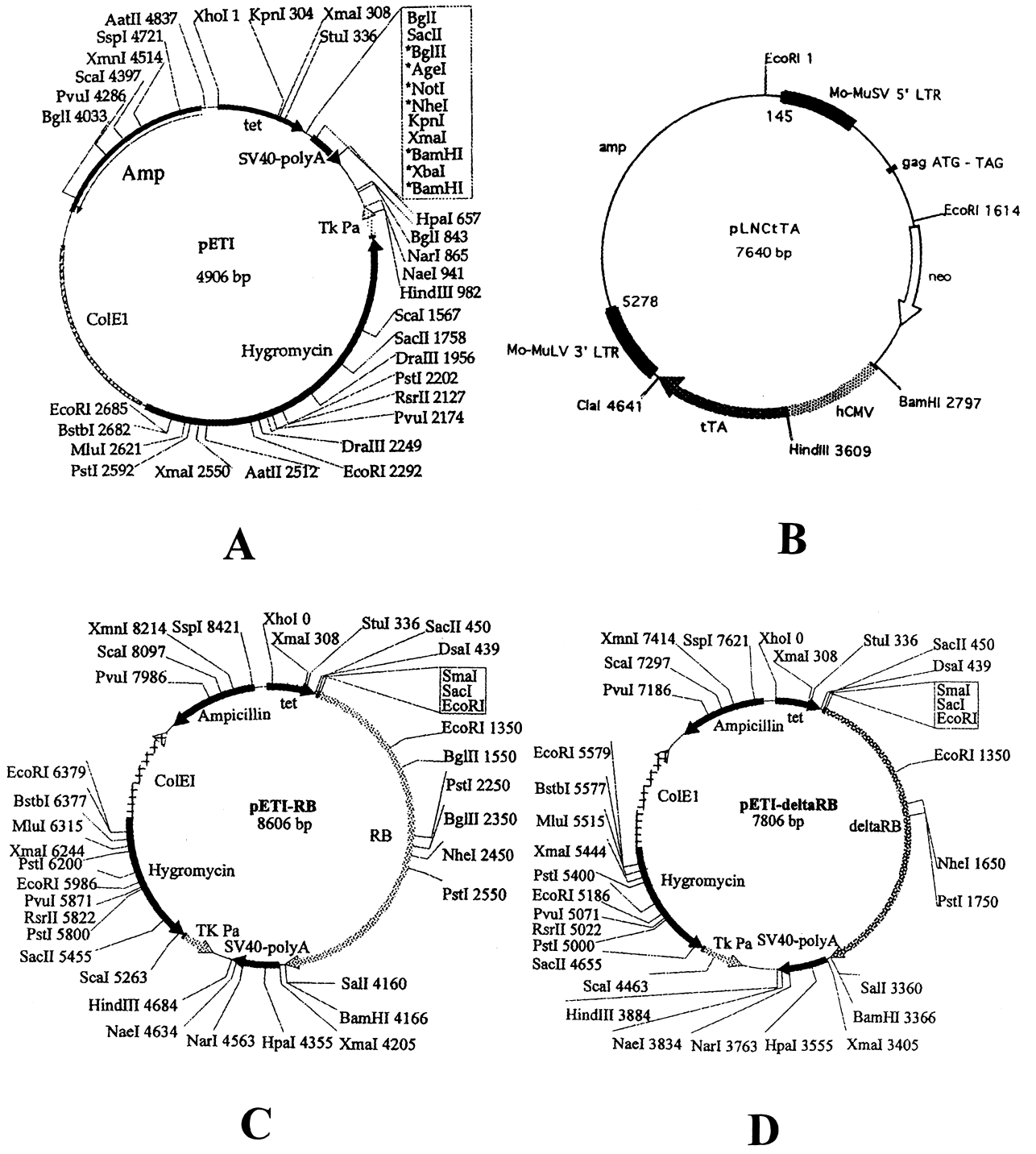

Fig. 1. Vectors used in the study. A: Map of vector pETI, cloning sites in the polylinker are marked by asterisks. B: Map of the retroviral vector pLNCtTA producing tTA. C: Map of pETI-RB. D: pETI-deltaRB. Sizes are not to scale.

2.2. Construction of tTA expressing T711 cell line and luciferase assay

Details of retrovirus construction are available upon request. The fragment of pUHD15-1 [12] containing the *tTA* open reading frame was amplified by PCR and then inserted into the *Cla*I-*Bam*HI sites of the pLNCx retroviral vector (A.D. Miller). The tTA primers used were: tTAF1 5′-CCATATATGGAGTTCCGCGTT-3′ and tTAR1 5′-TACTTTTGCTCCATCGCGAT-3′. The resulting plasmid, pLNCtTA, contained two genes between Mo-MuLV long terminal repeats (LTRs): the *tTA* gene under the control of the enhancer and promoter sequences of the immediate early gene of human cytomegalovirus (hCMV) and the *neo* gene under the control of the 5′ LTR (Fig. 1B). A stable provirus was generated in two steps. First, pLNCtTA was transfected into PE501 mouse ecotropic packaging cells using LipofectAMINE (Life Technologies, Gaithersburg, MD, USA; here and in other lipofection experiments we followed the manufacturer's protocols). Supernatants from clones selected with G418 (7 days) were used for infection of PA317 amphotropic packaging cells: PA317 cells were grown to 50% confluence, growth medium was replaced with a minimal volume of undiluted viral supernatant supplemented with 8 μg/ml hexadimethrine bromide (Sigma). Cells were incubated with retroviral particles in a cell culture incubator for 5 h. After 48 h, cells were selected for 10 days in the presence of 500 μg/ml of G418. The viral supernatant was collected and filtered through a 0.2 μm filter and either used immediately for infection or

stored at −80°C and used in further experiments. Finally, we selected the PA317 cell clone producing amphotropic retrovirus with the *tTA* gene, referred to as RVtTA7.

After growth as a SCID tumor, A9 mouse fibrosarcoma cells were explanted and infected with RVtTA7. A set of stable cell clones was established that expressed *tTA* constitutively (clones T71–T78). To check the level of *tTA* expression, plasmid pUHC13-3 [12], which carried the gene of firefly luciferase under the control of the hCMV minimal promoter with upstream tet operator, was transfected transiently. Luciferase activity was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) and a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturers' protocols. To reduce the variation, every sample was measured twice. Luciferase activity was calculated as relative light units per mg of total protein. The protein concentration of the lysates was determined by a Bradford assay.

2.3. Construction of pETI-RB and pETI-deltaRB and transfection to the T711 cell line

Plasmid pBABE-RB [13] was digested with *Sal*I and sticky ends were blunted using Klenow enzyme. After heat inactivation of enzymes (65°C, 20 min) DNA was digested with *Bam*HI and a 3.7 kb fragment containing the *RB* gene was electrophoretically purified in low melting point agarose. The tetracycline vector pETI was digested by *Xba*I, ends were filled in as described above for *Sal*I and then digested with *Bgl*II. The linear vector fragment (4.9 kb) was purified and eluted from low melting point agarose. Both purified fragments were ligated overnight and transformed into XL1-Blue cells. The final recombinant (pETI-RB) was confirmed by sequencing and its map is shown in Fig. 1C.

This recombinant (pETI-RB) was digested with *Bgl*II and ends were made blunt as indicated above. DNA was diluted to 1 μg/ml and self-ligated with T4 DNA ligase overnight. After transformation, a pETI-deltaRB clone with the structure shown in Fig. 1D was identified. The DNAs from both recombinants were digested with *Xho*I and transfected into T711 cells. Stable transfectants were selected during 10 days, using 400 mg/ml hygromycin (Calbiochem) and 1 μg/ml tetracycline (Stratagene). Two primers, exon23F 5′-TACCTCACATTCCTCGAAGCC-3′ and exon27R 5′-GCTTTTGCATTCGTGTTCGA-3′, were used to screen resistant clones.

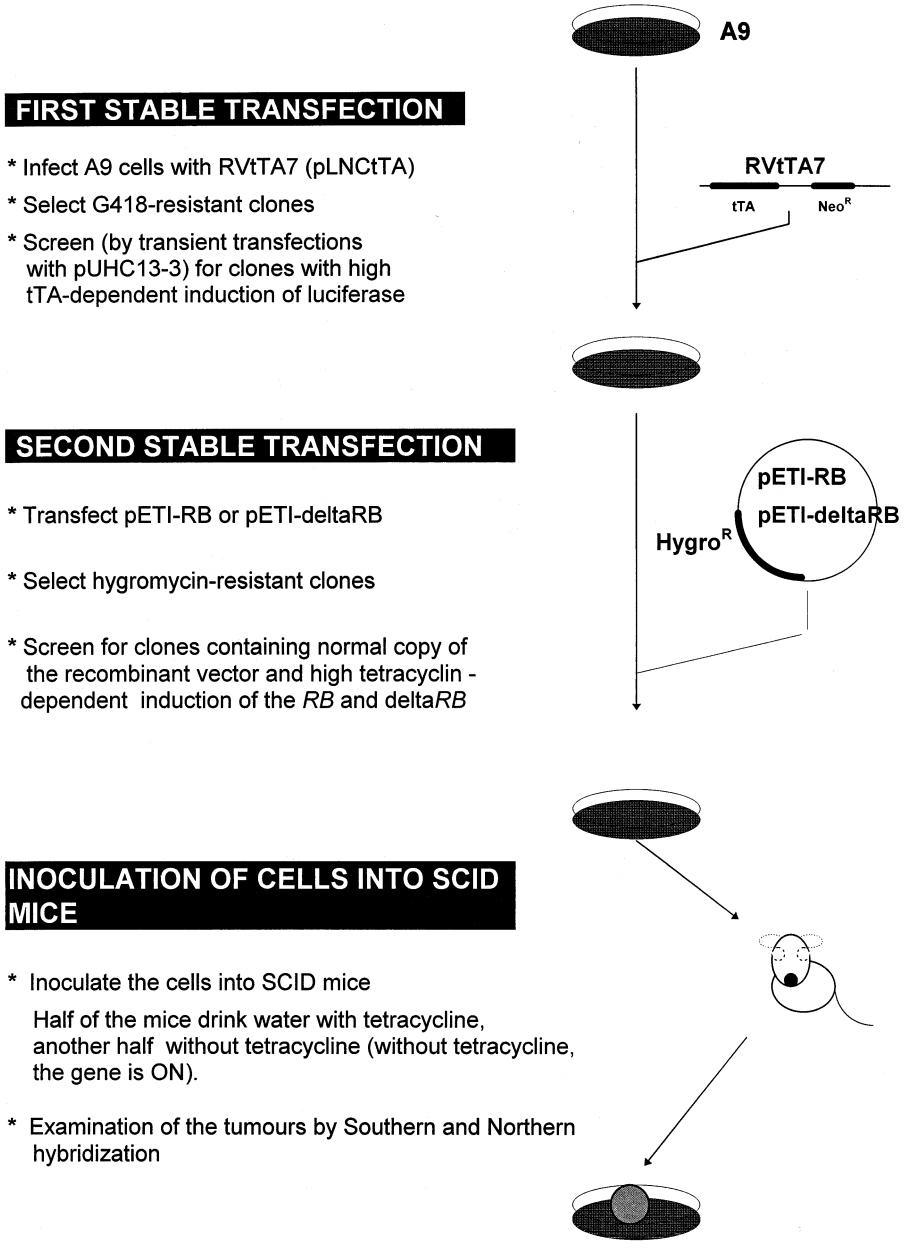

Fig. 2. General scheme of the experiments.

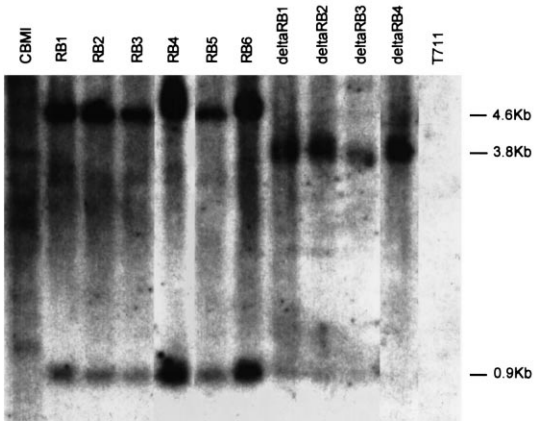

Fig. 3. Southern hybridization of RB+A9 (RB1–RB6) and deltaRB+A9 (deltaRB1–deltaRB4) transfectants after *Eco*RI digestion. Lanes with *Eco*RI digested DNA from T711 and CBMI-Ral-Sto (human LCL: lymphoblastoid cell line) are shown for comparison.

2.4. Southern and Northern hybridization

For Southern blotting genomic DNA was isolated from cells and biopsies according to standard procedures [14]. 10 μg of DNA was digested with *Eco*RI and fractionated by electrophoresis in 0.8% agarose gel. Hybridization was carried out [15] with ^{32}P-labeled DNA probes at 65°C in the presence of 0.75 M NaCl, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 200 μg/ml heat-denatured salmon sperm DNA in a hybridization tube with constant agitation for 20 h. The *Sal*I fragment (RB probe) from pBABE-RB containing the whole *RB* gene was labeled with [^{32}P]dCTP by the random oligonucleotide priming method [16].

For Northern analysis RNA isolation was performed via acid guanidinium thiocyanate-phenol-chloroform extraction. 20 μg of total RNA was loaded per lane on a 1.5% denaturing agarose gel with formaldehyde. After electrophoresis, the RNAs from the gel were transferred to Hybond-N and hybridized with a ^{32}P-labeled *RB* fragment of pBABE-RB using standard procedures [17].

2.5. Inoculation into SCID mice

Each transfectant clone was grown in IMDM medium with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), 400 U/ml hygromycin and 1 μg/ml tetracycline to obtain about 2×10^6 cells for one SCID mouse. Cells were treated with trypsin-EDTA and concentrated by centrifugation at 2000 rpm for 12 min. The cell pellet was suspended in 0.2 ml serum-free IMDM medium. Six SCID mice were used for each experiment. Three of them received water with tetracycline (1 mg/ml).

The tumor sizes (mm^3) were measured and DNA and RNA were isolated as described above.

3. Results and discussion

3.1. General design of the experiment

The strategy of the experiment is shown in Fig. 2. Our aim was to compare the transfected cells carrying wild type or mutated *RB*, respectively, for the presence and expression of the transgene, following growth in SCID mice with and without tetracycline. Delta*RB* was obtained from the *RB* gene by removing an 840 bp *Bgl*II-*Bgl*II fragment (see Section 2). One of the *Bgl*II sites is localized in the amino-terminal domain. Another *Bgl*II site is from the spacer sequences in the *RB* gene. The deleted region included 213 bp from the 3′ end of the amino-terminal domain, the whole 'A' pocket and 99 bp from the 5′ end of the spacer. The 'A' pocket is the functionally important domain with sequence similarity to the conserved C-terminus of the transcription factor TFIID [18,19]. The deleted delta*RB* is functionally deficient and was used as a control.

3.2. tTA expressing cell line

For final comparison, cells from clone T71 were explanted on a 24 well plate, one clone per well, in a growth medium without tetracycline. Parental A9 cells were used as a control. The tTA activation factor for transiently luciferase expressing T71 clones was about 1000-fold, depending slightly on the clone. This experiment was repeated twice. Clone T711 showed more than 10^3 times higher luciferase activity than the control and was selected for further experiments.

It is known that *tTA* regulated constructs retain some residual activity when the system is switched off [20]. This background activity depends on the number of gene copies per cell. Our data were consistent with these results. The background was lowest in the control A9 cells when the transfection efficiency was low.

3.3. Expression of RB and deltaRB in transfected clones in vitro

After the transfection of pETI-RB and pETI-deltaRB into T711 cells and hygromycin selection, the positive clones (RB+A9 and deltaRB+A9) were first analyzed by PCR using *RB* specific exon23F and exon27R primers (see Section 2, GDB database). Ten positive clones were analyzed by Southern hybridization (Fig. 3). All six RB+A9 clones gave the 4.6 kb and 900 bp bands expected. Three deltaRB+A9 clones analyzed also gave the 3.8 kb and 900 bp bands expected but deltaRB+A9 clone 4 lacked the 900 bp band. All clones were tested for tetracycline dependent regulation. The four RB+A9 and two deltaRB+A9 clones that had the best results were selected for further experiments. Fig. 4 shows that *RB* and delta*RB* were expressed in the absence of tetracycline. They were expressed weakly in the presence of tetracycline.

3.4. Tumor growth in SCID mice

Fig. 5 shows the growth of five tumors (two tumors with tetracycline and three tumors without tetracycline) from RB+A9 clone 1 and six tumors (three tumors with tetracycline and three tumors without tetracycline) from deltaRB+A9 clone 3 in 11 SCID mice. The growth of the *RB* transfected

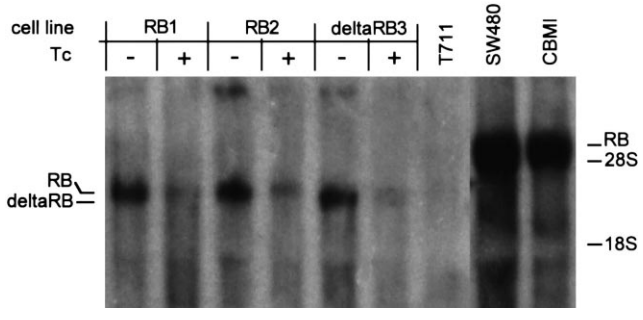

Fig. 4. Northern hybridization of RB+A9 (RB1 and RB2) and deltaRB+A9 (deltaRB3) transfectants with RB probe. The size of the endogenous human *RB* is 4.8 kb [18]. The transfected *RB* gene is about 3.8 kb, since 1 kb of non-coding sequence was deleted from it, compared to the endogenous *RB* gene. CBMI-Ral-Sto (human LCL) and SW480 (human lung cancer) lines were used as positive controls, the T711 (A9) line was used as a negative control. Tc, tetracycline; +, with tetracycline; −, without tetracycline.

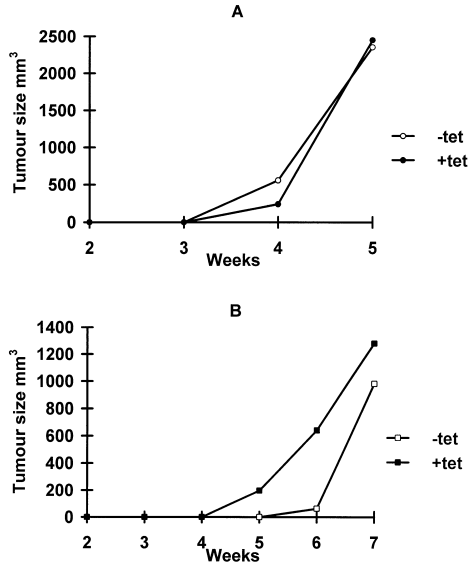

Fig. 5. Growth curve of six tumors from deltaRB+A9 clone 3 (A) and five tumors from RB+A9 clone 1 (B) in 11 SCID mice (drinking tetracycline or not). Each point corresponds to the geometric mean tumor volume (mm^3) from three mice (two mice drinking tetracycline in the case of RB+A9 clone 1).

tumors was delayed when tetracycline was absent. Delta*RB* transfected tumors grew equally well with and without tetracycline. Compared to the delta*RB* expressing tumors, the growth of *RB* expressing tumors was slightly delayed even in the presence of tetracycline. Other clones showed similar growth kinetics.

3.5. *RB and deltaRB DNA and RNA in SCID derived tumors*

Tumors that grew in SCID mice after the inoculation of RB+A9 and deltaRB+A9 cells were examined by Southern and Northern hybridization. These results are summarized in Table 1. Altogether 20 tumors were derived from four RB+A9 clones. Eleven were harvested from tetracycline drinking and nine from untreated mice. A total of 10 tumors

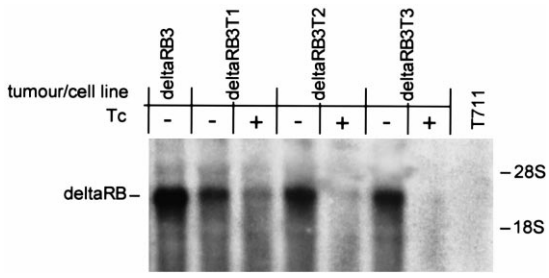

Fig. 6. Northern hybridization of tumors (deltaRB3T1–deltaRB3T3) derived from deltaRB+A9 clone 3 (deltaRB3) transfectant with RB probe. Tc, tetracycline; +, with tetracycline; −, without tetracycline.

were derived from two deltaRB+A9 clones, five of them were from tetracycline drinking and five from untreated mice.

All deltaRB+A9 clones retained the deleted gene after one passage in SCID mice. They expressed deleted *RB* RNA at a high level in the untreated mice and at a reduced level in the tetracycline drinking mice (Fig. 6). In contrast, *RB* was deleted in 15 of the 20 tumors, derived from three of four clones tested. Northern hybridization gave a positive signal with all four clones prior to inoculation, but none of them expressed detectable *RB* after mouse passage, either in the presence or the absence of tetracycline. Only one clone (clone 1) showed retention of the gene by Southern hybridization after mouse passage, but no expression was detected in any of the five tumors tested, irrespective of whether they were harvested from tetracycline treated or untreated mice.

The deviating behavior of clone 1 prompted further comparisons. The in vitro maintained clone 1 subline was compared with the derived tumors by Southern hybridization, following *Bgl*II digestion. There are two *Bgl*II sites in the *RB* gene. RB+A9 tumors generate one 800 bp *Bgl*II fragment and two additional fragments that are of variable size, depending on the integration of the transgene. Southern hybridization results showed that the *Bgl*II hybridization pattern was different for the parental RB+A9 clone 1 and SCID mice derived tumors (data not shown). This is probably due

Table 1
Southern and Northern results in A9 clones transfected with *RB*/delta*RB* in pETI and derived tumors

RB/deltaRB clone	Before inoculation			Tumors after inoculation			
	Southern	Northern		With Tc		Without Tc	
	With Tc	With Tc	Without Tc	Southern	Northern	Southern	Northern
Clone 1	+	−*	+	T1 +	T1 −	T1 +	T1 −
				T2 +	T2 −	T2 +	T2 −
				No tumor	No tumor	T3 +	T3 −
Clone 2	+	−*	+	T1 −	T1 −	T1 −	T1 −
				T2 −	T2 −	T2 −	T2 −
				T3 −	T3 −	T3 −	T3 −
Clone 4	+	−*	+	T1 −	T1 −	T1 −	T1 −
				T2 −	T2 −	No tumor	No tumor
				T3 −	T3 −	No tumor	No tumor
Clone 5	+	−*	+	T1 −	T1 −	T1 −	T1 −
				T2 −	T2 −	No tumor	No tumor
				T3 −	T3 −	T3 −	T3 −
Delta clone 1	+	−*	+	T1 +	T1 −*	No tumor	No tumor
				T2 +	T2 −*	T1 +	T1 +
				No tumor	No tumor	T2 +	T2 +
Delta clone 3	+	−*	+	T1 +	T1 −*	T1 +	T1 +
				T2 +	T2 −*	T2 +	T2 +
				T3 +	T3 −*	T3 +	T3 +

T, tumor; No tumor, tumor did not grow in vivo. +, positive signal; −, negative signal; −*, some leakage. Tc, tetracycline.

to the integration of several pETI-RB constructs into the parental RB+A9 clone 1 and the expression of only some of the integrated copies. Another explanation is that this cell line is polyclonal with regard to integrated pETI-RB. During tumor growth in SCID mice, cells with unexpressed *RB* copies would have a selective advantage over clones with an expressed *RB* gene.

In summary, our results suggest that a functional human *RB* gene is a growth handicap for A9 cells in SCID mice. It is therefore either deleted or inactivated in growing tumors. RB+A9 tumors that grew in the presence of tetracycline showed the same *RB* losses as tumors growing in untreated mice, indicating *RB* expression was not fully repressed in the tetracycline drinking mice. An episomal variant of the pETI vector may improve the system, since the influence of flanking host sequences on gene expression would be avoided. We have constructed such a vector (pETE). Our first experiments indicate that these constructs have a significantly decreased leakage but have the same level of expression in the absence of tetracycline.

The present findings indicate that tumor suppressor genes may be deleted or inactivated when transfected cells are passaged through immunodeficient SCID mice. We are now using this 'inactivation test' for analysis of 10 candidate TSGs isolated from the 3p21 region.

Acknowledgements: The authors are grateful to Dr. Ying Yang for the help in some experiments. Plasmids pUHD15-1 and pUHC13-3 were obtained from H. Bujard, University of Heidelberg, Germany. MMLV retroviral vector pLNCx was kindly provided by A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA, USA. This work was supported by research grants from the Swedish Cancer Society, Karolinska Institute and Åke Wiberg foundation. J.L., C.K. and V.I.K. were recipients of fellowships from the Concern Foundation in Los Angeles and the Cancer Research Institute in New York.

References

[1] Grander, D. (1998) Med. Oncol. 15, 20–26.
[2] Clurman, B. and Groudine, M. (1997) Nature 389, 122–123.
[3] Haber, D. and Harlow, E. (1997) Nature Genet. 16, 320–322.
[4] Nicolaides, N.C., Littman, S.J., Modrich, P., Kinzler, K.W. and Vogelstein, B. (1998) Mol. Cell Biol. 18, 1635–1641.
[5] Kohler, B., Schumacher, V., Schulte-Overberg, U., Biewald, W., Lennert, T., l'Allemand, D., Royer-Pokora, B. and Gruters, A. (1999) Pediatr. Res. 45, 187–190.
[6] Zhou, Y., Li, J., Xu, K., Hu, S., Benedict, W.F. and Xu, H. (1994) Proc. Natl. Acad. Sci. USA 91, 4165–4169.
[7] Szeles, A., Yang, Y., Sandlund, A.M., Kholodnyuk, I., Kiss, H., Kost-Alimova, M., Zabarovsky, E.R., Stanbridge, E., Klein, G. and Imreh, S. (1997) Genes Chromosomes Cancer 20, 329–336.
[8] Shimizu, M., Yokota, J., Mori, N., Shuin, T., Shinoda, M., Terada, M. and Oshimura, M. (1990) Oncogene 5, 185–194.
[9] Sumegi, J., Uzvolgyi, E. and Klein, G. (1990) Cell Growth Differ. 1, 247–250.
[10] Killary, A.M., Wolf, M.E., Giambernardi, T.A. and Naylor, S.L. (1992) Proc. Natl. Acad. Sci. USA 22, 10877–10881.
[11] Resnitzky, D., Gossen, M., Bujard, H. and Reed, S.I. (1994) Mol. Cell Biol. 14, 1669–1679.
[12] Gossen, M. and Bujard, H. (1992) Proc. Natl. Acad. Sci. USA 89, 5547–5551.
[13] Wang, X., Hafezparast, M. and Masters, J.R. (1997) Cancer Genet. Cytogenet. 98, 56–62.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Zabarovsky, E.R., Boldog, F., Thompson, T., Scanlon, D., Winberg, G., Marcsek, Z., Erlandsson, R., Stanbridge, E.J., Klein, G. and Sumegi, J. (1990) Nucleic Acids Res. 18, 6319–6324.
[16] Feinberg, A. and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267.
[17] Kroczek, R.A. (1993) J. Chromatogr. 618, 133–145.
[18] Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y. and Lee, E.Y. (1987) Science 235, 1394–1399.
[19] Hagemeier, C., Bannister, A.J., Cook, A. and Kouzarides, T. (1993) Proc. Natl. Acad. Sci. USA 90, 1580–1584.
[20] Gossen, M., Bonin, A. and Bujard, H. (1993) Trends Biochem. Sci. 18, 471–475.