

Transcriptional and Posttranslational Regulation of Cre Recombinase by RU486 as the Basis for an Enhanced Inducible Expression System

Stephanos Kyrkanides,^{1,2,*} Jen-nie H. Miller,¹ William J. Bowers,³ and Howard J. Federoff³

¹Department of Dentistry and ²Department of Neurobiology & Anatomy, University of Rochester School of Medicine & Dentistry, 625 Elmwood Avenue, Rochester, New York 14620

³Department of Neurology, Center for Aging & Developmental Biology, Aab Institute of Biomedical Science, University of Rochester, Rochester, New York 14642

*To whom correspondence and reprint requests should be addressed at the Eastman Dental Center, 625 Elmwood Avenue, Rochester, NY 14620.
E-mail: Stephanos_Kyrkanides@urmc.rochester.edu.

Genetic studies often require the employment of an inducible expression system, whereby the expression of a particular gene can be regulated by the exogenous administration of an inert ligand. Cre/loxP-based systems have been previously described as the basis for inducible expression systems by exerting site-specific DNA recombination. In our effort to enhance the properties of the RU486-responsive CrePr1 construct, we have developed the dual GLVP/CrePr system, in which RU486 confers activity control at both the transcriptional and the posttranslational level of CrePr1. This was achieved by placing CrePr1 transcriptional regulation under the control of the RU486-sensitive chimeric regulator GLVP. Stable cell lines harboring the dual GLVP/CrePr as well as the single CrePr1 system were developed. Our results indicate that the dually regulated system is highly inducible by RU486 while maintaining minimal basal activity ("leakage"), characteristics that can be employed in the development of transgenic mice in which genetic pathways can be turned on or turned off after exogenous administration of RU486 at physiologically inert doses.

Key Words: Cre, loxP, site directed, DNA, recombination

INTRODUCTION

The ability to turn on or turn off gene expression during selected time points in prenatal and/or postnatal development provides investigators the means to study specific genetic pathways *in vivo* while bypassing potential developmental compensatory mechanisms or lethal defects that often occur in transgenic or knockout animals. The Cre/loxP system [1] has been previously employed in generating desirable somatic genetic alterations via site-specific DNA recombination [2–7]. Several inducible Cre-recombinase systems have been previously developed, responsive to interferon [8], tetracycline [9,10], estrogen [11], and mifepristone [12]. In the latter case, the mutant progesterone receptor hPR891, which responds specifically to the synthetic steroid RU486 (mifepristone) but not to endogenous ligands [13], was combined with Cre recombinase, leading to the creation of the RU486-responsive fusion protein CrePr1 [12]. Some of the advantages of CrePr1 include its ability to be induced by RU486 at physiologically inert doses following po or iv administration (10^{-9} – 10^{-7} M), as well as the fact that CrePr1 does not respond to endogenous progesterone. Regulation of

loxP-directed DNA recombination by CrePr1 was demonstrated in the brains of mice *in vivo* [14]. However, detectable basal levels of Cre recombinase activity (leakage) were reported in the absence of RU486 *in vitro* and *in vivo* [12,14], making CrePr1 employment unsuitable in animal developmental studies in which tight gene regulation is paramount.

Utilization of a tightly regulated inducible DNA recombination system is paramount in sensitive developmental studies. We therefore focused on strategies that would abolish basal Cre activity. We hypothesized that placement of CrePr1 transcriptional expression under RU486 would eliminate leakage, whereby a single ligand would confer simultaneous regulation of CrePr1 at the transcriptional and posttranslational level. To this end, we employed the chimeric regulator GLVP [15], which is a fusion protein composed of the mutant progesterone receptor hPR891, the yeast transcriptional activator GAL4 DNA-binding domain, and the herpes simplex virus protein VP16 activator domain. We demonstrate here that this dually regulated CrePr/GLVP DNA recombinase system is highly inducible by RU486 and lacks any signifi-

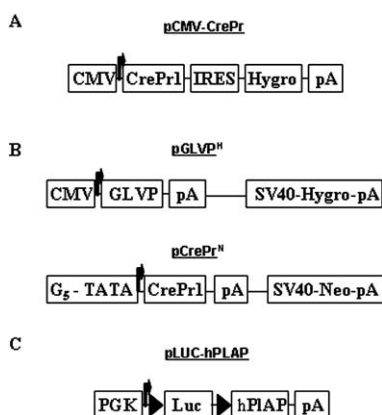


FIG. 1. Description of vector systems. (A) CrePr1, an RU486-regulated fusion protein of Cre recombinase and the mutant progesterone receptor hPR891, was cloned into the multiple cloning site of the pIRESHyg2 vector (Clontech). (B) The dually regulated system consists of the GLVP transcriptional activator, a fusion protein of hPR891, the yeast transcriptional activator GAL4, and the herpes simplex virus protein VP16, as well as the CrePr1 fusion gene driven by a minimal G_5 -TATA promoter (GAL4₅-TATA). (C) The dual recombination reporter plasmid consists of a PGK-driven loxP-flanked luciferase gene (Luc) followed by the human placental alkaline phosphatase gene (hPLAP). Successful loxP-directed Cre recombination would result in excision of the “floxed” Luc gene and subsequent expression of hPLAP.

cant basal activity; thus this new construct offers a tightly regulated system for control of gene expression via loxP-directed DNA recombination.

RESULTS

Transient Transfection

We treated BHK-21 cells transiently transfected with the pCrePr^N, pGLVP^H, and pLUC-hPLAP plasmids (Fig. 1) with RU486 (Fig. 2). Forty-eight hours posttreatment, cel-

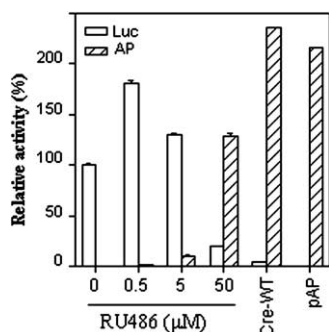


FIG. 2. RU486 regulates GLVP/CrePr activity. BHK-21 hamster kidney cell line was transiently transfected in triplicate with the vectors PGK-GLVP and G_5 -TATA-CrePr and the dual reporter pLUC-hPLAP. Following RU486 administration, Luc activity decreased concomitantly with increased AP activity as measured at 48 h posttreatment. Coexpression of pLUC-hPLAP and wild-type Cre recombinase (Cre-WT) resulted in AP upregulation at levels similar to those of the collapsed reporter pCMV-hPLAP-pA (pAP).

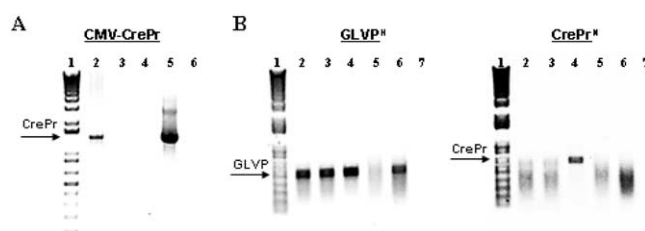


FIG. 3. Development of CrePr stable cell lines. (A) One of three CMV-CrePr-IRES-hygro clones selected with hygromycin expressed full-length CrePr transcript as demonstrated by RT-PCR (lane 2). Lane 1, 1 kb+ standard; lane 5, positive control (pCMV-CrePr-IRES-hygro plasmid); lane 6, primers control. (B) One of five dual GLVP^H/CrePr^N clones selected simultaneously with hygromycin and G418 expressed full-length transcripts for GLVP and CrePr as shown by RT-PCR (lanes 4) after addition of RU486 (10^{-7} M) to the culture medium.

lular alkaline phosphatase (AP) activity was increased, and luciferase activity (Luc) was concomitantly reduced by RU486 in a degree proportional to the amount added. We employed vectors coding for wild-type Cre recombinase and the collapsed reporter construct hPLAP (pAP) as controls (Fig. 2). Furthermore, individual transfection of pCrePr^N or pGLVP^H did not result in any changes in phosphatase or luciferase activity (data not shown).

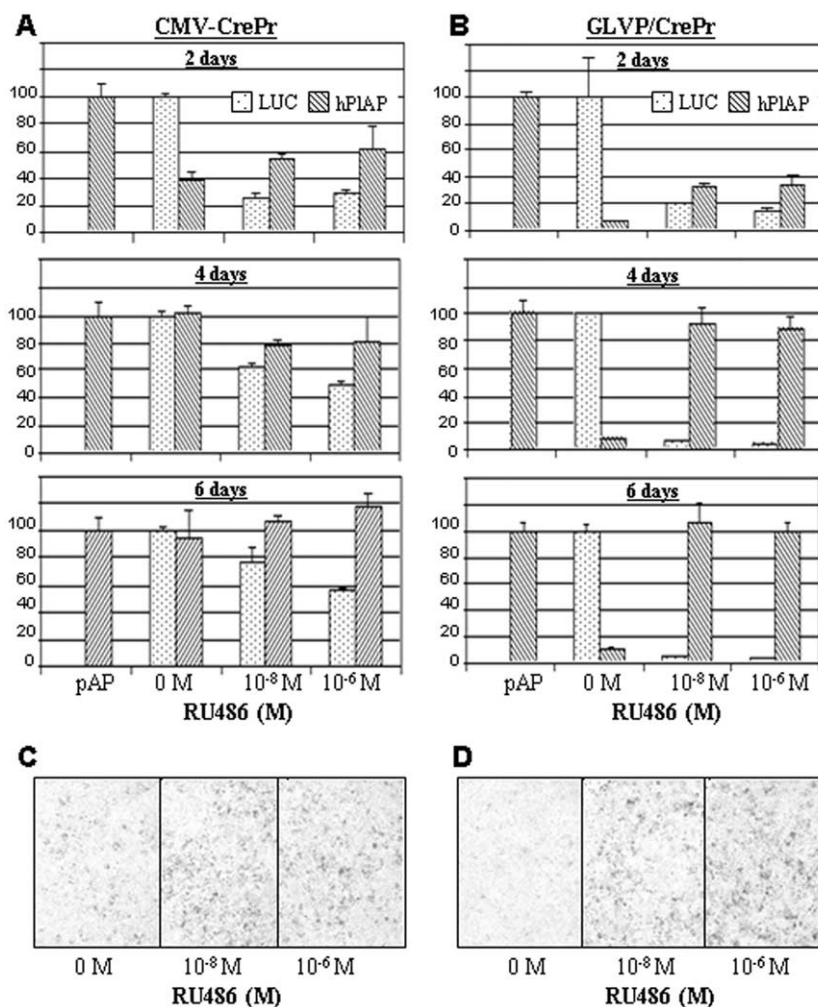
Stable Cell Lines

We selected stable 293H cell lines for (A) CMV-CrePr^H and (B) CMV-GLVP^H and G_5 -TATA-CrePr^N. We confirmed expression of full-length constructs by RT-PCR (Fig. 3). Subsequently, we temporally analyzed the regulation of these two systems by RU486 by employing the pLUC-hPLAP reporter. RU486 administration overall induced both the CMV-CrePr and the GLVP/CrePr systems as assessed by the increase in AP and decrease in Luc activities (Fig. 4). Results are depicted as relative AP and Luc activity (%) compared to pAP and unstimulated pLUC-hPLAP, respectively. At 2 days posttreatment, the CMV-CrePr system showed higher AP levels in response to RU486 than those observed in the GLVP/CrePr cell line; this observation may result from increased basal AP levels in the absence of RU486 (“leakage”). At posttreatment days 4 and 6, the GLVP/CrePr construct was tightly regulated by RU486 with low basal expression levels; in contrast the CMV-CrePr construct displayed a high degree of leakage. We also examined AP expression in these samples by means of BCIP/NBT histochemistry; our observations confirmed our earlier results (Figs. 4C and 4D). Specifically, administration of RU486 exerted an increase in the number of BCIP/NBT-positive cells in both systems; we noted a considerable number of positive cells in the unstimulated CMV-CrePr, indicating leakage compared to GLVP/CrePr.

RU486 Induces GLVP/CrePr DNA Recombinase Activity

We qualitatively evaluated the molecular properties of the dual GLVP/CrePr system by constructing a dormant bicis-

FIG. 4. Temporal analysis of Cre recombinase activity in the CMV-CrePr and GLVP/CrePr cell lines. The dual reporter pLUC-hPIAP was transiently transfected into the CMV-Cre and GLVP^H/CrePr^N cell lines in triplicate. Following RU486 administration, luciferase (LUC) and alkaline phosphatase (hPIAP) activity was measured at 2, 4, and 6 days posttreatment. Cells transfected with the collapsed reporter pCMV-hPIAP-pA (pAP) were used as positive controls. Results are depicted as percentages of activity. (A) Addition of RU486 to the culture medium of CMV-CrePr resulted in decrease in LUC and increase in AP activity as early as 2 days posttreatment. However, at 4 and 6 days posttreatment, significant levels of basal AP activity were detected in the absence of RU486, indicating "leakage" of CMV-CrePr. (B) Regulation of the GLVP/CrePr system by RU486 resulted in decrease in LUC and concomitant increase in AP activity. In contrast to CMV-CrePr, the dual GLVP/CrePr construct was tightly regulated by RU486 in all time points and showed a considerably smaller degree of basal activity (leakage). (C) AP histochemistry was detected in considerable levels in the CMV-CrePr cell line under naïve conditions and was upregulated by RU486 at 4 days posttreatment. In contrast, (D) absence of AP expression was noted in unstimulated GLVP^H/CrePr^N cells.



tronic gene, pHex^{XAT}, comprising both subunits of the human β -hexosaminidase, HexA and HexB (Fig. 5A). Following transient Hex^{XAT} expression in the GLVP^H/CrePr^N cell line, addition of RU486 (10^{-7} M) to the culture medium resulted in loxP-directed excisional DNA recombination as demonstrated by PCR amplification of DNA extracts from RU486-treated (Fig. 5B, lane 1) and unstimulated (lane 2) cells employing PCR primers flanking the transcription termination cassette (Fig. 5B). As anticipated, we detected GLVP expression by RT-PCR at constitutive levels (lanes 1–4, Fig. 5C); CrePr mRNA levels were induced after RU486 administration (lanes 1 and 3), leading to Hex^{XAT} transcriptional activation. Although we detected basal levels of β -hexosaminidase expression by RT-PCR (the 293H parent cell line is of human origin), addition of RU486 to the culture medium further increased the HexA and HexB mRNA levels (lane 1). Furthermore, RU486 (10^{-7} M) appeared to induce β -hexosaminidase expression in 293H cells in the absence of Hex^{XAT} (lane 3), indicating that the human β -hex-

osaminidase gene can be upregulated by RU486. Transient transfection of pHex^{XAT} also resulted in modest elevation of HexA and HexB mRNA levels in the absence of RU486 (lane 2), suggesting incomplete gene inactivation by the particular transcription termination cassette.

GLVP/CrePr Tightly Regulates the Expression of the Dormant Hex^{XAT} Gene

We further characterized the biological properties of the inducible GLVP/CrePr system by quantitative analysis of Hex^{XAT} regulation by RU486 in the GLVP^H/CrePr^N cell line. At 4 days posttreatment, RU486 induced HexA and HexB mRNA expression significantly as evaluated by semiquantitative RT-PCR (Fig. 6A). The cellular β -hexosaminidase activity increased fourfold on the 4th day after treatment compared to unstimulated controls as assessed by 4MUG fluorometry (Fig. 6B). Histochemical analysis of β -hexosaminidase activity *in situ* by X-Hex histochemistry showed upregulation of enzyme activity following RU486 treatment (Fig. 6C).

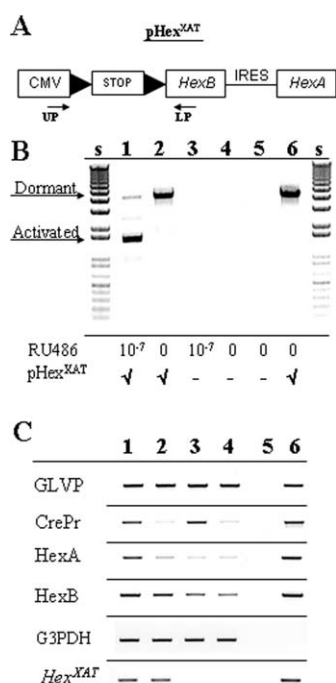


FIG. 5. Stimulation of GLVP/CrePr by RU486 resulted in site-directed DNA recombination and subsequent activation of a dormant transcriptional unit. (A) pHex^{XAT}, a dormant bicistronic transgene composed of both subunits of the human β -hexosaminidase, was transiently expressed in the GLVP^H/CrePr^N cell line in triplicate. (B) Addition of RU486 (10⁻⁷ M) to the culture medium resulted in loxP-directed DNA excisional recombination (lane 1). (C) In the presence of RU486, GLVP induced the synthesis of CrePr (lanes 1 and 3), which in turn resulted in transcriptional activation of pHex^{XAT} and synthesis of HexA and HexB mRNA. Results were normalized to G3PDH levels. Transcription efficiency was assessed by PCR employing primers designed to a plasmid-specific sequence (Hex^{XAT}).

DISCUSSION

Tightly regulated, nonleaky, site-specific DNA recombination systems for the generation of somatic mutations are highly desirable in the study of gene product function *in vivo*. Conditional regulation to control gain or loss of gene function affords a means to circumvent potential developmental compensatory mechanisms often described in transgenic animals or embryonic/perinatal lethality often seen in knockout mice. In our effort to enhance the regulatory properties of the RU486-responsive CrePr1 fusion protein [12], we placed CrePr1 transcriptional regulation under the control of the RU486-inducible GLVP system [15]. We hypothesized that dual control of CrePr1 activity by RU486 at the transcriptional and posttranslational levels would abolish any basal DNA recombinational activity (leakage) while maintaining high inducibility. In brief, we have developed a system in which the expression of the CrePr1 open reading frame is driven by a minimal promoter (GAL4₅-TATA). Since GAL4 is not found in mammalian cells and no mammalian target genes are known

to be activated by GAL4, the chimeric regulator GLVP cannot inadvertently transactivate endogenous genes. The GLVP, driven by the cytomegalovirus (CMV) promoter, encodes a chimeric gene product that can be activated by RU486 at low concentrations (nanomolar), but not progesterone or other endogenous hormones [13,20], to upregulate target gene cassettes containing the GAL4₅-TATA minimal promoter. The fusion of the mutated (42-amino-acid C-terminal deletion) human progesterone receptor (hPRB891) to Cre conferred inhibition of its recombinase activity [12,14], presumably resulting from masking of the catalytic site. Binding of RU486 to the hPRB891 makes this inactive enzyme catalytically capable.

Our data suggest that the dual GLVP/CrePr system is tightly regulated by RU486, lacking any significant leakage. Specifically, only minimal levels of basal activity were detected in both transient and stable expression experiments. The low AP basal activity observed was attributed to low-level readthrough of the pLUC-hPLAP reporter plasmid. In fact, based on transient transfection data, pLUC-hPLAP transfection into BHK-21 cells resulted in small but detectable levels of AP activity. Administration

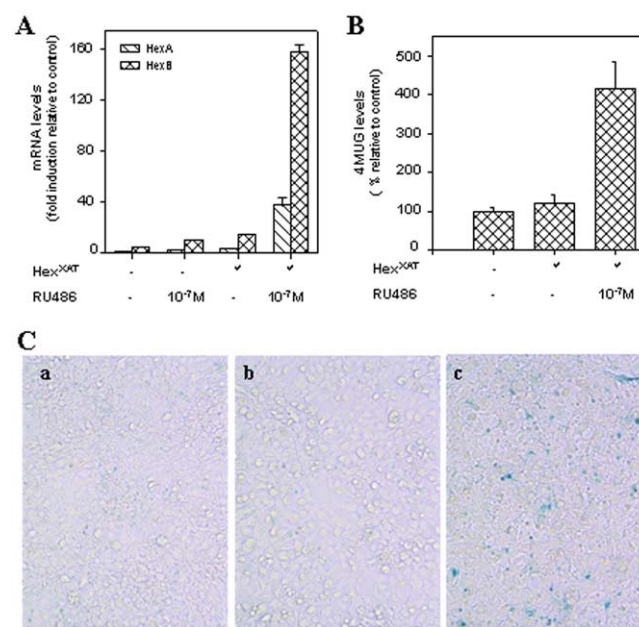


FIG. 6. GLVP/CrePr tightly regulates pHex^{XAT} transcription. The pHex^{XAT} dormant gene was transiently transfected into the GLVP^H/CrePr^N cell line and expression of HexA and HexB was evaluated at the mRNA and enzyme activity levels. RU486 (10⁻⁷ M) significantly upregulated (A) HexA and HexB at the mRNA level, as assessed by semiquantitative RT-PCR (normalized to G3PDH levels), as well as (B) β -hexosaminidase activity, as assessed by 4MUG fluorometry. Comparable transfection efficiency was confirmed after evaluating the levels of pHex^{XAT} DNA plasmid by PCR. (C) These results were confirmed by X-Hex histochemistry (blue staining): (a) naïve cells and pHex^{XAT}-transfected cells with (b) plain and (c) RU486-rich medium.

of RU486 to the culture medium consistently resulted in significant induction of AP concomitantly with reduction of LUC levels compared to untreated controls. In comparison, the ubiquitously expressed CrePr1 (CMV-CrePr) exhibited considerably higher basal activity levels in both the transient and the stable transfection experiments. This observation is in contrast to a previous report [12] and can be explained by the fact that different cell lines were utilized to test CrePr1 as well as that the results were based on different reporter constructs. Kellendonk *et al.* [12] utilized cells of the CV-1 line for their experiments and speculated that due to the low expression of CrePr1 in their stable cell line DNA recombination rates were low and therefore affected the overall inducibility of their construct by RU486. In comparison, GLVP-regulated synthesis of CrePr1 in our experiments was highly inducible by RU486, both in the transient transfection experiments utilizing the BHK-21 cell line and in the stable expression experiments employing the 293H cell line. In addition, the reporter system in their experiments was based on a β -galactosidase (*lacZ*) assay, whereas we employed methods evaluating alkaline phosphatase and luciferase enzymatic activities.

The data presented herein show clearly that placement of Cre recombinase under dual transcriptional and post-translational control leads to attenuation of basal leakage while maintaining high inducibility. These are highly desirable characteristics of an inducible expression system, which can be employed in the development of transgenic mice in which genetic pathways can be turned on or turned off after exogenous administration of RU486 at doses that cannot induce abortion, prevent nidation, or exert any teratogenic or mutagenic effect [21].

MATERIAL AND METHODS

Plasmid construction. The CrePr1 fusion gene was constructed by PCR, as previously described by Horton *et al.* [16]. Please refer to Fig. 1 for a summary of constructs. The Cre fragment was amplified from pMC1cre [17] utilizing (A) an N-terminal oligomer (5'-AGAATTCGCATGCCAAGAGAAGAG-3') containing an *EcoRI* restriction site (underlined) and a nuclear localization sequence and (B) a C-terminal oligomer (5'-AACTTTTATCGCCATCTCCAGCAGG-3') that is in part complementary to hPR891 (underlined). Furthermore, the hPR891 fragment was PCR amplified from pGLVP [15] utilizing (C) an N-terminal oligomer (5'-GATGGCGATAAAAAGTTCAATAAAGTCAG-3'), in part complementary (underlined) to the 3' end of the Cre fragment (above), and (D) a C-terminal oligomer (5'-CGAATCTAGAGTCAGCAGTACAGATG-3') containing an *EcoRI* restriction site (underlined). The overlapping fragments obtained were used in a fusion PCR with oligomers A and D as previously described by [16] to produce the fusion gene CrePr1. A minimal promoter comprising five copies of the yeast GAL4 recognition motifs (17-mer: CGGAGTACTGTCCTCCG) upstream of an SV40 TATA box (G_5 -TATA) [18] drives the expression of CrePr1. G_5 -TATA was amplified by PCR out of pG5BCAT (kindly provided by Dr. Howard Green, Harvard University) using a sense primer (5'-CCCAAGCTTCATGCCTGCA-3') containing a *HindIII* restriction site (underlined) and a lower primer (5'-GCGAATTCGAGCTCGGTACC-3') containing an *EcoRI* restriction site (underlined). The G_5 -TATA PCR product was isolated by agarose gel electrophoresis and cloned into the *HindIII* and *EcoRI* sites of pBS KS(+) (Stratagene). CrePr1 was subse-

quently inserted downstream into the *EcoRI* site. The accuracy of the construct was confirmed by multiple restriction enzyme digestions as well as by direct DNA sequencing. The *Sall*-*Apal* fragment containing G_5 -TATA-CrePr was then cloned into the pRc/CMV vector (Invitrogen, Carlsbad, CA) between *BglIII* and *NotI* by blunt ligation, creating pCrePr^N. The pGLVP^H plasmid was kindly provided to us by Dr. Bert O'Malley [15]. The CrePr fusion gene was also cloned into pRESHyg2 vector (Clontech, Palo Alto, CA) as follows. CrePr1 was excised from pCrePr^N by *EcoRI* digestion and cloned into the *NotI* site of pRESHyg2 by blunt ligation (pCMV-CrePr), the accuracy of which was confirmed by multiple restriction enzyme digestions as well as by direct DNA sequencing. The CMV promoter ubiquitously drives the expression of a bicistronic gene comprising CrePr1 and the hygromycin resistance gene (CMV-CrePr-IRES-Hygro). Expression of the second reading frame was facilitated by an internal ribosomal entry sequence (IRES). The dual recombination reporter CMV-loxP[LUC-pA]loxP-hPIAP-pA (pLUC-hPLAP) kindly provided by Dr. Barry Stripp (University of Rochester), whereby the luciferase reporter gene is flanked by loxP sites, target of the site-specific recombinase Cre, followed downstream by the human placental alkaline phosphatase gene.

A bicistronic transgene encoding both subunits of the human β -hexosaminidase, HexA and HexB, was constructed following isolation of the human HexB cDNA from the pHexB43 plasmid (ATCC, Manassas, VA) by *XhoI* digestion and insertion into the *XhoI* site of the pIRES expression vector (Clontech). The HexA cDNA was isolated from pBHA-5 (ATCC) following *XhoI* digestion and subsequently inserted into the *XbaI* site of the pIRES vector by blunt ligation. The CMV promoter drives transgene expression, and the translation of the second open reading frame, HexA, is facilitated by an IRES. The excisionally activated Hex^{XAT} gene was constructed by utilizing the pBigT vector containing a loxP-flanked translational termination cassette [19], which was kindly donated to us by Dr. Wei Hsu (University of Rochester). The CMV promoter was amplified from the pRc/CMV vector (Invitrogen) by polymerase chain reaction employing the following upper and lower primers: 5'-AATATCTTAATTAATCTCTAGATGCTTCGCGATGTACGGGC-3' and 5'-TAGTCATATATGATCTTAAT-TAAAAGCTTGGGTCTCCC-3' (*PacI* restriction enzyme sequence is underlined). The PCR product was gel purified, cloned into the pRCII-TOPO multiple cloning site (Invitrogen), and subsequently bidirectionally sequenced. The *PacI*-flanked CMV promoter sequence was then cloned upstream of the first loxP sequence in the pBigT vector (pCMV-BigT). Subsequently, the *NheI*-*NotI* pHex fragment containing the bicistronic gene HexB-IRES-HexA was cloned into the *NheI* and *NotI* sites of the pCMV-BigT vector, leading to the construction of pHex^{XAT}: CMV-loxP-STOP-loxP-HexB-IRES-HexA (Fig. 5).

Transient transfection. Transfection of BHK-21 cells (ATCC) was performed utilizing the LipofectAMINE 2000 reagent per the manufacturer's instructions (Invitrogen). In brief, BHK-21 cells were seeded in 12-well plates (10^4 cells/well) in DMEM growth medium with 10% FBS (Invitrogen) at 37°C and 5% CO₂ 24 h prior to transfection. A total of 1 μ g of DNA was added per well using this method, including the pCrePr, pGLVP, and pLUC-hPLAP plasmids. A 1 mM stock solution of RU486 (mifepristone; Sigma, St. Louis, MO) was prepared in 80% ethanol and was added to the culture medium 6 h following transfection. Cell samples were harvested 2 days posttreatment for luciferase and alkaline phosphatase analysis utilizing the luciferase (Promega, Madison, WI) and Phospha-Light (Tropix, Bedford, MA) reagents per the manufacturers' instructions using a Packard Lumnicont BL1000 plate reader (Meriden, CT). In another experiment, BHK-21 cells were transfected in 12-well plates with pCMV-CrePr and pLuc-AP plasmids (1 μ g total DNA per well) and subsequently treated with RU486 at 10^{-6} M final concentration, and samples were harvested 2 days posttreatment for luciferase and alkaline phosphatase analysis as described above.

Stable cell line generation. Stable cell lines were established by cotransfecting the pCrePr^N and pGLVP^H plasmids into 293-H cells (Invitrogen) using the LipofectAMINE 2000 reagent and simultaneously selecting with G418 (400 μ g/ml; Invitrogen) and hygromycin B (200 μ g/ml; Sigma) in DMEM growth medium with 10% FBS and 1 \times nonessential amino acid solution (Invitrogen) at 37°C and 5% CO₂. Appropriate cell lines were

selected following detection of CrePr and GL-VP mRNA by RT-PCR from total RNA extracts. In brief, 1 μ g of total RNA isolated from cells utilizing Trizol reagent (Invitrogen) per the manufacturer's instructions was reverse transcribed by employing the First Strand Synthesis kit (Invitrogen) and poly(T) primers per the manufacturer's instructions, followed by PCR utilizing primers specifically designed for CrePr1 (5'-CCAATTACTGACCGTACACC-3' and 5'-CCAACACCATTAAGCTCATCC-3') and GLVP (5'-CCAGAGATTCACATTTTTCACC-3' and 5'-TGACTTCGTAGCCCTTCC-3'). RT(-) samples were included in the experiment to control for possible DNA contamination of RNA extracts. Successful lines were then expanded and seeded on 12-well tissue culture dishes (10^4 cells/well) and maintained for 24 h. After a fresh medium change, the cells were transiently transfected with the pLUC-PLAP reporter plasmid (1 μ g) utilizing the LipofectAMINE 2000 reagent per the manufacturer's instructions. Six hours following pLUC-hPLAP transfection, RU486 was added to the culture medium at 10^{-6} M and 10^{-8} M final concentrations. Cell samples were harvested at days 2, 4, and 6 for luciferase and alkaline phosphatase analysis utilizing the luciferase (Promega) and Phospha-Light (Tropix) reagents per the manufacturers' instructions with a Packard Lumnicount BL1000 plate reader. In addition, for cytochemical detection of alkaline phosphatase activity, cells plated on poly-D-lysine-coated 12-well plates (Biocoat Cell Environment; Becton-Dickinson, Bedford, MA) were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min, followed by extensive PBS washes, and stained with BCIP/NBT (Vector Laboratories, Burlingame, CA) per the manufacturer's instructions. An Olympus BX51 light microscope with attached Spot RT digital camera connected to a personal computer was employed to capture digital images of BCIP/NBT histochemistry.

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