The Glucocorticoid Receptor and a Putative Repressor Protein 
Coordinately Modulate Glucocorticoid Responsiveness of the 
Mouse Mammary Tumor Virus Promoter in the Rat Hepatoma 
Cell Line M1.19*

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Hiroshi Tanaka§‡¶, Yu Dong§‡¶, Jacqueline McGuire§, Sam Okret§, Lorenz Pöllinger§, 
Isao Makino‡¶, and Jan-Åke Gustafsson$§

From the §Second Department of Internal Medicine, Asahikawa Medical College, Nishikagura-4-5-3, 
Asahikawa 078, Japan and the $Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital, Novum F-60, 
Huddinge S-141 86, Sweden

Signal transduction by glucocorticoid hormones is 
mediated by the intracellular glucocorticoid receptor 
protein. The mechanisms determining cell type- or 
tissue-specific differences in hormone responsiveness re- 
main, however, unclear. To address this issue we have 
used two different rat hepatoma cell lines, 762 and 
6.10.2, respectively, in which mouse mammary tumor 
virus has been stably integrated. Nuclear extracts from 
both of these cell lines contained a factor that bound to 
a sequence motif extending from -183 to -147 in the 
mouse mammary tumor virus promoter and that ap- 
ppeared to repress hormonal induction of viral mRNA 
expression. Transient transfection experiments indi- 
cated that the cellular levels of this putative repressor 
did not affect basal promoter activity; this factor ap- 
ppeared rather to determine cellular sensitivity to glu- 
cocorticoids. Moreover, in these experiments the re- 
lative levels of the glucocorticoid receptor appeared to 
be the main determinant of maximum inducibility of 
 virus expression by hormone. Taken together, these 
data indicate that the differential expression patterns 
of receptor versus the putative repressor protein may 
determine the level of hormonal responsiveness of tar- 
get genes in glucocorticoid-sensitive tissues.

Glucocorticoid hormones have been widely used as potent 
anti-inflammatory drugs. Their mode of action is rather 
complex, since they act at various levels of immune 
responses and subsequent inflammatory reactions (1). These hormones exert 
their regulatory effects by binding to the glucocorticoid recep- 
tor (GR), a member of the superfamily of nuclear receptors.

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† To whom correspondence should be addressed: Second Dept. of 
Internal Medicine, Asahikawa Medical College, Nishikagura-4-5-3, 
Asahikawa 078, Japan. Tel.: 81-166-65-2111 (ext. 2454); Fax: 81-166- 
65-1182.

‡ Present address: Cardiology Division, Dept. of Pediatrics, Washing- 
ton University School of Medicine, St. Louis, MO 63104.

1 The abbreviations used are: GR, glucocorticoid receptor; hsp90, 
90-kDa heat shock protein; LTR, long terminal repeat; MMTV, 
mouse mammary tumor virus.

In response to binding of ligand, these proteins modulate 
target gene transcription in either a positive or negative 
manner (reviewed in Refs. 2-5). Regulation of eukaryotic gene 
transcription is mediated by an interaction of trans-regulatory 
proteins with defined nucleotide sequences (6, 7). Upon bind- 
ing of hormone, GR undergoes a conformational change 
termed receptor activation involving dissociation of an in- 
hibitory protein, hsp90 (2-5). The activated hormone-receptor 
complex translocates to the cell nucleus and binds to target 
DNA sequences termed glucocorticoid response elements (2- 
5). Although GR has been demonstrated to interact function- 
ally with various ubiquitous transcription factors (8-14), it is 
largely unknown how the receptor communicates with the 
basal transcription machinery.

An important aspect of glucocorticoid mechanism of action is to clarify how hormone responsiveness is modulated in a cell- or tissue-specific manner. It is well known that GR is present in target tissues, some of which are more responsive than others to hormone. Earlier observations have strongly indicated that the cellular level of GR is a primary determi- 
nant of hormone sensitivity (15-17). Consequently, it has 
recently been shown that introduction of GR antisense RNA into cells results in decreased cellular responsiveness to glu- 
cocorticoids, most likely through blocking of cellular receptor 
mRNA processing and/or translation and subsequent de- 
crease in cellular GR levels (18). In vivo, cellular GR levels 
are down-regulated in an autologous manner, by means of 
which fine tuning of hormone action may be accomplished in 
cells under exposure to agonists (19-24). On the other hand, 
the inhibitory protein hsp90 associates with the receptor in 
the cytoplasm (2-5, 25), suggesting that cellular levels of 
hsp90 may influence GR function. This hypothesis is sup- 
ported by the observation that down-regulation of hsp90 
expression results in decreased sensitivity to glucocorticoid 
hormones (26). However, hsp90 appears to be in a vast excess 
relative to GR, indicating that its intracellular level is unlikely 
to be a physiological determinant of hormone sensitivity of 
target cells.

Recent reports on "squelching" or transcriptional interference 
have suggested that steroid hormone receptors function by 
interacting with putative cofactors present in limiting 
amounts in the cell (27, 28). In the case of the retinoic acid 
receptor, it has been suggested that a potentially large number 
of distinct nuclear proteins may determine both DNA binding 
specificity and function of the retinoic acid receptor in a cell 
type specific manner (29). For instance, the retinoid X recep- 
tor represents a coregulator of thyroid hormone, vitamin D,
and retinoic acid receptors (30-35). Moreover, GR function appears to be directly or indirectly modulated by cellular protooncogene products such as Fos/Jun or the transcription factors CREB/ATF, resulting in interference (also termed "crosstalk") of distinct cellular signal transduction pathways (36-43). Thus, glucocorticoid-inducible gene activation may be a consequence of complex interactions between a variety of protein factors: GR, hsp90, basal transcription factors, and additional, as yet unidentified, cellular coregulators.

We have previously reported that nuclear extracts from the variant rat hepatoma cell line 6.10.2 contain a specific DNA binding activity that recognizes a sequence motif extending from -163 to -147 (A element) in the promoter region of the long terminal repeat of mouse mammary tumor virus (MMTV LTR). We have preliminary evidence that the factor recognizing this sequence motif may represent a repressor protein that interferes with glucocorticoid-dependent activation of the MMTV promoter (44). Here we show that wild-type 762 hepatoma cells also contain this repressor activity, raising the possibility that the relative levels of either GR or the putative repressor may determine cellular responsiveness to glucocorticoids.

MATERIALS AND METHODS

Plasmids—Double-stranded oligonucleotides encompassing the A element (noncoding strand: 5' CGAGGATTGGACACACG-3') of the MMTV LTR were synthesized and (i) inserted into the polylinker region of pGEMZ (Promega Biotec) to give the construct pA2A9-2, or (ii) inserted in front of a minimal β-globin promoter/reporter construct, OVEC (45). Subsequently, stepwise dimerization of the A element in the front of the β-globin promoter was performed as described (45) (resulting in the constructs pOVa1 and pOVa2, respectively).

Cell Culture—The rat hepatoma cell line M1.19 is stably infected with MMTV, the expression of which is induced by glucocorticoids (46). 762 is an unselected subclone of M1.19. The variant NSM6.10 is a clonal derivative of M1.19 selected by fluorescence-activated cell sorting due to low levels of expression of the viral protein p52 following treatment with dexamethasone (47). 6.10.2 is an unselected subclone of NSM6.10. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO/BRL) at 37 °C in a 10% CO₂ atmosphere. Fetal calf serum was heat-treated at 55 °C for 30 min, and serum steroids were stripped by dextran-coated charcoal treatment.

Preparation of RNA and Blot Hybridization—Total cellular RNA was isolated from cultured cells by the acid-guanidinium thiocyanate-phenol chloroform method (48). RNA blots and hybridization with radiolabeled probes were performed as described (49). Recombinant plasmids used for MMTV and β-actin probes have been described before (16, 19, 44). For quantification, the autoradiograms were analyzed densitometrically, and results were expressed using β-actin as a standard.

Immunoblot Experiments—Whole cell extracts were prepared by homogenization of cells in 20 mM sodium phosphate, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, and 50 mM NaCl, followed by centrifugation at 54,000 x g for 15 min at 2 °C. Cellular protein (20 μg/lane) was separated on a 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose filters. Immunoreactivity was localized by incubation with an anti-hsp90 monoclonal antibody 2 and subsequent incubation with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin. Immunocomplexes were visualized using the enhanced chemiluminescence light-emitting, nonradioactive method according to the manufacturer's (Amersham Corp.) protocol.

Preparation of Nuclear Extracts—Cells were cultured and harvested at near confluence, and nuclear extracts were prepared essentially as described previously. The expression of which is induced by glucocorticoids was determined using a protein assay kit (Pierce Chemical Co.). Routinely, the nuclear extracts from 762 and 6.10.2 cells contained approximately 4 and 3 μg of protein/ml, respectively. The extracts were immediately frozen in liquid nitrogen and stored at -70 °C.

2 M. Rönholm, G. Akner, and A.-C. Wikström, manuscript in preparation.

D’Nase I Protection Experiment—Plasmid pS8’139 (50) was digested with SalI, dephosphorylated, and end-labeled with T4-polydeoxynucleotide kinase (Promega) in the presence of [γ-32P]ATP (Amersham). After digestion with EcoRI, a labeled 185-base pair SalI-EcoRI fragment containing MMTV LTR sequences from positions -264 to -109 relative to the transcription start site was purified on a 5% polyacrylamide gel and used for in vitro DNA binding studies. Binding reactions were performed in a total volume of 100 μl with 5 fmol of probe (20,000 cpm), 10 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia), 0-100 μg of nuclear extract in 5 mM Heps (pH 7.9), 2.5 mM EDTA, 0.25 mM dithiothreitol, 2.5 mM MgCl₂, 60 mM KCl, 10 mM spermidine, and 10% glycerol. The reaction mixture was incubated at 25 °C for 20 min, and 5 μl of freshly diluted DNsase I (10 ng/ml) was added. The material was digested for 2 min on ice. The reaction was terminated by the addition of 100 μl of a stop buffer (10 mM EDTA, 0.2% SDS, 20 μg/ml proteinase K) with 5 μg of carrier yeast tRNA. The sample was further incubated at 37 °C for 30 min, extracted by phenol/chloroform (1:1), precipitated by ethanol, and analyzed on a 7% denaturing urea-polyacrylamide gel. The gels were dried on Whatman 3MM paper and exposed on Amersham Hyperfilm overnight at -70 °C.

Gel Mobility Shift Assay—Protein-DNA complexes were analyzed on 7% low ionic strength polyacrylamide gels as described (45). Oligo(A) was end-labeled with [γ-32P]dATP using the Klenow fragment of DNA polymerase I (Promega) and used as a specific probe. Protein samples (10 μg/reaction) were incubated with 5 fmol of the 32P-labeled double-stranded DNA probe (20,000 cpm) in a binding buffer, the composition of which is described above. The incubation was terminated at 37 °C for 20 min, and samples were separated on a 4% polyacrylamide gel for 2 h at 4 °C. The gels were subsequently dried and autoradiographed.

Transfection—Cells were grown on 100-mm dishes to 70–80% confluence and transfected by lipofection as previously described (44, 51). The indicated amounts of plasmid DNA were transfected in 30 μl of lipofection (GIBCO/BRL).

S1 Nuclease Protection Assay—10 μg of plasmids OVEC (45), pOVA1, or pOVA2 were transfected with 2 μg of the β galactosidase expression vector pRSVβgal (kindly provided by Dr. Anders Ström, Karolinska Institute) as internal standard. Cytoplasmic RNA was isolated by Nonidet P-40-mediated lysis of the cells 24 h after transfection, and residual input plasmid DNA was digested with RNase-free DNase I as described (45). A 93-mer single-stranded oligonucleotide probe for S1 analysis extending from positions -18 to +75 bases relative to the transcription start site of the rabbit β-globin gene (45) was kindly provided by Dr. Scott Cuthill, Karolinska Institute. The 32P-labeled probe was hybridized with cytosolic RNA, and analyzed by S1 nuclease protection as described (45). Finally, the samples were analyzed on a 10% denaturing urea-polyacrylamide gel and autoradiographed. To quantitatively determine the intensity of the signals, the corresponding bands were excised and counted in a liquid scintillation counter.

RESULTS

Northern Blot Analysis of RNA Isolated from 762 and 6.10.2 Cells after Treatment with Dexamethasone—In glucocorticoid-sensitive cells, the relative levels of GR and hsp90 appear to be essential for normal hormone responsiveness (see the Introduction). We have studied 762 and 6.10.2 cells as a model system to investigate molecular mechanisms of glucocorticoid hormone action, since the induction of the stably infected MMTV serves as a good marker for hormone responsiveness (16, 17, 44) and the MMTV LTR is a very well characterized hormone responsive promoter (see Ref. 4, and references therein). We have previously shown by ligand binding and immunoblot experiments that 6.10.2 cells contain more than 5-fold lower levels of GR when compared with wild-type 762 cells and are, possibly as a consequence thereof, quite resistant to hormone treatment (16, 17). To test the levels of MMTV expression in these cells, total cellular RNA was isolated after an 18-h incubation in the absence or presence of 1 μM dexamethasone and analyzed by Northern blot hybridization (Fig. 1A). In the presence of dexamethasone, wild-type 762 cells showed a strong increase in MMTV expression (Fig. 1A). By contrast, in resistant 6.10.2 cells, the expression of MMTV...
6.10.2 cells. Cells were incubated in the absence or presence of dexamethasone blot analysis of whole cell extracts from the two cell lines 6.10.2 cells. Cellular protein was separated on a 9% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose filters. Each lane contained 20 μg of RNA and was hybridized with probes for MMTV. B, immunoblot analysis of cellular extracts from 762 and 6.10.2 cells. Cellular protein was separated on a 9% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose filters. Immunoactivity was localized by incubation with an anti-hsp90 monoclonal antibody. Molecular mass was determined by the marker run in parallel.

was poorly induced by hormone treatment in agreement with previous reports (16, 17) (Fig. 1A). On the other hand, no differences in hsp90 protein levels were detected in immunoblot analysis of whole cell extracts from the two cell lines (Fig. 1B), in line with the model that the cellular levels of hsp90 do not play a key role in determining the degree of hormone sensitivity of these cells.

Specific DNA Binding Activities of Nuclear Extract from 6.10.2 and 762 Cells—We have previously identified a putative repressor protein in nuclear extracts from 6.10.2 cells. This factor binds in vitro to MMTV promoter DNA in the region of the A element (−163 to −147 from the transcription start site), and may contribute to glucocorticoid resistance in 6.10.2 cells (16, 44). Interestingly, nuclear extracts from 762 cells also contained a similar specific DNA binding activity. Dnase I protection experiments in vitro demonstrated that nuclear extracts from either 6.10.2 or 762 cells contained a factor that protected the A element of the MMTV probe in a similar fashion (Fig. 2). Furthermore, gel mobility shift experiments were carried out using the oligonucleotide A as a probe to visualize protein-DNA complexes formed between the A element and nuclear extracts from the two cell lines. As demonstrated in Fig. 3, nuclear extracts from both cell lines generated a protein-DNA complex (C1) of identical relative mobility. Moreover, the relative amounts of the C1-forming factor(s) were very similar in both cell lines, since quantitation of the C1 complex by excision and scintillation counting demonstrated virtually identical levels of this complex. Therefore, only the relative levels of the GR protein itself appeared to account for the differences in hormone responsiveness between these two cell lines. No differences in the levels of hsp90 or in the concentration of the putative repressor protein could be detected in either cell line.

Role of GR and a Putative Repressor on the MMTV Expression—To study the dose relationship between glucocorticoid concentrations and hormone responsiveness, 762 and 6.10.2 cells were treated with increasing concentrations of dexamethasone, and the activation of the MMTV promoter was assayed by means of RNA slot-blot hybridization (Fig. 4A). Reportedly, GR in both 762 and 6.10.2 cells binds dexamethasone with the same affinity (0.6–1.6 nM; see Ref. 16). At 5 nm dexamethasone, MMTV RNA levels were not significantly induced in either cell line. However, at 20 nm or at higher concentrations of hormone, a significant induction of MMTV RNA expression was observed in a dose-dependent fashion in 762 cells, and this induction reached nearly maximal levels at about 100 nm dexamethasone (Fig. 4), indicating that hormonal inducibility of the MMTV promoter is related to the amounts of hormone-bound, activated receptor. By contrast,
relative increase in hormone-dependent induction of MMTV expression in 762 cells versus 6.10.2 cells. However, the concentration of hormone that was required to trigger this response differed in the two cell lines: 5 nM versus 1 μM dexamethasone, respectively. Treatment of 6.10.2 cells with 5 or 20 nM dexamethasone did not result in a significant induction of MMTV expression after transfection of pA (not shown). These results suggest that 6.10.2 cells or 762 cells treated with low concentrations of dexamethasone are resistant to hormone, possibly due to a low level of available, functional receptor sites. Consequently, the threshold level for MMTV activation is not overcome. The C1 factor may contribute to define this functional threshold level. Once GR functionally competes for the repressor activity of the C1 factor, MMTV promoter activity can be induced. On the other hand, transfection of pA did not increase MMTV inducibility when 762 cells were treated with 1 μM dexamethasone (Fig. 5, compare with Fig. 4B). The C1 factor, therefore, may not affect the maximum levels of MMTV induction.

**Effect of Putative Repressor on Basal Promoter Activity**—Since transfection of pA into either 762 or 6.10.2 cells did not affect the basal levels of MMTV expression (not shown), we speculated that the A element is usually silent but negatively modulates the MMTV expression only when cells are treated with glucocorticoids. To examine this hypothesis, we tested in transient transfection assays the effect of the A element on the activity of a heterologous minimal promoter construct. To this end, the A element was fused immediately upstream of the minimal β-globin promoter in OVEC (45). Following transfection of the promoter constructs pOVA1 or pOVA2 into 762 cells, the levels of correctly initiated β-globin transcripts were analyzed by an S1 nuclease protection assay (45). As shown in Fig. 6, the levels of correctly initiated transcripts were not affected by the presence of single or double copies

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**Fig. 4.** Dose dependence of the MMTV induction response. 762 and 6.10.2 cells were incubated in the absence or presence of the indicated concentrations of dexamethasone (Dex), and RNA was extracted. The RNA blot (10 μg of total RNA/slot) was hybridized with 32P-labeled MMTV or β-actin probes. The results were visualized by autoradiography (representative results are shown in panel A) and analyzed densitometrically (panel B; see “Materials and Methods”). For quantitation, data were normalized against β-actin mRNA (not shown). All data are expressed as -fold induction over basal levels. The data were summarized from three independent experiments. Vertical bar represents standard deviation.

**Fig. 5.** In vivo competition experiments to test the function of the putative repressor protein. 762 and 6.10.2 cells were transfected with various amounts of pA vector DNA and incubated in the absence or presence of the indicated concentrations of dexamethasone. The total amounts of transfected plasmids were kept constant (10 μg) by adding pGEM3Z vector. MMTV RNA expression was measured by slot-blot hybridization as described, and the results are shown as -fold increase against basal levels. The data were summarized from three independent experiments. Vertical bar represents mean ± standard deviation.

MMTV RNA was hardly induced in 6.10.2 cells (Fig. 4). Thus, these results suggest that although 6.10.2 cells were treated with saturating concentration of dexamethasone, the GR content in 6.10.2 cells was below the threshold level for triggering MMTV gene activation.

To test the function of the A element and C1 factor in vivo, we performed in vivo competition experiments using increasing concentrations of plasmid pA transfected into both cell lines. Inducibility of MMTV RNA expression was assayed after treatment with 5 nM, 20 nM, and 1 μM dexamethasone (Fig. 5). In 762 cells a dose-dependent increase in MMTV expression was observed at relatively low concentrations of dexamethasone (5 and 20 nM) after transfection of pA. Importantly, transfection of the pA DNA resulted in a similar
of the A elements, strongly supporting the model that even upon binding a putative repressor, the A element does not repress basal promoter activity.

**DISCUSSION**

Tissue-specific regulation of glucocorticoid action is generally believed to involve several mechanisms, i.e. differential expression of hormone receptor (17, 52) or hsp90 (26) and/or differential inactivation of hormone (53). Our present data suggest that differential expression of GR and a cellular negative factor that counteracts receptor functions may modulate target gene activity at a physiological range of hormone concentrations. At relatively low concentrations of dexamethasone, it is difficult to activate GR in 762 cells. In fact, we did not detect any hormonal induction of MMTV expression at 5 nM dexamethasone. This concentration of ligand is in the range of the Kd value for binding to the receptor protein. However, transfection of the cells with pA, which presumably resulted in sequestration of the C1 factor, restored hormonal inducibility. This phenomenon may be analogous to the observed hormone responsiveness in 6.10.2 cells following treatment with 1 μM dexamethasone and transfection with pA. Together, these data indicate that the balance between activated GR and the C1 factor may determine glucocorticoid-responsive regulation of MMTV expression in these two cell lines. In agreement with this model, transfection of GR cDNA (17) or cAMP treatment (16) increases cellular GR contents and subsequently restores glucocorticoid responsiveness in 6.10.2 cells.

Although the concentration of dexamethasone was critical for the magnitude of the MMTV induction response following transfection of the pA vector in both cell types, hormonal inducibility appeared to be nearly saturated in 762 cells at 0.1–1 μM dexamethasone irrespective of the cellular concentration of the C1 factor. Against this background it is tempting to speculate that at relatively low concentrations of dexamethasone, GR is only partially activated by the hormone to surpass the threshold level for induction of MMTV expression and that the magnitude of the hormone effect is largely dependent on the amount of ligand-activated receptor. Consistent with this model it has been shown that the cellular levels of GR determine target gene responsiveness (15, 52) and that introduction of GR antisense RNA results in reduced responsiveness to hormone (18).

It has previously been reported that several ubiquitous transcription factors functionally cooperate with GR and positively modulate hormone-inducible target promoters (8–14). Our data indicate a physiologically relevant mechanism of regulation of an inducible promoter, where variable levels of expression of a negative trans-regulatory factor in target tissues may result in cell type specificity with regard to the hormonal induction response. We propose that the C1 factor, a negative modulator of hormone-inducible MMTV expression, resides in cells a priori and binds to its recognition DNA sequences until it is displaced by increasing nuclear concentrations of the activated hormone-receptor complex resulting in gene activation. In *vitro*, the C1 factor binds to the A element, which is located in the very proximity of glucocorticoid response elements in the MMTV hormone-responsive promoter region (44), and competes with GR for binding to the MMTV promoter (44). Thus, there may simply be steric hindrance effects between GR and the C1 factor as a possible mechanism for displacement of GR. However, further studies are required to clarify the underlying mechanisms.

In the promoter of the tyrosine aminotransferase gene, proteins encoded by the tissue-specific extinguisher-1 serve as cellular antagonists of cAMP-mediated tyrosine aminotransferase gene activation (54). The CAMP-dependent signal is, at least in part, mediated by protein kinase A and subsequently by the transcription factor CREB (55). The protein encoded by the tissue-specific extinguisher-1 is suggested to be the catalytic subunit of protein kinase A (56). Therefore, the repression of the tyrosine aminotransferase promoter by the tissue-specific extinguisher-1 is not likely to be due to interaction between DNA-binding proteins. In any case, the presence of endogenous factors, which negatively modulate hormone receptor function or the function of other inducible trans-acting factors, could be essential for fine tuning of the cellular response to external stimuli. A more detailed understanding of cell type- or tissue-specific regulation of glucocorticoid hormone action awaits further biochemical characterization of such negative regulatory factors. In this context it is interesting to note that patients have been described who exhibit tissue hypersensitivity to glucocorticoids. Possibly, this might be ascribed to a deficiency of a C1-like factor negatively modulating glucocorticoid hormone action in a physiological situation (57). Thus, a further elucidation of the model presented here could also facilitate our understanding of human disorders exhibiting glucocorticoid hyper- or hyporesponsivity.

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