Nucleic Acids Research, 1997, Vol. 25, No. 21 **4323–4330**

Cre-mediated gene deletion in the mammary gland

Kay-Uwe Wagner, Robert J. Wall[2], Luc St-Onge[5], Peter Gruss[5], Anthony Wynshaw-Boris[1], Lisa Garrett[1], Minglin Li[3], Priscilla A. Furth[3,4] and Lothar Hennighausen*

Laboratory of Metabolism and Biochemistry, National Institute of Diabetes Digestive and Kidney Diseases and [1]Laboratory of Genetic Disease Research, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA, [2]United States Department of Agriculture, Beltsville, MD 20725, USA, [3]Division of Infectious Diseases, Department of Medicine, University of Maryland Medical School and the Institute of Human Virology and [4]Department of Physiology, University of Maryland Medical School, Baltimore, MD 21201, USA and [5]Department of Molecular Cell Biology (Abteilung 160), Max-Planck Institute for Biophysical Chemistry, Am Fassberg, Göttingen 37018, Germany

Received July 1, 1997; Revised and Accepted September 4, 1997

ABSTRACT

To delete genes specifically from mammary tissue using the Cre–*lox* system, we have established transgenic mice expressing Cre recombinase under control of the *WAP* gene promoter and the MMTV LTR. Cre activity in these mice was evaluated by three criteria. First, the tissue distribution of Cre mRNA was analyzed. Second, an adenovirus carrying a reporter gene was used to determine expression at the level of single cells. Third, tissue specificity of Cre activity was determined in a mouse strain carrying a reporter gene. In adult MMTV–*Cre* mice expression of the transgene was confined to striated ductal cells of the salivary gland and mammary epithelial cells in virgin and lactating mice. Expression of *WAP–Cre* was only detected in alveolar epithelial cells of mammary tissue during lactation. Analysis of transgenic mice carrying both the MMTV–*Cre* and the reporter transgenes revealed recombination in every tissue. In contrast, recombination mediated by Cre under control of the *WAP* gene promoter was largely restricted to the mammary gland but occasionally observed in the brain. These results show that transgenic mice with *WAP–Cre* but not MMTV–*Cre* can be used as a powerful tool to study gene function in development and tumorigenesis in the mammary gland.

INTRODUCTION

The Cre–*lox* recombination system has the potential to become a powerful tool for the conditional and cell-specific deletion of genes. The introduction of this system into transgenic mice should facilitate studies on the loss of function of genes in a particular cell type. The Cre recombinase from bacteriophage P1 (1) excises intervening DNA sequences located between two unidirectional *lox* sites positioned on the same linear DNA segment, leaving one *lox* site behind. Since the first introduction of the Cre–*lox* system into the eukaryotic genome (2) this excisive feature of the Cre recombinase has been used successfully to eliminate endogenous genes or to activate transgenes in mammalian cell culture systems as well as transgenic mouse models (3–6). Through insertion of *lox* sites via homologous recombination into the gene of interest and targeting Cre recombinase expression to a specific cell type using a tissue-specific promoter it will be possible to introduce predetermined deletions into the mammalian genome.

Several genes have been implicated in breast development and cancer. However, in many cases the unequivocal evaluation of these genes in breast function has been impeded because their deletion from the genome resulted in embryonic lethality or widespread physiological consequences (7,8). Therefore, application of the Cre–*lox* recombination system to inactivate genes, such as *brca*1 and *brca*2, in mammary epithelial cells appears to be an attractive method. The generation of transgenic mice expressing the Cre enzyme in mammary tissue is a critical and essential step towards the goal of deleting genes specifically from the mammary gland. In this study we have generated and evaluated transgenic mice carrying the *Cre* gene under the control of two different regulatory elements which target gene expression to the mammary gland, the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and the whey acidic protein (WAP) gene promoter. The use of these two promoters in combination with Cre recombinase also provided new insights into the regulation of these DNA elements during ontogenesis, since low expression and past events of Cre expression can be detected by recombination.

MATERIALS AND METHODS

Cre expression vectors

Two *Cre* expression vectors were generated. The *Cre* gene was placed under control of the MMTV LTR and the mouse *WAP* gene promoter. The 2.6 kb *WAP* promoter was isolated as an *Eco*RI–*Asp*718 restriction fragment (9,10) and the MMTV LTR

*To whom correspondence should be addressed. Tel: +1 301 496 2716; Fax: +1 301 496 0839; Email: mammary@nih.gov

was isolated as a 1.1 kb *Hin*dIII fragment (11). The vector containing the *Cre* gene, β-globin intron and poly(A) signal has been described earlier (6). The *Xho*I *tetop* fragment from the *tetop–Cre* vector (6) was deleted, the restriction site was filled in using T4 polymerase and the blunt-ended *WAP* gene promoter or MMTV LTR was inserted. Diagrams of the *Cre* transgenes are shown in Figure 1A.

In vitro expression of *Cre* and recombination analysis

HC11 cells (12) were used to analyze the integrity and functionality of the *WAP–Cre* and MMTV–*Cre* expression vectors. HC11 cells were maintained in RPMI-1640 medium (Biofluids) in the presence of 10% fetal calf serum (Gibco BRL) supplemented with 5 µg/ml insulin (Sigma), 10 ng/ml EGF (Sigma) and 50 µg/ml gentamycin (Sigma). To express *Cre* transiently under control of the *WAP* gene promoter or MMTV LTR, electrotransformations were performed on 1×10^6 cells using 10 µg *WAP–Cre* or MMTV–*Cre* plasmid and 10 µg salmon sperm DNA as carrier. After transformation the cells were grown to confluency for 2 days in the medium as described above. Expression of the *WAP–Cre* gene was induced in RPMI-1640 medium containing 2% fetal calf serum, 5 µg/ml insulin (Sigma), 5 µg/ml ovine prolactin (Sigma), 0.1 µM dexamethasone (Sigma) and 50 µg/ml gentamycin but no EGF. Treatment with lactogenic hormones was not essential for induction of the MMTV–*Cre* construct. After 3 days maintenance in induction medium the cells were either harvested for RNA preparation or infected for 4 h with an adenovirus reporter construct (13). Three days after infection the cells were fixed and stained as described earlier (14).

Propagation and maintenance of adenovirus

The adenovirus reporter vector (AxCALNLZ) used in this study for HC11 *in vitro* assay and injection into organs of *Cre* transgenic mice was generously provided by Dr Izumo Saito (13). The viral particles were propagated and scaled up in 293A cells according to a protocol from Quantum Biotechnologies Inc. (Canada). HC11 cells were infected with filtered crude viral lysate (~10^8 viral particles/ml). For *in vivo* transfer to the salivary and mammary gland the crude viral lysate was purified using standard CsCl gradient centrifugation. The final concentration was 10^{12} viral particles/ml as determined by spectrophotometry.

Generation of transgenic mice

The MMTV–*Cre* insert was released from the vector as a 3.1 kb *Aat*II–*Pfi*mI fragment. To isolate the *WAP–Cre* transgene we had to convert the *Nde*I site at position 1391 into a *Not*I site by partial digestion with *Nde*I, fill in with T4 polymerase and ligation of an oligonucleotide (5′-GTA CGC GGC CGC GTA C-3′), creating a new *Not*I restriction site. The 4.5 kb *WAP–Cre* transgene was then separated from the vector with *Aat*II and *Not*I and isolated from an agarose gel using a Qiagen gel purification kit.

The DNA fragments were injected into fertilized oocytes at a concentration of ~5–10 ng/µl. Transgenic mice were generated according to standard procedures and founder mice were detected by PCR. The MMTV–*Cre* transgene was identified by PCR analysis of tail tip DNA using primers corresponding to the MMTV LTR from –112 to –94 (5′-CTG ATC TGA GCT CTG AGT G-3′) and the sequence annealing to the *Cre* gene (5′-CAT CAC TCG TTG CAT CGA CC-3′). The resulting PCR product was 250 bp in size. Incorporation of the *WAP–Cre* transgene into the mouse genome was detected using a primer corresponding to the *WAP* promoter at position –88 to –68 (5′-TAG AGC TGT GCC AGC CTC TTC-3′) and the same *Cre* primer as described above. The resulting PCR product was 210 bp long.

Six MMTV–*Cre* founder mice tested positive by PCR for transgene integration into the genome. Unfortunately, one female founder was sterile and another one died just before delivering her first litter. Hence, four lines of transgenic mice carrying the MMTV–*Cre* transgene were analyzed. Regarding the *WAP–Cre*, construct 15 founder mice were identified. From those, 10 lines were established and eight examined in detail.

RNA preparation and RT-PCR

Total RNA was isolated from HC11 cells and tissues of adult Cre mice according to Chomczynski and Sacchi (15). One microgram of RNA was reverse transcribed using MLV reverse transcriptase (Gibco) and an oligo($dT_{12–18}$) primer. An aliquot of 10 µl RT reaction was used in a 100 µl PCR. Thirty cycles of PCR were performed in the presence of two specific primers (see Fig. 1) from the coding region of *Cre* (5′-GCC AGC TAT CAA CTC GCG CCC-3′) and downstream of the β-globin intron (5′-CCT TCT GAT AGG CAG CCT GCA CC-3′). To verify the specificity of the fragments, part of the amplified DNA was digested with *Ssp*I, producing two expected smaller fragments of 175 and 190 bp. The RT-PCR products were separated on a 2% agarose gel. A 365 bp band represented the spliced Cre mRNA and a 936 bp indicated unspliced mRNA.

In vivo functional assay of Cre recombinase using an adenovirus reporter

The adenovirus reporter construct (AxCALNLZ) contains promoter elements from chicken β-actin and regulatory sequences from the human cytomegalovirus (CMV) and a nuclear β-galactosidase gene (*lacZ*) (13). It is not the main strategy of adenovirus to stably integrate its viral DNA into the host cell genome, therefore, the chimeric promoter can be transiently activated at high levels in almost all cell types. The promoter sequences are separated from *lacZ* by transcriptional stop sequences flanked by two directly orientated *lox* sites. The construct can be activated in the presence of a functional Cre protein resulting in excision of the stop signal, which then leads to transcription of *lacZ*. The nuclear localization signal subsequently directs the β-galactosidase into the cell nucleus. β-Galactosidase activity in the nucleus and some in the cytoplasm can be visualized by X-gal staining. This functional analysis for Cre expression is very sensitive because it allows detection of very low levels of Cre expression.

About 5×10^8 particles were given in one application per gland. The mice were anesthetized and the viral particles delivered to the targeted organ by injection directly into the salivary gland or mammary gland respectively. For infection of the mammary gland only one inguinal gland (number 4) was injected and the other gland was used as an uninjected internal control for the X-gal assay.

Three days after delivery of the reporter construct the mice were killed by cervical dislocation and salivary and injected and uninjected mammary glands were removed and cut into 5 mm^3 cubes. The tissues were fixed for 1.5 h in 2% paraformaldehyde and 0.02% glutaraldehyde in PBS and X-gal staining was performed as described earlier (14).

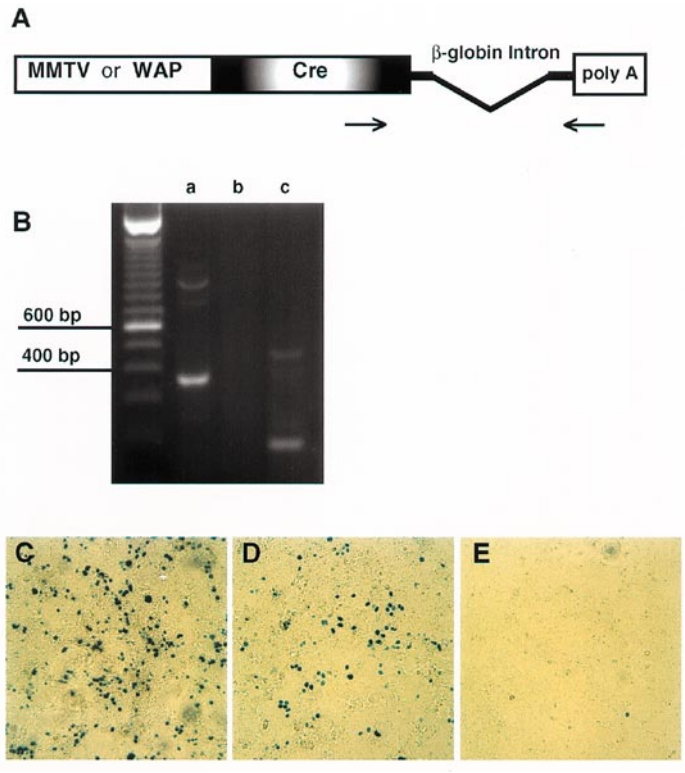

Figure 1. (**A**) Structure of the MMTV–*Cre* or *WAP–Cre* transgene. The MMTV LTR or *WAP* promoter is linked to the coding sequence of the *Cre* gene followed by an intron and a polyadenylation site from the rabbit β-globin gene. Arrows represent the primers used for RT-PCR assay. (**B**) Functional analysis of the MMTV–*Cre* transgene in HC11 cells using RT-PCR assay. (a) A 365 bp band represents the spliced Cre mRNA and a 936 bp indicates unspliced mRNA. (b) Control without reverse transcriptase. (c) Control digest of the amplified cDNA fragment with *Ssp*I. (C–E) *In vitro* functional analysis of the MMTV–*Cre* and *WAP–Cre* transgenes in HC11 cells using an adenovirus reporter vector (AxCALNLZ). (**C**) Cells transiently expressing MMTV–*Cre* were infected with AxCALNLZ and stained with X-gal. (**D**) HC11 cells expressing *WAP–Cre* were infected with AxCALNLZ and stained with X-gal. (**E**) X-Gal staining after electroporation with salmon sperm DNA and infection with AxCALNLZ.

Analysis of recombination in double transgenic mice carrying a reporter transgene and either MMTV–*Cre* or *WAP–Cre*

Mice from *WAP–Cre* and MMTV–*Cre* lines were crossed with a reporter strain carrying a CMV *lox*–stop–*lacZ* (*lox*–stop) transgene (6). Cre-mediated recombination in double transgenic mice was detected by PCR analysis using primers located in the CMV promoter (5′-GTA GGC GTG TAC GGT GGG AGG-3′) and the nuclear localization signal of *lacZ* (5′-CGG GAT CCC CCA TGC TCC CC-3′). The 1719 bp PCR fragment represents the sequence from unrecombined copies and a 344 bp fragment tags recombined copies of the reporter gene. The accuracy of the sequence of the recombined DNA segment containing the remaining *lox* site was verified by sequencing using the Perkin Elmer Cycle Sequencing protocol.

RESULTS

Analysis of the MMTV–*Cre* and *WAP–Cre* hybrid genes in HC11 cells

Integrity of the MMTV–*Cre* and *WAP–Cre* hybrid genes (Fig. 1A) was evaluated in the murine mammary epithelial cell line HC11. The cells were transfected with plasmids containing the MMTV–*Cre* and *WAP–Cre* transgenes respectively. Expression was analyzed at the level of RNA by RT-PCR and in a functional assay by infection of the HC11 cells with an adenovirus bearing a reporter transgene (13). RNA from the MMTV–*Cre*- and *WAP–Cre*-transfected HC11 cells was analyzed in a RT-PCR assay for the presence of mature Cre mRNA. After reverse transcription with MLV reverse transcriptase using oligo($dT_{12–18}$) primers the cDNA was amplified with primers spanning the globin intron and flanking sequences (Fig. 1A). A 365 bp fragment, corresponding to the predicted size of the mature Cre mRNA after excision of the β-globin intron, was detected (Fig. 1B). The faint fragment of 936 bp represents low levels of unspliced RNA. Additionally, the accuracy of the RT-PCR product was confirmed by *Ssp*I digestion (Fig. 1B). The results of the RT-PCR analysis demonstrate that the majority of the RNAs expressed from the MMTV–*Cre* and *WAP–Cre* transgenes were processed correctly.

Cre activity was evaluated with an adenovirus containing a *lacZ* reporter gene which had been inactivated through insertion of a *lox*–stop sequence. HC11 cells transfected with either the MMTV–*Cre* or *WAP–Cre* hybrid gene were infected with adenovirus bearing the reporter gene and cells were stained *in situ* for β-galactosidase activity (Fig. 1C–E). As expected, the MMTV–*Cre* gene exhibited higher expression than the *WAP–Cre* gene (Fig. 1C and D). Only one blue nucleus in more than 1 000 000 were detected in HC11 cells which had been electroporated with salmon sperm DNA only and infected with the adenovirus reporter (Fig. 1E). These results confirmed that

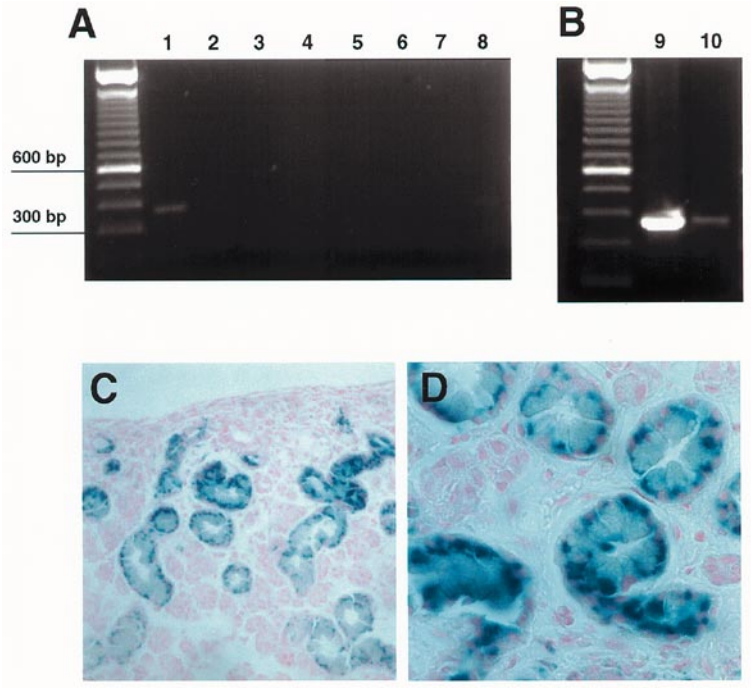

Figure 2. (**A** and **B**) Expression analysis of MMTV–*Cre* transgenic mice using RT-PCR. (**A**) MMTV–*Cre* expression was detected in the salivary gland (lane 1). The other organs are muscle (lane 2), liver (lane 3), heart (lane 4), kidney (lane 5), spleen (lane 6), tail (lane 7) and brain (lane 8). (**B**) MMTV–Cre mRNA was present in the virgin (lane 9) and lactating (lane 10) mammary gland. (**C** and **D**). X-Gal staining of striated ductal cells in the salivary gland expressing MMTV–*Cre* after infection with AxCALNLZ. (C) 200×; (D) 630×.

both the MMTV–*Cre* and *WAP–Cre* hybrid genes were functional and encoded active Cre.

Generation and analysis of transgenic mice carrying the MMTV–*Cre* transgene

Four lines of MMTV–*Cre* mice and eight lines of *WAP–Cre* mice were analyzed. Activity of the transgenes was evaluated on three levels. First, transcription of the MMTV–*Cre* and *WAP–Cre* transgenes was analyzed by RT-PCR. Second, functional analysis of Cre recombinase was assayed by injection of the adenovirus reporter into tissues. Finally, the activity of Cre was examined in a recombination assay by crossing MMTV–*Cre* and *WAP–Cre* lines into a reporter strain carrying a *lox*–stop transgene (6). Expression of the MMTV–*Cre* transgene in tissues of adult mice was evaluated by RT-PCR. Mature Cre RNA was detected in the salivary gland (Fig. 2A) of both sexes and in mammary tissue of virgin and lactating females (Fig. 2B). To determine Cre activity at the level of single cells we injected reporter adenoviral particles into the salivary and mammary glands. Since adenovirus infects all cell types, this strategy can be used to identify those cell types which express Cre recombinase. Nuclear X-gal staining was detected in striated ducts of the salivary gland (Fig. 2C and D) and mammary epithelial cells (not shown). These results are in agreement with our earlier studies that the MMTV LTR specifically activates transcription in striated ductal cells (14,16). No β-galactosidase expression was observed in the absence of the MMTV–*Cre* transgene (not shown).

The results of the RT-PCR and functional analysis using an adenovirus reporter indicate that the MMTV LTR directs expression of Cre recombinase in the secretory epithelium of adult mice, including the desired target organ, the mammary gland. However, these assays only display Cre activity in a narrow window during development. In mice carrying floxed alleles and a *Cre* transgene, the floxed allele will be subject to recombination because of Cre activity throughout development. Since the temporal and spatial activity pattern of the MMTV LTR is not known, we performed a recombination assay by crossing the MMTV–*Cre* lines into a reporter strain carrying a *lox*–stop transgene. The basic principle of this experiment is the same as the adenovirus approach, but X-gal staining was not successful because of insufficient activity of the HCMV IE1 gene enhancer when stably integrated into the genome (17). Recombined transgenes were identified by PCR using primers which flank the stop sequence, resulting in a 1719 bp fragment for non-recombined copies and a 344 bp fragment for recombined copies of the transgene when the stop sequence was deleted (Fig. 3A). Recombination of the *lox*–stop transgene was observed in all tissues examined (Fig. 3B). This indicates that the MMTV–*Cre* transgene is transcribed at some point early in development or at low levels constitutively. Recombination and accurate deletion of the *lox*–stop sequence was verified by sequencing (data not shown). We observed the same recombination event in all four MMTV–*Cre* lines, indicating that this *Cre* expression pattern is not the result of integration of the transgene into a specific locus. This expression event is not effected by the β-globin intron of the transgene. As shown later, *WAP–Cre* mice with the same intron show a different expression pattern.

To test whether *Cre* expression also occurs in the female germline, we crossed double transgenic females with non-transgenic mice and analyzed DNA taken from tail biopsies of the progeny. Offspring which carry only the reporter transgene exhibited extensive

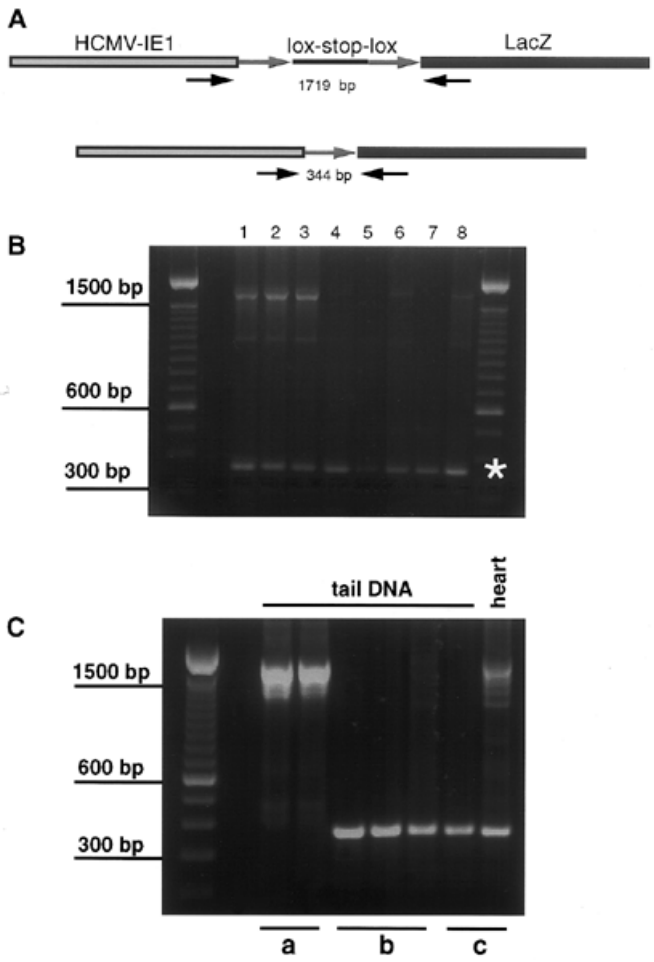

Figure 3. (**A**) Structure of the *lox*–stop reporter transgene. The CMV promoter and β-galactosidase coding sequences are separated by a floxed transcriptional stop sequence. Arrows represent primers used in PCR recombination analysis. The PCR product before recombination is 1719 bp in size. Cre-mediated recombination will excise the stop sequence, resulting in a 344 bp PCR fragment. (**B**) PCR recombination analysis in mice carrying MMTV–*Cre* and the *lox*–stop transgene. Deletion of transcriptional stop sequences of the reporter transgene were detected in all tissues: (lane 1) mammary gland, (lane 2) lung, (lane 3) heart, (lane 4) salivary gland, (lane 5) kidney, (lane 6) brain, (lane 7) spleen and (lane 8) muscle. (**C**) Transmission of the recombined *lox*–stop reporter transgene in the female germline. (a) Control PCR in the parental generation of the reporter strain. (b) Recombination was detected in tail DNA of mice carrying only the reporter transgene when they derived from a double transgenic parent bearing both the MMTV–*Cre* and the reporter construct. (c) Recombination analysis in a double transgenic mouse carrying the MMTV–*Cre* and *lox*–stop construct.

recombination (Fig. 3C), indicating that MMTV–*Cre* expression also occurred in the female germline.

The *WAP* promoter targets Cre recombinase to mammary epithelial cells

Expression of the *WAP–Cre* transgene in tissues of adult mice was initially analyzed by RT-PCR. From eight *WAP–Cre* lines analyzed, Cre RNA was detected exclusively in mammary tissue of one line. Expression was restricted to the mammary glands of late pregnant (Fig. 4A, lane 6) and lactating mice (Fig. 4B, lane 2). No expression was detected in mammary tissue from virgin mice (Fig. 4B, lane 1) or in any other organ, including the salivary gland (Fig. 4A, lane 5). Hence, activation of the transgene parallels expression of the endogenous *WAP* gene.

To verify that expression of the *WAP–Cre* transgene was restricted to the secretory epithelium of the mammary gland, the adenovirus reporter was injected into one number 4 gland of a lactating mouse. The β-galactosidase assay was performed 3 days later and the non-injected number 4 gland served as an internal control. The results shown in Figure 4C and D demonstrate that the *WAP* gene promoter directed expression of *Cre* exclusively to epithelial cells. No staining was observed in the fat cells. No blue staining of cell nuclei was observed in the absence of the adenovirus reporter (Fig. 4E).

To evaluate cumulative Cre activity and to address whether the *WAP–Cre* transgene was active during development in tissues other than the mammary gland, mice carrying both the *WAP–Cre* and the *lox*–stop transgenes were analyzed. In pregnant females recombination was observed in mammary tissue but not in other organs (Fig. 5A). Occasionally, Cre-mediated recombination was detected in brain (Fig. 5B), but not in testes. Germline recombination was never observed in either sex (data not shown). Sequence analysis of the PCR fragment confirmed the recombination event (data not shown).

Several lines did not exhibit *Cre* expression in mammary tissue. Recombination analysis of other tissues in these lines revealed a line-specific expression pattern (Table 1). Clearly, the *WAP–Cre* transgene can be activated in other tissues depending on the site of integration.

Table 1. Expression of the WAP–*Cre* transgene in eight different mouse lines

Line	Mammary gland	Brain	Testes	Muscle
11738	E	E	no	no
11743	no	no	E	no
11748	no	no	E	no
11763	no	no	no	no
11854	no	E	no	E
11858	no	no	E	no
11863	no	no	n.t.	n.t.
11886	no	no	E	E

E, *Cre* expression was detected by recombination analysis in mice carrying WAP–*Cre* and *lox*–stop transgenes; no, no expression observed, n.t., not tested.

WAP–Cre-mediated recombination during different stages of mammary development

The value and eventual application of Cre-mediated deletion of genes in specific tissues is partly dependent on the time point in the development of the organ at which a gene can be deleted. We therefore determined at which stage of mammary development the *WAP–Cre* transgene was active. No recombination was detected in mammary tissue of virgin mice (Fig. 5C). Low level recombination was detected at day 14 of pregnancy and increased during lactation. Cells which had undergone recombination were detected after 10 days involution, when the vast majority of epithelial cells have undergone apoptosis and the gland has been remodeled. Recombined copies were still present at 30 days after weaning the litter (data not shown). This suggests that the transgene had been

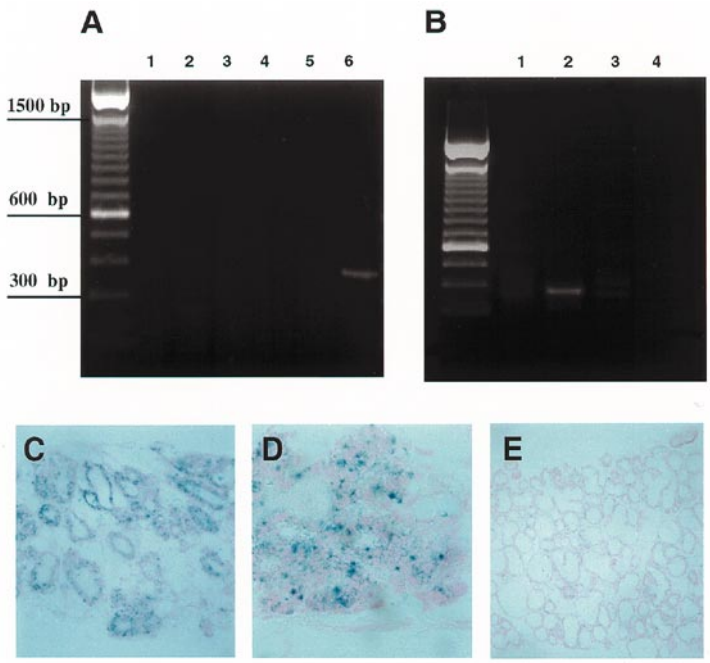

Figure 4. (**A** and **B**) Expression analysis of different tissues from *WAP–Cre* transgenic mice using RT-PCR. (**A**) *WAP–Cre* mRNA was detected in the mammary gland (lane 6) at day 18 of gestation, but not in liver (lane 1), heart (lane 2), kidney (lane 3), brain (lane 4) or salivary gland (lane 5). (**B**) *WAP–Cre* message was present in the lactating mammary gland (lane 2), but absent in the gland of a virgin mouse (lane 1). Lanes 3 and 4 are controls without reverse transcriptase. (**C** and **D**) X-Gal staining of mammary epithelial cells in gland number 4 of mice expressing *WAP–Cre* after infection with AxCALNLZ. (C) 200×; (D) 630×. (**E**) The uninfected gland number 4 of the same mouse did not show specific β-galactosidase activity. 200×.

active in putative stem cells. This is supported by the higher recombination rates in the second pregnancy (Fig. 5C).

DISCUSSION

The deletion of genes from embryonic stem cells by homologous recombination is a useful tool to study gene function *in vivo*. However, understanding gene function in a particular cell type may be compromised by the fact that the respective gene has been deleted from the germline and is therefore absent from all somatic cells. Inactivation of a gene can also result in embryonic lethality, which in turn prohibits functional studies in adult organs. Alternatively, it may be impossible to link complex phenotypes to a particular cell type. Introduction of the Cre–*lox* recombination system has the potential to alleviate many problems associated with the germline deletion of genes. While application of the Cre–*lox* recombination system in mouse genetics has been a milestone, further developments are required to make it widely applicable. First, it is necessary to direct *Cre* expression to specific tissues and defined cell types. Second, it is essential to express *Cre* in the majority of a given cell type and to control temporal activation. Third, it is necessary to minimize cumulative background activity throughout an animal's life. The latter point is of renewed interest, because recent experiments with 'tissue-specific' promoters have revealed that cumulative Cre activity in ectopic tissues can result in a widespread deletion of sequences flanked by *lox* sites.

The mammary gland provides an attractive developmental system because this organ is formed to a large extent in the adult organism. From a societal viewpoint, breast cancer is a major health problem. Hence, major efforts have focused on the biology of the mammary gland. However, many genes suspected to be important for breast development and cancer are also critical for embryonic development and their deletion from the mouse genome results in prenatal lethality (7,8). Therefore, it becomes increasingly important to generate mice in which *Cre* is expressed preferentially or exclusively in mammary tissue. Towards this goal we have generated and evaluated transgenic mouse strains in which Cre recombinase has been targeted to mammary tissue using the MMTV LTR and the mouse *WAP* gene promoter. We have focused on three questions. First, can *Cre* be expressed specifically in mammary epithelial cells of adult mice? Second, are the MMTV–*Cre* and *WAP–Cre* transgenes active at some point of mouse development which would lead to widespread Cre-mediated recombination in tissues other than the mammary gland? Third, can Cre activity in mammary tissue be obtained in appropriate time windows? The results of this study demonstrate that recombination in *WAP–Cre* mice is confined to mammary tissue of pregnant and lactating mice. In contrast, *Cre* expression under control of the MMTV LTR results in widespread recombination in many tissues.

WAP versus MMTV

Tissue specificity in adult animals. Both the *WAP* gene promoter and the MMTV LTR have been used successfully to activate transgenes in mammary tissue (14,18–22). The *WAP* gene promoter reliably targets gene expression to mammary tissue and expression in other tissues is normally orders of magnitude lower than in the breast (19). Expression of transgenes under control of the MMTV LTR is high in mammary tissue of pregnant and lactating females, but generally is also found in other secretory

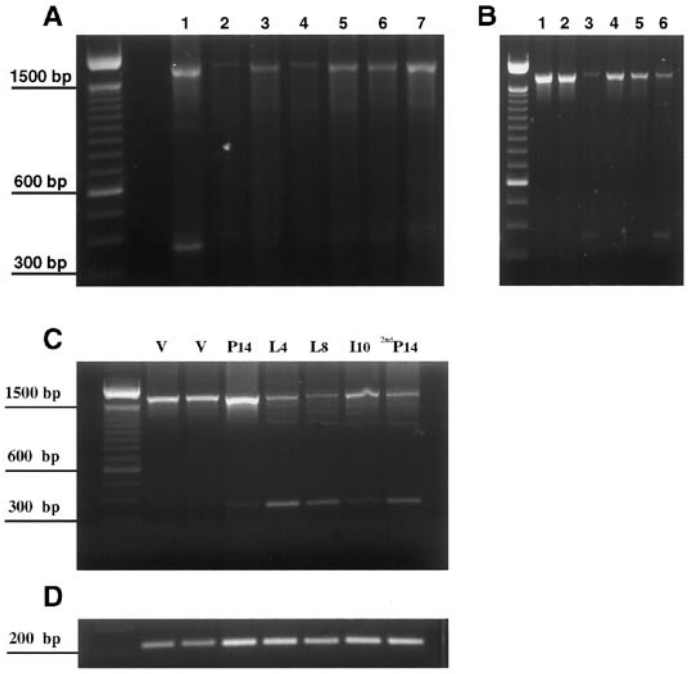

Figure 5. PCR recombination analysis in mice carrying the *WAP–Cre* and *lox*–stop transgenes. (**A**) Tissues from a female double transgenic mouse at day 18 of gestation. Recombination was observed in the mammary gland (lane 1), but not in DNA from tail (lane 2), liver (lane 3), heart (lane 4), kidney (lane 5), salivary gland (lane 6) or muscle (lane 7). (**B**) Selected tissues from two male double transgenic mice (lanes 1–3 and 4–6). Some Cre-mediated recombination was detected in brain (lanes 3 and 6), but not in testis (lanes 2 and 5) or tail DNA (lanes 1 and 4). (**C**) PCR recombination analysis during stages of mammary gland development in mice carrying the *WAP–Cre* and *lox*–stop transgenes. v, virgin; P, pregnant or days of gestation; L, days of lactation; I, involution or days after removing the litter; 2ndP, days of gestation during second pregnancy. (**D**) PCR detection of the presence of the *WAP–Cre* transgene.

organs, such as the salivary gland, the Harderian gland, seminal vesicles and lymphoid cells (20–22). We evaluated *Cre* expression in adult mice at both the levels of RNA and functionality using an adenovirus *lox*–stop reporter. The latter approach is fast and reliable and identifies cell types in which *Cre* is expressed. In adult MMTV–*Cre* mice *Cre* expression and activity was detected in the salivary and mammary glands. Expression in salivary tissue was confined to the striated ducts, which is in agreement with earlier studies that the MMTV LTR targets gene expression to this particular cell type (16). In *WAP–Cre* mice expression was confined to the mammary gland.

Cumulative and background expression. The analysis of mice carrying both the MMTV–*Cre* and the *lox*–stop transgenes permitted us to evaluate *Cre* expression that had occurred at any time prior to harvesting the tissues. Clearly, Cre-mediated recombination was detected in every single tissue including germ cells of mice carrying the MMTV–*Cre* transgene. In contrast, recombination in mice carrying the *WAP–Cre* and the *lox*–stop transgenes was confined to mammary tissue and to some extent brain. These results suggest that the MMTV LTR is active at an early embryonic stage or at low levels in many tissues. Since MMTV–*Cre*-mediated gene deletion is widespread, these animals may be of limited use to study gene function in the mammary gland. However, they may be of use to delete genes overall in a mosaic fashion. In addition, they may provide an alternative for deletion of genes in the germline, as described for CMV–*Cre* (23) and ZP3–*Cre* mice (24). Unfortunately, the HCMV–*lacZ* transgene in the reporter mice does not permit an analysis at the level of single cells. The HCMV enhancer is expressed at only very low levels when integrated as a transgene and it is virtually inactive in mammary tissue (25,26).

While the standard Cre–*lox* recombination system will struggle with background recombination, in combination with a time-sensitive gene expression approach, such as that using tetracycline-based transactivator protein (6), it should solve some of the problems. In that particular case the *Cre* gene would be under control of *tetop* sequences and activation would occur only after withdrawal of tetracycline (6). An obvious drawback of this approach would be the extensive breeding scheme to create mice carrying two transgenes, a null allele and a floxed allele.

Temporal activation. Depending on the gene under investigation, deletion may be required at various time points during mammary gland development. Since the majority of alveolar proliferation and differentiation occurs between days 10 and 18 of pregnancy, it is desirable to obtain mice which express *Cre* in this time window. High level expression of the endogenous *WAP* gene is confined to the last few days of pregnancy and lactation. Our experiments demonstrate that extensive recombination in mammary tissue is already present on day 14 of pregnancy, thus indicating robust activity of the *WAP–Cre* transgene at or before this time point. Precocious activation of *WAP* transgenes has also been observed in other studies (19,27,28). From our PCR results it is not clear whether the unrecombined transgenes originate from stromal cells which are not targeted by the *WAP* gene promoter or partially from epithelial cells. Based on the expression pattern of both the endogenous *WAP* gene and several *WAP*-based transgenes it is clear that transcriptional activity in mammary epithelial cells can be obtained at every stage of mammary development. *WAP* gene

activity has been seen transiently during the estrus cycle and heterogeneous expression is predominant throughout pregnancy (29). Homogeneous high level expression is confined to the lactational stage (29). Hence, it is not clear yet whether it is possible to achieve gene deletion during pregnancy in every single secretory cell. While this could affect interpretation of experiments which address the function of genes mediating mammary epithelial cell development and differentiation, this may not be a realistic scenario in experiments designed to inactivate tumor suppressor genes. In some experimental settings it is necessary to study tumor suppressor genes and tumor formation in the absence of pregnancy. This will also be possible using the *WAP–Cre* transgenic approach since the *WAP* gene promoter is active transiently during the estrus cycle (29).

The ratio of recombined to unrecombined genes increased during lactation, suggesting that increasing Cre activity resulted in more extensive recombination. Importantly, cells with recombined *lox*–stop alleles persisted even after 10 and 30 days involution. At this time terminal differentiated epithelial cells have died and the gland has been remodeled. It is conceivable that the transgene has been active in mammary stem cells which are retained after complete involution. The view that the *WAP–Cre* transgene is expressed in stem cells or lobulo-alveolar progenitor cells is supported by the finding that recombination during a second pregnancy is more extensive. As discussed previously (6), the PCR recombination assay may be a more qualitative than quantitative estimation of recombination. However, our experiments with adenovirus demonstrate that a large number of cells express active Cre. Southern blot analysis would greatly underestimate the efficiency of recombination in epithelial cells since effects of a variable amount of different cell types in the mammary gland (fat cells, fibroblasts, lymphocytes, ductal and myoepithelial cells, etc.) in conjunction with differences in the copy number of the reporter transgene between recombined epithelial cells and non-recombined stroma cells cannot be separated. The large number of *lacZ*-positive epithelial cells after injection of the reporter virus indicate that *Cre* is expressed during pregnancy and lactation in most if not all of the epithelial cells.

Use of adenovirus to evaluate mice carrying *Cre* transgenes

The results of our study have demonstrated that adenovirus can infect mammary and salivary epithelial cells. This experimental approach is not only a new and efficient way of identifying those mouse lines which express the *Cre* transgene, but it can also be used for additional applications. For example it should be possible to transiently overexpress specific proteins (oncogenes, dominant negative regulatory proteins or antisense transcripts). Since the adenovirus contains an inactive gene to be analyzed, the *Cre* expression pattern will determine in which tissue the inactive gene is processed and activated.

ACKNOWLEDGEMENTS

The authors would like to thank Mark Spencer (USDA) for DNA microinjection, Theresa Hernandez (NCHGR) for animal husbandry and technical assistance and Brian O'Connell and Beverely Handelman (NIDR) for introducing us to the preparation of recombinant adenovirus. We also thank Gertraud Robinson and Xiuwen Liu for stimulating discussions and reading of the manuscript and Edmund Rucker for technical help. This work was supported in part by a German Academic Exchange Service (DAAD) fellowship and through funding by the DFG (WA-1119/1-1) to KUW. LS-O was supported by the National Cancer Institute of Canada.

REFERENCES

1 Sternberg,N. and Hamilton,D. (1981) *J. Mol. Biol.*, **150**, 467–486.
2 Sauer,B. (1987) *Mol. Cell. Biol.*, **7**, 2087–2096.
3 Orban,P.C., Chui,D. and Marth,J.D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6861–6865.
4 Lakso,M., Sauer,B., Mosinger,B.,Jr., Lee,E.J., Manning,R.W., Yu,S.H., Mulder,K.L. and Westphal,H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6232–6236.
5 Kuhn,R., Schwenk,F., Aguet,M. and Rajewsky,K. (1995) *Science*, **269**, 1427–1429.
6 St-Onge,L., Furth,P.A. and Gruss,P. (1996) *Nucleic Acids Res.*, **24**, 3875–3877.
7 Hakem,R., de la Pompa,J.L., Sirard,C., Mo,R., Woo,M., Hakem,A., Wakeham,A., Potter,J., Reitmair,A., Billia,F., *et al.* (1996) *Cell*, **85**, 1009–1023.
8 Sharan,S.K., Morimatsu,M., Albrecht,U., Lim,D.S., Regel,E., Dinh,C., Sands,A., Eichele,G., Hasty,P. and Bradley,A. (1997) *Nature*, **386**, 804–810.
9 Campbell,S.M., Rosen,J.M., Hennighausen,L., Strech-Jurk,U. and Sippel,A.E. (1984) *Nucleic Acids Res.*, **12**, 8685–8697.
10 Gordon,K., Lee,E., Vitale,J.A., Smith,A.E., Westphal,H. and Hennighausen,L. (1987) *BioTechnology*, **5**, 1183–1187.
11 Hennighausen,L., McKnight,R.A., Burdon,T., Baik,M., Wall,R.J. and Smith,G.H. (1994) *Cell Growth Differentiat.*, **5**, 607–613.
12 Doppler,W., Villunger,A., Jennewein,P., Brduscha,K., Groner,B. and Ball,R.K. (1991) *Mol. Endocrinol.*, **5**, 1624–1632.
13 Kanegae,Y., Lee,G., Sato,Y., Tanaka,M., Nakai,M., Sakaki,T., Sugano,S. and Saito,I. (1995) *Nucleic Acids Res.*, **23**, 3816–3821.
14 Hennighausen,L., Wall,R.J., Tillmann,U., Li,M. and Furth,P.A. (1995) *J. Cell Biochem.*, **59**, 463–472.
15 Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
16 Ewald,D., Li,M., Efrat,S., Auer,G., Wall,R.J., Furth,P.A. and Hennighausen,L. (1996) *Science*, **273**, 1384–1386.
17 Furth,P.A., Hennighausen,L., Baker,C., Beatty,B. and Woychick,R. (1991) *Nucleic Acids Res.*, **19**, 6205–6208.
18 Andres,A.-C., Schönenberger,C.-A., Groner,B., Hennighausen,L., LeMeur,M. and Gerlinger,P. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1299–1303.
19 Pittius,C.W., Hennighausen,L., Lee,E., Westphal,H., Nichols,E., Vitale,J. and Gordon,K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5874–5878.
20 Hennighausen,L., McKnight,R., Burdon,T., Baik,M., Wall,R.J. and Smith,G.H. (1994) *Cell Growth. Differentiat.*, **5**, 607–613.
21 Sinn,E, Muller,W, Pattengale,P, Tepler,I, Wallace,R and Leder,P (1987) *Cell*, **49**, 465–475.
22 Muller,W.J., Sinn,E., Pattengale,P.K., Wallace,R. and Leder,P. (1988) *Cell*, **54**, 105–115.
23 Schwenk,F., Baron,U. and Rajewsky,K. (1995) *Nucleic Acids Res.*, **23**, 5080–5081.
24 Lewandoski,M., Wassarman,K.M. and Martin,G.R. (1997) *Curr. Biol.*, **7**, 148–151.
25 Furth,P.A., Hennighausen,L., Baker,C., Beatty,B. and Woychick,R. (1991) *Nucleic Acids Res.*, **19**, 6205–6208.
26 Furth,P.A., St.Onge,L., Boger,H., Gruss,P., Gossen,M., Kistner,A., Bujard,H. and Hennighausen,L. (1994) *Proc. Acad. Natl. Sci. USA*, **91**, 9302–9306.
27 Burdon,T., Sankaran,L., Wall,R.J., Spencer,M. and Hennighausen,L. (1991) *J. Biol. Chem.*, **266**, 6909–6914.
28 Pittius,C.W., Sankaran,S., Topper,Y. and Hennighausen,L. (1988) *Mol. Endocrinol.*, **2**, 1027–1032.
29 Robinson,G.W., McKnight,R.A., Smith,G.H. and Hennighausen,L. (1995) *Development*, **121**, 2079–2090.