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Highly inducible expression from vectors containing multiple GRE's in CHO cells overexpressing the glucocorticoid receptor

David I.Israel* and Randal J.Kaufman

Genetics Institute, 87 Cambridge Park Drive, Cambridge, MA 02140, USA

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ABSTRACT

A conditional glucocorticoid-responsive expression vector system is described for highly inducible expression of heterologous genes in mammalian cells. This host-vector system requires high level expression of the glucocorticoid receptor (GR) protein in the host cell and multiple copies of the receptor binding site within the expression vector. Transfection and selection of Chinese hamster ovary cells with expression vectors encoding the rat GR yielded cell lines which express functional receptor at high levels. Insertion of multiple copies of the MMTV enhancer (glucocorticoid responsive element, GRE) into an Adenovirus major late promoter (AdMLP) based expression vector yielded greater than 1000-fold inducible expression by dexamethasone (dex) in transient DNA transfection assays. The induced expression level was 7-fold greater than that obtained with an AdMLP based vector containing an SV40 enhancer, but lacking GRE's. Vectors containing the SV40 enhancer in combination with multiple GRE's exhibited elevated basal expression in the absence of dex, but retained inducibility in both transient assays and after integration and amplification in the CHO genome. This expression system should be of general utility for studying gene regulation and for expressing heterologous genes in a regulatable fashion.

INTRODUCTION

Glucocorticoids are members of a family of steroid hormones that act through specific intracellular receptor proteins. The structures of the various steroid hormones, which are all derived from cholesterol, are similar. Likewise, the amino acid sequences of members of the steroid hormone receptor family are also similar. Despite this similarity, a given steroid hormone will bind to its receptor with high affinity to elicit a specific biological effect that typically results from altered expression of a small number of genes. The cellular response to a given steroid hormone will therefore be determined by the presence of functional hormone receptor, as well as genes competent to respond to the hormone/receptor complex.

Glucocorticoid hormones may positively (1−6) or negatively (7−13) regulate expression of specific genes during development, and in response to environmental stress to maintain homeostasis. The expression of several viral genomes, typified by the mouse mammary tumor virus (MMTV), is under glucocorticoid regulation (14). In most of these cases, glucocorticoids and other steroid hormones exert their effect at the transcriptional level, although these hormones can also alter the stability of specific mRNA's (13) and alter protein processing (15).

Molecular clones encoding the rat (16), human (17), and mouse (18) glucocorticoid receptors have been isolated and characterized by functional studies using wild-type and mutant receptors. Like other steroid hormone receptors, the GR has distinct domains for

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hormone binding, DNA binding, transcriptional activation and nuclear localization (19–22). Cumulative results indicate that the hormone binding domain masks the transcriptional modulatory activities of the receptor in the absence of hormone. Upon hormone binding, a temperature-dependent change in the receptor occurs that elicits its transformation into a DNA binding protein that can modulate gene expression. Receptor activation may involve displacement of an hsp90 protein dimer from the non-activated 9S cytosolic complex (23).

Characterization of the transcription elements of MMTV, and of several cellular genes whose expression is regulated by glucocorticoids, have identified specific DNA sequences (24–26) that bind the hormone/receptor complex and function as hormone-responsive enhancer elements. The isolation of clones for both steroid receptor proteins and the responsive DNA elements has allowed detailed molecular studies of hormone-controlled gene expression, making this an excellent model system for studying eukaryotic gene regulation. The steroid hormone system may also be exploited to regulate the expression of heterologous genes.

In this paper, we describe a glucocorticoid inducible expression vector system. The rat glucocorticoid receptor gene was stably expressed at high levels in Chinese hamster ovary (CHO) cell lines by coamplification with dominant selectable markers. As a result, these cell lines have become highly responsive to glucocorticoid hormones. Furthermore, we have developed a series of expression vectors that contain multiple copies of the MMTV GRE. These vectors are hyperresponsive to glucocorticoids and can be used to obtain stable, inducible expression of heterologous genes. These cell lines and vectors should be useful for studying the molecular mechanisms of steroid hormone action, and for regulating the expression of amplified heterologous genes in stable cell lines.

MATERIALS AND METHODS

Construction of Expression Vectors

The rat glucocorticoid receptor cDNA was isolated from the plasmid pRBal117 (16) by XbaI digestion (all restriction endonucleases were obtained from New England Biolabs) and gel purification. The expression vectors pMT2T and pMT3SVA were linearized with EcoRI. The expression vector pMT2 (27) contains an SV40 enhancer adjacent to the adenovirus type-2 major late promoter (AdMLP), the tripartite leader from adenovirus, a hybrid intron containing a 5′ splice site from the first adenovirus late leader and 3′ splice site from a mouse IgG gene, a mouse DHFR cDNA, and the SV40 polyadenylation region. Plasmid pMT2T was derived from pMT2 (27) by inserting the sequence TGAGATCTAACTAACAATT immediately after the EcoRI site. This oligonucleotide introduces translation termination codons in each reading frame upstream from the DHFR coding region and downstream from the cDNA insertion site. This results in improved translation of DHFR from a bicistronic transcript (28). Plasmid pMT3SVA was derived from pMT2-Ada-vWF (27) by deleting the vWF and DHFR sequences contained between the EcoRI site and HpaI site within the SV40 polyadenylation region, and replacing it with an oligonucleotide containing the sequence AATTCCGTCGACTCTAGAG. The glucocorticoid receptor fragment and linearized expression vectors were treated with Klenow fragment of DNA polymeraseI (BRL) in the presence of deoxynucleoside triphosphates, and were ligated together. Bacterial colonies were screened with a glucocorticoid receptor cDNA labelled by nick-translation, and plasmid DNA from positive colonies was analyzed

by restriction endonuclease digestion and agarose gel electrophoresis. The expression vectors pMT2T-GR and pMT3GR-SVA (Fig.1) contain the glucocorticoid receptor cDNA under transcriptional control of the SV40 enhancer/Ad MLP.

A semisynthetic fragment of the MMTV LTR containing the GRE's was cloned into pSP64. The HaeIII/SacI fragment of the LTR (29), corresponding to nucleotides −225 to −108 relative to the start of transcription, was excised from paLTR2 (obtained from Keith Yamamoto). Synthetic oligonucleotides containing the sequences from −108 to −50 of the LTR followed by a BglII and EcoRI site were prepared, and ligated with the HaeIII-SacI fragment from the LTR into pSP64 which was previously linearized with SmaI and EcoRI. The resulting plasmid, pLTR−225:−50, contains 175 bases from the LTR flanked by restriction sites from pSP64, and the additional BglII site from the synthetic oligonucleotide.

The inducible mammalian cell expression vectors were obtained by inserting one or multiple copies of the LTR fragment from pLTR−225:−50 into pMT2. Inducible pMT2 derivatives containing a single copy of the GRE were made by ligating the BamHI-BglII LTR fragment from pLTR−225:−50 into one of the BamHI sites or the BglII site within pMT2. Head to tail multimers of the GRE were prepared by ligating the BamHI-BglII fragment from pLTR−225:−50 to itself, digesting with BamHI and BglII, and isolating the polymerized products after agarose gel electrophoresis. GRE multimers were inserted into the BamHI or BglII sites of pMT2 as described above. Appropriate restriction fragments from the pMT2 derivatives containing one or more GRE's were then combined to yield vectors containing GRE's at multiple sites around the vector. The plasmid pMT2MΔS was made by replacing the HinDIII to BamHI SV40 enhancer fragment from pMT2 with a HinDIII-BglII GRE fragment from pLTR-225:-50. Plasmid pMG18PC was derived from pMT2A9B5C4 by deleting approximately 100 base pairs from the 5′ untranslated end of DHFR. Plasmid pMG18ΔS was derived from pMG18PC by replacing the HinDIII to BamHI SV40 enhancer fragment with a HinDIII-BamHI oligonucleotide linker containing an internal XhoI site.

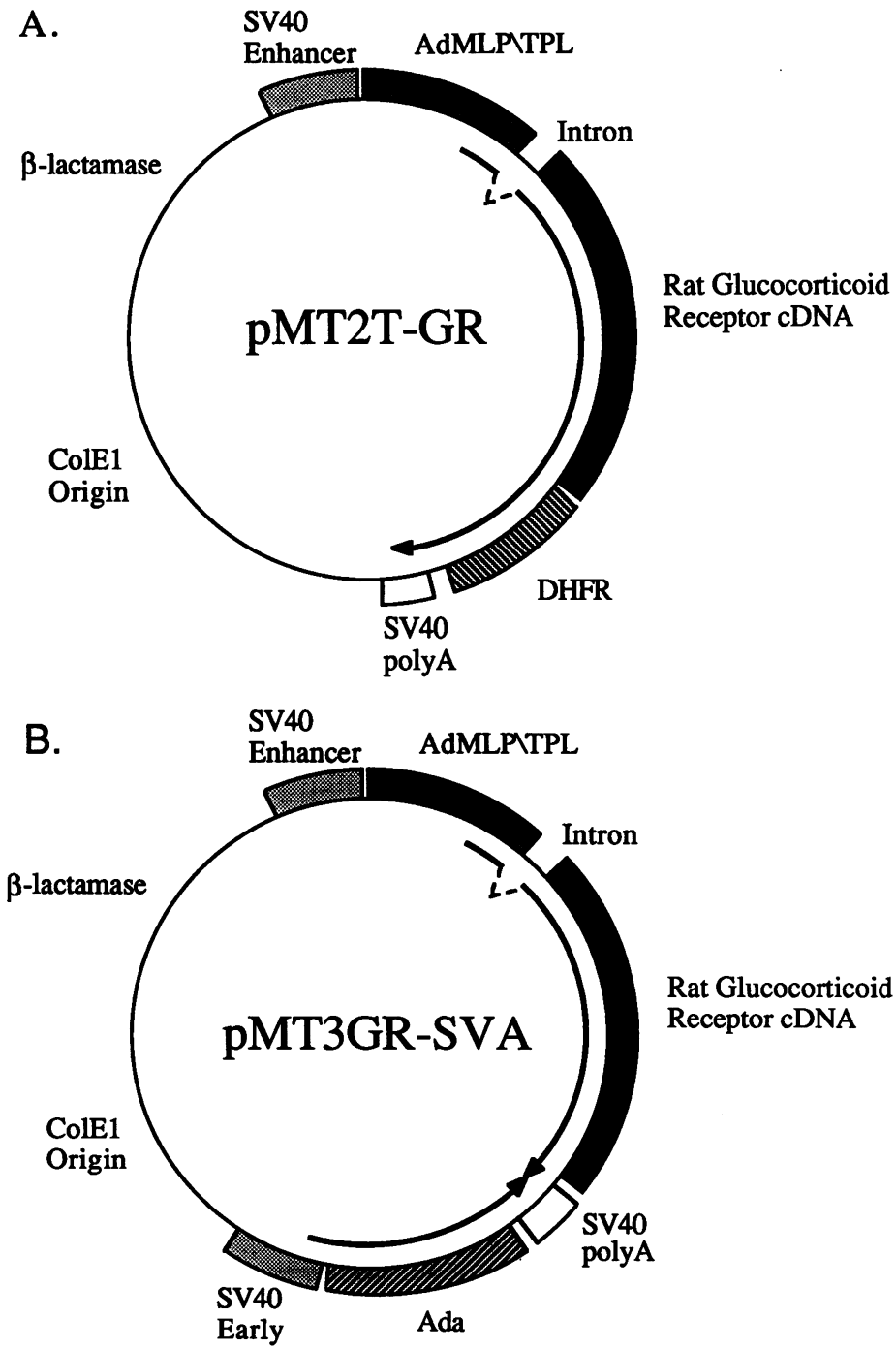
Inducible expression vectors for tPA and CSF-1 were made by introducing the cDNA clone encoding either tPA (30) or CSF-1 (31) into the EcoRI site of pMG18PC or pMT2A9B5C4, respectively. These vectors direct the synthesis of either tPA or CSF-1 and DHFR on a single bicistronic transcript.

Establishment of Stable Cell Lines Expressing GR

CHO DUKX cells and derivatives were grown in α-medium as described previously (30, 32). In all experiments, the fetal bovine serum (MA Bioproducts) was extensively dialyzed against PBS in order to remove endogenous steroids. CHO DUKX cells were transfected with pMT2T-GR or pMT3GR-SVA by electroporation (33) at 200 volts and 1250 μFarads. Two days later, cells were selected for DHFR expression from pMT2T-GR by growth in nucleoside-free medium, or for Ada expression from pMT3GR-SVA by growth in 0.1 μM 2-deoxycoformycin (dCF) (Sigma) and 1.1 mM adenosine, 50 μM alanosine, 1 mM uridine (11-AAU) (32). Cells transfected with pMT3GR-SVA were subsequently selected for resistance to 1.0 μM dCF in order to amplify the GR expression, and are termed GRA

Establishment of Stable GR Cell Lines Expressing Inducible Genes

The cell lines GRA1 or GRA2 were transfected with the inducible tPA or CSF-1 expression vectors by electroporation as described above, and selected two days later for DHFR expression in nucleoside-free medium. Cells were subsequently selected in increasing methotrexate (MTX) (Sigma) (30) in the presence of 1.0 μM dCF and 11-AAU.


A.
SV40
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polyA
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SV40
Enhancer
AdMLP\TPL
Intron
β-lactamase
pMT3GR-SVA
Rat Glucocorticoid
Receptor cDNA
ColE1
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Early
Ada

Transient Expression of Vectors

Cells were transfected as described (34), except α-medium was used, and cells were incubated with DNA for 4 hrs. Cells were maintained in the presence or absence of 1 μM dexamethasone (dex) (Sigma) following transfection, and were analyzed 36−40 hrs later. Expression of chloramphenicol acetyltransferase (CAT) activity was analyzed as described (35). For analysis of DHFR mRNA expression, RNA was prepared by the guanidine thiocyanate method (36) and analyzed using Northern blot hybridization procedures (37).

Analysis of Glucocorticoid Receptor

The synthesis of the glucocorticoid receptor protein was analyzed by labelling cells with [^{35}S]methionine (NEN, 7800 Ci/mMol) and lysing cells in RIPA buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1% SDS, 1% Na deoxycholate, 1% Triton X-100, 0.05% Na azide, 1 mM phenylmethanesulfonyl fluoride, and 1 mg/ml soybean trypsin inhibitor). Cell lysates were incubated with the BUGR1 monoclonal antibody (38) and protein A-Sepharose CL-4B (Pharmacia). Bound material was electrophoresed through SDS-8% polyacrylamide gels under reducing conditions. The binding of [^{3}H]dex (NEN, 39.4 Ci/mMol) to the receptor was assayed using the charcoal absorption method (39), except cytosol was prepared in 10mM Tris, pH 7.5, 1mM EDTA, 1mM DTT and 10% glycerol, and unbound hormone was removed with charcoal:dextran (10:1). Data was analyzed according to the method of Scatchard (40). The functional properties of the GR were determined using transient CAT and DHFR assays described above.

tPA and CSF-1 Assays

For both tPA and CSF-1 assays, cells were grown to approximately 50% confluence in 10-cm diameter tissue culture plates, and the medium was replaced with 10 ml fresh α medium containing 1μM dex (1 mM in DMSO), or 0.1% DMSO (minus hormone). Conditioned medium was harvested 24 hr. later. t-PA activity was assayed by a chromogenic assay with purified recombinant t-PA as the reference standard (30). For determination of CSF-1 antigen levels, an ELISA assay utilizing rabbit-anti-human CSF-1 polyclonal antibody was employed, with purified recombinant human CSF-1 (31) as the reference standard.

RESULTS

Expression of Glucocorticoid Receptor Protein

A cDNA clone for the rat glucocorticoid receptor (16) was inserted into the expression vector pMT2T (Figure 1A). The resulting plasmid pMT2T-GR contains a single bicistronic transcription unit containing both glucocorticoid receptor and DHFR coding regions expressed from the Adenovirus major late promoter. In addition, the plasmid contains an SV40 enhancer element and origin for replication in COS-1 monkey kidney cells. The

Fig. 1. Expression vectors for the glucocorticoid receptor cDNA clone−An XbaI fragment from pRBal 117 (Miesfeld et al, 1986), containing the entire coding region plus 24 nucleotides of 5′ noncoding sequences and 360 nucleotides of 3′ nonding sequences from the rat glucocorticoid receptor cDNA clone, was subcloned into the expression vectors pMT2T and pMT3SVA. The products, pMT2T-GR (Fig 1A) and pMT3GR-SVA (Fig 1B), contain the glucocorticoid receptor clone under transcriptional control of the SV40 enhancer and Adenovirus major late promoter. In pMT2T-GR, a second open reading frame encoding murine DHFR immediately follows the glucocorticoid receptor insert, and can be used to select for DHFR expression in CHO cells. The DHFR sequences have been removed in pMT3GR-SVA, allowing for expression of glucocorticoid receptor in the absence of DHFR. In this vector, a second transcription unit in the opposite orientation as the glucocorticoid receptor cDNA encodes human adenosine deaminase, and serves as a dominant, amplifiable marker (Kaufman et al., 1986).

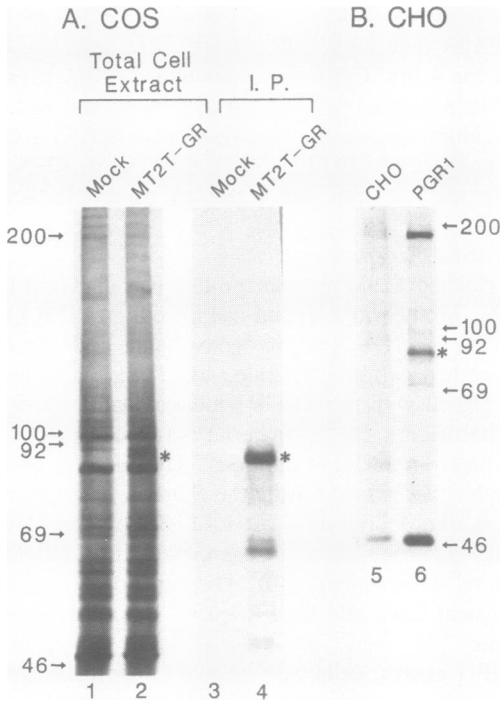


Fig. 2. Expression of the glucocorticoid receptor protein in COS-1 and CHO cells – Cells were pulse labelled (1 hr) with [^{35}S]methionine and lysed in RIPA buffer. Approximately 10^7 cpm of TCA-precipitable counts were immunoprecipitated overnight with the BUGR1 monoclonal antibody, and immunoabsorbed on protein A-sepharose. Following reduction, samples were electrophoresed on 8% polyacrylamide gels. The gels were fixed, treated with Enhance, dried, and autoradiographed at −80°C. Fig 2A) Expression of glucocorticoid receptor protein in COS cells. Cells were transfected with pMT2T-GR, or mock transfected. Approximately 66 hr post-transfection, cells were analyzed for expression of glucocorticoid receptor protein. Fig 2B) Stable Expression of glucocorticoid receptor protein in CHO DUKX and the PGR1 cell line.

expression of glucocorticoid receptor from pMT2T-GR was analyzed by transfection of COS-1 cells and immunoprecipitation of [^{35}S]methionine labelled cells using a GR specific monoclonal antibody, BUGR1 (38). The results (Figure 2A) show a predominant 90 kDa protein expressed in COS-1 cells transfected with pMT2T-GR. This protein can be detected in total cell extracts (lane 2, asterisk), and is enriched after immunoprecipitation with BUGR1 (lane 4, asterisk). Several minor immunoreactive GR species are also detected in the transfected COS-1 cells. Similar observations of multiple forms of endogenous (41) and *in vitro* produced (16) glucocorticoid receptor have been noted by others. Glucocorticoid receptor expressed from pMT2T-GR can stimulate transcription from a cotransfected MMTV-based vector in COS-1 cells upon exposure to dex (data not shown). Thus, relatively high levels of functional glucocorticoid receptor protein are obtained in COS cells transfected with this expression vector.

PMT2T-GR was stably introduced into DHFR-deficient CHO DUKX cells by electroporation and selection for DHFR expression by propagation in nucleoside-free medium. Pools of transformants were analyzed for expression of glucocorticoid receptor mRNA. PGR1, a pool from three transformants, contained the highest level of rat

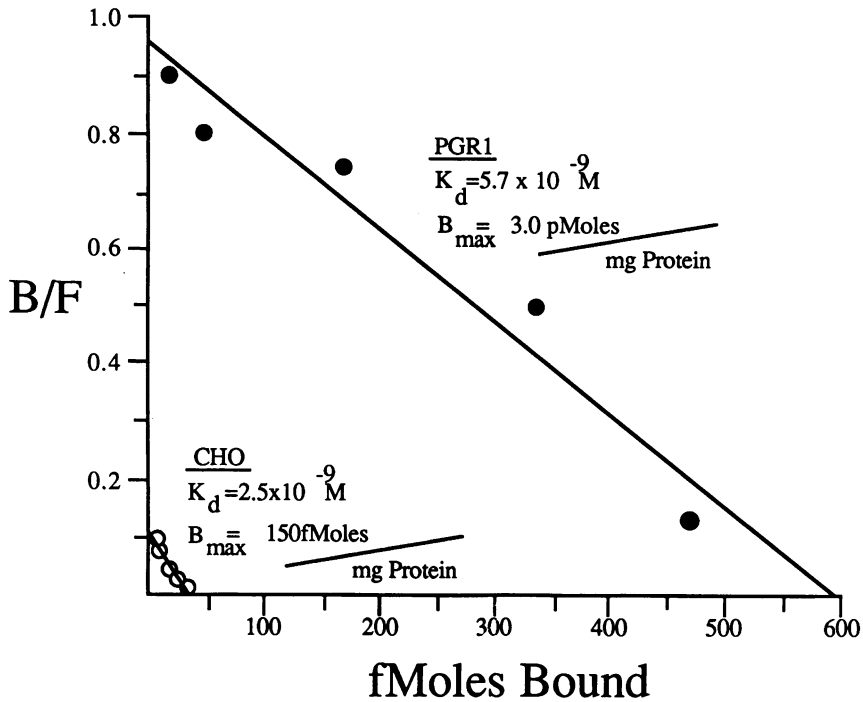


Fig. 3. Dexamethasone binding activity in cytosol from CHO DUKX and PGR1 cell lines – Cytosol preparations (100 μl, 1.3–2.3 mg/ml protein) were incubated with increasing concentrations (4×10^{-10}M – 4×10^{-8}M) of [^{3}H]dex. Duplicate samples also contained 10^{-5} M cold dexamethasone. Following extraction with dextran-coated charcoal, specific binding was defined as the amount of [^{3}H]dex binding that was inhibited by the large excess of non-radioactive hormone. Data was plotted by the method of Scatchard (Scatchard, 1949).

glucocorticoid receptor mRNA (data not shown) and was chosen for further analyses. PGR1 cells were labelled with [^{3}5S]methionine, and cell extracts were immunoprecipitated with BUGR1 for analysis by SDS-PAGE. A 90 kDa molecular weight protein is present in cell extracts from PGR1 cells, but not CHO DUKX (Figure 2B, asterisk). This protein is similar in size to that produced in transfected COS cells (Figure 2A), and to endogenous receptor protein (16, 41). The bands of approximately 46 kDa and 180 kDa are background CHO proteins that occasionally bind to the BUGR1/protein A-sepharose.

Hormone Binding Properties of Recombinant Rat GR

The binding properties of GR expressed in PGR1 cells were studied by measuring the specific interaction of [^{3}H]dex to cytosolic preparations. The parental CHO DUKX cells contain very low cytosolic binding activity (Figure 3). In contrast, cytosol from PGR1 cells binds approximately 3 pMoles[^{3}H]dex/mg protein with a K_d of 6×10^{-9}M. This is similar in affinity and 5 to 10-fold more abundant than the glucocorticoid receptor protein present in rat liver (42) and hepatoma cells (39). Thus, GR expressed in the PGR1 CHO cell line has similar binding properties to native receptor present in rat liver cells.

Transient Expression of CAT Activity

The functional properties of the rat GR expressed in PGR1 cells were examined by a transient expression assay for CAT activity. CHO and PGR1 cells were transfected with CAT expression vectors, incubated in the presence or absence of dex for 48 hr, and assayed

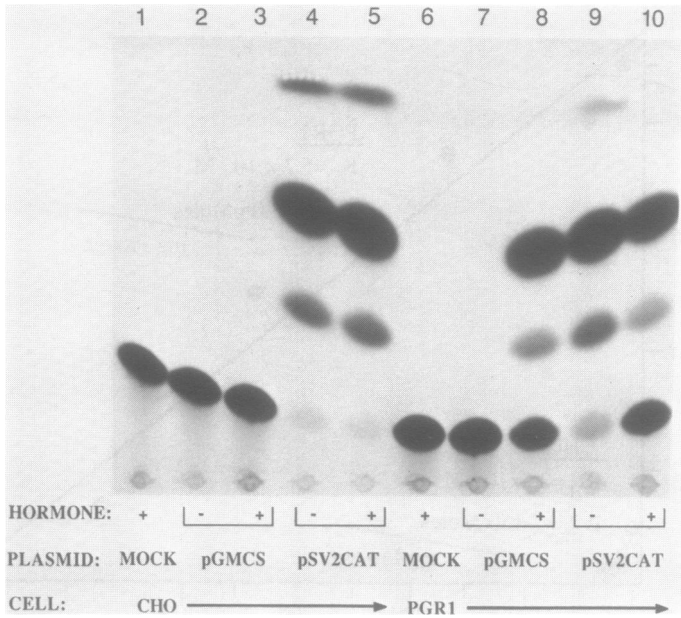


Fig. 4. Transient expression of CAT activity in CHO and PGR1 cells – Cells were transfected with the indicated plasmids for 4 hr, followed by a 2 hr incubation with chloroquin. Following transfection, the cells were treated for 48 hr in the absence or presence of 1μM dex, scraped into PBS, and sonicated. The CAT assay was performed using cell lysates from 1/4 of a 10 cm tissue culture plate, as described previously (Gorman et al., 1982).

for CAT activity. CAT expression in pGMCS is under control of the MMTV-LTR and a second GRE from the Moloney sarcoma virus present within the 3′ end of the CAT transcription unit (43). For comparison, cells were transfected with pSV2CAT, a vector which expresses CAT constitutively (35). No CAT activity is detected by transfection of pGMCS into CHO cells in the presence or absence of dex (Figure 4, lanes 2 and 3), or into PGR1 cells in the absence of dex (Figure 4, lane 7). Addition of dex to PGR1 cells transfected with pGMCS elicits a dramatic increase in the amount of CAT activity (Figure 4, lane 8). Northern blot analysis of cells transfected with the same vectors shows that CAT mRNA levels parallel the activity seen in Figure 4 (data not shown). Since basal CAT activity and CAT RNA in PGR1 cells transfected with pGMCS is undetectable even after a prolonged exposure of the autoradiogram, a minimum estimate of the induction is 1000-fold. These results demonstrate that at least three components are necessary for glucocorticoid-regulated gene expression; 1) a cell line that expresses functional hormone receptor; 2) an expression vector that contains a glucocorticoid-responsive element(s) (GRE), and; 3) the inducing steroid hormone. Interestingly, dex treatment of PGR1 cells, but not CHO cells, results in a decrease in CAT activity (Figure 4, lane 10) and CAT RNA (data not shown) after transfection with pSV2CAT. Subsequent experiments have indicated that glucocorticoids repress expression of vectors containing the SV40 enhancer element (manuscript in preparation).

Design of Inducible Vectors For Expression at High Levels

The large induction ratio observed above (Figure 4) and by others (14, 19) is a desirable feature of a conditional expression system. However, the maximum induced expression

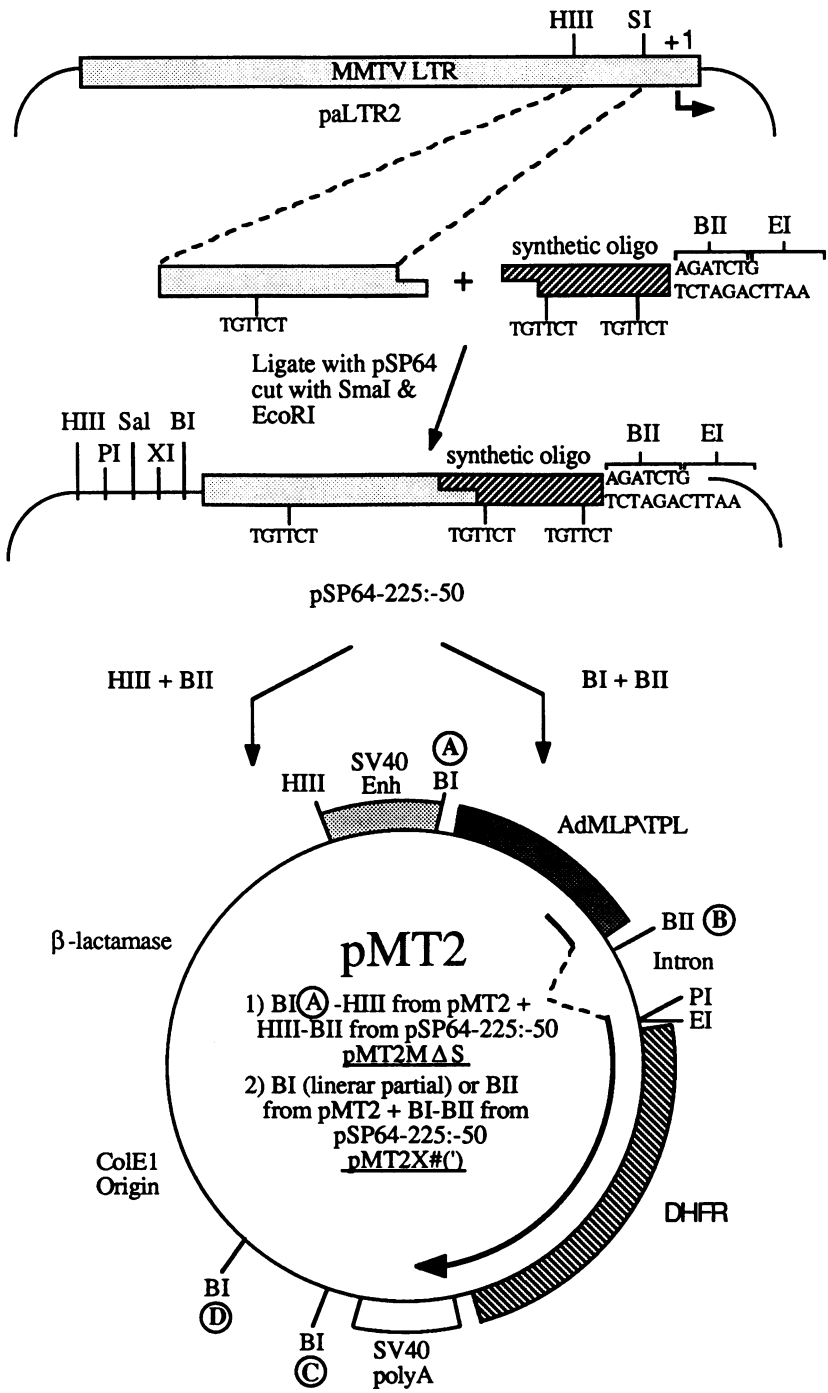
levels from several different vectors containing the MMTV LTR were always significantly lower than that obtained with constitutive expression vectors utilizing the SV40 enhancer/early promoter or SV40 enhancer/AdMLP (Figure 4, unpublished observations). We therefore constructed a series of plasmids designed to combine the inducible properties of MMTV-based vectors with the high expression levels obtained with SV40/AdMLP based vectors.

The experimental strategy was to insert one or multiple copies of the inducible enhancer element from the MMTV LTR at various sites within the vector pMT2 (Figure 5). A 180 bp BamHI/BglII fragment containing the MMTV GRE's was prepared and ligated as a monomer or multimer to various BamHI and BglII sites within the pMT2 expression vector as indicated in Figure 5. The different GRE-containing pMT2 derivatives were assayed by measuring DHFR mRNA production after transient transfection of PGR1 cells (Figure 6A). The DHFR transcript from the transfected pMT2 test plasmid is smaller than the GR/DHFR transcript expressed from pMT2T-GR in PGR1 cells. Expression from the original pMT2 vector which lacks GRE's is inhibited by dex (Figure 6A, lanes 2 and 3), consistent with the inhibition of the pSV2CAT vector observed previously (Figure 4, lanes 9 and 10). Every derivative of pMT2 that contains one or more GRE's plus the SV40 enhancer is induced by addition of dex. Furthermore, the maximum expression level after hormone treatment is approximately five-fold greater than that obtained by constitutive expression of pMT2 in several of the inducible pMT2 derivatives. The highest expression levels and induction ratios are obtained with vectors that contained multiple GRE's at several different sites within the vector. All vectors that contain the SV40 enhancer are constitutively expressed in the absence of dex, although this basal expression decreases 3-fold when 9 GRE's are located between the SV40 enhancer and AdMLP. The results, summarized in Table I, demonstrate that both hormonal responsiveness and high expression levels are retained when the SV40 enhancer and MMTV GRE's are combined within the same vector.

To reduce the basal level of hormone independent expression, the SV40 enhancer was deleted from the vector pMG18PC, which contains 18 copies of the GRE fragment, to generate the vector pMG18ΔS. Expression of DHFR mRNA from pMG18ΔS is not detectable in transfected cells in the absence of dex (Figure 6B, lane 11). However, in the presence of dex, DHFR mRNA is induced to levels greater than that obtained with the parental pMT2 vector containing the SV40 enhancer, but lacking GRE's (Figure 6B, compare lanes 1 and 12). Thus, mRNA expression from a vector containing 18 copies of the MMTV GRE but lacking a constitutive enhancer is not detectable in the absence of dex and in the presence of dex is induced to levels greater than that observed with the SV40 enhancer alone. By analyzing serial 10-fold dilutions of RNA from induced cells, we estimate the minimum induction to be 1000-fold (data not shown).

Stable, Inducible Expression of Heterologous Proteins

The expression of CAT activity (Figure 4) and DHFR mRNA (Figure 6) demonstrates that hormonal responsiveness is retained in transiently transfected plasmids that contain GRE's. We next determined if high expression and induction levels are retained upon stable integration and amplification of these vectors into the host chromosome. In order to generate a GR expressing CHO cell line which can be used as a host for DHFR co-transfection and co-amplification of glucocorticoid responsive heterologous genes, a second GR expression vector, pMT3GR-SVA (Figure 1B), was constructed to lack DHFR, and contain a second transcription unit directing Ada expression. CHO cells were transfected with pMT3GR-SVA (Figure 1B), selected for Ada expression, and subsequently selected for


HIII
SI
+1
MMTV LTR
paLTR2
BII
EI
synthetic oligo
AGATCTG
TCTAGACTTAA
TGTTCT
TGTTCT
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Ligate with pSP64
cut with SmaI &
EcoRI
HIII Sal BI
PI XI
BII
EI
synthetic oligo
AGATCTG
TCTAGACTTAA
TGTTCT
TGTTCT
TGTTCT
pSP64-225:-50
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BI + BII
HIII
SV40
Enh
BI
A
AdMLP\TPL
BII B
Intron
PI
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1) BI A -HIII from pMT2 +
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2) BI (linerar partial) or BII
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amplification in increasing concentrations of the Ada inhibitor dCF. Several cell lines, termed GRA, were found to express GR RNA and GR protein at levels comparable to PGR1 cells (Table II). Since GRA cells are DHFR deficient, they can be used as a host for selection and amplification of heterologous genes with DHFR. GRA cells were transfected with a vector containing a dex-inducible transcription unit encoding either tPA or CSF-1 with a DHFR coding region within the 3′ portion of the bicistronic mRNA, and were selected for stable expression of DHFR. While translation of DHFR in this position is inefficient relative to the upstream open reading frame (28), it is sufficient for the selection of stable cell lines for growth in nucleoside-free medium. Cells were selected for DHFR expression in the absence of dex by virtue of the basal expression that occurs with vectors containing both the SV40 enhancer and GRE's (Figure 6, Table I). A majority of cell lines assayed for either tPA or M-CSF activity showed a moderate (2−5 fold) increase in secretion of these proteins after a 24 hr induction by dex (data not shown). Several cell lines possessed higher induction responses to dex, or expressed the heterologous protein at a high level, as shown in Table III. Furthermore, amplification of the inducible genes by selection for increased methotrexate resistance resulted in cells that retained inducibility at higher absolute expression levels (Table III). Thus, stable, inducible expression of amplified genes at relatively high levels can be achieved with this system.

DISCUSSION

We have developed an inducible host-vector system for mammalian cells suitable for either transient or stable expression of heterologous genes at high levels. Stable CHO cell lines that are competent for glucocorticoid-regulated gene transcription were established by high level expression of a rat GR clone. Our results with transient DNA transfection into these cell lines demonstrated that addition of multiple GRE's to the AdMLP yields 1000-fold inducible expression to mRNA levels 7-fold greater than that observed by addition of an SV40 enhancer alone to the AdMLP. Addition of both the SV40 enhancer and GRE's increased basal expression with little effect on the maximal induced level of expression. Upon stable integration of vectors containing both GRE's and the SV40 enhancer into the chromosome, cell lines can be obtained which exhibit dex-responsiveness. We are presently studying the inducibility of stably integrated vectors lacking the SV40 enhancer.

Several laboratories have demonstrated that expression of a steroid receptor cDNA clone confers hormonal responsiveness to a previously nonresponsive cell (16, 18, 19, 44). We

Fig. 5. Construction of inducible derivatives of the pMT2 expression vector−A 175 bp fragment corresponding to nucleotides −225 through −50 relative to the start site of RNA transcription in the MMTV LTR was excised from pSP64−225:−50 by digestion with BglII and either HinDIII or BamHI, and was purified by extraction after agarose gel electrophoresis. This fragment contains a portion of the MMTV LTR that has three binding sites for the activated glucocorticoid receptor (Payvar et al., 1983). The GRE-containing fragment was ligated into pMT2 at the indicated positions. In order to obtain multimers of the LTR fragment, the BamHI to BglII fragment was ligated to itself, digested with BamHI and BglII, gel purified, and ligated with pMT2. Various restriction fragments from the inducible pMT2 derivatives containing one or more LTR inserts at a single site were then combined to yield vectors with LTR sequences at several sites. Derivatives of pMT2 were named by indicating the location (A-D) and number of the GRE element within pMT2. In some cases, the orientation of the GRE fragment(s) within the expression vector is reversed relative to its orientation within the MMTV LTR, and is indicated with an asterisk. In pMT2MΔS, the SV40 enhancer was removed and replaced with the HinDIII to BgIII fragment from pSP64−225:−50. The location of recognition sites for the endonucleases BamHI (BI), BgIII (BII), EcoRI (EI), HinDIII (HIII), PstI (PI), SacI (SI), SalI (Sal), and XbaI (XI) are indicated.

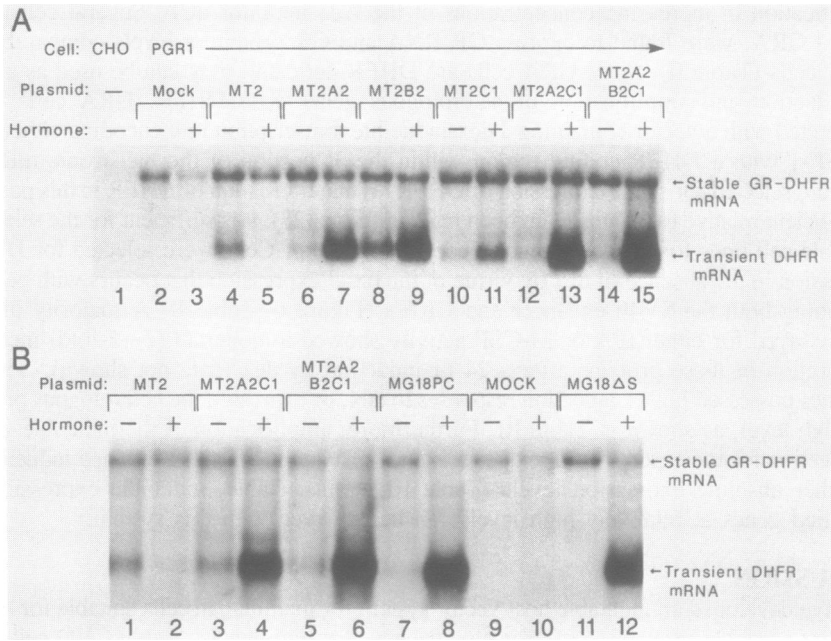

Fig. 6. Transient expression from inducible pMT2-based vectors in PGR1 cells−PGR1 cells were transfected with the indicated plasmids as described in the legend to Fig 4. Half of the plates recieved 1 μM dex for 40 hrs. Control plates were treated with 0.1% DMSO. Total cellular RNA was prepared, electrophoresed on a 1% agarose/formaldehyde gel (10 μg/lane), transferred to a nitrocellulose filter, and hybridized to a DHFR cDNA probe labelled by nick-translation. The positions of the stably expressed glucocorticoid receptor/DHFR bicistronic transcript and the transiently expressed DHFR mRNA from pMT2 and derivatives are indicated. A) Expression from vectors containing the SV40 enhancer alone (lanes 4 and 5), or in combination with GRE's (lanes 6−15). Lane 1 represents original untransfected CHO cells; B) Expression from vectors containing GRE's with (lanes 3−8) and without (lanes 11 and 12) the SV40 enhancer.

Table I

Plasmid	DHFR Expression −Dex	+Dex	Plasmid	DHFR Expression −Dex	+Dex
pMT2	1.0	0.2 (0.2)	pMT2B4	0.9	3.9 (4.3)
pMT2A1	1.2	2.0 (1.7)	pMT2B5	1.1	4.1 (3.7)
pMT2A1′	0.9	4.3 (4.7)	pMT2B13′	0.8	3.5 (4.4)
pMT2A2	1.2	4.4 (3.7)	pMT2C4	1.0	3.7 (3.7)
pMT2B1′	0.9	1.6 (1.8)	pMT2D3′	1.2	3.8 (3.2)
pMT2B2	1.3	2.6 (2.0)	pMT2A9	0.3	2.8 (9.3)
pMT2B2′	0.9	1.9 (2.1)	pMT2A2C1	0.6	3.5 (5.8)
pMT2C1	0.9	2.4 (2.7)	pMT2A2B2C1	0.6	4.0 (6.7)
pMT2C1′	1.9	4.3 (2.3)	pMT2A9B5C4	0.3	5.3 (17.7)
pMT2D1	1.8	2.5 (1.4)	pMT2MΔS	<0.1	0.2 (>10)
pMT2D1′	0.9	1.5 (1.7)	pMG18ΔS	<0.1	7.6 (>1000)

PGR1 cells were transfected with the indicated plasmids and analyzed for expression of DHFR mRNA as decribed in the legend to Fig. 6. The amount of transiently expressed RNA was quantitated by scanning densitometry of the autoradiographs, and is expressed relative to the amount of DHFR expressed from the pMT2 vector in the absence of hormone. The fold induction for each plasmid is shown in parentheses.

Table II

Cell Line	K_d	Binding Capacity
CHO DUKX	2.1×10^{-9}M	160 fMoles/mg protein
PGR1	6.6×10^{-9}M	2700 fMoles/mg protein
GRA1 (0.1 dCF)	6.0×10^{-9}M	1200 fMoles/mg protein
GRA1 (1.0 dCF)	5.4×10^{-9}M	3500 fMoles/mg protein
GRA2 (1.0 dCF)	5.8×10^{-9}M	3000 fMoles/mg protein
GRA8 (1.0 dCF)	4.5×10^{-9}M	2400 fMoles/mg protein
M1.19 (HTC cells)	6.4×10^{-9}M	600 fMoles/mg protein

Cytosol was incubated with various concentrations of [^{3}H]dexamethasone, and extracted with dextran-coated charcoal. Specific binding was determined as described in the legend to Fig. 3, and used to determine binding affinity and capacity by the method of Scatchard.

have extended these initial observations by expressing the glucocorticoid receptor protein at high levels using efficient, amplifiable vectors. In addition, a series of expression vectors were developed that show both high induction ratios and maximum expression levels in cells containing amplified receptors.

In CHO cells, endogenous glucocorticoid receptor is present at low levels, and fails to detectably induce transcription from the MMTV LTR in a transient assay system. Other established cell lines retain some glucocorticoid responsiveness, apparently due to the presence of endogenous functional glucocorticoid receptor (1, 3, 45–48). It has previously been reported that CHOk1 cells contain functional glucocorticoid receptors (46). We have also observed a low level of inducible expression in a stable G-418-resistant CHO cell line cotransfected with a MMTV-based CAT vector and an SV40-based Neo vector (unpublished data). We do not know why the MMTV LTR fails to respond to hormone in a transient assay, yet possesses some inducibility when integrated. Due to its weak promoter strength in CHO cells, the MMTV LTR may need to be integrated adjacent to either an endogenous enhancer or an enhancer element on the cotransfected plasmid in order to detect induced expression.

The recombinant glucocorticoid receptor protein stably expressed in CHO cells is similar by gel analysis to the purified receptor from rat liver (41). The binding affinity of dex is indistinguishable from endogenous receptor (39, 42). In addition, the ED_{50} for induction of tPA or M-CSF activity by dex in the cell lines shown in Table III is approximately 10^{-9}M (unpublished data), which is very similar to the biological responsiveness observed with endogenous receptor from several cell lines (5, 12, 39, 43). Thus, by all criteria tested, the overproduced glucocorticoid receptor protein is identical to endogenous receptor

Table III

	0 MTX		.005 MTX		.02 MTX		0.1 MTX	
Cell Line	−	+	−	+	−	+	−	+
G1F39c1 (tPA−units/ml)	24	258	63	673	192	1289	−	
G1M18c3 (CSF-1−μg/ml)	0.11	0.33	−		1.53	2.88	6.5	15.0

Cell lines were selected to the indicated concentration (μM) of methotrexate (MTX) resistance. Expression of tPA activity or CSF1 protein in the presence (+) or absence (−) of dex was determined as described in Materials and Methods.

present in liver or tissue culture cells. The amount of receptor expressed in the PGR1 and GRA cell lines (~ 3pMoles/mg cytosoloic protein) is approximately 5 – 10 fold higher than that found in rat liver (42) or hepatoma cell lines (39). These cell lines should provide a rich source of glucocorticoid receptor protein for biochemical and functional studies of glucocorticoid action.

Many of the vectors generated by the strategy diagrammed in Figure 5 contain both the constitutively active SV40 enhancer and one or more copies of the inducible MMTV enhancer. Expression from these vectors in both transient and stable assays demonstrates that properties of both enhancer elements are retained, since high constitutive expression is further increased by treatment with glucocorticoids. A decrease in basal expression occurs only when a large number of GRE's are between the SV40 enhancer and AdMLP. This decrease may result from the physical seperation of the SV40 enhancer from the promoter and not the GRE's per se, since basal expression is not changed when 13 GRE's are at another site in the plasmid. Thus, the MMTV enhancer does not silence the SV40 enhancer in the absence of hormone, nor does the constitutive activity of the SV40 enhancer obliterate hormone-dependant transcriptional activation by the MMTV enhancer. Therefore, the actions of these two types of enhancer elements appear additive. Many endogenous genes have similar combinations of constitutive and/or conditional transcription elements (49 – 52). The combination of enhancer elements with different functional properties may therefore be a general strategy for regulating gene expression.

The additivity of multiple GRE's in stimulating transcription has been reported previously (51, 53). We have extended these observations by inserting up to 18 copies of the GRE fragment into a single expression vector. While there is a general tendency towards increased inducible expression with progressively greater numbers of GRE's, there does not appear to be a strict linear relationship between GRE number and transcriptional strength. The reiteration of binding sites for transcription factors occurs with many cellular and viral genes (54) and may be one evolutionary mechanism for assuring efficient gene expression. In fact, the 175 bp GRE fragment used here contains three glucocorticoid receptor binding sites (55), and is contained in both the 5′ and 3′ LTR's of the MMTV provirus (29).

Several other inducible systems have been developed for expression of heterologous genes. The signals for induction in these other systems include heat shock, double-stranded nucleic acid, heavy metals, and steroid hormones (reviewed in 56). The system presented here offers several advantages over these other systems. First, the genetic events involved in glucocorticoid induction of gene transcription are well understood. We have used molecular clones of the cis-acting DNA elements to construct expression vectors that are highly inducible by hormone. Inducible vectors are available that allow some basal expression, or that are tightly regulated (pMG18ΔS) by glucocorticoids. We have also developed cell lines that express at high levels the transcription factor that permits efficient hormone-dependent expression from these vectors. Secondly, switching to the induced state merely involves the addition of an inexpensive and readily available hormone that is relatively nontoxic to cells. Following addition of the hormone, induction is rapid and persistant. Finally, the inducible vectors can be amplified using methotrexate selection, allowing for stable inducible expression at high levels.

We have encountered several limitations with this system. The fold-induction in stable cell lines is variable, as has been observed previously with integrated MMTV proviruses (14). Therefore, many cell lines may have to be screened in order to obtain one which is highly inducible. In addition, the growth rate of CHO cells containing highly amplified

receptors is approximately 32% slower in the presence of inducing steroid (unpublished observations). We are currently refining several aspects of this system to try to overcome these limitations.

The system described here should have several useful scientific and biotechnological applications. Stable cell lines containing amplified copies of both hormone receptor genes and inducible genes should be useful for analyzing the molecular events that occur upon glucocorticoid stimulation. This system also offers the opportunity to stably express and study proteins that are toxic to the cell. In some systems, it may be desireable to vary the levels of a particular protein in order to study its role in a cell structure or function. By using different concentrations of hormone, we can easily vary the level of expression obtained with the inducible vectors (unpublished data). It will also be possible to study gene activation and RNA processing in finer detail, since addition of hormone should synchronously and rapidly turn on gene transcription. The early events of RNA synthesis, processing, and transport can then be studied as a function of time in cells sychronized with regards to transcription of the induced gene. Finally, this system may be of use for the efficient production of proteins from heterologous genes.

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*To whom correspondence should be addressed

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