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Inducible site-directed recombination in mouse embryonic stem cells

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ABSTRACT

The site-directed recombinase Cre can be employed to delete or express genes in cell lines or animals. Clearly, the ability to control remotely the activity of this enzyme would be highly desirable. To this end we have constructed expression vectors for fusion proteins consisting of the Cre recombinase and a mutated hormone-binding domain of the murine oestrogen receptor. The latter still binds the anti-oestrogen drug tamoxifen but no longer 17β-oestradiol. We show here that in embryonic stem cells expressing such fusion proteins, tamoxifen can efficiently induce Cre-mediated recombination, thereby activating a stably integrated LacZ reporter gene. In the presence of either 10 μM tamoxifen or 800 nM 4-hydroxy-tamoxifen, recombination of the LacZ gene is complete within 3–4 days. By placing a tamoxifen-binding domain on both ends of the Cre protein, the enzymatic activity of Cre can be even more tightly controlled. Transgenic mice expressing such an tamoxifen-inducible Cre enzyme may thus provide a new and useful genetic tool to mutate or delete genes at specific times during developement or in adult animals.

INTRODUCTION

Genes can be mutated or deleted from the mouse genome by homologous recombination in embryonic stem (ES) cells. Many genes, however, are essential for embryonic development and a homozygous deletion of these genes will therefore often result in non-viable embryos. To circumvent this problem, strategies have been developed that aim at deleting or mutating genes in the animal rather than in ES cells (1–3). Such strategies employ site-specific recombinases like the Cre recombinase of bacteriophage P_1 (4–6) or the FLP recombinase from yeast (7). The 38 kDa Cre recombinase can delete genes which are flanked on each site by a 34 base pair (bp) Cre recombination signal sequence called *loxP* site. Such genes can be generated by homologous recombination in ES cells (8). These genes will be deleted only in cells expressing the Cre recombinase. Indeed, transgenic mice carrying a gene flanked by *loxP* sites and expressing the Cre recombinase under the control of a tissue-specific promoter, show a high percentage of gene deletion in the tissue where Cre is expressed (9,10).

For a better understanding of the role of genes during murine development, it would be desirable to delete genes by a Cre recombinase system which is not only tissue-specific but also inducible (11). The available tissue-specific promoters, however, are not easy to combine with inducible promoter elements. Another way to control the activity of a protein is to fuse it with regulatory domains like the hormone-binding domain (HBD) of steroid receptors. Several transcription factors (12,13), as well as other proteins, for example the RAF kinase (14), or the recombinases FLP (15) and Cre (16) have been rendered functionally hormone-dependent by such a strategy (reviewed in 17). In the absence of ligand, the HBD-fusion proteins are bound and inactivated by heat shock proteins like Hsp90. Ligand binding releases the receptors from the inhibitory complexes (reviewed in 18,19).

The HBD of the murine oestrogen receptor is well studied and often employed in HBD-fusion proteins. Such fusion proteins, however, can not easily be used as control elements in transgenic mice because the ligand of HBD, 17β-oestradiol, is abundant in murine plasma. Recently the murine oestrogen receptor HBD has been mutated (20) so that the glycine at position 525 is replaced by arginine (G525R). This mutated HBD has lost its binding activity for 17β-oestradiol but retains binding to the anti-oestrogen drugs tamoxifen or 4-hydroxy-tamoxifen (21) and we therefore refer to it here as the tamoxifen-binding domain (TBD). Functional analysis of a c-Myc/TBD fusion protein has shown that it is activated by 4-hydroxy-tamoxifen yet totally unresponsive to 17β-oestradiol (22). We have constructed expression vectors encoding fusion proteins that consists of the Cre recombinase and TBD; we show here that the recombinase activity of these proteins can be induced by tamoxifen.

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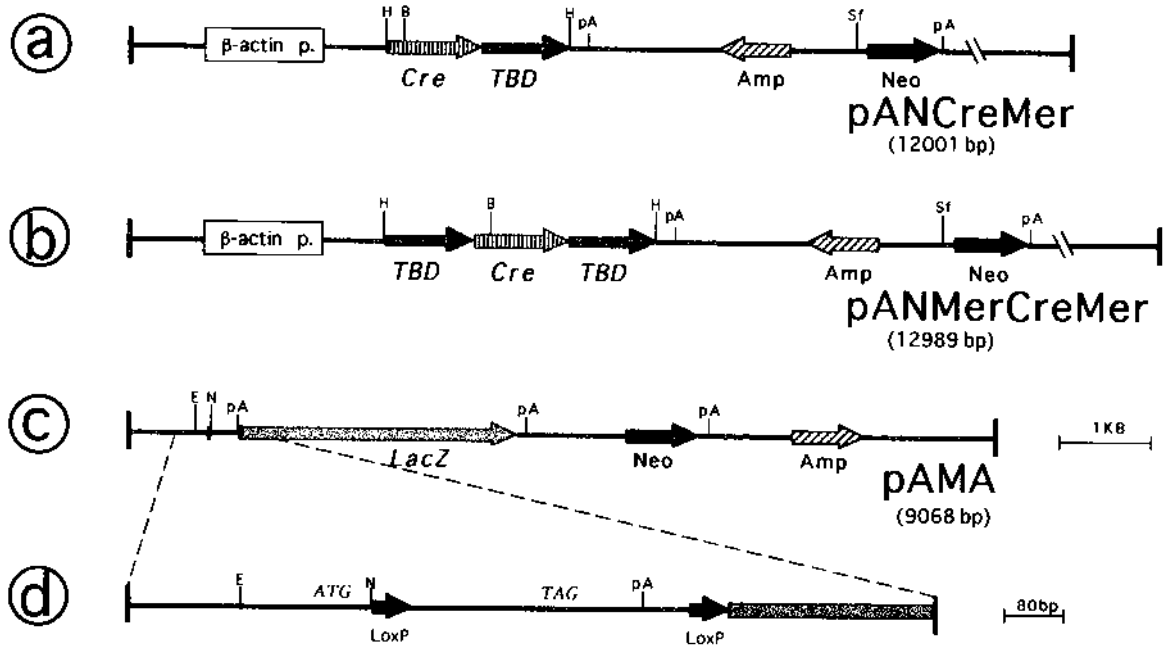

Figure 1. Maps of the expression vectors pANCreMer (**a**) and pANMerCreMer (**b**) as well as the Cre-*loxP* recombination indicator-construct pAMA (**c**) carrying the ATG-*loxP*-stop-pA-*loxP*-LacZ fusion gene (**d**).

MATERIALS AND METHODS

Vectors

The plasmid pBSKS+MerG525R (22) contains the mutated (G525R) coding sequence of the ligand-binding domain of the murine oestrogen receptor (amino acid 281–599) cloned into the *Bam*HI–*Eco*RI sites of Bluescript (Stratagene). The plasmid pTZ-creN carrying the 1050 bp coding sequence of the Cre recombinase was a kind gift of Lars Nitschke. At the 3′ end of the Cre coding sequence, a glycine–serine encoding linker and a *Bgl*II site were introduced by PCR using a 5′ primer (5′-ACTCGCGCCCTGGAAGGGATT-3′) lying just 13 bp upstream of the *Cla*I site of pTZ-creN and a 3′ primer (5′-ACGGA-GATCTCCAGAACCTCCATCGCCATCTTCCAG-3′) carrying the *Bgl*II site (underlined). After a fill-in reaction, the PCR product was digested with *Cla*I and *Bgl*II. This fragment, and a *Bam*HI–*Kpn*I fragment of pBSKS+MerG525R (partially digested with *Kpn*I), were co-ligated into *Cla*I and *Kpn*I-digested pTZ-creN to yield pTZ-CreMer.

The expression vector pAN-H carries the human β-actin promotor followed by a *Hin*dIII cloning site and the polyadenylation site of SV40 as well as ampicillin and neomycin resistance genes. To obtain the Cre-TBD expression vector pANCreMer (Fig. 1a), the CreMer coding sequence of pTZ-CreMer was cloned as a *Hin*dIII fragment into the *Hin*dIII-linearized pAN-H plasmid. The plasmid pUCHMR carrying the hygromycin resistance gene has been described previously (23). To construct the pANMerCreMer vector, we cloned a linker containing an ATG start codon and *Bgl*II + *Sal*I sites into pTZ-Cre just in front of the Cre reading frame. The modified pTZ-Cre plasmid was linearized with *Bgl*II + *Sal*I digestion and a PCR fragment of the murine oestrogen receptor (Mer) cDNA derived from pBSKS+MerG525R was ligated into this plasmid to yield pTZMerCre. From the latter plasmid, we isolated a *Hin*dIII–*Bam*HI fragment which was coligated with a *Bam*HI–*Hin*dIII fragment from pANCreMer into the *Hin*dIII linearized pAN-H plasmid to obtain the vector pANMerCreMer (Fig. 1b).

Cell lines

The cell line, MS4pAM (A.-M. Ayral *et al*., in preparation), is a derivative of the 129/Ola embryonic stem cell line E14.1 and carries a single copy of the Cre activity indicator construct pAMA. The pAMA vector carries a neomycin resistance gene and a modified β-galactosidase (ATG-*loxP*-stop-pA-*loxP*-LacZ) gene (Fig. 1c), the reading frame of which is disrupted after the second codon by two *loxP* sites flanking a 304 bp sequence with a translational stop and a polyadenylation site (Fig. 1d). Transcription of the ATG-*loxP*-stop-pA-*loxP*-LacZ gene is initiated at a polyoma enhancer-Tk promoter cassette and terminates at the polyA site present between the two *loxP* sites. Cre-*loxP*-mediated recombination will lead to the deletion of the translational stop and poly-A site and generate the ATG-*loxP*-LacZ gene expressing β-galactosidase.

Cell culture

ES cells were cultured in high-glucose (4.5 mg/ml) DMEM medium containing 10 mM sodium pyruvate, 20 mM L-glutamine, 120 μM β-mercaptoethanol, 50 U/ml penicillin, 50 U/ml streptomycin and 1× MEM non-essential amino acids supplemented by 15% fetal calf serum and supernatant of LIF-producing CHO cells at a 1:300 dilution. The ES cells were grown either on a dense monolayer of mitomycin C-treated, G418-resistant primary mouse embryo fibroblasts or on gelatin-coated Petri dishes.

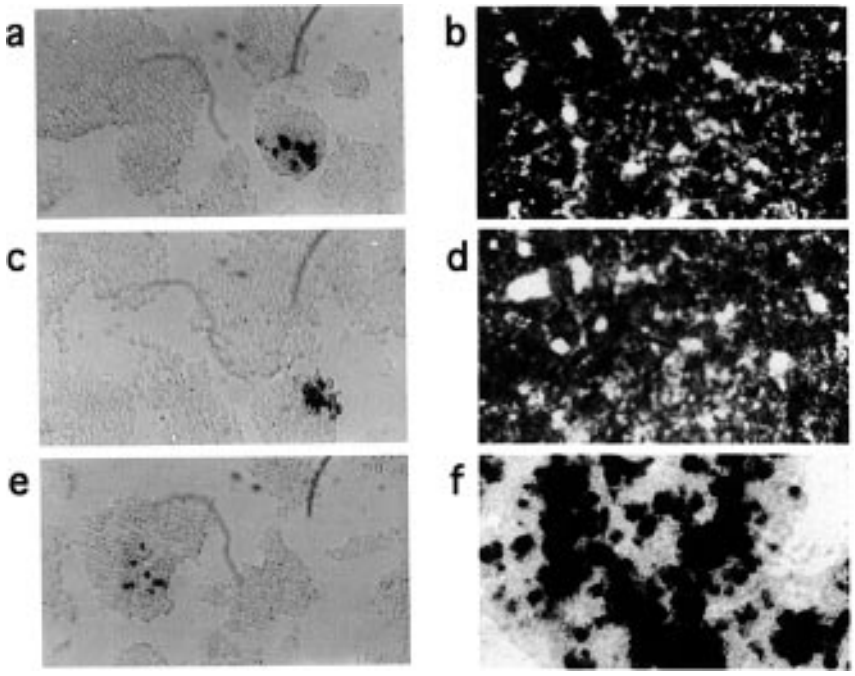

Figure 2. X-Gal staining of the ES cell clones ES33 (**a** and **b**), ES41 (**c** and **d**) and ES5 (**e** and **f**) for β-galactosidase activity. Cells were cultured for 3 days either without (a, c and e) or with 10 μM tamoxifen (b, d and f).

Transfection and β-galactosidase detection

The MS4pAM cells were harvested by trypsinisation, washed once with phosphate-buffered saline (PBS) and resuspended in PBS at 2×10^7 cells/ml. For stable transfection, 30 μg of *Sfi*1-linearized vectors pANCreMer or pANMerCreMer and 10 μg of *Hin*dIII-linearized pUCHMR were added to 1 ml of the cell suspension and the cells were transfected by electroporation as described previously (24). After 1 day in culture, the transfectants were selected in medium containing 200 U/ml hygromycin. Growing transfectants were split and incubated either in the absence or presence of 10 μM tamoxifen. The cells were then stained with X-Gal by the method of Buhler *et al.* (25). The amount of β-galactosidase in the lysate of the transfectants was determined by the β-Galactosidase Enzyme Assay System (Promega).

RESULTS

Expression of the Cre-TBD fusion protein

To obtain a tamoxifen-inducible Cre recombinase we constructed the expression vector pANCreMer in which the reading frame of the Cre gene was fused with that of TBD, the mutated (G525R) HBD of the murine oestrogen receptor (Fig. 1a). Compared with the wild-type, the mutated TBD has a 1000-fold lower affinity for 17β-oestradiol, whereas its affinity for tamoxifen or 4-hydroxy-tamoxifen remains unchanged (20).

The ES cell line MS4pAM had been previously transfected with the recombination-dependent β-galactosidase expression vector pAMA (Fig. 1c). In this vector, the reading frame of the β-galactosidase (LacZ) gene is disrupted after the second codon by two *loxP* sites flanking a 304 bp sequence with a translational stop and a polyadenylation site (Fig. 1d). As a consequence, this vector cannot express β-galactosidase. Cre-*loxP*-mediated recombination, however, would lead to deletion of the stop codon and the polyadenylation site, thereby restoring the open reading frame of the ATG-*loxP*-LacZ fusion gene and allowing β-galactosidase expression. The MS4pAM cell line can thus be used as an indicator line for the activity of the Cre recombinase.

The MS4pAM cells were co-transfected with pANCreMER and the hygromycin vector pUCHMR. Two weeks after selection in hygromycin-containing medium, we obtained 52 hygromycin-resistant transfectants of MS4pAM. These cells were cultured for 2 days in the presence or absence of 10 μM tamoxifen and analyzed for Cre recombinase activity by X-Gal staining. Five transfectants scored Cre-positive in this assay and three (ES41, ES33 and ES5) were chosen for further analysis. Cultures of these stable transfectants with normal medium contained only a few blue cells (Fig. 2a, c and e) whereas after a 3 day culture in the presence of tamoxifen, most cells of ES41 and ES33 (Fig. 2b and d) and >50% of ES5 (Fig. 2f) stained with X-Gal. This result showed that the recombinase activity of Cre-TBD in three ES cell transfectants could be regulated by tamoxifen in a majority of the cells.

Induction of Cre-mediated recombination by tamoxifen or 4-hydroxy-tamoxifen

We next cultured 2×10^3 cells of either ES5 or ES41 for 3 days with various doses of tamoxifen (Fig. 3a). After culture, the cells were lysed and the amount of β-galactosidase activity in the lysate was determined by an enzymatic assay. An optimum of β-galactosidase activity was reached in both Cre-TBD transfectants at 8 μM tamoxifen. Higher concentrations of tamoxifen were toxic to the cells and resulted therefore in declining β-galactosidase activity.

In Figure 3b, the Cre-TBD transfectants ES33 and ES41 were cultured for various times in the presence of either 10 μM

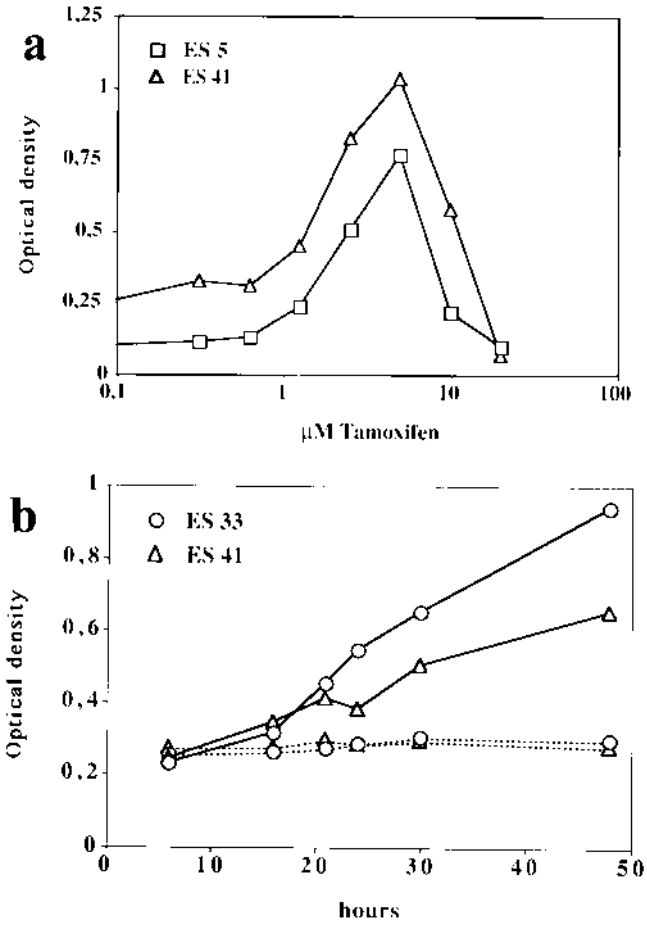

Figure 3. Induction of Cre recombination by tamoxifen. (**a**) Dose–response experiment of Cre/*loxP* recombination induced by tamoxifen. The ES cell transfectants ES41 (triangle) and ES5 (square) were cultured (2×10^3 cells/well) for 3 days in the presence of increasing amounts of tamoxifen. (**b**) Time-course experiment of tamoxifen-induced Cre/*loxP* recombination in the ES cell transfectants ES33 (circle) and ES41 (triangle) The cells (2×10^3 cells/well) were cultured for the indicated time in the presence of either 10μM tamoxifen (solid line) or 10 nM oestradiol (dashed line). The amount of β-galactosidase expressed from the Cre-recombined ATG-*loxP*-LacZ fusion gene was measured by an enzymatic assay.

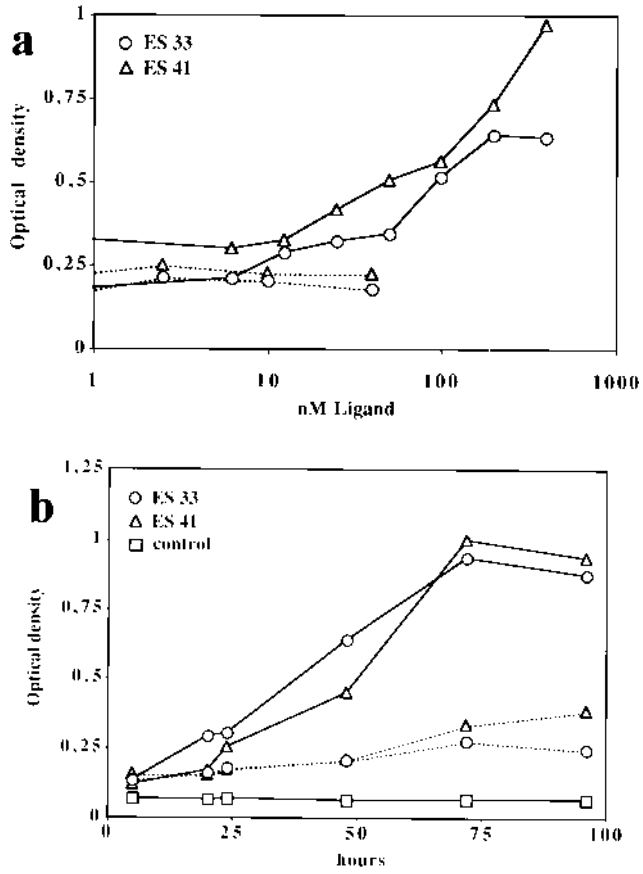

Figure 4. Induction of Cre recombination by 4-hydroxy-tamoxifen. (**a**) Dose–response experiment of Cre/*loxP* recombination induced by 4-hydroxy-tamoxifen. The pANCreMer transfectants ES33 (circle) and ES41 (triangle) were cultured (2×10^3 cells/well) for 3 days with increasing amounts of either 4-hydroxy-tamoxifen (solid line) or oestradiol (dashed line). (**b**) Time course experiment of Cre-mediated recombination induced in the ES cell transfectants ES33 (circle) and ES41 (triangle) by 4-hydroxy-tamoxifen. The cells (2×10^3 cells/well) were cultured for the indicated time in the presence of either 800 nM 4-hydroxy-tamoxifen (solid line) or 80 nM 17β-oestradiol (dashed line). As control the untransfected MS4pAM cells (square) were also cultured in 800 nM 4-hydroxy-tamoxifen. The amount of β-galactosidase expressed from the Cre-recombined ATG-*loxP*-LacZ fusion gene was measured by an enzymatic assay.

tamoxifen or 10 nM 17β-oestradiol, a dose which is sufficient to stimulate maximally the wild type Mer (20,26). After 15 h the β-galactosidase activity increased only in cultures containing tamoxifen but not in those containing 17β-oestradiol. This result shows that the recombinase activity of the Cre-TBD fusion protein can be efficiently induced by tamoxifen while 17β-oestradiol is ineffective.

When the Cre-TBD transfectants ES33 and ES41 were cultured for 3 days in various doses of 4-hydroxy-tamoxifen (Fig. 4a) 800 nM of 4-hydroxy-tamoxifen was found to induce the same amount of β-galactosidase activity as a 10-fold higher dose of tamoxifen (compare Fig. 3a with Fig. 4a). A culture of the same transfectants with β-oestradiol at a concentration of 80 nM did not result in any increase of β-galactosidase activity (dashed line in Fig. 4a).

A time-course experiment (Fig. 4b) in which ES33 and ES41 cells were cultured for various times in the presence of either 800 nM 4-hydroxy-tamoxifen or 80 nM β-oestradiol showed that the β-galactosidase activity reached a plateau after 75 h. In this experiment, the cells cultured with β-oestradiol also showed a slight increase of β-galactosidase activity in comparison to the control MS4pAM cells not transfected with the Cre-TBD construct (squares in Fig. 4b). This background β-galactosidase activity showed that in the absence of its ligand, the Cre-TBD protein was not completely silent. Indeed after a prolonged culture (8–10 weeks) in normal medium, the β-galactosidase activity in ES33 and ES41 cells increased (up to an absorbance of 0.5).

The basal activity of a TBD-Cre-TBD fusion protein is under tighter control than that of a Cre-TBD fusion protein

To control the Cre enzyme more tightly, we constructed the vector pANMerCreMer (Fig.1b) expressing a fusion protein in which a TBD is appended to both ends of the Cre enzyme. This vector was stably transfected into MS4pAM cells and 12 of 92 obtained

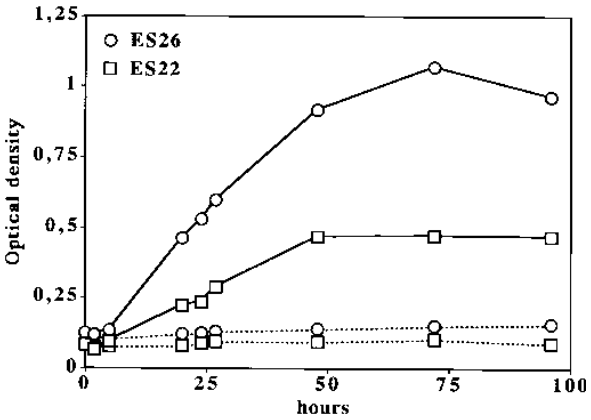

Figure 5. Time course of site-directed recombination mediated by a TBD-Cre-TBD fusion protein after exposure to 4-hydroxy-tamoxifen. The pANMer-CreMer transfectants ES22 (square) and ES26 (circle) were cultured (2×10^3 cells/well) for the indicated time in the presence of either 800 nM 4-hydroxy-tamoxifen (solid line) or 80 nM 17β-oestradiol (dashed line). The amount of β-galactosidase expressed from the Cre-recombined ATG-*loxP*-LacZ fusion gene was measured by an enzymatic assay.

hygromycin-resistant transfectants scored Cre-positive. Two of these transfectants (ES22 and ES26) were analyzed for the induction of Cre-activity. Surprisingly, the TBD-Cre-TBD protein was still enzymatically active. When exposed for various times to 800 nM 4-hydroxy-tamoxifen, both TBD-Cre-TBD transfectants showed a kinetic of Cre induction similar to that of the Cre-TBD transfectants (Fig. 5). The Cre activity, however, did not increase when these cells were cultured in the presence of 80 nM 17β-oestradiol. Furthermore, when ES22 and ES26 cells were kept in culture for >8 weeks, the background level of Cre activity did not increase (data not shown). This demonstrates that the TBD-Cre-TBD protein is under a more stringent control and should therefore be more suitable when expressed in transgenic mice than the Cre-TBD protein.

DISCUSSION

The functional analysis of Cre-TBD-expressing ES cells demonstrates that the Cre recombinase can be fused at its C-terminus to another protein without losing its enzymatic activity. By fusion to the TBD derived from the oestrogen receptor, a Cre-mediated recombination can be rendered ligand-dependent. In two independent CreTBD transfectants, ES33 and ES41, Cre-mediated recombination of the LacZ-reporter gene could be induced by the anti-oestrogen drugs tamoxifen and 4-hydroxy-tamoxifen in the micromolar and nanomolar range, respectively. The different sensitivities of the two drugs is in agreement with the finding that the 4-hydroxy-tamoxifen binds to HBD of the oestrogen receptor with a 10–100-fold higher affinity than tamoxifen (21,27). In the presence of these HBD-ligands, the Cre-mediated LacZ gene recombination and activation occurred in most cells of the ES33 and ES41 culture showing that the ligand-bound Cre-TBD fusion protein is an efficient recombinase.

The β-galactosidase activity in the cell culture increased from the 15 h time point onward and followed a time course which was very similar to that observed in cells expressing a fusion protein (FLP-LBD) in which the FLP recombinase of yeast was fused to the ligand-binding domain of the human oestrogen receptor (15). Furthermore, rat fibroblasts expressing a myc-TBD fusion protein undergo apoptosis with similar kinetics when cultured in the presence of 4-hydroxy-tamoxifen (22). Therefore, after ligand binding and release from the Hsp90 complex, the different HBD-containing fusion proteins apparently can exert their diverse biological functions with similar efficiency.

The control exerted by the TBD on the Cre enzyme is tight, but not absolute. When the Cre-TBD-transfected ES cells were kept for >10 weeks in normal medium, between 5% and 10% of the cells stained with X-Gal (data not shown). The background Cre activity may be due to a cleavage of the linker between the Cre and TBD by intracellular proteases. To overcome this problem, we designed a Cre enzyme carrying a TBD at both of its termini. ES cells expressing this TBD-Cre-TBD fusion protein showed a much lower basal Cre activity. The TBD-Cre-TBD protein, however, was still a functional enzyme which upon ligand binding could mediate site-directed recombination with a kinetic similar to that exerted by the Cre-TBD protein.

We have designed the Cre/TBD system for the purpose of inducing the deletion or expression of genes in animals. The Cre-TBD or TBD-Cre-TBD recombinases can be activated by tamoxifen or 4-hydroxy-tamoxifen but not by the endogenously expressed oestrogen. Therefore, these proteins should be better suited for *in vivo* experiments than Cre fusion proteins carrying the wild-type oestrogen binding domain (16). The two drugs can be easily administered in drinking water to transgenic mice. At the dose required for Cre-TBD or TBD-Cre-TBD activation, these compounds are not toxic. Indeed breast cancer patients (28), as well as mice (29,30), have been treated with these drugs over prolonged time.

As these fusion proteins are efficient recombinases, the deletion or activation of a murine gene flanked by *loxP* sites should require only a few days of exposure to the drug. With such a short period of treatment, the side effect of these anti-oestrogen drugs should remain relatively mild and should rapidly vanish upon withdrawal of the drug. However, whether Cre fusion proteins also work efficiently in different cell types of an animal has to await the analysis of Cre/TBD-trangenic mouse lines. By using a tissue-specific promotor in front of the CreMer or MerCreMer cDNA the fusion proteins can be expressed not only in an inducible but also in an tissue-specific manner. Similar to the inducible tetracyclin repressor systems (31,32), the Cre/TBD system thus may be developed into a useful new genetic tool to mutate or delete genes from the genome of a living organism.

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