MOLECULAR PHARMACOLOGY, 23:779–785

Analysis of Glucocorticoid-Inducible Genes in Wild-Type and Variant Rat Hepatoma Cells

JAMES L. VANNICE,[1] J. RUSSELL GROVE,[2] AND GORDON M. RINGOLD

Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

Received September 22, 1982; Accepted November 22, 1982

SUMMARY

We present pharmacological and genetic evidence that regulation of different genes by glucocorticoid hormones in the rat hepatoma cell line, HTC, occurs in a coordinate manner. We have analyzed the responses of four different glucocorticoid-inducible proteins, tyrosine aminotransferase [L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5)], glutamine synthetase [L-glutamate:ammonia ligase (EC 6.3.1.2)], a secreted glycoprotein Belt I, and the mouse mammary tumor virus (MMTV)-encoded protein (gp52) in these cells. The concentration of dexamethasone necessary for half-maximal induction of each of these proteins is 10–20 nM, the same concentration necessary to half-saturate glucocorticoid receptors. Furthermore, glucocorticoids of varying potency elicit parallel inductions of these markers. MSN5.3, a glucocorticoid receptor-defective cell line selected for its inability to induce gp52, is also unable to induce the other three cellular gene products. In contrast, another class of variants incapable of gp52 induction retains inducibility of the other three markers. We show here by "superinfection" with MMTV that these cells harbor a defect in the original integrated provirus itself and not in the cellular induction machinery. The results presented here suggest that the induction of glucocorticoid-responsive genes in these cells is mediated by a single glucocorticoid induction pathway.

INTRODUCTION

Steroid hormones probably exert most of their effects by altering the rates of transcription of specific genes (for reviews see refs. 1 and 2). The currently accepted two-step model for steroid action, originally proposed by Jensen *et al.* (3) and Gorski *et al.* (4), can be summarized as follows: the hormone first binds to a soluble, cytoplasmic receptor protein that undergoes a structural alteration ("activation") followed by accumulation of the steroid-receptor complex in the nucleus. Within the nucleus, interaction of this complex with specific sites on DNA or chromatin is presumed to be responsible for the observed changes in gene expression. The exact nature of these nuclear acceptor sites, however, remains obscure.

Within any particular cell type only a limited number of genes is under the control of a given class of steroid hormone. For example, glucocorticoid hormone treatment of tissue culture cells derived from rat hepatomas alters the rate of synthesis of only 10 or 12 out of more than 2000 proteins visible by 2-dimensional PAGE[3] (5). Similar observations have been made in studies of various cell types responsive to other classes of steroids (2).

We have recently used MMTV-infected HTC rat hepatoma cells to analyze the actions of glucocorticoid hormones by genetic and molecular techniques. In previous reports (6, 7) we demonstrated that cells selected for their inability to induce gp52 in response to dexamethasone (a potent glucocorticoid) exhibit two classes of defects. Variants derived from M1.19, a clone of MMTV-infected HTC cells harboring multiple copies of the MMTV provirus, contain little or no glucocorticoid receptor (6). In contrast, nonresponsive variants derived from J2.17, an HTC clone which contains only a single MMTV provirus, have normal receptors but appear to be specifically defective in hormonal regulation of viral gene expression (7). In addition to the viral gp52, HTC cells contain several cellular gene products whose levels are altered by glucocorticoid hormones. These include the enzymes tyrosine aminotransferase [L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5)] and glutamine

This work was supported by National Institutes of Health Grants GM25821 and GM17368, and was aided by a Basil O'Connor Starter Research Grant from the National Foundation-March of Dimes.

[1] Supported by National Institutes of Health Predoctoral Training Grant GM07149.

[2] Recipient of a Predoctoral Fellowship from the Pharmaceutical Manufacturers' Association. Present address, Department of Molecular Biology, Massachusetts General Hospital, Boston, Mass. 02114.

[3] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MMTV, mouse mammary tumor virus; gp52, mouse mammary tumor virus-encoded glycoprotein of M_r 52,000; FACS, fluorescence-activated cell sorter; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; DMP, 6α,16α-dimethylprogesterone.

0026-895X/83/030779-07$02.00/0

synthetase [L-glutamate:ammonia ligase (EC 6.3.1.5)], as well as a secreted glycoprotein known as Belt I, so-named by Ivarie and O'Farrell (5) because it forms a series (or belt) of spots on 2-dimensional gels; the function of this protein is unknown. Detailed studies have not been reported comparing the hormonal specificity and/or extent of induction of these three proteins; thus, it is not clear whether the same receptor pathway is used for induction of these diverse gene products.

We have analyzed in greater detail the glucocorticoid responsiveness of both viral and cellular gene products in wild-type and variant HTC cells. Our results strongly suggest that a single receptor mediates the induction of the glucocorticoid-responsive genes in these cells; however, the absolute extent of induction varies among the four markers we have studied. In addition, we present evidence that one class of glucocorticoid-unresponsive variants is defective at the level of the MMTV provirus itself and not in the cellular machinery required for hormone action.

MATERIALS AND METHODS

Cell lines. All cell lines used were derived from the rat hepatoma cell line, HTC (8), which was infected with MMTV, giving rise to a number of clonal cell lines containing different numbers of MMTV proviral DNA copies (9). One resulting cell line, J2.17, which contains one proviral copy of MMTV, was mutagenized with ethyl-methane sulfonate as described (7), giving rise to a population of cells called JZ. This cell population was then subcloned, giving rise to the cell line JZ.1. The cell line JZN3.7 was selected from the JZ population using the FACS as described (7) and exhibits little, if any, glucocorticoid-mediated gp52 induction. The glucocorticoid-unresponsive cell line, MSN5.3, was derived from another MMTV-infected HTC cell line, M1.19, which contains approximately 10 proviral copies, as described (6). MSC1 is a subpopulation of M1.19 cells exhibiting wild-type hormonal responsiveness.

Cell culture. Cells were grown in monolayer culture in DMEM (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 5% newborn calf serum and 3% fetal calf serum (both from Irvine Scientific, Santa Ana, Calif.) at 37° in 5% CO_2. Cells were passaged by removing cells from tissue culture dishes with PBS containing 2.5 mM EDTA (pH 7.4) and plating them onto new tissue culture dishes at a dilution of 1:6. After 1 day of growth and attachment to the dishes, hormone was added to a concentration of 1 μM (from a 10 mM stock in 95% ethanol) for a period of 48 hr, unless otherwise noted. All hormones were obtained from Sigma Chemical Company (St. Louis, Mo.).

Tyrosine aminotransferase assay. Tyrosine aminotransferase enzymatic activity was determined by a colorimetric assay essentially as described by Diamondstone (10) which monitors the conversion of tyrosine to *p*-hydroxyphenylpyruvate. About 10^6 cells were harvested, washed once with PBS, and lysed in 250 μl of 0.25 M sucrose/10 mM Tris (pH 7.4)/10 mM EDTA (pH 7.4) by subjecting them to three cycles of freezing in liquid nitrogen and thawing in a 37° water bath. This lysate was then centrifuged for 3 min in an Eppendorf Microfuge, and the supernatant was used as the cell extract as described (10). The fold induction was calculated by dividing the specific activity of tyrosine aminotransferase in cells treated with hormone by the specific activity of cells from parallel cultures without hormone treatment. Protein content was determined by the procedure of Bradford (11).

Glutamine synthetase assay. Glutamine synthetase activity was measured by the colorimetric assay described by Kulka *et al.* (12), which monitors the formation of γ-glutamylhydroxamic acid in the presence of glutamine, ADP, and hydroxamic acid. Instead of filtering the protein precipitate from the reaction products, the assay tubes were centrifuged at 1500 × *g* in a Sorvall GLC2B centrifuge for 10 min and the absorbance of the supernatant was measured. As with tyrosine aminotransferase, fold induction indicates the quotient of the specific activity of cells grown in the presence of hormone divided by the specific activity of cells grown in the absence of hormone. Protein content was measured by the method of Bradford (11).

Belt I assay. Belt I is a secreted glycoprotein produced by HTC cells. It has been shown to be a glucocorticoid-inducible protein using 2-dimensional PAGE analysis (4). For routine assays of this protein, 2-dimensional gel electrophoresis proved somewhat unwieldy. Since this protein sticks tightly to tissue culture dishes, we have been able to develop a simple 1-dimensional gel electrophoretic procedure to monitor the synthesis of this product in HTC cells in culture. Cells were grown in the presence or absence of hormone as described above for 48 hr. The medium was removed and the cells were washed three times with PBS and once with DMEM (methionine-free). Cells were then incubated for 1 hr in DMEM (methionine-free) containing [^{35}S]methionine (250 μCi/ml) (900–1500 Ci/mmole; Amersham) and the appropriate concentration of hormone. The labeling medium was then removed, and the cells were washed three times with PBS (to remove as much free [^{35}S]methionine as possible) and removed from the plate with PBS/2.5 mM EDTA (pH 7.4). The plates were washed vigorously three or four times with PBS/EDTA (2.5 mM) and allowed to air-dry for 15–20 min. The proteins that remained on the plate after this treatment were removed by scraping with a rubber policeman into SDS/PAGE sample buffer. An aliquot of this preparation was electrophoresed on a 10% denaturing polyacrylamide gel as described by Laemmli (13). Typically, a 60-mm tissue culture dish of cells was labeled with 0.75 ml of [^{35}S]methionine-containing medium, and one-fourth of the resulting preparation was loaded on the gel. The gel was treated with salicylate as described (14), dried, and autoradiographed overnight at −70° using Kodak XAR-5 film. Quantitation of Belt I was done by scanning the gel lane with an optical densitometer (E. C. Instruments) and measuring the area under the Belt I peak.

Superinfection with MMTV. Confluent cultures of GR mouse mammary tumor cells were treated with 1 μM dexamethasone for 24 hr, medium from these cultures was collected and clarified (1,500 × G for 10 min), and the virus was pelleted by ultracentrifugation (54,000 × *g* for 1 hr). The virus pellet was resuspended in fresh medium at 5–10 times the original concentration; cells were infected as previously described (9). Superinfected cultures were assayed for induction of gp52 after one or two passages (1–2 weeks after superinfection).

FACS analysis of gp52 induction. The amount of gp52 present on the surface of cells was determined by analysis on the FACS after binding rabbit antibody to gp52 (a gift from D. Robertson and H. Varmus, University of California, San Francisco) and staining with fluorescein-conjugated goat anti-rabbit immunoglobulin (Miles Laboratories) as described previously (6). In the FACS, the fluorescence of a single cell is measured in arbitrary logarithmic units. Ten thousand cells were analyzed and the data were plotted as a histogram of the fraction of total cells in gated windows of increasing fluorescence intensity. Induction was measured in Δ units, which represents the difference between the mean value (in arbitrary logarithmic units) of cells grown in the presence and absence of 1 μM dexamethasone. We cannot convert these Δ values in JZ.1 and JZN3.7 to a fold induction, because the uninduced level is below the limit of sensitivity of the assay. In the M class of cells, 100 arbitrary units represent at least 20-fold induction.

RESULTS

Induction of tyrosine aminotransferase, glutamine synthetase, and Belt I by glucocorticoids. JZ.1 is a clone of mutagenized J2.17 cells (see Materials and Methods) that exhibits glucocorticoid-inducible expression of several proteins, including tyrosine aminotransferase, glutamine synthetase, Belt I, and MMTV gp52. We have found (data not shown) that glucocorticoid-induced tyrosine aminotransferase activity is maximal by 12 hr, glutamine synthetase activity by 24–36 hr, and Belt I

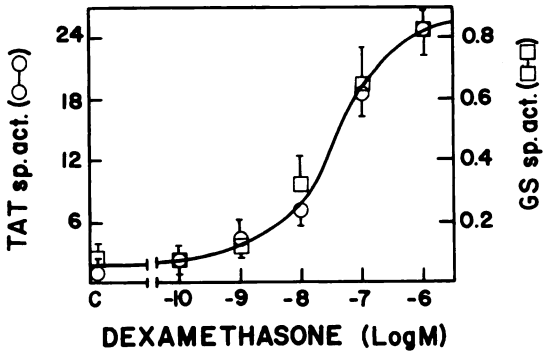

FIG. 1. *Log-dose response curves of tyrosine aminotransferase (TAT) and glutamine synthetase (GS) to dexamethasone in JZ.1 cells*

Tyrosine aminotransferase and glutamine synthetase assays were carried out as described under Materials and Methods. Tyrosine aminotransferase specific activity is expressed as nanomoles of *p*-hydroxybenzaldehyde formed per minute per milligram of protein. Glutamine synthetase specific activity is expressed as micromoles of γ-glutamylhydroxamic acid formed per hour per microgram of protein. The tyrosine aminotransferase data points represent means of three independent experiments; the glutamine synthetase data are means of four independent experiments. Protein content was determined by the method of Bradford (11), using bovine γ-globulin as standard.

synthesis by 6 hr. MMTV accumulation has been shown to be maximal by 16 hr (15). By making our measurements 48 hr after the addition of hormone we are measuring maximal levels of induction of all four markers. To ascertain whether these proteins are induced in a coordinate fashion, cells were exposed to concentrations of dexamethasone ranging from 0.1 nM to 1 μM for 48 hr. The results of tyrosine aminotransferase and glutamine synthetase assays of cells treated in this way are shown in Fig. 1 and indicate that half-maximal induction of both tyrosine amintotransferase and glutamine synthetase occurs at approximately 20 nM dexamethasone. Moreover, the log dose-response curves are virtually superimposable for these two enzymatic activities. The induction of Belt I was measured by PAGE of pulse-labeled proteins bound tightly to tissue culture plates after removal of growing cells. The results of such an experiment are shown in Fig. 2A; the band appearing in the 50–55 K_d region of the gel migrates at the position expected for known Belt I (5). While there is some variability in the amounts of other proteins in each lane, presumably arising from more or less vigorous removal of cell debris, we have observed very consistent recovery of Belt I in over 20 independent experiments. Thus each lane was loaded with an amount of plate-bound proteins recovered from equivalent numbers of cells. Quantitation of labeled Belt I was achieved by optical densitometry of the autoradiogram, and the area under the peak from each hormone concentration was determined. These data, presented as a log dose-response curve (Fig. 2B), indicate that half-maximal induction of Belt I occurs at about 10 nM dexamethasone. Since there is variability in the amounts of proteins in bands near Belt I (e.g., Lane −6 versus Lane −7), optical densitometry may not be quantitatively precise in the measurement of Belt I. The data points at *C*, *−8*, and

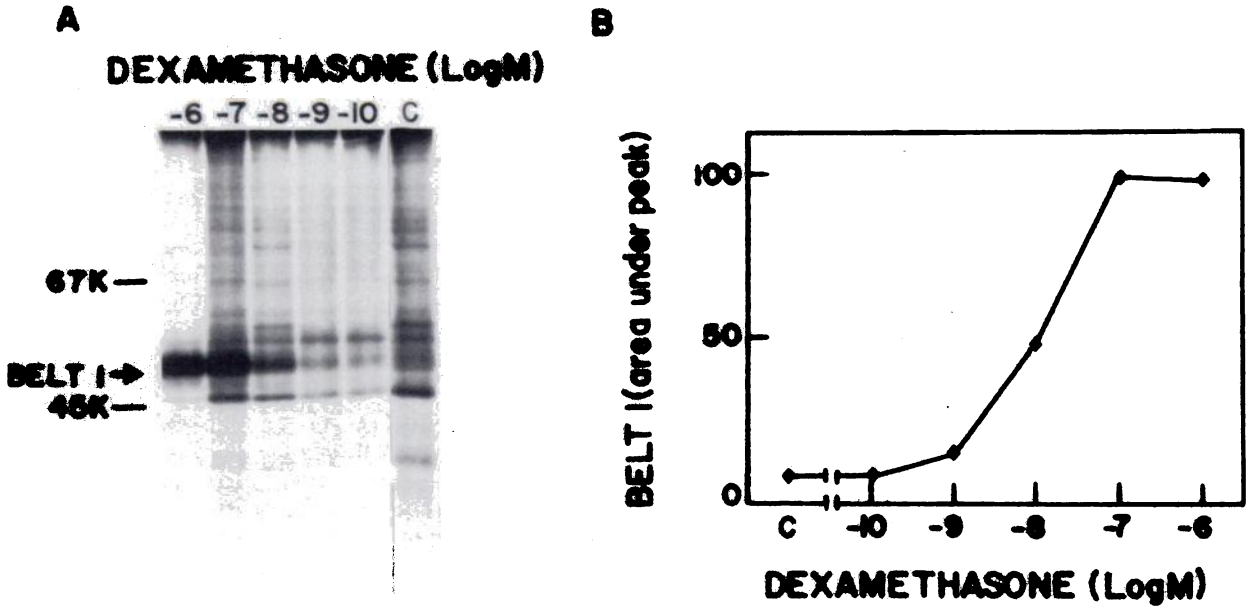

FIG. 2. *Log-dose response of Belt I to dexamethasone in JZ.1 cells*

Cell induction and labeling with [^{35}S]methionine were carried out as described under Materials and Methods. A. The gel was a 10% denaturing polyacrylamide gel. The molecular weight markers, bovine serum albumin (*67K*) and chicken ovalbumin (*45K*), were run in a separate lane and stained with Coomassie brilliant blue in a methanol/acetic acid (10% v/v each) solution. After destaining, the gel was dried and autoradiographed on Kodak XAR-5 film. Each lane represents 25% of the total labeled protein isolated from one 6-cm tissue culture dish. The last lane, labeled *C*, represents a control culture where no hormone was added. B. A graphic representation of the Belt I induction is shown. Each lane was scanned with an optical densitometer, and the area under the Belt I peaks of the resulting tracing was cut out and weighed. Various exposures of this gel were scanned to ensure that there was linearity of response and that saturation of the film had not occurred. The data are expressed as milligrams under the peak.

TABLE 1

Induction of tyrosine aminotransferase, glutamine synthetase, and Belt I in JZ.1 cells with hormones of varying glucocorticoid potency

Assays were carried out as described under Materials and Methods. Cells were incubated for 48 hr in the presence and absence of 1 μM of each of the indicated hormones. Fold induction was determined by dividing the enzymatic activity (tyrosine aminotransferase and glutamine synthetase) or the amount of protein in the gel lane (Belt I) in the presence of hormone by the amount in the absence of hormone. The data for tyrosine aminotransferase and glutamine synthetase represent mean values ± standard error measurements with the number of experiments indicated in parentheses. The Belt I data represent one determination each.

Hormone	Tyrosine aminotransferase	Glutamine synthetase	Belt I
	-fold induction	*-fold induction*	*-fold induction*
Progesterone	1.0, 1.6 (2)	0.9, 1.1 (2)	1.7
DMP	3.4 ± 0.6 (6)	2.1 ± 0.3 (5)	2.7
Aldosterone	9.8 ± 2.1 (4)	4.4 ± 1.1 (5)	6.5
Cortisol	21.5 ± 5.5 (4)	7.8 ± 1.8 (3)	15.6
Dexamethasone	24.5 ± 4.5 (4)	8.3 ± 1.8 (7)	10.1

−7 may be slightly disproportionately high for this reason, but this will not make a large difference in the determination of a half-maximal concentration. We have shown previously that half-maximal induction of gp52 also occurs at 10–20 nM dexamethasone in MMTV-infected HTC cells (5).

Samuels and Tomkins (16) have categorized different steroid hormones with regard to their ability to induce tyrosine aminotransferase in HTC cells as optimal, suboptimal, or anti-inducers. We have analyzed the response of JZ.1 cells to two hormones of the first class, dexamethasone and cortisol; two of the second class, aldosterone and DMP; and one of the anti-inducer class, progesterone. Table 1 shows the results of glutamine synthetase, tyrosine aminotransferase, and Belt I assays in JZ.1 cells incubated for 48 hr in the presence of 1 μM of each of these hormones. Dexamethasone and cortisol show maximal induction of each of these three markers. Aldosterone and DMP exhibit partial induction of all three markers, aldosterone induction being about 60% that of dexamethasone induction, and DMP induction being about 25–35% that of dexamethasone induction of these three markers. The anti-inducer, progesterone, exhibits very little, if any, induction of any of these markers. Similar results have been obtained by Young *et al.* (15) for the induction of MMTV RNA in a mouse mammary tumor cell line.

TABLE 2

Concentration of glucocorticoids needed to elicit half-maximal induction of tyrosine aminotransferase and glutamine synthetase

Log dose-response curves were constructed for both glutamine synthetase and tyrosine aminotransferase for each of these hormones as shown in Fig. 1. Concentrations of up to 10 μM were administered, and a plateau of induction was achieved in each case. The molar concentration necessary for half-maximal induction was determined graphically, using a semilogarithmic plot for each, and is listed. The results represent the mean ± standard error measurements with the number of experiments indicated in parentheses.

Hormone	Tyrosine aminotransferase	Glutamine synthetase
Dexamethasone (nM)	18 ± 5 (4)	12 ± 3 (4)
Cortisol (μM)	0.1, 0.3 (2)	0.1, 0.2 (2)
Aldosterone (μM)	0.3, 0.5 (2)	0.4, 0.7 (2)
DMP (μM)	0.5, 0.7 (2)	0.2, 0.5 (2)

The data in Table 1 represent experiments performed with a single, high concentration of each hormone. We also carried out dose-response studies of tyrosine aminotransferase and glutamine synthetase induction with each of the partial inducers. The results of these experiments, summarized in Table 2, confirm that these enzymes behave similarly in response to suboptimal as well as optimal inducers.

Analysis of the glucocorticoid domain in variant HTC cells. We have recently described the isolation of MMTV-infected HTC cells (by selection in an FACS) that are defective in their ability to induce viral gp52 in response to dexamethasone (6). One class of variants derived from the cell line M1.19, which contains about 10 copies of the MMTV provirus, exhibits markedly decreased levels of glucocorticoid receptor. Earlier studies indicated that, in addition to the loss of gp52 inducibility, these cells are unable to induce tyrosine aminotransferase when treated with dexamethasone. We have analyzed these cells in further detail and find that they are also incapable of inducing glutamine synthetase or Belt I. Figure 3A shows the results of a Belt I assay in which induction is obvious in MSC1 cells, a subpopulation of the parental M1.19 cells, whereas little or no induction is observed in MSN5.3 cells, a variant clone described previously (6). There are two other proteins on these gels that exhibit dexamethasone inducibility: one migrates at $M_r \simeq 47$Kd and the other at $M_r \simeq 35$Kd. The behavior of these two proteins seems to parallel that of Belt I (see also Fig. 2A and Fig. 3B). We have not yet identified these protein products, although the 35Kd protein could be the protein called Belt II by Ivarie and O'Farrell (5). Table 3 summarizes the results of tyrosine aminotransferase, glutamine synthetase, Belt I, and gp52 assays, which demonstrate coordinate loss of glucocorticoid-inducible markers in these receptor-deficient cells.

A second class of nonresponding variants has been identified using JZ, which contains a single MMTV provirus, as the parental population (7). Several of the clonal isolates from this selection demonstrated reduced or no induction of gp52 with retention of tyrosine aminotransferase inducibility. In Table 3 and Fig. 3B we extend this observation in variant clone JZN3.7 by demonstrating that, although gp52 is not glucocorticoid-inducible, all three cellular genes remain as responsive to the hormone in this clone as in the normal parental population.

Superinfection of JZN3.7 with MMTV. The results of the studies described above suggest that the defect in gp52 inducibility in JZN3.7 resides in the MMTV DNA itself or in a cellular factor (e.g., a chromosomal acceptor protein) that is specifically required for the hormonal responsiveness of the viral genes. In order to distinguish between these alternatives, JZN3.7 and JZ cells were superinfected with MMTV as described under Materials and Methods. This introduces one or a few additional copies of the MMTV provirus inserted randomly into the chromosomes of the recipient cells (17). If the defect in JZN3.7 resides in the original copy of MMTV DNA (i.e.,

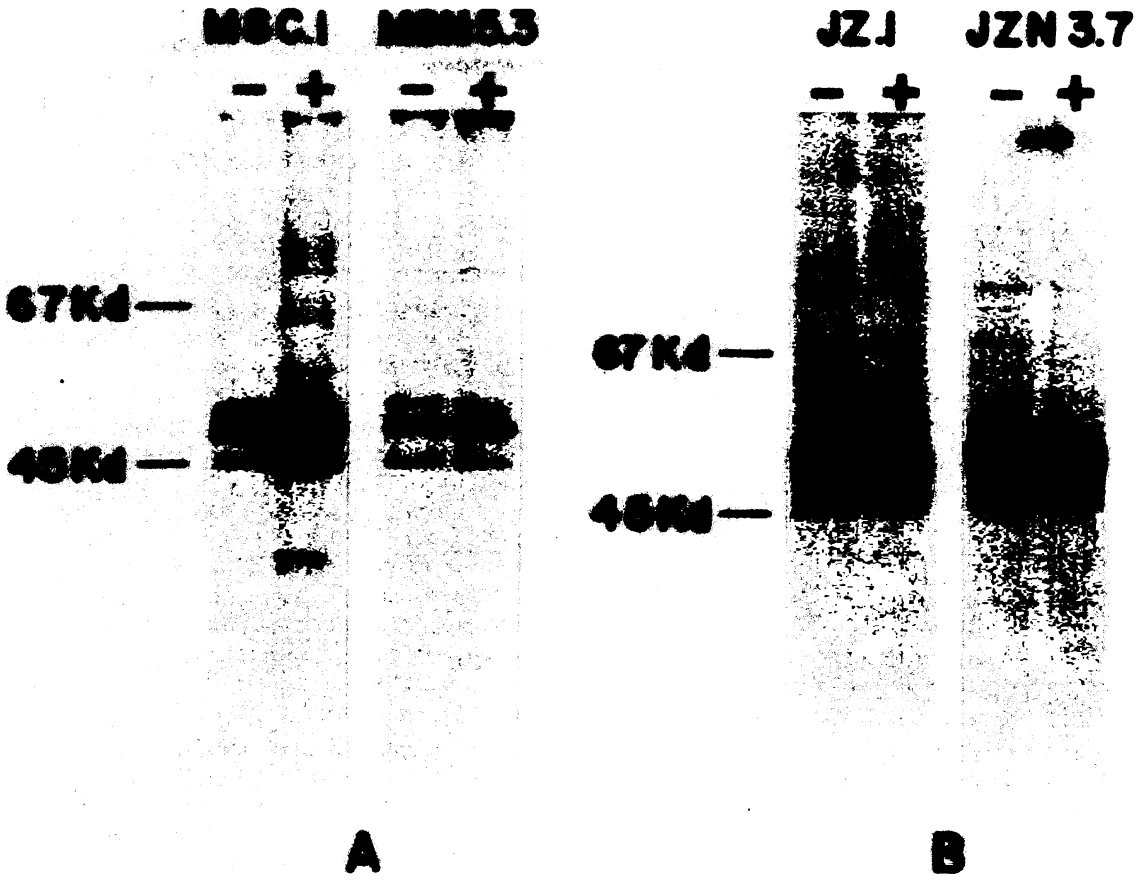

FIG. 3. *Belt I assays of normal and variant cell lines*

Belt I assays were carried out on parental and variant cells as described under Materials and Methods and in Fig. 1. Each pair of lanes represents the induction in one cell line. The *left lane* of each pair represents the plate proteins from control cells, and the *right lane* represents plates of cells incubated in the presence of 1 μM dexamethasone for 48 hr. Belt I assays of MSN5.3 and its parental cell line MSC1 are shown in A. Assays of JZN3.7 and its parental cell line, JZ.1, are shown in B.

is *cis*-acting), then the newly introduced copies should be responsive to hormone. Alternatively, if the defect is in a cellular protein that acts upon MMTV DNA (i.e., is *trans*-acting), the newly acquired proviruses will also be unable to respond to glucocorticoids. The results of such an experiment, shown in Fig. 4, clearly demonstrate that the superinfected JZN3.7 cells exhibit glucocorticoid-inducible gp52. Table 4 summarizes assays of both gp52 and tyrosine aminotransferase, which show that there is no alteration in tyrosine aminotransferase inducibility in the superinfected cells. Thus the incorporation of new copies of MMTV DNA into this type of glucocorticoid-unresponsive cell line leads to restoration of gp52 induction. Similar experiments performed with MSN cells that are defective in receptor do not, as one would expect, restore gp52 inducibility (data not shown).

TABLE 3

Inductions of gp52, tyrosine aminotransferase, glutamine synthetase, and Belt I in parental and nonresponsive cells

Tyrosine aminotransferase, glutamine synthetase, and Belt I assays and inductions were determined as described under Materials and Methods. The gp52 was analyzed in the FACS, where Δ represents the difference of the mean value of fluorescence intensity of 10,000 cells in the presence versus the absence of dexamethasone in arbitrary logarithmic units of fluorescence (see Fig. 4) and does not reflect a fold induction. The data are represented as the mean ± standard error measurements with the number of experiments indicated in parentheses. The Belt I data show the fold induction using optical densitometry of an autoradiogram.

Cells	Tyrosine amino-transferase	gp52	Glutamine synthetase	Belt I
	-fold induction	Δ	*-fold induction*	*-fold induction*
MSC1	4.8 ± 1.0 (5)	105 ± 7.2 (7)	2.7, 3.5 (2)	7.9 ± 1.2 (3)
MSN5.3	1.0 ± 0.1 (4)	8.8 ± 2.1 (5)	0.9, 1.1 (2)	1.3 ± 0.4 (3)
JZ	10.6 ± 1.3 (16)	32.7 ± 3.7 (15)	5.5, 8.5 (2)	8.7 ± 1.6 (4)
JZN3.7	8.3 ± 1.2 (9)	6.4 ± 1.1 (15)	6.4 ± 0.6 (4)	7.6 ± 1.0 (3)

DISCUSSION

Coordinate regulation of the glucocorticoid domain. We have described genetic and pharmacological experiments whose results support the contention that a single receptor type mediates the induction of all glucocorticoid-inducible gene products in HTC cells. Our conclusion is based on the following observations. (a) The same concentration of dexamethasone (10–20 nM) is required to elicit half-maximal induction of four independent markers (glutamine synthetase, tyrosine aminotransferase, Belt I, and MMTV gp52); moreover, this is very similar to the concentration of dexamethasone required to half-saturate receptors (18). (b) MMTV (15), as well as the three cellular inducible genes, respond in a similar fashion to optimal, suboptimal, and anti-inducers. (c)

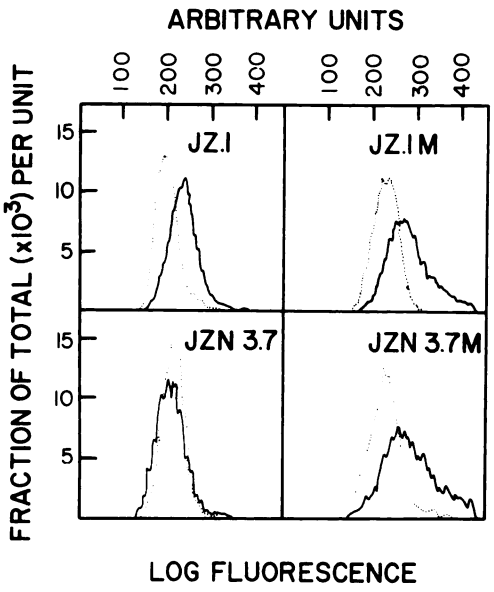

FIG. 4. *Induction of gp52 in superinfected JZN3.7*

Cell surface gp52 levels were determined in the FACS as previously described (5). The data are presented as histograms of fluorescence intensity on a logarithmic scale. The control level of fluorescence (····) and the dexamethasone-induced level (——) are depicted for JZ.1 and JZN3.7 for their superinfected counterparts, JZ.1M and JZN3.7M.

Receptor-defective cells (6) selected for their inability to induce the MMTV glycoprotein, gp52, simultaneously lose the capacity for inducing all three cellular gene products.

Our results are in contrast to the findings of Thompson *et al.* (19). In HTC variants defective in the induction of tyrosine aminotransferase, this group found that the ability of glucocorticoids to suppress phosphodiesterase activity was also lost. Since all of the cells that they analyzed originated from a single tyrosine aminotransferase non-inducer clone, it is possible that the original clone had two defects similar to those seen in JZN3.7, one in the tyrosine aminotransferase gene and one in the phosphodiesterase gene. In this regard, we have isolated a clone of JZN cells, JZN3.11(7), that is defective in the induction of both gp52 and tyrosine aminotransferase, yet maintains normal Belt I and glutamine synthetase inducibility. We suspect that this is a double mutant since (a) the loss of gp52 induction does not necessarily lead to loss of tyrosine aminotransferase induction (as in JZN3.7); (b) the glucocorticoid receptors from JZN3.11 do not differ from those of JZ in either number or affinity; and (c) superinfection of this clone with MMTV leads to restoration of gp52 inducibility, but not tyrosine aminotransferase inducibility.[4] These data support the suggestion that a single receptor-mediated mechanism regulates the glucocorticoid domain.

TABLE 4

Induction of gp52 and tyrosine aminotransferase in cells superinfected with MMTV

Tyrosine aminotransferase and gp52 were analyzed as described under Materials and Methods. Tyrosine aminotransferase is expressed as the mean fold induction ± standard error measurements with the number of experiments indicated in parentheses. The gp52 induction is expressed as Δ units as described in Table 3, under Materials and Methods, and in Fig. 4. The superinfected cell lines are designated as the starting cell line followed by an M.

Cells	gp52	Tyrosine aminotransferase
	Δ	*-fold induction*
JZ.1	33	10.6 ± 1.3 (16)
JZ.1M	57	10.1 ± 2.2 (3)
JZN3.7	6	8.3 ± 1.2 (9)
JZN3.7M	55	5.8 ± 1.8 (3)

In the case of MMTV, direct nucleic acid hybridization studies have demonstrated that the effect of glucocorticoids is to increase the rate of synthesis of viral RNA (20, 21). Until such time as cDNA probes become available for the glucocorticoid-inducible gene products of HTC cells, we are unable to document whether the effect of the hormone on these genes is truly at the transcriptional level. In preliminary experiments we have recently found that the mRNA coding for the acute phase protein, α_1-acid glycoprotein, is induced by dexamethasone in HTC cells.[5] Thus it should now be possible to ascertain whether this additional marker is regulated coordinately with MMTV at the transcriptional level.

In considering mechanisms by which the glucocorticoid-receptor complex activates specific genes, one must take into account the fact that in a given cell line not all inducible gene products are induced to the same extent. For example, in the MSC1 cells described here, glutamine synthetase is induced 3-fold, tyrosine aminotransferase 5-fold, Belt I 8-fold, and gp52 at least 20-fold. This may reflect differences in the affinity of the steroid-receptor complex for a regulatory region associated with each of these genes or could possibly result from chromosomal proteins or DNA modifications that alter the ability of the steroid-receptor complex to activate transcription at their respective promoters. Cloning and identification of the hormone regulatory regions of genes such as tyrosine aminotransferase, glutamine synthetase, and Belt I are required to answer these questions.

Analysis of glucocorticoid response variants. A major class of HTC cells defective in the ability to respond to dexamethasone contains little or no functional glucocorticoid receptor (6). Such cells, exemplified by clone MSN5.3, may contain defects in the receptor protein itself, in the regulation of receptor production, or perhaps in receptor modification required for hormone binding. For example, recent studies by Pratt and colleagues (22) suggest that phosphorylation of the glucocorticoid receptor may be an important factor in its function. Whatever the defect may be, the lack of functional glucocorticoid receptor results in cells unable to induce any of the four gene products we have assayed. Although only one clone of the receptor-defective class was analyzed here in detail, 12 other clones of this type simultaneously lose gp52 and tyrosine aminotransferase inducibility (6). Several of these have now been analyzed for inducibility of glutamine synthetase and Belt I, and all exhibit the same noninducible phenotype.

[4] J. R. Grove, J. L. Vannice, and G. M. Ringold, unpublished observations.

[5] J. L. Vannice, J. Taylor, and G. M. Ringold, manuscript in preparation.

The cell line JZN3.7, in contrast to the receptor-defective cells, exhibits levels of induction of tyrosine aminotransferase, glutamine synthetase, and Belt I nearly identical with those of its parental line JZ, yet is deficient in induction of the MMTV gp52. Our previous studies implicate a defect in transcription of the MMTV provirus in JZN3.7 since no viral RNA could be detected in hormone-treated cells (6). Here we have demonstrated that MMTV per se is capable of induction by glucocorticoids in these cells since, after superinfection, gp52 levels markedly increase in response to hormone. The defect in this class of nonresponsive variants, therefore, most likely resides in the provirus itself and not in a cellular component required for MMTV induction. Preliminary restriction endonuclease mapping of the viral DNA in JZN3.7 (and related clones) has not revealed any gross alterations in the structure of the viral DNA.[6] Thus, it seems plausible that the defect in the hormone-stimulated production of MMTV gene products is the result of a point mutation or a small deletion or insertion in a viral DNA sequence required for the induction mechanism; such a region could be a site for high-affinity binding of the glucocorticoid-receptor complex. In this regard, we have recently demonstrated that a gene fusion between the promoter region of MMTV and a cDNA of dihydrofolate reductase exhibits glucocorticoid-responsive production of dihydrofolate reductase in hamster cells (23). The fragment bearing the hormone regulatory region in these recombinant molecules contains approximately 1200 nucleotides of MMTV DNA upstream of the start of transcription; however, we do not yet know the precise location of the regulatory signal within this fragment. We are in the process of recovering the corresponding DNA fragments from JZ and JZN3.7 cells by molecular cloning in order to determine whether alterations within this region of the viral DNA are responsible for the nonresponsive phenotype observed in the variant cells.

[6] J. L. Vannice, J. R. Grove, and G. M. Ringold, unpublished observations.

ACKNOWLEDGMENTS

We thank Drs. J. Whitlock, D. Dobson, and H. Schulman for careful reading of the manuscript, and the Pharmacology secretarial staff for typing it.

REFERENCES

1. Yamamoto, K. R., and B. M. Alberts. Steroid receptors: elements for modulation of eukaryotic transcription. *Annu. Rev. Biochem.* **45**:721–746 (1976).
2. Higgins, S. J., and U. Gehring. Molecular mechanisms of steroid hormone action. *Adv. Cancer Res.* **28**:313–397 (1978).
3. Jensen, E. V., T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut, and E. R. DeSombre. A two-step mechanism for the interaction of estradiol with rat uterus. *Proc. Natl. Acad. Sci. U. S. A.* **59**:632–638 (1968).
4. Gorski, J., D. O. Taft, G. Shyamala, D. Smith, and A. Notides. Hormone receptors: studies on the interaction of estrogen with the uterus. *Recent Prog. Horm. Res.* **24**:45–80 (1968).
5. Ivarie, R. D., and P. H. O'Farrell. The glucocorticoid domain: steroid-mediated changes in the rate of synthesis of rat hepatoma proteins. *Cell* **13**:41–55 (1978).
6. Grove, J. R., B. S. Dieckmann, T. A. Schroer, and G. M. Ringold. Isolation of glucocorticoid-unresponsive rat hepatoma cells by fluorescence-activated cell sorting. *Cell* **21**:47–56 (1980).
7. Grove, J. R., and G. M. Ringold. Selection of rat hepatoma cells defective in hormone-regulated production of mouse mammary tumor virus RNA. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4349–4353 (1981).
8. Thompson, E. B., G. M. Tomkins, and J. F. Curran. Induction of tyrosine α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Proc. Natl. Acad. Sci. U. S. A.* **56**:296–303 (1966).
9. Ringold, G. M., R. D. Cardiff, H. E. Varmus, and K. R. Yamamoto. Infection of cultured rat hepatoma cells by mouse mammary tumor virus. *Cell* **10**:11–18 (1977).
10. Diamondstone, T. I. Assay of tyrosine transaminase activity by conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde. *Anal. Biochem.* **16**:395–404 (1966).
11. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
12. Kulka, R. G., G. M. Tomkins, and R. B. Crook. Clonal differences in glutamine synthetase activity of hepatoma cells. *J. Cell Biol.* **54**:175–179 (1972).
13. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
14. Chamberlain, J. P. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**:132–135 (1979).
15. Young, H. A., E. M. Scolnick, and W. P. Parks. Glucocorticoid receptor interaction and induction of murine mammary tumor virus. *J. Biol. Chem.* **250**:3337–3343 (1975).
16. Samuels, H. H., and G. M. Tomkins. Relation of steroid structure to enzyme induction in hepatoma tissue culture cells. *J. Mol. Biol* **52**:57–74 (1970).
17. Ringold, G. M., P. R. Shank, H. E. Varmus, J. Ring, and K. R. Yamamoto. Integration and transcription of mouse mammary tumor virus DNA in rat hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **76**:665–669 (1979).
18. Ringold, G. M., K. R. Yamamoto, G. M. Tomkins, J. M. Bishop, and H. E. Varmus. Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. *Cell* **6**:299–305 (1975).
19. Thompson, E. B., D. K. Granner, T. Gelehrter, J. Erickson, and G. L. Hager. Unlinked control of multiple glucocorticoid-induced processes in HTC cells. *Mol. Cell. Endocrinol.* **15**:135–150 (1979).
20. Ringold, G. M., K. R. Yamamoto, J. M. Bishop, and H. E. Varmus. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. *Proc. Natl. Acad. Sci. U. S. A.* **74**:2879–2883 (1977).
21. Young, H. A., T. Y. Shih, E. M. Scolnick, and W. P. Parks. Steroid induction of mouse mammary tumor virus: effect upon synthesis and degradation of viral RNA. *J. Virol.* **21**:139–146 (1977).
22. Leach, K. L., M. K. Dahmer, N. D. Hammond, J. J. Sando, and W. B. Pratt. Molybdate inhibition of glucocorticoid receptor inactivation and transformation. *J. Biol. Chem.* **254**:11884–11890 (1979).
23. Lee, F., R. Mulligan, P. Berg, and G. Ringold. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. *Nature (Lond.)* **294**:228–232 (1981).

Send reprint requests to: Dr. Gordon Ringold, Department of Pharmacology, Stanford University School of Medicine, Stanford, Calif. 94305.