EMBO reports

scientific report

A highly efficient ligand-regulated Cre recombinase mouse line shows that *LoxP* recombination is position dependent

Marc Vooijs[1], Jos Jonkers[1] & Anton Berns[1,2,+]

The Netherlands Cancer Institute, [1]Division of Molecular Genetics and [2]Center for Biomedical Genetics, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Received September 21, 2000; revised January 12, 2001; accepted January 26, 2001

Conditional gene inactivation using the Cre/*loxP* system is widely used, but the difficulty in properly regulating Cre expression remains one of the bottlenecks. One approach to regulate Cre activity utilizes a mutant estrogen hormone-binding domain (ER^T) to keep Cre inactive unless the non-steroidal estrogen analog 4-hydroxytamoxifen (OHT) is present. Here we describe a mouse strain expressing Cre-ER^T from the ubiquitously expressed ROSA26 (*R26*) locus. We demonstrate efficient temporal and spatial regulation of Cre recombination *in vivo* and in primary cells derived from these mice. We show the existence of marked differences in recombination frequencies between different substrates within the same cell. This has important consequences when concurrent switching of multiple alleles within the same cell is needed, and highlights one of the difficulties that may be encountered when using reporter mice as indicator strains.

INTRODUCTION

Methods that permit specific gene modifications *in vivo* in mice provide a powerful approach to assess gene function. However, germline modification often results in embryonic lethality or has pleiotropic effects interfering with the analysis of specific disease aspects. The use of the bacterial (Cre/*loxP*) and yeast-derived (FLP/*Frt*) site-specific DNA recombination systems permits the analysis of gene function *in vivo* in a cell-type restricted fashion (Gu *et al.*, 1993; Dymecki, 1996; Shibata *et al.*, 1997; Vooijs *et al.*, 1998; Marino *et al.*, 2000). These systems are based on the ability of Cre and FLP recombinases to catalyze the excision of DNA flanked by *loxP* or *Frt* recognition sequences (Sauer and Henderson, 1988; O'Gorman *et al.*, 1991). *LoxP* or *Frt* sites can be inserted around one or more exons of the cognate gene via homologous recombination in embryonic stem (ES) cells. Conditional mutant mice can be crossed with transgenic mice expressing Cre- or FLP recombinase under the control of tissue-specific promoters. This strategy ensures normal development of the animal, fixes the window of gene inactivation to defined cell types and is now widely used in a broad range of biological settings (Rajewsky *et al.*, 1996; Metzger and Feil, 1999).

The lack of control over the onset of Cre expression in classical transgenic mice has important consequences for the phenotypic outcome. If switching occurs early in a particular cell lineage, the phenotypic aberrations reflect the accumulation of defects from the earliest stage at which the gene is required, complicating the analysis of later developmental stages. Temporally controlled Cre-mediated recombination overcomes this and permits analysis of gene function at selected timepoints.

Spatial and temporal control of Cre-mediated recombination *in vivo* has been achieved through the use of adenoviruses expressing Cre (Rohlmann *et al.*, 1996; Wang *et al.*, 1996; Akagi *et al.*, 1997; Shibata *et al.*, 1997). However, the immune response elicited by these viruses compromises the persistence of infected cells and affects the general health status of mice (Akagi *et al.*, 1997). Recently, temporal regulation of Cre recombinase *in vivo* has been accomplished using tetracycline-controlled gene expression (Utomo *et al.*, 1999) and interferon-inducible expression (Kuhn *et al.*, 1995).

An alternative approach utilizes engineered recombinases fused to the hormone-binding domain (HBD) of the mutated estrogen receptor (ER^T). The fusion protein becomes active upon administration of the synthetic estrogen antagonist 4-hydroxytamoxifen (OHT), but not in the presence of the natural ligand

+Corresponding author. Tel: +31-20-5121991; Fax: +31-20-5122011; E-mail: tberns@nki.nl

17β-estradiol (Feil *et al.*, 1996). Transgenic mouse lines have been generated that express *cre-ER*^{T} fusion genes controlled by tissue-specific promoters, which show ligand-dependent recombination (Brocard *et al.*, 1997; Danielian *et al.*, 1998; Schwenk *et al.*, 1998; Vasioukhin *et al.*, 1999). These approaches depend on properly regulated Cre expression from tissue-specific promoters in transgenic mice. However, the random integration of transgenes into the mouse genome often results in mosaic gene expression (Garrick *et al.*, 1998; Henikoff, 1998) and offers little control over the level of gene-expression obtained. This requires the screening of a large number of independent transgenic lines to obtain one with appropriate expression characteristics.

To date, no mouse strains exist that permit highly efficient ubiquitous and inducible gene inactivation *in vivo*. To achieve this we used gene targeting to insert a single copy of a ligand responsive *cre* gene into a locus that is expressed ubiquitously throughout mouse development and in the adult.

RESULTS

To express a ligand-dependent Cre recombinase we used a fusion gene encoding Cre and the mutated ligand binding domain (LBD) of the human estrogen receptor (G521R) (Schwenk *et al.*, 1998). A targeting vector containing the *cre-ER*^{T} fusion gene preceded by a β-globin splice acceptor site was inserted into the *R26* locus (Figure 1A). This strategy is nearly identical to that reported by Soriano for the generation of the *R26R* mouse (Soriano, 1999). Mouse ES cells were electroporated and Southern analysis revealed that the majority of clones had undergone homologous recombination (Figure 1B). Clone C2 was used for the studies described below.

We first determined whether introduction of *cre-ER*^{T} into the *R26* locus resulted in ligand-dependent recombination in ES cells. *R26cre-ER*^{T} ES cells already harbored *loxP* sites surrounding exon 11 of breast cancer susceptibility gene-2 (*Brca2*)(e.g. *Brca2*^{F11F}; J. Jonkers, R. Menwissen, H. van der Gulden, H. Peterse, M. van der Valk and A. Berns, in preparation). *R26cre-ER*^{T};*Brca2*$^{F11F/wt}$ ES cells, cultured in the absence of ligand showed no recombination. Addition of OHT to the medium for 60 h resulted in a dose-dependent recombination of *Brca2*$^{F11F/wt}$ reaching 100% at 400 nM (Figure 1C). Targeted ES cells were injected into blastocysts and heterozygous mice were obtained. *R26cre-ER*^{T};*Brca2*$^{F11F/wt}$ mice were healthy and fertile and showed no evidence for excision of *Brca2*^{F11F} in the absence of ligand. This indicates that physiological levels of estrogens present in chimeric mice and subsequent offspring do not promote recombination of *Brca2*^{F11F}.

Next, we tested if *R26cre-ER*^{T} mice could be used as a source of primary cells in which conditional alleles could be efficiently switched in culture. Primary keratinocytes from newborn *Brca2*$^{F11F/wt}$;*R26cre-ER*^{T} mice were isolated and after 48 h of culture, OHT was added to the medium for 24 h followed by culturing in normal medium for an additional 24 h (24+, 24–). When cultured in the presence of 100 nM of OHT *Brca2*^{F11F} could be switched with nearly 100% efficiency (Figure 1D). Analysis of recombination efficiencies directly after removal of OHT (24+) showed that recombination was incomplete with 100 nM suggesting residual Cre activity after removing ligand from the medium. As expected a further reduction in the concentration of OHT reduced the frequency of recombination on *Brca2*^{F11F}.

Conditional gene targeting permits the introduction of specific mutations in mice similar to those found in human diseases. Modeling of multifactorial disease in mice requires concurrent switching of floxed alleles *in vivo*. To date it is not known whether different conditional alleles within the same cell show a comparable switching efficiency. To investigate this we crossed *R26cre-ER*^{T} mice with mice carrying conditional alleles for the retinoblastoma susceptibility gene (*Rb*) (*Rb*^{F19F}; Vooijs and Berns, 1999; Marino *et al.*, 2000), *Brca2*^{F11F} and *p53* (Marino *et al.*, 2000). *Rb*$^{F19F/wt}$;*Brca2*$^{F11F/wt}$;*p53*$^{F2\text{-}10F/wt}$;*R26cre-ER*^{T} mice were injected for three days with 8 mg of OHT (i.p.) and tissue DNA was isolated 7 days later. Southern blot analysis showed that recombination of *Rb*^{F19F} approached 100% in most tissues, whereas moderate recombination frequencies were observed of *Brca2*^{F11F} and *p53*$^{F2\text{-}10F}$ in the same samples (Figure 2A). A consistent low level of OHT-independent recombination in some tissues was seen on *Rb*^{F19F} but not on *Brca2*^{F11F} or *p53*$^{F2\text{-}10F}$.

Since Cre reporter mice are widely used to monitor Cre activity (Akagi *et al.*, 1997; Soriano, 1999), we considered it important to directly compare *in vivo* the behavior of a reporter allele with an independent conditional allele. *R26R* mice that express *LacZ* after Cre-mediated excision of a *neo* cassette (Soriano, 1999), were crossed with *R26cre-ER*^{T};*Brca2*$^{F11F/wt}$ mice to obtain *R26R;Brca2*$^{F11F/wt}$;*R26cre-ER*^{T} mice. These mice were readily obtained and we did not observe significant effects on their viability (Zambrowicz *et al.*, 1997). Mice were injected i.p. with 3× 8 mg of OHT and tissue DNA was analyzed for recombination at both alleles. Whereas both loci behaved similarly in lung and duodenum, significant differences were seen in the testis where *R26R* recombined to a much greater extent than *Brca2*^{F11F} (Figure 2B). To investigate whether these differences were related to tissue-specific differences in the expression of Cre-ER^{T} we performed northern blot analysis on poly(A)$^{+}$ RNA extracted from *R26cre-ER*^{T} mice (Figure 1E). This analysis demonstrated that Cre-ER^{T} is expressed in all tissues examined albeit at different levels. Most notably, expression in the cerebellum was found to be relatively low.

Having demonstrated that *R26cre-ER*^{T} mice permit efficient deletion of floxed alleles in most tissues *in vivo*, we investigated whether local administration of OHT led to more restricted switching (Vasioukhin *et al.*, 1999). We applied a single dose of 0.5 mg or three consecutive doses of 8 mg of OHT, respectively, to the shaved back of *R26R;R26cre-ER*^{T} mice. Treated and untreated skin and various internal organs were processed for β-galactosidase activity 7 days after OHT application. At the lowest concentration (0.5 mg OHT) blue-stained cells were detected in cells of the follicular and interfollicular epidermis in the area where OHT was applied (Figure 2D). Increasing the topical dose to 3× 8 mg resulted in widespread staining throughout the treated area (Figure 2F). Under these conditions no appreciable staining was detected in non-treated adjacent skin nor in the underlying dermis (Figure 2C and E).

To investigate further the ligand-independent recombination seen in the absence of ligand at the cellular level we analyzed frozen-tissue sections from *R26R;R26cre-ER*^{T} mice by staining for β-galactosidase activity. In most tissues analyzed (skeletal muscle, skin, liver, lungs, heart, kidney, spleen, stomach, eye, pituitary, testis, cerebellum and cerebrum) <0.1% of stained

M. Vooijs, J. Jonkers & A. Berns

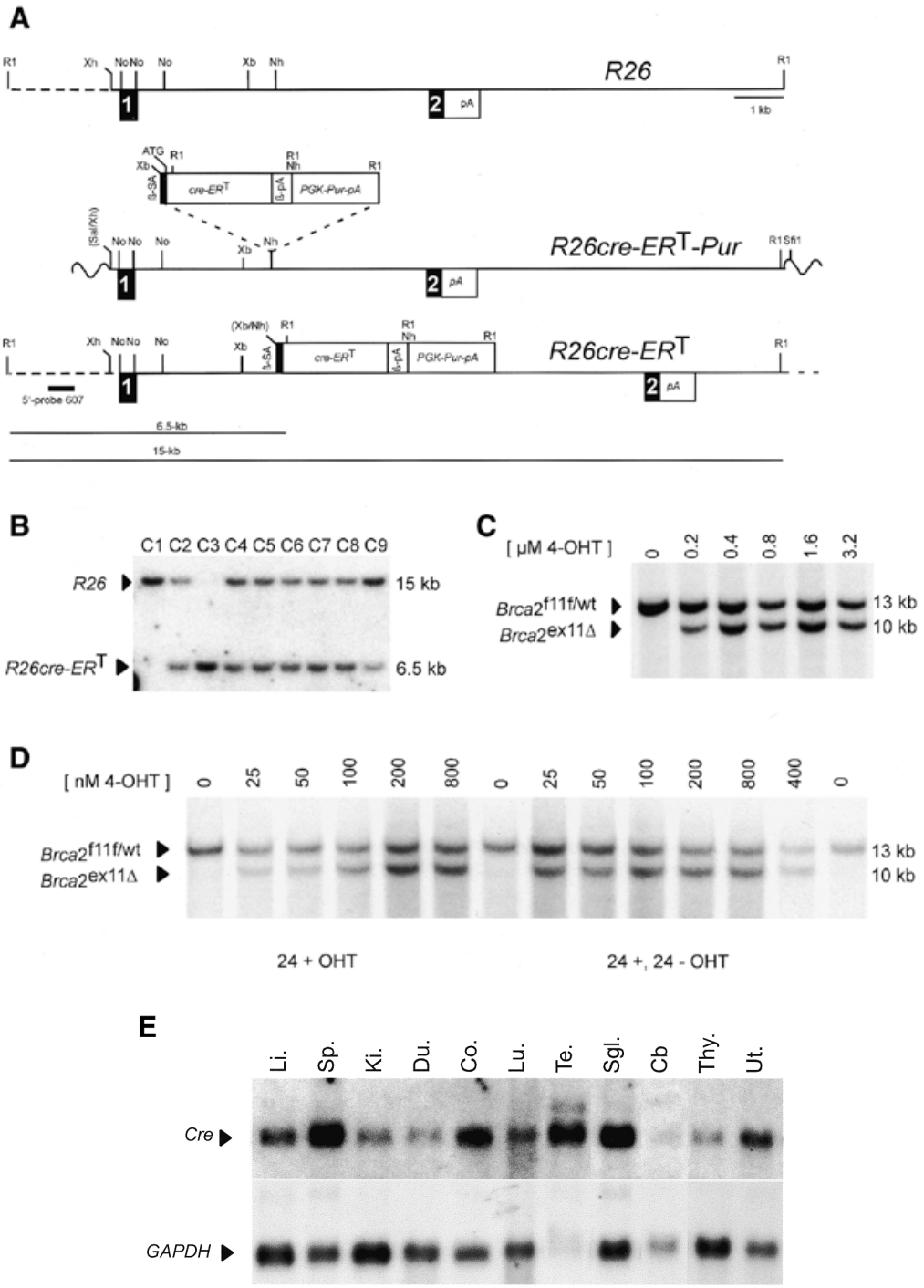

Fig. 1. Knock-in of *cre-ER*^{T} fusion gene into the *ROSA26* locus and ligand-inducibility *in vitro*. (**A**) Structure of the *ROSA26* locus, the *R26cre-ER*^{T} targeting vector and targeted locus. R1, *Eco*R; *Xh*, *Xho*I; *No*; *Not*I; *Xb*, *Xba*I; *Nh*, *Nhe*I. (**B**) Southern blot analysis with probe pHA607 (black box) on *Eco*RI digested DNA from puromycin resistant clones C1–C9. Wildtype (15 kb) and targeted (6.5 kb) *R26* alleles are indicated. Clone C1 a non-targeted clone, C2 and C4–C9 heterozygous targeted clone, and C3 a homozygous targeted clone. (**C**) Ligand-inducible recombination of *Brca2*^{F11F} in ES cells cultured for 60 h in the presence of OHT analyzed by Southern blot. Unrecombined *Brca2*$^{F11F/wt}$ (13 kb) and the recombined *Brca2*^{ex11} (10 kb) fragments are indicated. (**D**) Ligand-inducible recombination of *Brca2*^{F11F} in primary keratinocytes analyzed as in (C), directly after removal of OHT (24+) or after culturing another 24 h without OHT (24+; 24–). (**E**) Northern blot analysis of poly(A)$^{+}$ isolated RNA from *R26cre-ER*^{T} mice. Upper panel shows hybridization to Cre probe and lower panel control hybridization to GAPDH. Tissues are Li (Liver), Sp (Spleen), Ki (Kidney), Du (Duodenum), Co (Colon), Lu (Lung), Te (Testis), Sgl (Salivary Gland), Cb (Cerebellum), Thy (Thymus) and Ut (Uterus).

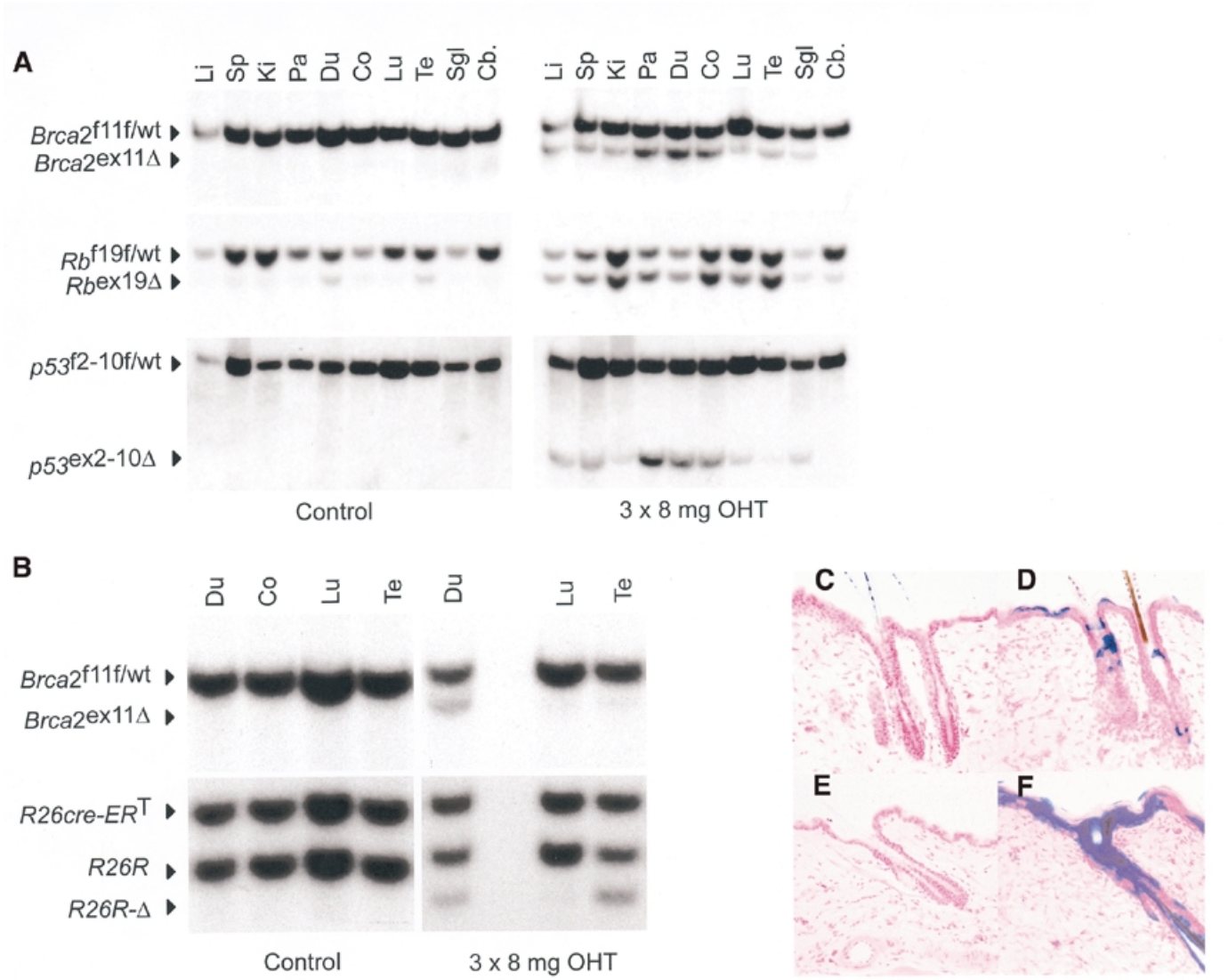

Fig. 2. Differential recombination efficiencies *in vivo* between Rb^{F19F}, $p53^{F2\text{-}10F}$ $Brca2^{F11F}$ and *R26R* alleles. (**A**) Southern blot analysis of tissue DNA from OHT-treated $Rb^{F19F/wt}$;$Brca2^{F11F/wt}$;$p53^{F2\text{-}10F/wt}$;*R26cre-ER*^{T} mice. Fragment sizes are 5.0 kb ($Rb^{F19F/wt}$), 4.5 kb ($Rb^{ex19\Delta}$), 15 kb ($p53^{F2\text{-}10F/wt}$) and 7 kb ($p53^{ex2\text{-}10\Delta}$). Tissues are as in Figure 1C. (**B**) Southern blot analysis of tissues from $Brca2^{F11F}$;*R26R*; *R26cre-ER*^{T}. Fragments are 4.8 kb (*R26cre-ER*^{T}), 4.2 kb for the unrecombined and 3.8 kb for the recombined *R26R* allele. (C–F) Dose-dependent local Cre-mediated recombination in *R26R;R26cre-ER*^{T} analyzed by X-Gal staining on frozen sections of treated (**D**, **F**) and non-treated (**C**, **E**) skin. Mice received 1× 0.5 mg OHT (C, D) or 3× 8 mg OHT (E, F) in DMSO. Counterstain is neutral red. Magnification 10×.

cells were identified. In contrast in duodenum and pancreas patches of stained cells were consistently found (1–5%, not shown).

DISCUSSION

The introduction of defined mutations in the mouse in a spatially and temporally controlled manner is considered an important next step in generating better mouse models of human diseases (Metzger and Feil, 1999; Porter, 1999; Vooijs and Berns, 1999). Our findings show that introduction of a ligand responsive *cre* gene (*cre-ER*^{T}) under the transcriptional control of a broadly expressed gene fulfills this requirement.

Using *R26cre-ER*^{T} mice in conjunction with *Brca2*, *Rb*, *p53* and *R26R* conditional mutants, we show that Cre-mediated recombination *in vivo* can be efficiently regulated in a temporal, spatial and dose-dependent manner by OHT. The reduced activity observed in cerebellum may be explained by a lower or less uniform expression of *R26-cre-ER*^{T} or less efficient activation of Cre-ER^{T} due to a lower local concentration of OHT (Robinson *et al.*, 1991). The ligand-independent Cre activity observed on the Rb^{F19F} and *R26R* alleles may be a consequence of inappropriate nuclear transport or proteolysis of the Cre-fusion protein, sufficient to catalyze a low level of recombination. We extended the use of *R26cre-ER*^{T} mice by showing that restricted switching could be achieved with localized application of OHT on the skin. The

short half-life of OHT *in vivo* (Robinson *et al.*, 1991) should also permit localized switching at other tissue sites.

A comparison of recombination frequencies of different alleles *in vivo* showed marked differences. The distance between *LoxP* sites in the various targets tested may in part explain these differences. These distances are 0.45 kb for *Rb*, 2.5 kb for *R26R*, 6.6 kb for *Brca2* and 7.9 kb for *p53*. However, the distance between *loxP* sites cannot solely explain the differences in recombination frequency as we also found a tissue-specific effect on recombination efficiency of the *R26R* allele compared to the *Brca2*^{F11F} allele. Possibly these differences may be related to the expression level of the target gene or the local chromatin structure. *R26* and *Brca2* are both expressed in testes (Sharan and Bradley, 1997; Zambrowicz *et al.*, 1997). However, whereas *R26* is expressed ubiquitously, *Brca2* is only expressed in proliferating cells (Blackshear *et al.*, 1998). As these cells only constitute a fraction of the total number of cells used for the analysis, it is possible that recombination in *Brca2*-expressing cells is below the limit of our detection sensitivity. Further experiments are required to determine the relationship between target gene expression and Cre-mediated gene excision. Thus, not only are the expression characteristics an important variable in Cre-reporter strains, but also the susceptibility between different conditional alleles to be switched by Cre.

Finally, we have shown that in ES cells and primary keratinocytes complete control over gene switching can be exercised using *R26cre-ER*^{T}. This system has significant advantages, since experimental and control cells only differ in their exposure to OHT. This difference (i) abrogates the effect of experimental variation between independent isolates, (ii) eliminates the effect of genetic differences between isolates from non-inbred strains, (iii) ensures rapid and controlled gene-excision without the need for selection, and (iv) permits gene-inactivation in both dividing and non-dividing cells.

Together, our results show that the introduction of *cre-ER*^{T} in the *R26* locus results in non-variegated OHT-inducible Cre activity, yielding a mouse strain that is of more general use than the currently available *cre* transgenic mice.

METHODS

ROSA26 targeting. A 13 kb genomic *Xho*I–*Eco*RI fragment from the *ROSA26* locus was subcloned in the *Sal*I–*Eco*RI sites of a modified pBR322 vector. A *Nhe*I site was used to introduce a 1.6 kb PGK-Puromycin selection cassette (p*R26*-MCS13-*Pur*). A 2 kb *Hin*dIII–*Kpn*I fragment of pNPK*Cre*ER1c (Schwenk *et al.*, 1998) containing the *cre-ER*^{T} fusion gene was fitted with *Eco*RI sites and subcloned into the *Eco*RI site of a plasmid CAGGS containing the 3′ splice and polyadenylation signal from the rabbit β-globin gene (Niwa *et al.*, 1991). A 2.2 kb *Xba*I–*Nhe*I fragment containing the 3′ splice site, the *cre-ER*^{T} gene and the polyadenylation signal was inserted into the *Nhe*I site of p*R26*-MCS13-*Pur* to obtain the targeting vector *R26cre-ER*^{T}*-Pur*. The resulting targeting vector was linearized with *Sfi*I and purified by agarose gel electrophoresis.

Cell culture. All ES manipulations were performed as described previously (Clarke *et al.*, 1992; Vooijs *et al.*, 1998). E14 (129/Ola) ES cells were electroporated with *R26cre-ER*^{T}*-Pur* and selected for 1 week on gelatin-coated dishes in BRL-conditioned medium containing 1.8 μg/ml of puromycin. Clones were expanded in triplicate and 1/3 was used for DNA analysis, the remainder was frozen in liquid N_2. Homologous recombination at the *R26* locus was confirmed by Southern blot analysis using a probe (pHA607) located outside of the targeting vector. One clone (C2) was karyotyped and injected into recipient blastocysts to obtain chimeras and heterozygous *R26cre-ER*^{T} mice.

Primary mouse keratinocytes were isolated by trypsin flotation overnight at 4°C from newborn mice (Hennings *et al.*, 1980; Missero *et al.*, 1995). Cells were plated on mouse collagen type IV (1.5 μg/cm^2, Becton Dickinson Labware, MA) coated plastic dishes in EMEM containing 4% chelexed FBS (Gibco-BRL), 10 ng/ml EGF (Upstate Biotechnology, NY) and 0.2 mM $CaCl_2$. The next day cells were re-fed with the same medium containing only 0.045 mM $CaCl_2$. Cells were cultured for another 24 h before adding OHT (Sigma H-6278, 30 mM stock solution in 100% EtOH) to the medium. DNA isolation from cultured cells was as described below for tissue DNA.

Genotyping. Mice were genotyped by PCR analysis on tail tip DNA (Laird *et al.*, 1991). Genotyping of the *Brca2*^{F11F} was performed using a primer in intron 11 *int11F* (5′-CTCATCATTTGTTGCCTCACTTC-3′) and *int11R* (5′-TGTTGGATACAAGGCATGTACAC-3′) yielding products of 529 and 450 bp for the floxed and wildtype alleles, respectively. Deletion of *Brca2* was monitored using primers *int10F* (5′-GGCTGTCTTAGAACTTAGGCTG-3′) and *int11R*, yielding a 324 bp fragment. p53-*int10-fwd* (5′-AAGGGGTATGAGGGACAAGG-3′) and p53-*int10-rev* (5′-GAAGACAGAAAAGGGGAGGG-3′) primers were used to identify wildtype (391 bp) and *p53* floxed alleles (461 bp). *R26cre-ER*^{T} mice were screened with Cre1 (5′-GCACGTTCACCGGCATCAAC-3′) and Cre2 (5′-CGATGCAACGAGTGATGAGGTTC-3′). Genotyping of *Rb*^{F19F} mice (Vooijs *et al.*, 1998) and of *R26R* Cre-reporter strain was performed by PCR as described (Soriano, 1999).

Ligand administration. For i.p. injection (300 μl) of mice, OHT was dispersed in sunflower seed oil by sonication. For topical administration OHT was dissolved in DMSO at 100 μg/μl and applied to the shaved back of mice.

Tissue DNA/RNA analysis. For Southern blot analysis of tissue DNA, tissues were isolated, minced and digested in lysis buffer (Laird *et al.*, 1991). For the detection of *Brca2* deletion, DNA was digested with *Sac*I, separated by gel electrophoresis, transferred to Hybond N$^+$ (Amersham) and hybridized with an exon-14 probe. *p53* deletion was monitored using a *Bgl*II digest and the *Xba*I probe. Deletion of the *Rb* floxed and *R26R* alleles were monitored as described as described (Vooijs *et al.*, 1998; Soriano, 1999). For northern blot analysis poly(A)$^+$ RNA was isolated [Promega, poly(A)$^+$-tract system] from total tissue RNA (Trizol, Gibco-BRL) separated by gel electrophoresis, transferred to Hybond N$^+$ and hybridized to hGAPDH and Cre probes.

Probes. Southern analysis were performed for *Brca2* with a 255 bp probe generated by PCR with *ex14F* (5′-GCTTCTGTCTAAAGGGCATC-3′) and *ex14R* (5′-TCTTCCCTGTCTCCATCTG-3′) primers and for *p53* with a 700 nt *Xba*I fragment located ~1 kb upstream of *p53* exon-1. Deletion of the *Rb* floxed allele was detected with probe pHA153 (Clarke *et al.*, 1992; Vooijs *et al.*, 1998) and recombination at the *R26* locus was analyzed using as a probe a *Pst*I–*Sal*I fragment subcloned in pGEM11 and amplified by PCR using T3 and T7 primers. Cre expression was analyzed using a Klenow-labeled probe comprising the complete Cre open reading frame.

β-galactosidase staining. Tissues were fixed in 2% paraformaldehyde/PBS, equilibrated overnight in 30% sucrose/PBS at 4°C, frozen in OCT compound (Miles Scientific) and stored at –80°C. Sections, 15 μM, were air-dried for 20 min, postfixed in 0.2% paraformaldehyde, washed in PBS and stained with X-gal reaction buffer according to standard procedures (Akagi *et al.*, 1997).

ACKNOWLEDGEMENTS

We acknowledge Jan-Hermen Dannenberg and Hein te Riele (Division of Molecular Carcinogenesis, NKI, Amsterdam) for the *ROSA26* genomic clone, Jurjen Bulthuis for technical assistance, Phil Soriano (Fred Hutchinson, Seattle, WA) for reagents and *R26R* mice, Francis Stewart (EMBL, Heidelberg) for pNPKCreER1c and Erik Danen (Division of Cell Biology, NKI, Amsterdam) for critically reading the manuscript. This work was supported by the organization for Medical Research (M.V.) and the Dutch Cancer Society (J.J.).

REFERENCES

Akagi, K., Sandig, V., Vooijs, M., van der Valk, M., Giovannini, M., Strauss, M. and Berns, A. (1997) Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res.*, **25**, 1766–1773.

Blackshear, P.E. *et al*. (1998) Brca1 and Brca2 expression patterns in mitotic and meiotic cells of mice. *Oncogene*, **16**, 61–68.

Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J.L., Chambon, P. and Metzger, D. (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc. Natl Acad. Sci. USA*, **94**, 14559–14563.

Clarke, A.R., Robanus-Maandag, E., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A. and te Riele, H. (1992) Requirement for a functional *Rb-1* gene in murine development. *Nature*, **359**, 328–330.

Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K. and McMahon, A.P. (1998) Modification of gene activity in mouse embryos *in utero* by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.*, **8**, 1323–1326.

Dymecki, S.M. (1996) Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc. Natl Acad. Sci. USA*, **93**, 6191–6196.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996) Ligand-activated site-specific recombination in mice. *Proc. Natl Acad. Sci. USA*, **93**, 10887–10890.

Garrick, D., Fiering, S., Martin, D.I. and Whitelaw, E. (1998) Repeat-induced gene silencing in mammals. *Nature Genet.*, **18**, 56–59.

Gu, H., Zou, Y.R. and Rajewsky, K. (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell*, **73**, 1155–1164.

Henikoff, S. (1998) Conspiracy of silence among repeated transgenes. *BioEssays*, **20**, 532–535.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S.H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*, **19**, 245–254.

Kuhn, R., Schwenk, F., Aguet, M. and Rajewsky, K. (1995) Inducible gene targeting in mice. *Science*, **269**, 1427–1429.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R. and Berns, A. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.*, **19**, 4293.

Marino, S., Vooijs, M., van Der Gulden, H., Jonkers, J. and Berns, A. (2000) Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev.*, **14**, 994–1004.

Metzger, D. and Feil, R. (1999) Engineering the mouse genome by site-specific recombination. *Curr. Opin. Biotechnol.*, **10**, 470–476.

Missero, C., Calautti, E., Eckner, R., Chin, J., Tsai, L.H., Livingston, D.M. and Dotto, G.P. (1995) Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. *Proc. Natl Acad. Sci. USA*, **92**, 5451–5455.

Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**, 193–199.

O'Gorman, S., Fox, D.T. and Wahl, G.M. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science*, **251**, 1351–1355.

Porter, A. (1999) Controlling your losses: conditional gene silencing in mammals. *Trends Genet.*, **14**, 73–79.

Rajewsky, K., Gu, H., Kuhn, R., Betz, U.A., Muller, W., Roes, J. and Schwenk, F. (1996) Conditional gene targeting. *J. Clin. Invest.*, **98**, 600–603.

Robinson, S.P., Langan-Fahey, S.M., Johnson, D.A. and Jordan, V.C. (1991) Metabolites, pharmacodynamics, and pharmacokinetics of tamoxifen in rats and mice compared to the breast cancer patient. *Drug Metab. Dispos.*, **19**, 36–43.

Rohlmann, A., Gotthardt, M., Willnow, T.E., Hammer, R.E. and Herz, J. (1996) Sustained somatic gene inactivation by viral transfer of Cre recombinase. *Nature Biotechnol.*, **14**, 1562–1565.

Sauer, B. and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl Acad. Sci. USA*, **85**, 5166–5170.

Schwenk, F., Kuhn, R., Angrand, P.O., Rajewsky, K. and Stewart, A.F. (1998) Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res.*, **26**, 1427–1432.

Sharan, S.K. and Bradley, A. (1997) Murine Brca2: sequence, map position, and expression pattern. *Genomics*, **40**, 234–241.

Shibata, H. *et al*. (1997) Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science*, **278**, 120–123.

Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature Genet.*, **21**, 70–71.

Utomo, A.R., Nikitin, A.Y. and Lee, W.H. (1999) Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nature Biotechnol.*, **17**, 1091–1096.

Vasioukhin, V., Degenstein, L., Wise, B. and Fuchs, E. (1999) The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc. Natl Acad. Sci. USA*, **96**, 8551–8556.

Vooijs, M. and Berns, A. (1999) Developmental defects and tumor predisposition in Rb mutant mice. *Oncogene*, **18**, 5293–5303.

Vooijs, M., van der Valk, M., te Riele, H. and Berns, A. (1998) Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. *Oncogene*, **17**, 1–12.

Wang, Y., Krushel, L.A. and Edelman, G.M. (1996) Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. *Proc. Natl Acad. Sci. USA*, **93**, 3932–3936.

Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., Kerr, W.G. and Soriano, P. (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of β-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl Acad. Sci. USA*, **94**, 3789–3794.

DOI: 10.1093/embo-reports/kve064