Tissue- and time-dependent estrogen receptor activation in estrogen reporter mice

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Abstract

With the aim of developing an *in vivo* model that directly detects activation of estrogen receptors (ERs), transgenic mice carrying a luciferase reporter gene were generated. The luciferase reporter gene was under the control of three consensus estrogen-responsive elements (EREs) coupled to a minimal TATA-box, with or without flanking chick β -globin insulators. By using this model in combination with the IVIS imaging system, *in vivo* ER activation was measured. Dose- and time-dependent luciferase activity was induced in various organs of adult transgenic male mice exposed to diethylstilbestrol (DES) (10–1000 $\mu\text{g/kg}$) and 17 β -estradiol dipropionate (EP) (10–1000 $\mu\text{g/kg}$), when luciferase activity was measured *ex vivo*. The highest (>10 000-fold) induction of luciferase was measured in bone and kidney 24 h after exposure to 1000 $\mu\text{g/kg}$ EP. Other highly responsive organs include liver, testis, pituitary, brain, prostate and colon, which show different activity profiles. This *in vivo* model for detecting estrogenic activity can be used to assess tissue-specific action of ER agonists and antagonists. These could include selective ER modulators and environmental estrogens. In combination with the IVIS imaging system, this *in vivo* model is a powerful tool for assessing the kinetics of gene activation by estrogenic compounds.

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Introduction

Steroidal estrogens exert their physiological actions by activating target genes via estrogen receptor α and/or β (ER α and ER β). Estrogens are known to influence numerous target tissues in the female and male reproductive systems, such as mammary gland, uterus, vagina, ovary, testes, epididymis and prostate (Korach *et al.* 1994). In addition, estrogens have been shown to have a function in bone homeostasis and the central nervous system as well as in the cardiovascular system (Turner *et al.* 1994, Farhat *et al.* 1996, Iafrati *et al.* 1997, LeBlanc *et al.* 1997).

In addition to physiological steroidal estrogens, a broad number of studies have identified exogenous

compounds that exert estrogenic activity. Many of these studies are based on *in vitro* assays (Grese *et al.* 1997, Shang & Brown 2002, Harrington *et al.* 2003). Such assays basically evaluate ER-binding affinities as well as ER transactivation potential, using various mammalian cell lines and yeast (Andersen *et al.* 1999, Legler *et al.* 1999). A common property of these assays is quickness and low cost. However, important aspects for *in vivo* activity of a compound, such as uptake, distribution, bioavailability and metabolism, are not taken into account in these *in vitro* assays. Therefore, animal models are necessary to confirm the estrogenic potential of compounds found to be positive in *in vitro* assays. Two classical *in vivo* bioassays, the uterotrophic and vaginal cornification assays, have often been used for this purpose. In these

assays, rodents are exposed to the test compounds, and either the uterus wet weight or the extent of vaginal cornification is assessed (Ashby *et al.* 2000, Schlumpf *et al.* 2001). The specific mechanisms underlying these effects are not known and theoretically could be effective without a direct interaction between the compound and the ER. A different approach that is increasingly used as a marker for ER activation is induction of target genes *in vivo* (Diel *et al.* 2000, Khurana *et al.* 2000). However, a drawback of these assays is that target genes are often studied only in no more than one organ or tissue at a time. An *in vivo* model involving direct detection of activated ERs in a broad range of tissues would combine the advantages of *in vivo* and *in vitro* assays.

To develop an *in vivo* model with direct detection of activated ERs, transgenic mice carrying a luciferase reporter gene under the control of three consensus estrogen-responsive elements (EREs) coupled to a minimal TATA-box (3xERE-TATA-Luc) were generated. In addition, to improve expression and minimize effects of DNA sequences surrounding the transgene, transgenic mice carrying a similar construct flanked by two copies of the chick β -globin insulator (Chung *et al.* 1993, Wang *et al.* 1997, Potts *et al.* 2000) were generated. Although other estrogen reporter mice have been generated, these models do not exclude ERE-independent activation because of the presence of other promoter sequences (Ciana *et al.* 2001, Nagel *et al.* 2001, Toda *et al.* 2003). The use of only a minimal TATA-box in the construct used in the present study will avoid activation of the construct via other promoter sites than the EREs. Our generated transgenic animals were exposed to 17 β -estradiol (E₂), 17 β -estradiol-dipropionate (EP) or diethylstilbestrol (DES) to compare and characterize their ability to activate the reporter construct, luciferase, via endogenous ERs. In addition, to test ER dependency of the luciferase induction, the ER antagonist ICI 182,780 (ICI) was tested alone and in combination with DES. A new *in vivo* luciferase imaging system (IVIS; Xenogen, Alameda CA, USA) was used to follow the kinetics of luciferase activity *in vivo* after exposure to the estrogens. The activity of luciferase in a broad range of tissues and organs was measured *ex vivo* after *in vivo* exposure to estrogens.

Materials and methods

Transgenic constructs

Two estrogen-responsive reporter gene constructs were used (Fig. 1A). One (3xERE-tata-luc), carrying three copies of a consensus ERE and a TATA-box in front of the luciferase cDNA, is described in more detail elsewhere (Legler *et al.* 1999). The second construct (3xERE-tata-luc-insulated) was made by cloning 3xERE-tata-luc between chicken β -globin insulators. From the pJC13-1 construct (Chung *et al.* 1993), the locus control region was removed by EcoRI restriction and self-ligation. Subsequently, the neo cassette was removed by BamHI restriction. The 3xERE-tata-luc construct was inserted in the blunted BamHI site of pJC13-1. Transient transfections of the constructs together with ER α in HEK293 cells were performed as described previously (Kuiper *et al.* 1998).

Generation of transgenic animals

Female F1 mice from CBA $\times$ C57Bl/6J crosses were superovulated by intraperitoneal (i.p.) injection of pregnant mare serum (5IU) and, 46 h later, human gonadotropin (5IU). Before zygote injection, constructs were linearized and cleaned by dialysis. DNA was dissolved in injection buffer (10 mM Tris, pH 7.6, and 0.1 mM EDTA) to a final concentration of approximately 2 ng/ μ l. Of this, 5 μ l were injected into the male pronucleus of one-cell zygote embryos. Injected embryos were cultured in M16 medium until transfer into the oviduct/uterus of CBA $\times$ C57Bl/6J F1 females on day 0.5 of pseudopregnancy. After weaning, ear-cut material of the pups was collected, and DNA was isolated as described before (Legler *et al.* 2000). PCR for detecting reporter construct integration in the mouse genome was performed with primers located within the transgenic construct (Legler *et al.* 2000). With the 3xERE-tata-luc construct, three founders (A–C) were obtained; with the 3xERE-tata-luc-insulated construct, nine founders (INS1–9) were obtained. Founders were subsequently crossed with C57Bl/6J mice to obtain F1 animals. Animals used for estrogen-exposure experiments described were 8–16-week-old male F1 animals from founder INS7.

Estrogens and *in vivo* exposure

17 β -Estradiol (E₂), 17 β -estradiol-dipropionate (EP) and diethylstilbestrol (DES) were all purchased from Sigma-Aldrich (Roosendaal, The Netherlands). ICI 182,780 (ICI) was obtained from Tocris Cookson Ltd (Bristol, UK). Stock solutions of estrogens (10 mg/ml) and ICI (25 mg/ml) were prepared in corn oil (Sigma-Aldrich) and were subsequently diluted further to final test concentrations in corn oil. Exposure of transgenic mice was done by i.p. injection. The doses for EP and DES exposure (10–1000 μ g/kg) were chosen in order to see the dose response in the IVIS system, which is less sensitive than the *in vitro* measurements on lysates. ICI was given 1 h prior to DES in the combination exposure. The numbers of animals for each exposure group are indicated in each figure legend. The mice were maintained under constant conditions with a photoperiod of 12 h light (beginning at 0700 h) and 12 h darkness. Food and water were supplied, and animals were allowed to feed *ad libitum*. All animal experiments were done with approval of the Netherlands Academy of Arts and Sciences Animal Ethics Committee (protocol HL01.09 and HL02.15). The IVIS experiments were done with additional approval from the Animal Ethics Committee of NV Organon.

Luciferase measurement *in vivo*

With the Xenogen IVIS imaging system, the luciferase protein was monitored in living animals. Animals were injected subcutaneously with the luciferase substrate, luciferin (100 μ l of stock 30 mg/ml in PBS). After 15 min, the animals were placed in a dark imaging chamber under isoflurane anesthesia. Photon emission resulting from the luciferin/luciferase reaction was detected with a sensitive CCD camera. This photon image was superimposed on a normal video image of the mouse with Living Image software (Xenogen). With IGOR software (WaveMetrics Corporation, Lake Oswego, OR, USA), the photon signal was quantified. The number of detected photons correlates with the amount of luciferase protein.

Tissue isolation and *in vitro* luciferase measurement

Animals were killed by CO₂/O₂ asphyxiation 8 or 24 h after estrogen exposure. Subsequently, tissues (pituitary, esophagus, testis, brain, colon, adrenal, liver, prostate, small intestine, bone (femur), heart, kidney and lung) were isolated and frozen at –80 °C. For luciferase analysis, tissues were thawed on ice, and lysis buffer (1% (v/v) Triton X-100, 2.5×10^{-2} M glycylglycine, 1.5×10^{-2} M MgSO₄, 4×10^{-3} M EGTA and 1×10^{-3} M dithiothreitol (DTT)) was added. The tissues were homogenized with an Eppendorf micropestle, and the lysate was centrifuged and the supernatant collected. Duplicate samples (25 μ l) were measured for luciferase enzyme activity on a luminometer (LUMAC/3 M BV, Schaesberg, The Netherlands), using 100 μ l of luciferin substrate solution (33 mM DTT, 20 mM tricine, 1 mM (MgCO₃)Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 270 μ M coenzyme A, 470 mM luciferin and 530 μ M ATP, pH 7.8). Protein correction was performed after protein measurements using the Biorad (München, Germany) kit according to the manufacturer's instructions.

Statistical analysis

For the transfection experiments, the EC₅₀ values were calculated by determining the concentration by which 50% of maximum activity was reached, using the sigmoidal fit equation. The E₂ curve was fitted using the sigmoidal fit ($y = a_0 + a_1 / (1 + \exp(-(x - a_2)/a_3))$) in Slidewrite Plus for Windows 3.0, which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least-squares criterion). For the IVIS experiments, significant differences of treatment groups from oil-exposed controls were determined by Kruskal–Wallis analysis followed by Dunn's post-test. In addition, the presence of a linear trend in the dose response was determined by ANOVA followed by Dunn's post-test for linear trends. For the IVIS time-course experiments, Friedman's test followed by Dunn's post-test was performed. For the luciferase measurements on organ lysates (see Fig. 4) after log transformation of the data, significant differences between all groups were determined by ANOVA followed by Tukey's post-test. All analyses were carried out with GraphPad Prism 3.02.

Results

In vitro comparison of estrogen-responsive reporter constructs

The newly generated insulated construct (3xERE-tata-luc-insulated; Fig. 1A) was compared with the previously described estrogen-responsive 3xERE-tata-luc construct (Legler *et al.* 1999) in transient transfection experiments with ER α . After E₂ exposure of transiently transfected 293 cells, a dose-dependent activation of each construct was observed (Fig. 1B). The dose-response profile was similar for both reporter constructs, reaching maximal induction at 1 nM E₂ (Fig. 1B), whereas total luciferase activity was highest for the insulated construct (Fig. 1C). Fold induction was also higher for the insulated construct, 19.1 times over solvent control in comparison to 13.8 times for the noninsulated construct. The concentrations of E₂ needed for half-maximal activation (EC₅₀) of the constructs were 3.8×10^{-12} and 1.9×10^{-11} M respectively, for the insulated and noninsulated construct.

Characterization and selection of transgenic lines

With the 3xERE-tata-luc construct, three founder mice were obtained, lines A, B and C. Nine founder mice were obtained with the 3xERE-tata-luc-insulated construct, lines INS1–9. Transgenic lines with germ-line transmission of the constructs were characterized for luciferase induction after DES exposure (1000 µg/kg). With the IVIS system, background luciferase activity was detected in testes

of nonexposed animals from lines C and INS9. This activity was further enhanced by exposure to DES (Table 1). In lines INS3, INS7 and INS9, luciferase activity was observed in the liver only after DES exposure (Table 1). In the paws of animals from lines INS3 and INS7, a constitutive luciferase activity was detected, which was unaltered by DES exposure (Table 1). When luciferase activity was measured in tissue lysates, the tissue distribution of DES-induced luciferase activity was similar in animals from lines INS3 and INS7 (Table 1) and could be detected in a broad range of tissues. After DES exposure, animals from INS9 showed a tissue distribution of luciferase activity partially similar to lines INS3 and INS7, whereas animals from line C showed expression in liver and testis, and animals from line INS4 and INS5 showed luciferase activity in the liver only (Table 1). Male animals from line INS7 were selected for further experiments due to their high induction of luciferase in various tissues after DES exposure when measured *ex vivo*. In addition, with the IVIS system, it was possible to follow *in vivo* activation of luciferase in this line, making its animals suitable for studying kinetics of gene activation by estrogens. Male mice were chosen to avoid having to perform ovariectomy to bring down background levels of circulating estrogens.

Kinetics of luciferase induction measured *in vivo* after DES and EP exposure

The advantage of using the IVIS system is that the induction of luciferase in a single animal can be followed in time, making it a very useful tool in

Figure 1 *In vitro* response of insulated and noninsulated estrogen reporter constructs to 17 β -estradiol (E₂). (A) Schematic illustration of the constructs used for generating transgenic reporter mice. The first construct consists of three consensus estrogen-responsive elements (EREs) from the vitellogenin gene coupled to a minimal TATA-box and luciferase. The second construct is essentially the same, but flanked on both sides by chicken β -globin insulator sequences. (B) E₂ activation of the reporter constructs shows a similar dose-dependent induction of luciferase activity, when taken as percentage of maximal induction by E₂. Transient transfections were carried out with either construct together with ER α in HEK 293 cells. Cells were exposed to E₂ or medium/ethanol controls as indicated. Luciferase activity was measured 24 h after exposure. Abscissa: log M of E₂, medium alone (DCC) or ethanol (solvent) control. Ordinate: luciferase activity as percentage of maximal induction by E₂. Shown is the average of three independent experiments performed in triplicate. (C) Photon emission (c.p.m.) resulting from the luciferin/luciferase reaction was higher when the insulated reporter construct was transfected than with the noninsulated reporter construct. Photon emission correlates with the amount of luciferase protein. Transient transfections and exposure were performed as described under B. Abscissa: log M of E₂, medium alone (DCC) or ethanol (solvent) control. Ordinate: c.p.m. of photon emission correlating with absolute luciferase protein amounts. Shown is a representative experiment. c.p.m., counts per minute; DCC, medium with dextran-coated, charcoal-stripped serum; E₂, 17 β -estradiol; Etoh, 0.1% ethanol (solvent) control.

Table 1 Luciferase activity in transgenic lines with germ-line transmission of ERE-reporter genes

		Transgenic lines											
		3×ERE-tata-luc			3×ERE-tat-luc-insulated								
		A	B	C	INS1	INS2	INS3	INS4	INS5	INS6	INS7	INS8	INS9
Background				Testis			Paws				Paws		Testis
IVIS ^a	DES	nd	nd	Testis(+)	–	–	Liver(+) Paws	–	–	nd	Liver(+) Paws	–	Liver(+) Testis(+)
Lysates ^b	Liver	–	–	+/-	–	–	+++	+/-	+/-	–	+++	–	+
	Testis	–	–	+/-	–	–	++	–	–	–	++	–	+
	Uterus	–	–	–	–	–	+	–	–	–	+	–	–
	Brain	–	–	–	–	–	+	–	–	–	+	–	+

^aOrgans with detectable luciferase activity with the IVIS system. Background: nonexposed animals; DES: exposure to 1000 µg/kg. (+) indicates higher luciferase activity after DES exposure when compared to background levels.
^bAnimals were exposed to 1000 µg/kg DES, and organs were isolated 24 h after exposure. nd, not determined; +/-, luciferase units after DES exposure 3–10 times background (lysis buffer); +, luciferase units after DES exposure 10–100 times background; ++, luciferase units after DES exposure 100–10 000 times background; +++, luciferase units after DES exposure >10 000 times background.

obtaining information of luciferase activation kinetics. In addition, collecting data from the same animal over multiple time points is likely to reduce the statistical variability compared with data from different groups of animals that must be killed at different time points. Dose dependency of induction of luciferase activity was examined in male mice from line INS7. Mice were exposed to increasing doses of either DES or EP. The dose response of induction of luciferase activity in the liver was measured *in vivo* by the IVIS system. In Fig. 2A, the image of photon activity 8 h after oil or estrogen exposure is shown. In an elliptical area encompassing the liver, the photon signal was subsequently quantified (Fig. 2B). No luciferase activity was observed after treatment with the solvent control (corn oil). After DES exposure, luciferase activity was measurable at the 10 µg/kg dose and increased further after exposure to 100 and 1000 µg/kg. Luciferase activity after EP exposure was measurable only after exposure to 100 and 1000 µg/kg. The time-dependent induction of luciferase activity was measured *in vivo* with the IVIS system (Fig. 3A and B). DES-induced luciferase activity in the liver peaked at the 8-h time point for all concentrations tested (Fig. 3A and B; data not shown). In contrast, luciferase activity induced by EP was maximal 24 h after exposure (Fig. 3A and B; data not shown). In addition, one

dose of E₂ (1000 µg/kg) was tested with the IVIS system, showing a peak in luciferase activity 8 h after injection. These data show that it is possible with these transgenic mice in combination with the IVIS system to detect the relative potency of estrogens to activate ERs, as well as the kinetics of this activation.

Tissue-dependent luciferase induction measured *in vitro* after DES and EP exposure

After the *in vivo* measurement of luciferase activity by the IVIS system, the animals were killed, and 13 different organs were isolated for *in vitro* detection of luciferase activity. Selection of tissues was based on induction of luciferase activity after exposure to DES, as identified during the selection of the transgenic lines. The *in vitro* measurements showed a considerable variation between the different organs in the basal levels of luciferase activity after oil exposure. In pituitary, esophagus, testis and brain, luciferase activity was relatively high, whereas it was moderate in colon, adrenal, liver, prostate, small intestine and bone, and absent in heart, kidney and lung (Fig. 4). After estrogen exposure, induced luciferase activity was measured in all organs isolated, but induction patterns of luciferase activity were dependent on both the

Figure 2 *In vivo* activation of the estrogen-responsive reporter construct by estrogens. (A) *In vivo* activation of the estrogen-responsive reporter construct by diethylstilbestrol (DES) and 17 β -estradiol dipropionate (EP) in adult transgenic male mice from line INS7 measured with the IVIS system. Dose-dependent activation of the reporter construct was measured 8 h after DES or EP exposure (10–1000 $\mu\text{g/kg}$). Number of photons produced by the reaction between luciferase and luciferin is depicted in a false-color image superimposed on a video image of the animal. (B) Quantification of the photon signal produced in the liver of the transgenic male mice after DES and EP exposure. Shown is average $\pm$ S.E.M. For oil, DES and EP, $n=5$. Abscissa: dose of DES, EP or solvent (oil) control. Ordinate: photons per s/cm² normalized for each animal on $t=0$, measured in an area encompassing the liver. Significant differences from oil-exposed control, as determined by Kruskal–Wallis analysis followed by Dunn's post-test, is indicated with asterisks; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 3 Time course of *in vivo* activation of the estrogen-responsive reporter construct by estrogens. (A) *In vivo* activation time course of the estrogen-responsive reporter construct by diethylstilbestrol (DES), 17β-estradiol-dipropionate (EP) and 17β-estradiol (E₂) in adult transgenic male mice from line INS7 measured with IVIS. After exposure to DES, EP and E₂ (1000 µg/kg), a time course of activation was measured. The number of photons produced by the reaction between luciferase and luciferin is depicted in a false-color image superimposed on a video image of the animal. (B) Quantification of the photon signal produced in the liver of transgenic male mice at various time points after DES, EP and E₂ exposure (1000 µg/kg). Shown is average ± S.E.M. For all, *n*=5. Abscissa: time (h). Ordinate: photons per s/cm² normalized for each animal on *t*=0 measured in an area encompassing the liver. Significant increase in luciferase activity, determined by Friedman test followed by Dunn's post-test, is indicated by asterisks; **P*<0.05, ***P*<0.01, ****P*<0.001.

ligand and the tissue (Fig. 4). The highest induction of luciferase activity was found in bone and kidney; in both organs, it was more than 10 000 times induction 24 h after exposure to 1000 µg/kg EP (Fig. 4). It is also likely that doses lower than 10 µg/kg would elicit significant induction in organs such as liver, bone, kidney and prostate, where the 10 µg/kg is still highly significant from the oil-exposed control group (Fig. 4). These data show that the reporter mice are responsive to estrogens in a broad range of tissues and organs.

ER dependency of estrogen-induced luciferase activity

The ER dependency for estrogen-induced luciferase activity was confirmed by exposing mice to the ER antagonist ICI 182,780 (ICI). Luciferase activity induced by endogenous estrogens in all organs except the testis was blocked by ICI exposure (data not shown). Additionally, the ability of ICI to block the luciferase induction in the liver after exposure to DES (10 µg/kg) was examined

Figure 4 Tissue-specific induction of the estrogen-responsive reporter construct by estrogens. Tissue-specific induction of the estrogen-responsive reporter construct measured *in vitro* in lysates of different tissues, which were isolated after 24 h in adult transgenic male mice from line INS7 to various concentrations of diethylstilbestrol (DES), 17 β -estradiol-dipropionate (EP) and 17 β -estradiol (E_2). Shown are the relative luciferase units (RLU) per mg protein induced by exposure to oil, DES, EP and E_2 . Average $\pm$ S.E.M. of the RLUs is shown. For all groups, $n=7$, except for EP10 and 1000, for which $n=8$. Significant differences from oil-exposed control as determined by ANOVA followed by Tukey's post-test are indicated by asterisks; * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Note scale differences. Abscissa: oil or dose of DES, EP and E_2 . Ordinate: RLU per mg protein.

with the IVIS system (Fig. 5A and B). A dose of 250 $\mu\text{g/kg}$ ICI was not sufficient to block the DES-induced luciferase activity completely in all animals, but one of 2500 $\mu\text{g/kg}$ ICI was. These data were confirmed by measuring luciferase activity in liver lysates (Fig. 5B). These results show that the ability of ICI to block both endogenous and DES-activated luciferase activity is due to activated ERs.

Discussion

Transgenic mice carrying an estrogen-responsive reporter construct coupled to luciferase were generated to study direct activation of ERs *in vivo*. With this *in vivo* model, it is possible to assess efficiently the ability of different compounds to activate ERs in a broad range of tissues.

Figure 5 Inhibition of diethylstilbestrol (DES)-induced reporter expression by ICI 182,780 (ICI). (A) Inhibition of DES-induced *in vivo* activation of the estrogen-responsive reporter construct by the antiestrogen ICI, as measured with IVIS in adult transgenic male mice from line INS7. Pretreatment with ICI (2500 $\mu\text{g}/\text{kg}$) for 60 min resulted in a complete block of DES activation. Photon number produced by the reaction between luciferase and luciferin is depicted in a false-color image superimposed on a video image of the animal. (B) Quantification of the photon signal produced in livers of transgenic male mice at 8 h after hormone exposure. Shown is average $\pm$ S.E.M. For the IVIS data, $n=2-5$. Abscissa: hormone exposure in $\mu\text{g}/\text{kg}$. Ordinate: photons per s/cm² normalized for each animal on $t=0$ measured in an area encompassing the liver. (C) Quantification of luciferase measured *in vitro* in liver lysates of transgenic male mice at 24 h after hormone exposure. Shown is average $\pm$ S.E.M.; $n=2-5$. Abscissa: hormone exposure in $\mu\text{g}/\text{kg}$. Ordinate in LUMAC: relative luciferase units per mg protein. I=ICI 182,780; D=DES. Significant differences from control exposed to DES 10 $\mu\text{g}/\text{kg}$, as determined by Kruskal–Wallis analysis followed by Dunn's post-test, is indicated by asterisks; * $P<0.05$, ** $P<0.01$.

After *in vitro* characterization of the constructs, transgenic lines were generated with these constructs. With the noninsulated and insulated construct, one and five expressing lines were obtained respectively. The level of expression in the noninsulated line was similar to the two weakest insulated lines. It is possible that the higher level of luciferase activity after *in vitro* stimulation of the

insulated construct contributed to the ability to detect expression of luciferase in the lines with this construct. Insulator sequences are believed to prevent possible integration effects, such as silencing or unregulated expression of the transgenic construct (Wang *et al.* 1997). Whether the use of insulators has played a role in the higher number of expressing lines, the more ubiquitous expression

and high inducibility in these lines cannot be directly assessed due to the different number of founders obtained with the two constructs.

Both DES and EP induced luciferase activity dose-dependently when measured *in vivo* with the IVIS, as well as *ex vivo* in tissue extracts. The results of comparing maximal luciferase activity in isolated tissues showed that EP often induced higher levels than the same dose of DES 24 h after exposure. However, as DES induces luciferase activity at lower doses than EP and the peak of DES-induced luciferase activity is earlier than that of EP, it is difficult to conclude which hormone is to be considered more potent in our *in vivo* model. *In vitro* studies have shown DES to be as potent as E₂ (Kuiper *et al.* 1998) and as EP (our unpublished data). In a 3-day uterotrophic assay, DES was slightly more potent than E₂ (Jefferson *et al.* 2002). No reports comparing target gene expression *in vivo* after DES and E₂ exposure are available for mice. Differences in induction of a target gene between E₂ and DES could be explained by the fact that coactivators and corepressors are differentially expressed. Moreover, some cofactors might have a better interaction with DES-bound ER and others with E₂-bound ER (Pennie *et al.* 1998).

The kinetic data obtained with the IVIS system showed that the transactivation activity of DES peaked 8 h after exposure for all doses tested. In adult rats treated with ¹⁴C-DES, plasma values were found to peak at 4 h (Bengtsson & Ullberg 1963). In the plasma of pregnant mice exposed to ¹⁴C-DES, a rapid disappearance of DES was found with a subsequent peak of DES conjugates after 8 h (McLachlan 1977), consistent with the peak we observed in luciferase activity in the transgenic mice. Exposure of the transgenic mice to E₂ resulted in a luciferase activity peak after 8 h. Similarly, target gene activity in transgenic pMAR mice peaked in liver and bone 6 h after E₂ exposure (Ciana *et al.* 2001, 2003). With respect to EP, its transactivation activity was highest 24 h after exposure, as shown with the IVIS system. EP induced a peak in luciferase activity later than E₂, consistent with the view that the added propionate groups must first be removed before the active E₂ is available.

Background levels of luciferase activity varied extensively between different organs studied. This could be due to a difference in the level of endogenous estrogens in the tissues. Local

production of estrogens is a likely reason for the high background activity observed in the testis (Carreau 2001). High background levels of luciferase activity in brain, pituitary and testis could possibly also reflect ER expression, which is high in these tissues (Kuiper *et al.* 1997). In all tissues except the testis, background activity was blocked after treatment with ER antagonist ICI 182,780. The reason for the lack of reduction of luciferase activity in the testis might be due to the inability of ICI 182,780 to pass the blood–testis barrier efficiently (Setchell & Main 1978).

Sensitivity to estrogen exposure varied for the various tissues. Organs showing a high fold induction of luciferase after estrogen exposure include bone, adrenal, liver, prostate, kidney, colon and lung. Some of these organs were also found to be relatively responsive in pMAR mice, although the fold induction was lower than in INS7 mice (Ciana *et al.* 2001, Di Lorenzo *et al.* 2002), while adrenal and colon tissue was not described. In ERIN mice, high inducibility was found in the kidney and slight induction in the adrenal gland, but no induction was found in lung, while bone, prostate and colon were not examined (Nagel *et al.* 2001). In ERE-EGFP reporter mice, E₂-regulated induction of the reporter was found in pituitary, ovary, uterus, adrenal gland and gonadal fat pad (Toda *et al.* 2003). The use of male mice versus ovariectomized female mice (Ciana *et al.* 2001, Nagel *et al.* 2001) or aromatase knockout females (Toda *et al.* 2003) could explain the lower induction observed in pMAR and ERIN mice, because low estradiol levels might still be present. However, this does not explain the low induction in the ERE-EGFP mice with aromatase knockout females, which have no circulating estradiol; here the use of EGFP might have influenced the inductions measured. In addition, the use of a very minimal TATA promoter in the present study, instead of the TK promoter used for the ERIN (Nagel *et al.* 2001) and pMAR (Ciana *et al.* 2001) mice or the hsp68-promoter for the ERE-EGFP mice (Toda *et al.* 2003), could have resulted in lower background activation and therefore higher fold inductions in the INS7 mice. In brain and testis of INS7 mice, the fold induction after estrogen exposure was relatively small, suggesting that background levels were present due to local activity of estrogens. In general, in organs where estrogen signaling plays an important role, tight

feedback mechanisms might be in place, keeping the effects of exogenously administered estrogens within certain limits, which in the present context means low induction of luciferase activity. In contrast, in organs with low endogenous estrogen signaling, in addition to lower background levels, the regulatory mechanisms may be less tight and therefore high luciferase activity can be induced already after exposure to low doses. Other mechanisms possibly involved are downregulation of ERs and/or coactivators, and upregulation of corepressors.

Our *in vivo* model for detecting estrogenic activity is a promising tool for various lines of research. Tissue-specific action of ER agonists and antagonists, including ER α /ER β specific ligands, environmental estrogens and SERMs, can be assessed. In addition, in combination with the IVIS imaging system, this *in vivo* model will be an effective means for assessing kinetics of receptor-mediated gene activation. Currently, experiments assessing placental transfer and activation of embryonal ERs by classical estrogens and environmental estrogens are carried out with this model.

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