Identification of genes differentially regulated by glucocorticoids and progestins using a Cre/loxP-mediated retroviral promoter-trapping strategy

Y Wan and S K Nordeen
Department of Pathology and Program in Molecular Biology, University of Colorado Health Sciences Center, Denver, Colorado 80262, USA

(Requests for offprints should be addressed to S K Nordeen, Department of Pathology B216, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, Colorado 80262, USA; Email: steve.nordeen@uchsc.edu)

Abstract

Glucocorticoids and progestins are two classes of steroid hormone with very distinct biological functions. However, the glucocorticoid receptor (GR) and the progesterone receptor (PR) share many structural and functional similarities. One way that glucocorticoids and progestins can exert different biological effects is through their different abilities to regulate the expression of certain target genes. A strategy employing a retroviral promoter-trap and Cre/loxP-mediated site-specific recombination has been developed to identify genes that are differentially regulated by glucocorticoids and progestins. A mouse fibroblast cell line (4F) stably expressing both GR and PR and containing a single copy of a multifunctional selection plasmid is generated. This line is transduced with a self-inactivating retroviral promoter-trap vector carrying coding sequences for Cre-recombinase (Cre) in the U3 region. Integration of the provirus places Cre expression under the control of a genomic flanking sequence. Activation of Cre expression from integration into active genes results in a permanent switch between the selectable marker genes that converts the cells from neomycin-resistant to hygromycin-resistant. Selection for hygromycin resistance after hormone treatment yields recombinants in which Cre sequences in the U3 region are expressed from hormone-inducible upstream cellular promoters. Because Cre-mediated recombination is a permanent event, the expression of the selectable marker genes is independent of ongoing Cre expression. Thus this system permits the identification of genes that are transiently or weakly induced by hormone.

Journal of Molecular Endocrinology (2002) 28, 177–192

Introduction

Glucocorticoids and progestins are two classes of steroid hormones with very distinct and complex biological functions. Many biological actions of glucocorticoids in mammals have been described (Porterfield 1996). These include: (i) regulation of metabolism, (ii) anti-inflammatory and immunosuppressive actions, (iii) stimulation of bone loss by stimulating bone resorption and inhibiting bone formation, (iv) catabolic actions in muscle, skin and connective tissue, (v) actions in the kidney, cardiovascular and gastrointestinal system, and (vi) promotion of milk protein production in the mammary gland. Abnormal function of glucocorticoids leads to complicated and pleiotropic symptomology; adrenocortical insufficiency causes Addison’s disease whereas adrenocortical excess results in Cushing’s syndrome. Thus, appropriate glucocorticoid action is critical to the maintenance of homeostasis. In addition, glucocorticoids have been widely employed as pharmaceuticals. For example, the immunosuppressive properties of glucocorticoids have been exploited to treat chronic inflammatory diseases such as asthma, rheumatoid arthritis, systemic lupus erythematosus, and transplant rejection. Glucocorticoids are also used in the treatment of leukemias and lymphomas. Nonetheless, in many cases, the molecular mechanisms underlying the physiological and...
pharmacological actions of glucocorticoids are poorly understood.

Progestins play major physiological roles, particularly in female reproductive function (Graham & Clarke 1997). These include: (i) release of mature oocytes from the ovary, facilitation of implantation, maintenance of pregnancy by promotion of uterine growth and suppression of myometrial contractility, (ii) promotion of lobular-alveolar development in the mammary gland in preparation for milk secretion and suppression of milk protein synthesis before parturition, (iii) mediation of signals required for sexually responsive behavior, and (iv) prevention of bone loss. Antiprogestins have been applied to prevention of breast cancer and for safe abortion. However, as for glucocorticoids, the mechanisms that tie these biological actions of progestins in target tissues to genes regulated by progestins are not well defined.

Despite the different spectrum of physiological actions mediated by glucocorticoids and progestins, the respective receptors, glucocorticoid receptor (GR) and progesterone receptor (PR), are closely related members of the nuclear receptor family of transcription factors (Thornton 2001). GR and PR share many similar structural and functional characteristics. First, they have a similar domain structure. Both receptors consist of a nonhomologous (<15% sequence identity) N-terminal domain containing a ligand-independent transcription activation function (AF-1), a highly conserved (86% sequence identity) zinc finger DNA-binding domain (Hollenberg et al. 1985, Misrahi et al. 1987, Evans 1988, Kastner et al. 1990), a hinge region where the major nuclear-localization signal is located, and a homologous (54% sequence identity) ligand-binding domain containing a ligand-dependent transcription activation domain (AF-2). PR naturally exists as two isoforms, PR-B and PR-A, that are transcribed from two promoters in a single gene (Kastner et al. 1990, Sartorius et al. 1994). The only difference between PR-A and PR-B is that PR-A lacks 164 N-terminal residues that contain a unique activation function (AF-3). Secondly, the high sequence identity of the DNA-binding domains of the two receptors indicates that they have little or no ability to distinguish individual target sites (Lieberman et al. 1993). Both GR and PR bind as homodimers to an inverted repeat (optimal sequence PuGNACA) separated by 3 bp (Hynes et al. 1983, Payvar et al. 1983, Scheidereit et al. 1983, Cato et al. 1986, Lieberman et al. 1993). Thirdly, they share a similar mode of activation. In the absence of ligand, the receptors form an inactive complex with molecular chaperones. After ligand binding, the receptors dissociate from the chaperones and undergo dimerization (Pratt & Toft 1997). The dimerized receptors bind to target promoters, recruit coactivators and general transcription machinery, leading to regulation of transcription from target genes (Gronemeyer 1991, Tsai & O’Malley 1994). In addition, a number of natural and synthetic ligands bind to both receptors.

How can two receptors with such remarkable similarities mediate such dramatically different biological functions? One mechanism is the tissue-specific expression of receptors (Strahle et al. 1989). GR is broadly expressed in many tissues, but PR has a restricted pattern of expression that includes ovary, uterus, mammary gland, brain and bone (Graham & Clarke 1997). However, in certain tissues that express both GR and PR, these two hormones exert opposite effects under certain circumstances. For example, in mammary gland, glucocorticoids promote milk protein synthesis and lactation (Doppler et al. 1989, Groner et al. 1994, Groner & Gouilleux 1995), while progestins inhibit milk production and secretion (Graham & Clarke 1997). In bone, glucocorticoids stimulate bone resorption (Lane & Lukert 1998, Rackoff & Rosen 1998, Ziegler & Kasperk 1998) while progestins prevent bone loss (Nomura et al. 1989, Prior 1990).

We propose that glucocorticoids and progestins must exert different biological effects, at least in part, through their different abilities to regulate the expression of certain target genes. There could be multiple potential mechanisms for this differential gene regulation. (i) Receptor-specific binding to certain hormone response elements (HREs) in the target promoters (Nelson et al. 1999). In the context of multiple GR/PR-binding sites that together make up an HRE, mutation of a given site can differentially affect glucocorticoid and progestosterone responses (Thackray et al. 1998). (ii) Receptor-specific abilities to remodel chromatin structure. For example, the different abilities of GR and PR to activate a stably integrated mouse mammary tumor virus (MMTV) promoter at certain chromosomal locations is due to the different abilities of GR and PR to remodel the chromatin structure.
(Archer et al. 1994, Bartsch et al. 1996, Beato 1996, Truss et al. 1996, Smith et al. 1997, Lambert & Nordeen 1998). (iii) Receptor-specific coactivators and/or corepressors. Many activated nuclear receptors function by recruiting cofactors that bridge the steroid receptors and the general transcription machinery (Horwitz et al. 1996, Glass et al. 1997). There could be receptor-specific cofactors that mediate receptor-specific transcription regulation. (iv) Receptor-specific interactions with other transcription factors or receptor-specific crosstalk with other signaling pathways. There are increasing examples of ligand-dependent and -independent crosstalk of steroid receptors with other signaling pathways (Beato & Sanchez-Pacheco 1996). These interactions are probably sources of receptor-specific interactions that result in receptor-specific target gene regulation (Boonyaratankornkit et al. 2001). The different abilities of GR and PR to regulate target genes may be mediated through the divergent part of the receptors, particularly the N-terminal domain.

Many of the studies on the mechanisms of GR and PR action have been performed using the MMTV promoter, but only a handful of cellular promoters that are regulated by GR or PR have been identified. In particular, there is a paucity of cellular promoters reported to be differentially regulated by GR and PR. In this study, we have utilized a promoter-trapping system to conduct a genome-wide search for the genes that can be induced by glucocorticoids or progestins, particularly those that can be differentially induced by the two hormones. The systematic identification of such genes and the characterization of the mechanisms that underlie differential hormone regulation will enhance our understanding of steroid physiology and give insight into the larger question of differential gene regulation by different members of transcription factor families.

Materials and Methods

Plasmid construction

Plasmid ppgkltkneo/hygro and ppgkltkneo/luc were constructed by modifying plasmid ppgkltkne oIL3 (a generous gift from Dr Harald von Melchner, University of Frankfurt Medical School, Germany) (Russ et al. 1996). In ppgkltkneo/hygro, hygromycin phosphotransferase gene was subcloned from plasmid pCMVHygro into ppgkltkne oIL3. In ppgkltkneo/luc, firefly luciferase gene was subcloned from plasmid pXP2 (Nordeen 1988) into ppgkltkneoIL3. U3Cre retroviral vector pGgU3Cre(−) was a gift from Dr Harald von Melchner (Russ et al. 1996). Plasmid pCMVCre was constructed by cloning Cre gene from pGgU3Cre(−) into pCMV6C vector (Chapman et al. 1991).

Cell culture and transient transfection

The 4F and derived cell lines were maintained in Minimum Essential Medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (HyClone, Logan, UT, USA), 10 mM Hepes, and nonessential amino acids. Transient transfections of 4F-derived cell lines were performed using a diethylaminoethyl/dextran method as previously described (Nordeen et al. 1998). After 20–24 h, cells were treated with vehicle, dexamethasone (100 nM) or R5020, a synthetic progestin, (10 nM). Cells were harvested 44–48 h after treatment. Cell extracts were prepared by first washing the cells, harvesting them in 0.5 ml cell lysis buffer, then pelleting debris (Nordeen et al. 1998). For luciferase assays, 25 µl soluble lysate were used and for β-galactosidase assays 2.5 µl. Luciferase and β-galactosidase assays were assessed using a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Sparks, MD, USA) as previously described (Nordeen et al. 1998). Data are reported as luciferase activity normalized to β-galactosidase activity in the same transfection.

The PA317 amphitropic packaging cell line was maintained in Dulbecco’s Modified Eagle’s Medium with high glucose (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (HyClone).

The T47D/A1–2 cell line was maintained in Minimum Essential Medium supplemented with 5% fetal bovine serum (HyClone), 10 mM Hepes, nonessential amino acids and 200 µg/ml G418.

Generation of 4Ftkneo/hygro21 clonal cell line

Plasmid ppgkltkneo/hygro was stably transfected into 4F cells by electroporation as described (Baum et al. 1994). Cells resistant to G418 (1 mg/ml) were cloned and subcloned. Genomic DNA Southern
blot analyses were performed to determine the copy number and integrity of the tkneo/hygro insert in each clone. To test integration copy number, a restriction enzyme that cuts only once in the reporter construct (BamHI) was used to digest the genomic DNA. To test insert integrity, a restriction enzyme that cuts the plasmid both upstream and downstream of the tkneo/hygro cassette (AlwNI) was used to digest the genomic DNA. Digested DNA was separated by agarose gel electrophoresis and transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The membrane was probed with either neomycin-resistance gene or hygromycin-resistance gene. One of the clones that contain a single copy of the full-length ppgkxtkneo/hygro cassette (4Ftkneo/hygro21) was selected.

**U3Cre virus packaging and infection**

The U3Cre retroviral vector was stably transfected into an amphitropic packaging cell line PA317 (Miller & Buttimore 1986) by a calcium phosphate coprecipitation method. Because the U3 Cre plasmid lacks a selectable marker, plasmid pRSV-neo was cotransfected at 1/10 amount of the retroviral plasmid. Stable clones were selected in G418 media (1 mg/ml). Clones were screened for the existence and integrity of Cre gene by PCR amplification of genomic DNA followed by Southern blot analysis. The ability of these packaging cell clones to produce U3Cre retrovirus was tested by infecting the 4Ftkneo/hygro21 cells and selecting for hygromycin resistance. Viral titer was determined in the same way. Because the U3Cre provirus lacks a constitutively expressed drug-resistance marker, viral titers were derived by multiplying the number of hygromycin-resistant colonies by the average frequency of integrations that enable U3 gene activation of other similar gene-trap vectors (von Melchner & Ruley 1989, von Melchner et al. 1990). The packaging cell clone (clone 4) that produced U3Cre retrovirus at the highest titer (>10^7 cfu/ml) was selected and used to prepare viral supernatant. Viral infection of the 4Ftkneo/hygro21 cells was performed at a multiplicity of infection (MOI) of 0.5 to ensure single viral integration in each cell. A library consisting of approximately 4 × 10^6 independent proviral integrations was generated.

**Sequential drug selection procedure**

Sequential drug selections were performed to isolate clonal cell lines representing trapped, hormone-inducible and differentially hormone-inducible genes as described later in Fig. 3. Retrovirus-transduced cells were first placed under G418 (1 mg/ml) selection for 5 days. Those cells in which the Cre gene is integrated downstream of a constitutively active cellular promoter will be eliminated due to the expression of Cre and thus the deletion of the tkneo gene. The survivors were split into two groups in media without G418. One group was treated with R5020 (10 nM) and the other with dexamethasone (100 nM) for 24 h. The cells are then subjected to hygromycin (500 µg/ml) selection to select for cells in which Cre expression has been induced by hormone induction. Thus this selection will enrich for cells in which the Cre gene was integrated downstream of a progestin-inducible promoter (group A) or a glucocorticoid-inducible promoter (group B). To maximize the identification of genes preferentially induced by one of the two hormones, an additional step was added to the selection scheme. After the initial G418 selection to eliminate constitutively expressed promoters, another selection was added to eliminate promoters induced by progestins (group C) or glucocorticoids (group D). This was done by adding the appropriate hormone for 24 h and continuing G418 selection for 5 days. Survivors in the progestin-treated group C were removed from G418 and treated with dexamethasone for 24 h. Similarly, the glucocorticoid-treated group D was removed from G418 and treated with R5020 for 24 h. The two groups were then placed under hygromycin selection to enrich for cells expressing Cre under the control of the appropriate hormone. A total of 120 clones, 40 each from groups A and B, and 20 each from groups C and D, were isolated for further analysis.

**Amplification and cloning of proviral upstream sequences (inverse PCR)**

Genomic DNAs from U3Cre-infected 4Ftkneo/hygro21 cell clones were digested with restriction enzyme (MseI or HinfI) and ligated at 5 µg/ml to obtain circular molecules. After cleavage with PvuII, 1 µg of each sample was used for PCR. PCR reactions were performed using an Expand high fidelity PCR system (Roche Diagnostics...
Corporation, Indianapolis, IN, USA) as previously described (Russ et al. 1996). Amplification products were cloned into the TA cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced.

**Semi-quantitative RT-PCR**

Total RNA was isolated from each sample using a Purescript RNA isolation kit (Genta Systems, Minneapolis, MN, USA) according to the manufacturer’s instruction. RNA was treated with RNase-free DNaseI (Ambion, Inc., Austin, TX, USA) to remove genomic DNA contamination. RNA was reverse transcribed into single-stranded cDNA using random primers and M-MLV reverse transcriptase (Life Technologies). For semi-quantitative PCR, first, the best cycle number for linear amplification of the cDNA using each gene-specific primer pairs was determined. One cDNA sample was serially diluted and used as template for PCR, which was performed with Taq DNA polymerase (Promega, Madison, WI, USA). PCR products were run on agarose gel and stained with ‘SYBR Gold’ Nucleic Acid fluorescent stain (Molecular Probes, Inc., Eugene, OR, USA). Each PCR product was quantitated with the Molecular Probes fluorescent scanner (Molecular Probes, Inc., Eugene, OR, USA). The PCR output was plotted against the relative cDNA input and the cycle that gave the best linear relationship was chosen to PCR amplify each experiment sample. The linear standard cDNA and the cDNA from each experiment sample were PCR amplified side by side. The PCR output for each sample was quantitated and used to calculate the input cDNA amount using the linear relationship derived from the standard.

Primers used for amplifying β-actin were from Promega. The Cre gene-specific primer used to amplify cellular-Cre fusion transcript was 5'-GGGTACCGTCAGTA-3'. Primers used for amplifying genes identified in the indicated clonal cell line are as follows. Clone 32 gene: 32-A 5'-GAAGATCTGAACCTCGTGG-3', 32-B 5'-ATACCCAAAGGATAGATGC-3'; clone 43 gene: 43-A 5'-ACTAGCCAGTATAGTGCT-3', 43-B 5'-CATGTAATCTCAAAAAAGTG-3'; clone 42 gene: 42-A 5'-CTTCTAAAGCGGAGTG-3', 42-B 5'-CATGACCAAGAAGATGTG CAC-3'; clone 66 gene: 66-A 5'-GATGTCATGTC-3', 66-B 5'-GTCCCTGAGAAGT GGAATCTG-3'.

**Figure 1** Schematic of the multifunctional selection plasmid (ppgklxtkneo/hygro) before and after Cre-mediated recombination at loxP sites. Functional components are labeled as follows: pPGK, mouse phosphoglycerate kinase promoter; tkneo, herpes simplex virus 2 thymidine kinase (tk)–neomycin phosphotransferase (neo) fusion gene; hygro, hygromycin-B phosphotransferase; pA, a pair of polyadenylation signals from the growth hormone gene; loxP, Cre recombination target sequences.

**Results**

**Establishment of a Cre/loxP-mediated retroviral promoter-trapping system**

In order to study the actions of both GR and PR in the same cells, a mouse fibroblast-derived cell line, 4F, was generated by stably introducing exogenous PR-B expression into the glucocorticoid-responsive Ltk− fibroblast cells as described (Thackray et al. 1998). Although 4F cells are responsive to both glucocorticoids and progestins, PRs are at least 10-fold more abundant than GRs in each 4F cell (Thackray et al. 1998).

To obtain a cell line that can sensitively detect Cre recombinase expression, the multifunctional reporter plasmid (ppgklxtkneo/hygro) (Fig. 1) was stably transfected into 4F fibroblasts by electroporation. The ppgklxtkneo/hygro reporter plasmid consists of two, tandemly arrayed selective marker genes that are transcribed from a pgk promoter. The 5′ selective marker gene encodes for a fusion protein between herpes simplex virus 2 thymidine kinase (tk) and the neomycin phosphotransferase (neo), and is flanked by direct-repeats of the loxP recombination target site (Russ et al. 1996). The 3′ selective marker gene encodes for hygromycin phosphotransferase (hygro) and is located downstream of the second loxP site. In the absence of Cre, the tkneo gene is expressed from the constitutive pgk promoter. The hygro gene does not have a promoter immediately upstream and chimeric transcripts initiated at the pgk promoter are suppressed by two tandem copies of the bovine growth hormone polyadenylation sequence upstream of hygro.
Therefore, the cells containing a single copy of pgk\textit{ltkneo/hygro} are G418-resistant but hygromycin-sensitive. However, when Cre is expressed, it will catalyze recombination between the two \textit{loxP} sites resulting in the deletion of \textit{tkneo}. This places \textit{hygro} immediately downstream of the \textit{pgk} promoter rendering the cells G418-sensitive but hygromycin-resistant (Fig. 1). Thus, 4F cells with a stably integrated pgk\textit{ltkneo/hygro} selection plasmid report even transient expression of Cre by undergoing a permanent switch in their drug-resistance phenotype.

The 4F cells were stably transfected with the selection plasmid. A G418-resistant clone containing a single copy plasmid (4Fneo/hygro21) was selected for further analysis (see Materials and methods). First, we determined whether the 4Fneo/hygro21 cells were sensitive to hygromycin by placing \(5 \times 10^5\) cells under hygromycin (500 µg/ml) selection. Because no colony formed within 12 days, we concluded that neither leaky \textit{hygro} translation nor spontaneous recombination occurred in these cells at levels that would interfere. Secondly, we determined whether the 4Fneo/hygro21 cells would become hygromycin-resistant and neomycin-sensitive after Cre expression. An expression plasmid, pCMVCre, was transiently transfected into the cells by a calcium phosphate coprecipitation method. Cells were selected for hygromycin (500 µg/ml) resistance. Then the hygromycin-resistant cells were pooled and placed under G418 (1 mg/ml) selection for 12 days to test neomycin sensitivity. Because all the hygromycin-resistant cells were neomycin-sensitive, we concluded that the cells could undergo a clean drug-resistance phenotype switch after Cre expression.

In this gene-trapping system, we employed a self-inactivating retroviral gene-trap vector (U3Cre) in which a promoterless Cre gene is inserted in the U3 region of an enhancer-deleted long terminal repeat (LTR) (Fig. 2). Viral replication and LTR-mediated duplication place the promoterless Cre sequence just 30 nucleotides downstream of the flanking cellular DNA (Fig. 2)


Figure 2 Structure and replication of the U3Cre retroviral vector. Functional components are labeled as follows: Cre, modified bacterial phage P1 Cre recombinase; U3, R and U5 are segments of the viral LTR, ‘-enh’ indicates ‘enhancer deleted’. Infection of 4Fneo/hygro21 target cells generates provirus in which the 5’ Cre-coding sequence is immediately downstream of cellular sequence. Neo\textsuperscript{R}, G418-resistant.
Isolation of cell lines representing trapped, hormone-inducible and differentially hormone-inducible promoters

The U3Cre plasmid was stably transfected into an amphi or tropic packaging cell line PA317 (Miller & Buttimore 1986). Supernatants from lines producing high titers of recombinant virus were used to infect 4Fneo/hygro21 cells (see Materials and methods) (von Melchner & Ruley 1989, von Melchner et al. 1990). Therefore, Cre is not expressed unless the virus integrates into an active cellular promoter. In this work, we employ this feature to identify hormone-regulated promoters.

Figure 3 Schematic illustration of the sequential selection procedure to identify 4F cell lines representing a trapped promoter that is induced or differentially induced by glucocorticoids and progestins. GC, glucocorticoids; PG, progestins; Dex, dexamethasone, a synthetic glucocorticoid; R5020, a synthetic progestin. Specifics on the selections are detailed in Material and methods.

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In the clonal cell lines with a stably integrated U3 Cre provirus, typically there are two Cre transcripts: a cellular-proviral fusion transcript initiating from the upstream cellular promoter and terminating at the polyadenylation site of the 5′ LTR, and a ‘viral genomic’ transcript initiating from the 5′ proviral LTR and terminating at the
polyadenylation site of the 3′ LTR (Russ et al. 1996). The double Cre transcripts can complicate the analysis of the selected cell lines since the viral genomic transcript can obscure the Cre transcript from the cellular promoter. However, translation of Cre from the ‘viral genomic’ transcript within the 3′ LTR is unlikely, due to multiple short open reading frames positioned between the LTRs. Furthermore, several previous studies have shown that, in most cases, activation of the promoter-trap is associated with the translation of Cre from the cellular-proviral fusion transcripts (von Melchner & Ruley 1989, von Melchner et al. 1990, Reddy et al. 1991, Chang et al. 1993, Russ et al. 1996).

To determine the expression pattern of the gene represented in each clonal cell line isolated, we developed a reporter assay to quantitate the Cre protein level based on Cre-recombinase activity (Fig. 4). A reporter plasmid (ppgklxtkneo/luc) (Fig. 4A) was constructed in which the hygro gene of the multifunctional selection plasmid was replaced by luciferase (luc). Luciferase expression from this plasmid was dependent on Cre expression. A titration of Cre expression vector demonstrated that the higher the input of Cre, the higher the output of luciferase expression (Fig. 4B). Therefore, this sensitive and quantitative luciferase reporter strategy can determine the exact regulation pattern of the trapped promoters in each isolated cell clone and allow the identification of the lines that represent genes that are differentially regulated by the two hormones.

Each clonal cell line with a potentially hormone-regulated, trapped promoter was transfected with ppgklxtkneo/luc and treated with dexamethasone or R5020 24 h later. The induction of Cre activity was assessed by the deletion of sequences flanked by loxP sites as measured by the appearance of luciferase activity. In 58 cell lines, luciferase expression was induced by either glucocorticoids or progestins by more than 2-fold. In 19 cell lines, luciferase expression was differentially regulated by the two hormones by more than 1·5-fold (Fig. 4C).

Hormone regulation of selected promoters

In order to determine whether the endogenous cellular genes trapped in the clonal cell lines are indeed hormone-regulated, genomic DNA sequences upstream of the proviral integration site of several clonal isolates were retrieved by inverse PCR and sequenced. They range from 154 to 874 bp (Fig. 5, also see Materials and methods) (von Melchner et al. 1990, Russ et al. 1996). Sequences from all four clones tested showed a typical cellular DNA–provirus junction (Fig. 5B) (Varmus 1988). Blast searches using different databases revealed that the entire sequence upstream of the proviral integration site in clone 43 (441 bp) is 98% identical with the expressed sequence tag (EST) ic84f11.x1 in the cDNA library ‘Melton Normalized Mixed Mouse Pancreas 1 N1-MMS1’ (dbEST ID 9280663, GenBank accession number BI438077). However, the sequences upstream of the proviral integration sites in clone 32, 42 and 66 showed no significant homology to known genes or ESTs at the time of analysis (see Discussion). These sequences have been deposited in GenBank and accession numbers are AF465703 (for clone 32 gene), AF465704 (for clone 42 gene) and AF465705 (for clone 66 gene).

Three of the four proximal flanking sequences (clone 32, 42 and 43) were able to be amplified by RT-PCR from the RNA isolated from the parental cell line (4F), suggesting that the upstream sequences are within an exon of the gene (Fig. 6 and 7). The primers designed for the upstream sequences of clone 66 did not give a RT-PCR product even though they can amplify the genomic DNA (data not shown). The integration site in this clone may not be in an exonic sequence or the 5′ primer could be upstream of the transcription start site.

The mRNA level of the gene represented by clone 32 (clone 32 gene) in the parental cell line (4F) was measured by semi-quantitative RT-PCR (see Materials and methods). Serially diluted cDNAs were amplified by PCR to select the cycle number that gave the best linear relationship between the cDNA input (x value) and the PCR output (y value) for each specific primer set (Fig. 6A and 6B). Then the cDNAs in each hormone-treated sample were amplified at this cycle number, with the linear standard run at the same time. The PCR output in each sample was quantitated and the amount of cDNA input was calculated using the linear relationship derived from the standard (Fig. 6C). The result demonstrated that clone 32 gene was preferentially induced by glucocorticoids (5-fold) compared with progestins (2·5-fold). Although this is only a 2-fold difference in the
induction, it is more impressive when one considers that PRs are 10-fold more abundant than GRs in these cells. Expression of the genes represented by the other two clones were quantitated in the same way (Fig. 7). Clone 43 gene was also preferentially induced by glucocorticoids. Glucocorticoids induced gene 43 by 2-fold while progestins gave no induction or a slight inhibition. Clone 42 gene was induced by both hormones to a similar level. For all three clones, the mRNA regulation patterns of the endogenous genes agree with the Cre protein regulation patterns shown in Fig. 4C, suggesting that the genes identified using this strategy are indeed regulated by hormones.

For two clones (32 and 43), we further examined the expression of the cellular-Cre fusion transcript to ascertain whether it is regulated by hormones in the same fashion as the endogenous gene. Clonal

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**Figure 4** Quantitation of Cre protein expression in clonal cell lines by transfection of ppgklxtkneo/luc. (A) Schematic of ppgklxtkneo/luc reporter construct before and after Cre-mediated recombination at loxP sites. Gene sequences are labeled as follows: pPGK, mouse phosphoglycerate kinase promoter; tkneo, herpes simplex virus 2 thymidine kinase (tk)–neomycin phosphotransferase (neo) fusion gene; pA, a pair of polyadenylation signals from the growth hormone gene; loxP, Cre recombination target sequences. (B) A dose curve demonstrating that relative levels of Cre activity can be analyzed by recombination of ppgklxtkneo/luc and concomitant production of luciferase activity. Increasing amounts of the expression plasmid pCMVCRe were transiently transfected into the 4Fneo/hygro21 cell line. A constant amount of the reporter tkneo/luc (1 µg/ml) and an internal control plasmid pCMVβ-gal (0.1 µg/ml) were cotransfected. Cells were harvested 72 h later. The result is shown as luciferase activity normalized to β-galactosidase activity. (C) Identification of clonal cell lines that represent promoters differentially regulated by glucocorticoids and progestins by quantitating Cre protein expression. The reporter plasmid ppgklxtkneo/luc (1 µg/ml) was transiently transfected into each clonal cell line with an internal control plasmid pCMVβ-gal (0.1 µg/ml). After 20–24 h, cells were treated with vehicle, dexamethasone (Dex, 100 nM) or R5020 (10 nM). Cells were harvested 48 h after treatment. The results are illustrated as the fold of induction of the luciferase activity normalized to β-galactosidase activity. Shown are 19 cell lines that represent promoters differentially regulated by the two hormones by more than 1.5-fold: Dex>R5020 indicates that the promoters are preferentially induced by dexamethasone, Dex<R5020 indicates that the promoters are preferentially induced by R5020. In addition, four examples of the cell lines that represent promoters induced by both hormones by more than 2-fold are also shown (Dex=R5020).
cell lines 32 and 43 were treated with hormone for 2 h and total RNA was isolated. Semi-quantitative RT-PCR analyses were performed with a 5’ primer from the endogenous gene and a 3’ primer from the Cre sequence. A PCR product of the predicted size was amplified from cDNA. Furthermore, both

**Figure 5** Retrieval of sequences upstream of the proviral integration site by inverse PCR. (A) Schematic of inverse PCR procedure. The restriction enzyme used in this example was MseI. Experimental details are given in Materials and methods. (B) Summary of the upstream sequences retrieved from selected clonal cell lines. Proviral sequences are underlined.
fusion transcripts were preferentially induced by glucocorticoids compared with progestins, as was observed for the endogenous genes (Fig. 8).

**Glucocorticoid induction of clone 32 gene is dependent on GR and does not require de novo protein synthesis**

Further characterizations of the glucocorticoid induction were performed using the clone 32 gene as an example. In order to assess the specificity of the induction of clone 32 gene by glucocorticoid, a dose–response study was performed. 4F cells were treated with increasing concentrations of dexamethasone for 2 h. Total RNA was isolated from each sample and the expression of clone 32 gene was quantitated by semi-quantitative RT-PCR (Fig. 9A). The result demonstrated that even at a concentration as low as 1 nM, dexamethasone can induce the expression of the clone 32 gene, suggesting that the induction of clone 32 gene by glucocorticoid is specific.

In order to determine whether the induction of clone 32 gene by glucocorticoid is dependent on GR, 4F cells were treated with 100 nM RU486 or ZK112993 in addition to hormone treatment for 2 h. RU486 is an antagonist that can block both GR and PR function, while ZK112993 is a much stronger antagonist for PR than for GR (data not shown). Quantitation of clone 32 gene expression by semi-quantitative RT-PCR (Fig. 9B) demonstrated that the induction of clone 32 gene by either dexamethasone or R5020 was completely abolished following RU486 treatment. In contrast, following
ZK112993 treatment, the induction by R5020 is abolished while induction by dexamethasone is only slightly reduced. These results indicate that the induction of clone 32 gene by glucocorticoid is dependent on GR.

In order to determine if the induction of clone 32 gene by glucocorticoid is a direct response, 4F cells were treated with de novo protein synthesis inhibitor cycloheximide for 15 min prior to hormone treatment. Quantitation of clone 32 gene expression by semi-quantitative RT-PCR (Fig. 9C) demonstrated that the induction of clone 32 gene by dexamethasone was maintained following cycloheximide treatment. This suggests that the glucocorticoid induction is a direct response that does not require de novo protein synthesis.

**Glucocorticoid induction of clone 32 gene is an early response and is cell-type specific**

The kinetics of the hormone regulation of clone 32 gene in 4F cells was assessed by semi-quantitative RT-PCR (Fig. 10A). Glucocorticoid induction of clone 32 gene peaked as early as 30 min, again indicating that this is a direct response of hormone regulation. The hormone induction lasted for at least 22 h.
Hormone regulation of clone 32 gene in two other cell types was also assessed. L929 cells are mouse fibroblasts like the 4F cells and their parent line, Ltk<sup>−</sup>. L929 cells express only GR but not PR. Semi-quantitative RT-PCR assays demonstrated that the clone 32 gene was also induced by glucocorticoids in L929 cells (Fig. 10B). T47D/A1-2 cells were derived from T47D human mammary carcinoma cells and have been engineered to express high levels of rat GR (Nordeen et al. 1989, Moyer et al. 1993). We have previously estimated that these cells express about 100 000 molecules of GR per cell, about half the number of endogenously expressed PR. The clone 32 gene was induced by neither glucocorticoids nor progestins in T47D/A1-2 cells (Fig. 10C). These results indicate that the hormone regulation of clone 32 gene is cell-type specific.

**Discussion**

In this study, we employed a strategy that combines a retrovirally delivered promoter-trap and Cre/loxP-mediated site-specific recombination to identify hormone-regulated genes, especially those that are differentially regulated by glucocorticoids and progestins. This strategy has several significant advantages. (i) It can detect transiently hormone-activated promoters. Since the Cre-mediated recombination at loxP sites is an irreversible event, even transiently activated promoters can produce enough Cre-recombinase to create a permanent switch of drug-resistance phenotype. Thus screening can be accomplished even when a brief...
induction period is used to avoid cytotoxicity or induction of apoptosis that might result from a longer treatment (Russ et al. 1996). (ii) It is not strongly biased toward highly expressed genes. Little Cre expression is required to catalyze recombination at the loxP sites. Therefore, unlike many conventional strategies, e.g. cDNA library screening, RNA differential display, and microarray analysis, this Cre/lox selection strategy can detect weakly expressed genes. (iii) It allows discovery of unknown genes. (iv) It allows a near-saturation screening of the whole genome. If we infect the target cell line with the U3 Cre retrovirus at an MOI of 0·5 to ensure single integration events and perform selections on 10^7 integrants, this will yield 1 integration per 300 bp, assuming random integration.

In addition to documenting this promoter-trapping system, we describe a sensitive and quantitative luciferase reporter strategy that can determine the regulation pattern of the trapped promoters in the isolated cell clones. Sequences upstream of the proviral integration site in selected clones were retrieved by inverse PCR. Hormone regulation of the endogenous genes identified in selected clones has been confirmed by semi-quantitative RT-PCR, indicating that many of the genes identified with these strategies are indeed hormone-regulated. Most significantly, we have successfully identified genes that are differentially regulated by two closely related transcription factors, the GR and the PR.

For three of the four genes analyzed, database searches of the sequences at the site of proviral integration reveal no homology to known genes or ESTs. This was not a great surprise, as the genes identified had extremely low basal expression. Therefore, identification of these transcription units might be easily missed by conventional gene discovery approaches. Another reason that cDNAs for these transcription units may not be found in the databases stems from the design of the U3 Cre retroviral construct that selects for integration sites in 5'-nontranslated regions. An amber stop codon (TAG) is located 24 nucleotides upstream and in frame with the initiation codon AUG for Cre. This imposes a strong selection for integration events in which Cre provided the first initiating AUG in the resulting hybrid transcript (von Melchner et al. 1990). For mammalian genes, the average distance between transcription start site and the first AUG initiation codon is 50–100 nucleotides (Kozak 1987). The 5'-most part of the transcription unit is most likely to be absent from all but full-length cDNA clones in the databases.

In this study, a group of 19 clonal cell lines representing genes differentially regulated by glucocorticoids and progestins have been isolated. Two genes (clone 32 and 43 genes) have been confirmed to be preferentially induced by glucocorticoids compared with progestins using RT-PCR. Despite the disparate biological activities of the two receptors, there is little information on what genes may be differentially regulated by the two receptors and how this may be accomplished. Our studies document a novel approach toward addressing this
void. Further studies on the mechanisms underlying the differential regulation will enhance our understanding of how glucocorticoids and progestins function as specific physiological regulators or therapeutic agents.

Acknowledgements

We would like to thank Dr Harald von Melchner for providing plasmids ppgkltxkneoIL3 and pGgU3 Creen[−]), and Dr James DeGregori for insightful discussion. ZK112993 was a generous gift from D Henderson (Schering A. G., Berlin, Germany). We also acknowledge the assistance of the DNA sequencing core of the University of Colorado Cancer Center. This work has been supported by grant award DK37061 from the National Institutes of Health to S K N and predoctoral fellowship award DAMD17-99-1-9445 from the Department of Defense to Y W.

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Received 9 January 2002
Accepted 11 February 2002