A Novel Drug-Regulated Gene Expression System Based on the Nuclear Receptor Constitutive Androstane Receptor (CAR)

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Purpose. To develop and characterize a new drug-regulated gene expression system based on the nuclear receptor constitutive androstane receptor (CAR).

Methods. Both transient and stable transfection into HEK293 cells of luciferase plasmids under the control of either drug- and steroid-responsive nuclear receptor CAR or the tetracycline-sensitive transactivator tTA were used in development of stable cell lines.

Results. A stable first-generation cell line that expresses luciferase gene under the control of nuclear receptor CAR was developed. The luciferase expression in CAR-producing cells could be suppressed by androstanes and reactivated by structurally unrelated drugs chlorpromazine, metyrapone, phenobarbital, and clotrimazole. The kinetics of luciferase expression in CAR-producing cells and the tTA system were comparable. The overall regulation of CAR system was improved by modifications to the DNA binding domain and site.

Conclusions. Because of its wide ligand selectivity and transferable ligand binding domain, CAR expands the repertoire of regulated gene expression systems.

KEY WORDS: HEK293 cells; transactivator tTA; nuclear receptor CAR; 1,4-*bis*(2-(3,5-dichloropyridyloxy))benzene; tetracycline; androstenol.

INTRODUCTION

Gene expression systems that can be reversibly controlled by extracellular signals are valuable in experimental biology and applied research. Such expression systems allow detailed studies on the physiological effects of any given cellular protein and potentially aid in the development of, for example, gene therapy. Early gene expression systems relying on mammalian heat shock-, heavy metal-, or hormone-responsive components suffered from undesirable pleiotropic effects (1) which were mostly circumvented by the increasingly popular system based on the bacterial *tet* operon and its repressor (2,3). In the original bacterial system, a fusion protein (tetracycline [TET]-sensitive transactivator [tTA]) between the *tet* repressor and the activation domain of viral protein 16 (VP16) can bind to its cognate DNA element and activate transcription only in the absence of tetracycline (TET). Several versions of this system are now available (4). On the other hand, the VP16 moiety may be toxic to some cells, the accumulation and slow removal of TET from cells and tissues may hamper regulation, and finally, cells do not necessarily respond gradually to changes in TET concentration (3,5–8). Therefore, alternative gene expression systems would be valuable.

Eukaryotic nuclear receptors are ligand-dependent transcriptional activators (9) that have also been employed to control gene expression. In these systems (10–12), the gene expression can be turned on either by a progesterone antagonist mifepristone (RU486) or by ecdysteroids that are cell-permeable chemicals with a relatively fast rate of elimination. Both TET and the nuclear receptor expression systems are, however, currently restricted to a single ligand or structurally very similar ligands. If the ligand has undesired properties or unknown side effects in a particular experimental setting, that would necessitate the change of the entire system.

To expand the selection of available gene expression systems, we used a nuclear receptor called constitutive androstane receptor (CAR) as a regulator that is sensitive to diverse ligands. *In vivo,* CAR controls the drug- and xenobiotic-inducible expression of mammalian cytochrome P450 *CYP2B* genes through its binding to the phenobarbital-responsive enhancer (PBREM) (13–15) (see Fig. 1). On the basis of transient transfection studies, some androstane metabolites are suppressors of CAR (15–17). Several structurally diverse chemicals, including clinically used drugs such as phenobarbital, chlorpromazine, and metyrapone, can reactivate suppressed CAR (15,17) and activate PBREM *in vivo* (13). At least 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is a direct agonist for CAR (17). Although it may be predicted that compounds able to induce *CYP2B* genes would also be CAR agonists, detailed structural requirements of CAR ligands are not yet known.

We describe stable cell lines carrying genes for both CAR and a PBREM-driven luciferase (activity) (LUC) reporter in comparison with the tTA-expressing system. CAR- and tTA-based systems performed equally well in LUC expression assays. We also show that replacement of the CAR DNA binding domain by the yeast GAL4 domain improved the range of regulation in transient transfection assays. Because of its wide ligand selectivity and transferable ligand-binding domain, CAR can be improved and it will complement other regulated gene expression systems. Furthermore, our CAR-based system is valuable in elucidation of the CAR ligand specificity.

MATERIALS AND METHODS

Chemicals

The synthesis of 1,4-*bis*[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) has been described (13). Steroids were purchased from Steraloids Inc. (Newport, RI) or Sigma Chemical Co. (St. Louis, MO). G418 and hygromycin B were from Calbiochem (La Jolla, CA). Other chemicals were at least analytical grade from Sigma. Deoxyoligonucleotides were synthesized and purified at the Nucleotide Synthesis

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ABBREVIATIONS: ANDR, 16,5α-androsten-3α-ol; CAR, constitutive androstane receptor; LUC, luciferase (activity); PBREM, phenobarbital-responsive enhancer module; RU486, mifepristone; TCPOBOP, 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene; TET, tetracycline; tk, thymidine kinase; tTA, TET-sensitive transactivator; VP16, activation domain of viral protein 16.

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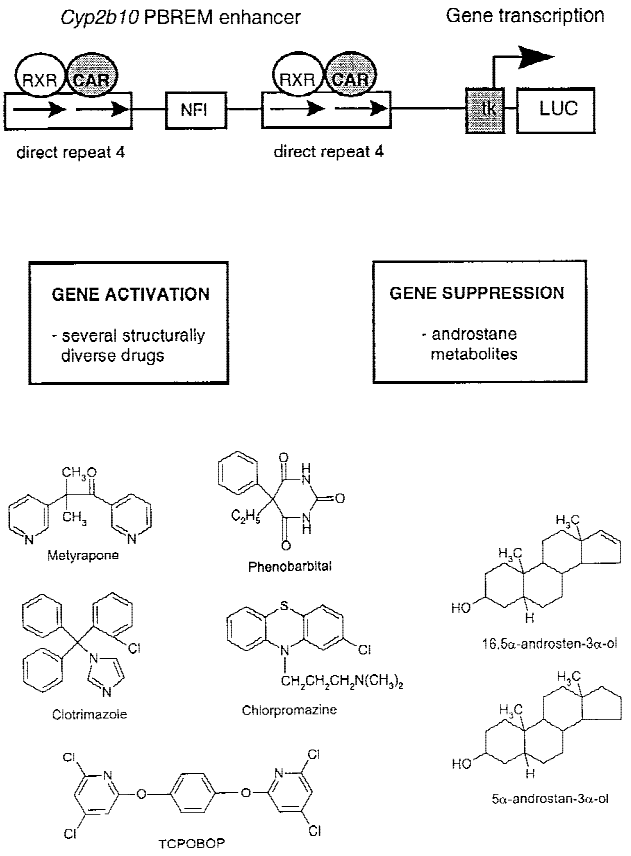

Fig. 1. The expression system based on nuclear receptor CAR. (A) The *Cyp2b10* gene contains a drug-responsive PBREM enhancer that consists of an NFI site surrounded by two direct repeat 4 motifs (double arrows) to which CAR binds as a heterodimer with retinoic X receptor (RXR) (13–15). In this work, the PBREM enhancer has been linked to a thymidine kinase (tk) promoter/luciferase reporter (LUC) construct. (B) The activity of the PBREM enhancer can be regulated by several CAR-activating drugs and CAR-inhibiting steroids (see references 13, 14–17 and the Results section).

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Plasmids

Plasmids coding for tTA (pTetOff), tTA-responsive LUC (pTREluc) (2), pTKhyg, and pCMVβ were purchased from Clontech Inc. (Palo Alto, CA). The LUC reporter plasmid pPBREMluc was constructed by *Bgl*II excision of the PBREM element containing CAR binding sites and the thymidine kinase promoter from pPBREMtkCAT (13), and insertion into *Bgl*II site of pGL3-Basic plasmid (Promega, Madison, WI). The CAR cDNA (15) was released as a *BamH*I (blunt)-*Xho*I fragment and inserted in *Eco*RI (blunt) and *Sal*I sites of pCI-neo vector (Promega). The ligand binding domain (amino acids 118–358) of mouse CAR was amplified from CAR cDNA with proof-reading *Pfu* DNA polymerase and inserted in-frame at the *Eco*RI and *BamH*I sites of the CMX-GAL4 plasmid (reference 18, courtesy of Dr. R. M. Evans). The UASx4-tk-luc reporter plasmid (18) was donated by Dr. D. Mangelsdorf and Dr. R.M. Evans. The plasmids were purified with Qiagen columns (Hilden, Germany) and verified by dideoxy sequencing and restriction mapping.

Generation of Cell Lines Expressing CAR- or tTA-Regulated LUC

HEK293 cells (ATCC CRL-1573) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin-100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD). Cells were transfected with the pCI-neo/CAR plasmid by the calcium phosphate method (19) and transformed cells were selected with 0.4 mg/ml G418. Twenty-four colonies were expanded and tested for expression of CAR by transient transfection as follows. Replicate cell aliquots were seeded on 24-well plates and transfected at 50% confluence with pCMVβ plus pPBREMluc plasmids (100 ng each). Cells were then cultured for 40 h in the presence of either 5 μM 16,5α-androsten-3α-ol (ANDR) or 0.5 μM TCPOBOP to repress or activate CAR-regulated LUC activity, respectively (15–17). Several colonies yielding CAR-dependent responses stronger than or equal to transient transfection assays (15- to 20-fold) were transfected with pPBREMluc *plus* pTKhyg (in 20:1 ratio) and selected with 0.1 mg/ml hygromycin B. Twenty-four colonies resistant to both G418 and hygromycin B were tested for ANDR-repressed and TCPOBOP-activated LUC. Colonies expressing both the tTA transactivator and the TET-responsive LUC were generated similarly using pTetOff and pTREluc *plus* pTKhyg plasmids, transient transfections, and TET (2 μg/ml) treatments according to manufacturer's instructions. Four to 5 colonies out of 24 exhibited good CAR- or tTA-regulated LUC activity, and sublines HEK293:CAR/PBREMluc (M29) and HEK293:tTA/TREluc (31-4) were expanded and characterized in further studies. Their growth and morphological characteristics did not differ from the parent HEK293 cells.

Treatments with Inducing Chemicals

To assess the reactivation potential of various chemicals, M29 cells were first grown in the presence of 5 μM ANDR for 24 h to suppress CAR completely. Then, known PBREM- or CAR-activating chemicals (13–15) or vehicles were added for 48 h. In dose and time response experiments, ANDR-pretreated cells were treated with 3.2–2000 nM TCPOBOP for 48 h or with 500 nM TCPOBOP for up to 48 h.

Treatments with Suppressing Chemicals

The inhibitory potential of various chemicals was evaluated using untreated M29 cells. In time response experiments, M29 cells were grown for up to 48 h with 5 μM ANDR. In dose response experiments, ANDR concentration was varied from 0.03 to 10 μM for 48 h. Several other steroids were also tested for their ability to suppress CAR. The 31-4 cells were grown in the absence of TET and then treated with 2 μg/ml TET for up to 48 h. In dose response experiments, TET concentration was varied from 0.01 to 1000 ng/ml. To test the derepression of LUC, 31-4 cells were grown in the presence of 10 or 100 ng/ml TET. Because TET is suspected to adhere to cellular calcium stores (3,4,11), cells were washed thoroughly with medium and fresh medium without TET was added for up to 48 h.

Analytical Assays

LUC was measured (20) with the Bio-Orbit II luminometer standardized with *P. pyralis* luciferase from Sigma (19×10^6 RLU/mg protein). Protein (21) and β-galactosidase (22) assays were performed as before (14).

RESULTS AND DISCUSSION

LUC Expression in M29 and 31-4 Cell Lines

The selection process yielded stable cell lines M29 and 31-4 with morphology and growth characteristics indistinguishable from the parent HEK293 cells. This suggests no overt toxicity by the expressed proteins. During cell development, similar numbers of cell colonies were obtained with CAR- and tTA-expressing plasmids, indicating no apparent bias in the selection process. However, the range of regulation varied among individual colonies from unresponsive to more than 20-fold induction, and in some cases, colonies grew slowly or appeared abnormal. This variation reflects the random manner in which transgenes are inserted in the cell genome and is inherent to the process (23).

In M29 cells, the estimated half-maximal concentrations for LUC suppression and reactivation were about 0.3 μM for ANDR and 80 nM for TCPOBOP, respectively (Fig. 2A). In 31-4 cells, the half-maximal concentration for repression was about 1 ng/ml TET (Fig. 2B). These values are in line with previous results with transiently transfected CAR (15,16) or tTA (2,6,8). Suppression of LUC by ANDR or by TET follows similar kinetics in M29 and 31-4 cells, respectively (Fig. 2C). Figure 2D indicates that LUC could be induced more than 12-fold by TCPOBOP in ANDR-pretreated M29 cells. This result matches previously observed 6- to 8-fold differences between ANDR-repressed and TCPOBOP-activated expression by CAR (15) and 10- to 13-fold activation of PBREM by CAR in transient transfections (14,15). The half-maximal increase occurred at 12 h. In TET-pretreated 31-4 cells, derepression of LUC occurred with a similar or slightly

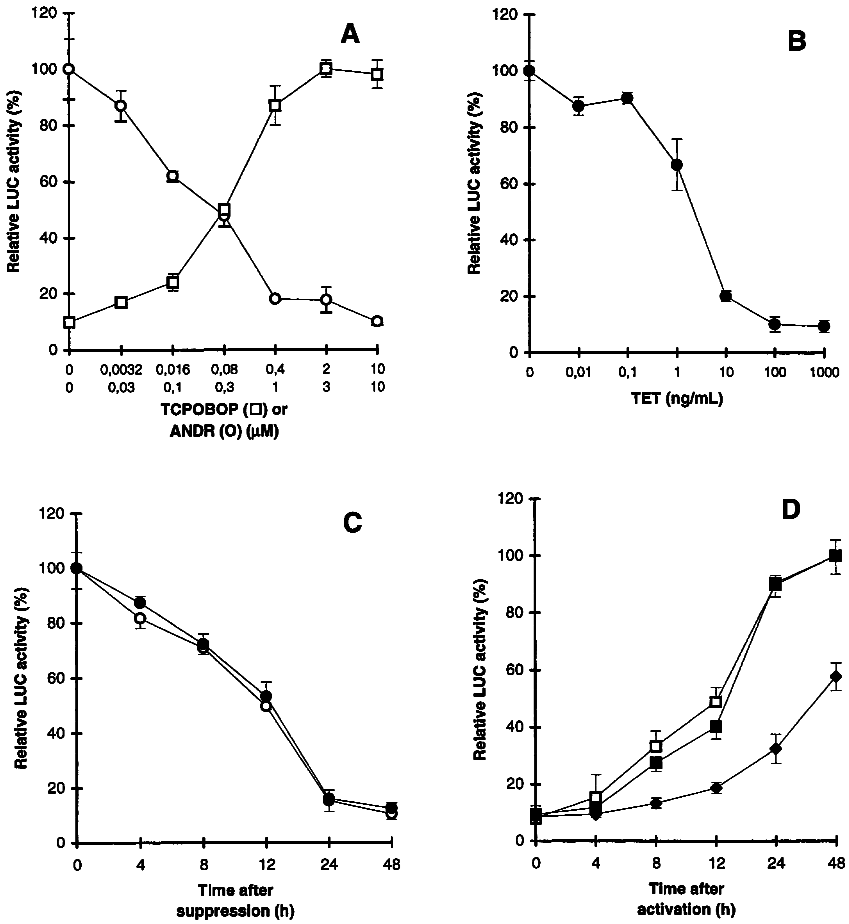

Fig. 2. Dose and time response of LUC expression. (A) M29 cells were cultured in the presence (□) or absence (○) of suppressing 5μM ANDR and then incubated for 48 h with indicated concentrations of TCPOBOP (□, upper values on *x*-scale) or ANDR (○, lower values on x-scale). (B) 31-4 cells (●) were incubated for 48 h with indicated concentrations of TET. (C) M29 or 31-4 cells were incubated for indicated times with suppressing 5 μM ANDR (○) or 2 μg/ml TET (●), respectively. (D) M29 cells (□) or 31-4 cells (■, ♦) were pretreated with suppressing 5 μM ANDR or 10 ng/ml TET (■) or 100 ng/ml TET (♦), and then for indicated times in the presence of activating 500 nM TCPOBOP or after TET removal, respectively. Data are expressed as relative to unsuppressed LUC (=100) and mean ± SD of 3-4 determinations. The specific unsuppressed, ANDR-suppressed, and TCPOBOP-activated LUC produced in M29 cells were approximately 1000 ± 120, 98 ± 10, and 1550 ± 200 ng LUC/mg protein, respectively. The corresponding values for unsuppressed, TET-suppressed, and reactivated LUC produced in M29 cells were approximately 3000 ± 280, 320 ± 25, and 2850 ± 310 ng LUC/mg protein, respectively.

delayed time scale. When TET concentrations higher than 10 ng/ml were used for pretreatment, a significant delay in derepression occurred despite washing of the cells (Fig. 2D). This indicates that, in some cases, kinetics of TET removal may hamper the tTA system as compared to CAR.

Ligand Specificity of CAR

CAR-dependent LUC can be suppressed by ANDR, its 16α-reduced, and 3-keto derivatives, but not by some other steroids or by TET in M29 cells (Fig. 3A). The presence of extra serum in cell culture resulted in only 15% decrease in LUC. For tTA, only TET but not ANDR-like steroids suppressed the activity. In addition, some serum batches apparently contain residual TET because we have observed variable 20–60% decreases in LUC produced by 31-4 cells upon serum change (Fig. 3A and data not shown). Figure 3B shows that in addition to TCPOBOP, CAR-dependent LUC can be reactivated by structurally diverse, clinically used drugs but not by TET. tTA-dependent LUC was not affected by the most potent CAR ligands. These data with stable cell lines and previous work with transiently transfected genes (13–16) indicate that CAR can be regulated by a wider spectrum of structurally unrelated ligands than the select chemicals currently available in other gene expression systems (4,10–12). The wide ligand specificity of CAR may be advantageous in eliminating the need to change the expression system if a chemical used to regulate CAR has undesirable properties or unintended effects. More detailed studies of the CAR ligand specificity are, however, needed and our M29 cell line will be valuable in such experiments.

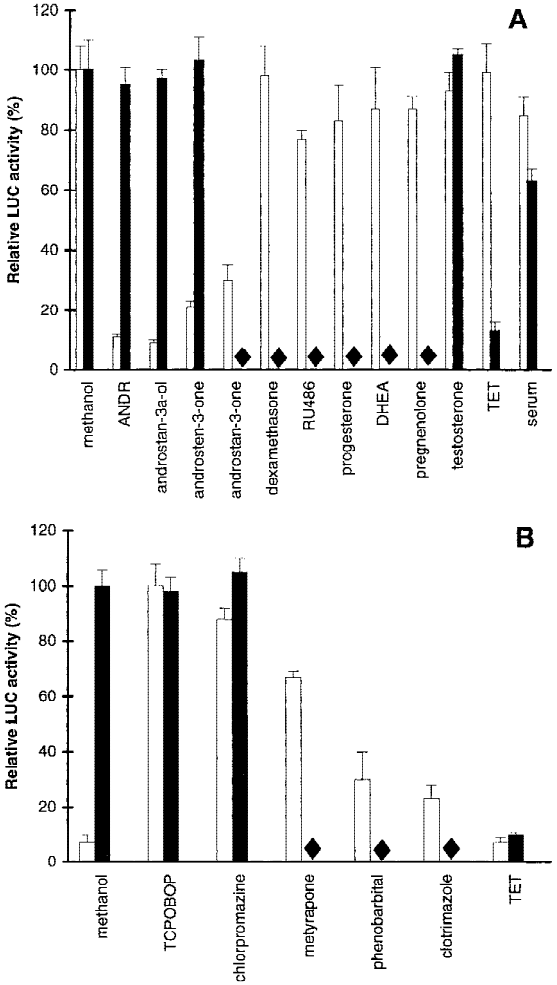

Fig. 3. Selected steroids and drugs regulate LUC activity in M29 cells. (A) M29 cells (*w*hite columns) or 31-4 cells (black columns) were treated for 48 h with 10 μM of indicated compounds (2 μM for ANDR, 2 μg/ml for TET, or 20% serum). (B) M29 cells were pretreated with suppressing 5 μM ANDR (white columns) or untreated 31-4 cells (black columns) were subsequently treated for 48 h with 500 nM TCPOBOP, 10 μM chlorpromazine, 200 μM metyrapone, 300 μM phenobarbital, 2 μM clotrimazole, or 2 μg/ml TET for 48 h. ♦, not done. Data are expressed as relative to unsuppressed LUC (100 = TCPOBOP for M29, methanol for 31-4 cells) and mean ± SD of 3-4 determinations.

Potential Improvements of the CAR System

The first-generation, unmodified CAR system was regulated about 12-fold in stable cell lines. This response may not be sufficient for applications where more robust regulation is needed. Therefore, in an attempt toward improving the response, we fused the ligand binding domain of CAR to the yeast DNA-binding protein GAL4 and replaced the PBREM enhancer with four copies of the 17-mer GAL4 binding site (18). Figure 4 shows that in transient transfections of original CAR and pPBREMluc plasmids, ANDR treatment resulted in 10-fold suppression of LUC. Activation of untreated cells by TCPOBOP alone was 1.6-fold, resulting in 16-fold overall regulation. When GAL4-CAR was used as the regulator, ANDR suppressed LUC to 7% of control values. It is significant that TCPOBOP alone upregulated LUC by more than 3-fold, resulting in 46-fold overall regulation (Fig. 4). Similarly, the response to metyrapone improved from a 30% increase to more than 2-fold activation by the GAL4-CAR fusion protein. This demonstrates that, in order to enhance the range of regulation, the ligand-binding domain of CAR can be transferred to heterologous DNA binding proteins without changing its specificity.

Additional improvements will also be possible in analogy with other nuclear receptors. Further modification of the re-

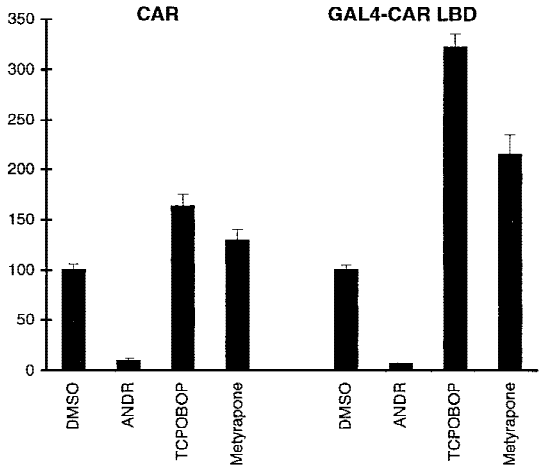

Fig. 4. Modification of the CAR system improves LUC regulation. HEK293 cells were transfected with pCI-neo/CAR (25 ng), pPBREMluc (50 ng), and pCMVβ (100 ng) (left, mCAR) or CMV-GAL4/mCAR (25 ng), UASx4-tk-luc (50 ng), and pCMVβ (100 ng) (right, GAL4-mCAR LBD) and treated with dimethylsulfoxide (DMSO), ANDR (10 μM), TCPOBOP (0.5 μM), or metyrapone (200 μM) for 48 h. Data are expressed as relative to DMSO-treated cells (=100) and mean ± SD of four determinations.

porter construct, such as substituting the thymidine kinase promoter with the TATA box region only (11), might lower the basal LUC expression. Second, the ligand-binding domain of CAR could be fused with stronger activation domains (11,12). In principle, such modifications would enhance the extent of inducibility but retain the ligand specificity. For comparison, the ecdysone receptor-based system was enhanced from a modest 3-fold regulation for first-generation plasmids to more than 200-fold induction in the final, VP16-containing receptor (12). Finally, when knowledge about CAR ligands increases, it may be possible to modify the CAR ligand binding site and to design novel high-affinity ligands.

Potential Drawbacks in the Original CAR System

First, the original PBREMluc plasmid contains a binding site for nuclear factor I and two sites for CAR in the enhancer. It is possible that endogenous nuclear receptors might bind to CAR sites, interfering with the activation. However, among seven nuclear receptors, only CAR could activate the PBREM enhancer (14). The nuclear factor I site appears to bind activators and could be eliminated without affecting the extent of induction response (15). Total replacement of the PBREM enhancer by, for example, the GAL4 binding site circumvents the potential interference by endogenous factors.

A second problem (inherent to all systems that include mammalian components) is the parallel regulation of endogenous genes by CAR ligands. *In vivo,* CAR is expressed only in the liver (24) and its expression is lacking in most continuous cell lines including HepG2 (15). Therefore, concerns about parallel phenomena would probably be limited to differentiated liver tissue in gene therapy applications. Finally, CAR ligands may have other effects unrelated to their binding to CAR. Assessment of the real impact of these issues requires more information on CAR ligand specificity and on CAR target genes.

CONCLUSIONS

We developed first-generation cell lines expressing LUC under the control of drug-responsive nuclear receptor CAR and showed the general utility of the system in gene regulation. We also demonstrated that the CAR system can be improved by using a heterologous DNA-binding protein. The CAR system could become a valuable addition in regulated gene expression because ligands that have already undergone extensive clinical, pharmacokinetic, and safety testing are available to control gene activity. The generated cell lines should also help elucidate the CAR ligand specificity.

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