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Selection of rat hepatoma cells defective in hormone-regulated production of mouse mammary tumor virus RNA

(cell sorting/glucocorticoid action)

J. RUSSELL GROVE AND GORDON M. RINGOLD

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

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ABSTRACT **We have been studying the mechanism of glucocorticoid hormone action by using mouse mammary tumor virus (MMTV)-infected rat hepatoma cells as a model system. J2.17, a clonal cell line that contains one MMTV provirus, induces tyrosine aminotransferase (TyrATase; L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5), viral RNA, and the cell surface viral glycoprotein gp52 in response to dexamethasone. Using a fluorescence-activated cell sorter and a rabbit antiserum directed against gp52, we selected a cell population that displays a reduced hormone-mediated increase in cell surface gp52. Fourteen clones of this population were assayed for induction of viral gp52 and RNA and of cellular TyrATase. The results of these assays revealed that the clones display a variety of responses to hormone. One clone has retained wild-type responses of both TyrATase and gp52. Six clones exhibit coordinately reduced or abolished responses of both markers. Seven clones show normal induction of TyrATase but reduced or undetectable induction of gp52. These latter clones exhibit reduced production of MMTV RNA and thus may represent a unique class of variants defective in the regulation of MMTV gene expression.**

Steroid hormones are thought to exert most of their effects by altering the rates of transcription of specific genes (for review see refs. 1 and 2). Upon entering a target cell, the steroid binds to a soluble receptor protein that undergoes a structural alteration (called "activation"), resulting in the translocation of the steroid–receptor complex to the nucleus (3, 4). The nuclear sites with which this complex interacts have not yet been defined. Whatever they may be, it is presumed that binding of the complex within the nucleus is associated with the observed changes in gene expression.

The early steps in the mechanism of steroid hormone action seem well documented (1, 2, 5). For example, a variety of pharmacological, biochemical, and genetic experiments have demonstrated that the response to glucocorticoids in HTC rat hepatoma or S49 mouse lymphoma cells requires the cytoplasmic receptor as well as translocation of the steroid–receptor complex to the nucleus (6–10). That steroids alter gene expression is also well documented. Direct measurements have demonstrated alterations in both the steady-state levels and, in some cases, the rates of synthesis of specific steroid-inducible messenger RNAs (11–15). Despite these observations, little is known about the nuclear actions of the steroid–receptor complex. In particular, the biochemical events that are initiated by the interaction of the complex with nuclear sites remain obscure.

Using HTC cells infected by mouse mammary tumor virus (MMTV), we have undertaken to analyze the actions of glucocorticoid hormones by genetic means. In a previous report (10), we demonstrated that M1.19 cells (a clone of MMTV-infected HTC cells) selected for their inability to induce the MMTV glycoprotein (gp52) in response to dexamethasone coordinately lost the ability to induce the cellular enzyme tyrosine aminotransferase (TyrATase; L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5). These cells were found to be devoid or severely depleted of glucocorticoid receptors. M1.19 cells contain approximately 10 MMTV proviruses per cell. Therefore, it was our belief that the selection procedure in which we enriched for cells exhibiting decreased levels of gp52 after hormone treatment, as measured by immunofluorescence in a fluorescence-activated cell sorter (FACS II), would invariably yield variants with defects in the receptor or some other as yet undefined component that would act on several genes coordinately. Because alterations in a single MMTV provirus in M1.19 cells would have reduced the expression of gp52 marginally, we did not expect to recover mutants in the viral promoter or in putative sites required for hormonal regulation of transcription.

In this communication we describe the isolation of glucocorticoid-response variants from J2.17, a line of HTC cells containing a single MMTV provirus (16). In addition to obtaining cells with a coordinate inability to induce gp52 and TyrATase, we have succeeded in identifying cells that appear to harbor defects specific to the transcription of MMTV DNA.

MATERIALS AND METHODS

Cells. J2.17, a MMTV-infected HTC cell line that contains one provirus per cell (16), was treated with the mutagen ethyl methanesulfonate at 1.5 mg/ml for 2.5 hr, a treatment that resulted in the killing of about 70% of the exposed cells. The surviving population was designated JZ. Cells were grown as monolayers in Dulbecco's modified Eagle's medium (Irvine Scientific, Irvine, CA) supplemented with 5% newborn calf serum and 3% fetal bovine serum (Irvine) in a humidified atmosphere containing 5% CO_2. For induction experiments, cells were fed medium containing 1 μM dexamethasone for 36–48 hr.

Selection of Unresponsive Cells. Hormone-treated and control cells were stained by indirect immunofluorescence, with rabbit antiserum directed against the major MMTV glycoprotein gp52 (anti-MTV or anti-gp52) and fluorescein-conjugated goat anti-rabbit IgG (fl-GARIG) as described (10). Stained populations were analyzed in a FACS II (17). Fluorescence intensity data were collected from the portion of the population consisting of live single cells (50–90% of the total signal); an electronic scatter gating was used to exclude most cell debris, dead cells, and cell aggregates from the analysis. For the selection of cells with altered hormonal response, hormone-treated JZ cells that represented the lowest 2.5% of the fluorescence intensity distri-

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Abbreviations: MMTV, mouse mammary tumor virus; TyrATase, tyrosine aminotransferase; FACS, fluorescence-activated cell sorter; DPT, diazophenyl thioether; NaCl/Cit, 0.15 M NaCl/0.015 M trisodium citrate.

bution were collected under sterile conditions. This population was expanded and reanalyzed for gp52 induction. Two additional sorts were required to produce a population sufficiently enriched for cells displaying reduced expression of cell surface gp52.

Data from analyses of stained cell populations were displayed as histograms of fluorescence intensity, measured on a logarithmic scale in which 100 arbitrary units represent a 10-fold increase in fluorescence intensity. Such histograms were used to determine the mean fluorescence intensities for various cell populations, allowing quantitative comparisons of induced and uninduced levels of gp52.

TyrATase Assay. TyrATase specific activity was determined in extracts of hormone-treated and control cells by using a simple colorimetric assay (18). Protein was measured by the method of Bradford (19), using bovine gamma globulin as standard.

Receptor Assays. Glucocorticoid receptor content and receptor affinity for dexamethasone were determined by using an intact cell binding assay previously described (10).

Analysis of Viral RNA. Cytoplasmic RNA was prepared from control and induced cells by using Nonidet P-40 and urea, as described by Berk *et al.* (20). Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography (21). RNA samples were denatured by treatment with glyoxal and dimethyl sulfoxide (22) and resolved by electrophoresis in 1.2% agarose gels buffered with 10 mM $NaPO_4$, pH 7.0. The gel was treated briefly with alkali to produce partial hydrolysis of the RNA and to increase the efficiency of transfer of large RNA species (23). The RNA was transferred to diazophenyl thioether paper (DPT-paper), developed by B. Seed, using the method described for transfer of nucleic acids to diazobenzyloxymethyl-paper (24). After transfer, the paper was wet thoroughly with preannealing mix [50% (vol/vol) formamide/3× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M trisodium citrate)/250 μg of salmon sperm DNA per ml/5× Denhardt's buffer (1× Denhardt's buffer is 0.02% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll)/0.1% $NaDodSO_4$/1% glycine], wrapped in Saran Wrap, and incubated at 42°C for 2–18 hr. The RNA on the preannealed paper was hybridized with MMTV DNA, labeled with ^{32}P by nick-translation (25) to about 2×10^7 cpm/μg, in annealing mix (50% formamide/3× NaCl/Cit/250 μg of salmon sperm DNA per ml/1× Denhardt's buffer/0.1% $NaDodSO_4$/10% dextran sulfate) at 42°C for 12–24 hr. The paper was washed 1–2 hr in wash buffer (50% formamide/5× NaCl/Cit/0.1% $NaDodSO_4$) at 42°C and exposed to x-ray film for 1–7 days, using an intensifying screen at −70°C.

RESULTS

Enrichment for Cells that Induce gp52 Poorly in Response to Dexamethasone. Mutagenesis of J2.17 with ethyl methanesulfonate yielded a population designated JZ. These cells retain hormonal responsiveness as evidenced by their ability to induce TyrATase and MMTV gp52 when treated with dexamethasone (Table 1 and Fig. 1). The amount of gp52 is measured by the fluorescence of cells in a FACS after staining with rabbit antibody directed against MMTV and fluorescein-conjugated goat anti-rabbit IgG (10). Whereas the absolute levels of TyrATase in JZ cells can be measured in the absence and presence of hormone (thereby yielding an induction ratio), this is not the case for gp52. The gp52-specific fluorescence exhibited by J2.17 and JZ cells (or their subclones) grown in the absence of dexamethasone is indistinguishable from the nonspecific fluorescence exhibited by uninfected HTC cells (Table 2). Thus, we cannot calculate an absolute induction ratio of gp52 in these cells and have therefore presented all of the data for induction of gp52 as the difference in number of fluorescein units exhibited by the hormone-treated and control cells.

Table 1. Induction of gp52 and TyrATase in parental cells

Cells	gp52, Δ	TyrATase, fold induction
J2.17	32 ± 6.0 (4)	13.2 ± 4.7 (6)
JZ	38 ± 5.4 (10)	12.3 ± 2.1 (10)

Cells were grown in the presence or absence of 1 μM dexamethasone for 2 days, removed from culture dishes with EDTA, and assayed for induction of gp52 and TyrATase. The results of gp52 assays are expressed as the difference in arbitrary units between the mean fluorescence intensities of hormone-treated and untreated cell populations. TyrATase assay results are expressed as the ratio of TyrATase specific activities in extracts of hormone-treated cells to those in extracts of untreated cells. The results represent mean and SEM for the number of determinations in parentheses.

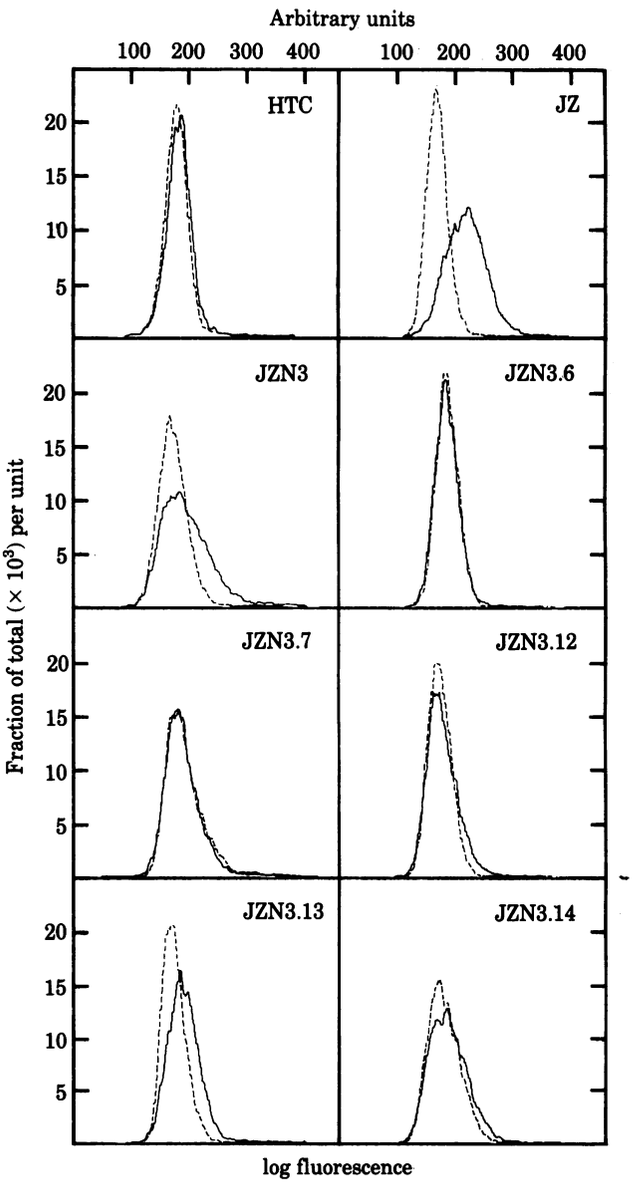

FIG. 1. Measurement of cell surface MMTV gp52. Cells, grown in the presence (——) or absence (----) of 1 μM dexamethasone, were stained with antibody to the MMTV gp52 and fluorescein-conjugated goat anti-rabbit IgG, and then analyzed in a FACS. The data are displayed as histograms of fluorescence intensity on a logarithmic scale in which 100 arbitrary units represent a 10-fold difference in fluorescence intensity.

Table 2. FACS analysis of MMTV-infected and uninfected cells

Cells	Dexamethasone	Mean fluorescence intensity, arbitrary units: Unstained	NRS + fl-GARIG	α-MTV + fl-GARIG
HTC 4.1	−	136	171	177
	+	144	171	182
JZ.1	−	137	173	178
	+	149	168	220

Cells were grown and analyzed for gp52 induction as described in the legend to Table 1 and in *Materials and Methods*. NRS, normal rabbit serum; fl-GARIG, fluorescein-conjugated goat anti-rabbit IgG; α-MTV, rabbit antiserum to MMTV gp52.

In an attempt to enrich for cells that are unable to induce gp52, dexamethasone-treated JZ cells exhibiting low fluorescence intensity in the FACS after indirect staining for the viral antigen were collected under sterile conditions and expanded to mass cultures. Three cycles of this enrichment procedure in which approximately 2% of the cells were collected at each step produced a population, JZN3, that displays markedly reduced levels of hormone-induced gp52 (Fig. 1).

Induction of gp52 and TyrATase in Clones of JZN3. In order to ascertain whether the residual induction of gp52 in JZN3 represented a partial response in a homogeneous population of cells or a varied response in a heterogeneous population, 16 single-cell clones of JZN3 were isolated for further study (two of these clones were lost during propagation). As is evident from Table 3, these clones display a panoply of phenotypes. Some clones (e.g., JZN3.2 and JZN3.6) show little or no induction of gp52, several display partial induction ranging from low (JZN3.14) to nearly normal (JZN3.4), while one clone (JZN3.8) shows wild-type levels of induction. Representative fluorescence intensity distributions of several of these variants grown in the presence and absence of dexamethasone are shown in Fig. 1. We have arbitrarily assigned the JZN3 clones to phenotypic categories with respect to gp52 induction in the following manner: clones displaying less than a 9.0-unit difference in the presence and absence of hormone are designated nonresponders (N); clones displaying greater than a 9.0- but less than a 25-unit difference are partial responders (P); JZN3.8, which exhibits a difference of 47 units, is an inducible (I) clone.

Table 3. Induction of gp52 and TyrATase in variant cells

Cells	gp52, Δ	TyrATase, fold induction	Phenotype: gp52	Phenotype: TyrATase
JZ	38 ± 5.4 (10)	12.3 ± 2.1 (8)	I	I
JZN3.2	2.3 ± 2.6 (3)	1.2 ± 0.4 (4)	N	N
JZN3.3	11 ± 0.9 (3)	2.9 ± 0.8 (3)	P	P
JZN3.4	22 ± 5.0 (5)	29.4 ± 10 (4)	P	I
JZN3.5	19 ± 3.2 (5)	16.4 ± 3.9 (5)	P	I
JZN3.6	−3.5 ± 2.4 (4)	1.3 ± 0.2 (7)	N	N
JZN3.7	6.0 ± 1.3 (5)	8.9 ± 1.3 (3)	N	I
JZN3.8	47 ± 7.0 (2)	20.5 ± 6.8 (4)	I	I
JZN3.9	19 ± 2.0 (5)	12.0 ± 4.6 (3)	P	I
JZN3.11	7.2 ± 0.6 (4)	1.4 ± 0.1 (7)	N	N
JZN3.12	10 ± 4.2 (4)	4.8 ± 1.6 (7)	P	P
JZN3.13	18 ± 6.7 (3)	11.4 ± 2.6 (4)	P	I
JZN3.14	9.0 ± 1.3 (6)	9.3 ± 1.0 (9)	P	I
JZN3.15	22 ± 3.1 (5)	10.1 ± 1.9 (6)	P	I
JZN3.16	9.7 ± 2.8 (3)	2.1 ± 1.1 (5)	P	P

The results were derived and are expressed as described in the legend to Table 1. The phenotype designations (N, nonresponder; P, partial inducer; I, inducer) were assigned as described in the text.

To determine whether the low levels of gp52 induction observed in the variants are due to general defects in hormonal responsiveness, we determined the specific activities of TyrATase in extracts of hormone-treated and control cells. Uninduced levels of TyrATase activity ranged between 0.5 and 3.0 milliunits/mg of protein and were highly reproducible; values in extracts of induced cells, which varied widely among the clones, ranged to a high of 50 milliunits/mg of protein. These results are tabulated in Table 3 for the parental population and all of the clones. Although many of the clones exhibit coordinate loss of inducibility of TyrATase and gp52 (e.g., JZN3.2 and JZN3.6), several of the variants display reduced induction of gp52 with no concomitant reduction in TyrATase induction (e.g., JZN3.5, JZN3.7, and JZN3.14). As for gp52 induction, we have arbitrarily assigned the variants to phenotypic categories according to their ability to induce TyrATase as follows: glucocorticoid nonresponders (N) exhibit less than 2-fold induction of TyrATase; partial responders (P) exhibit between 2- and 8-fold induction of TyrATase; complete responders or inducible cells (I) exhibit greater than 8-fold induction. The phenotypes of the JZN3 variants are summarized in the last column of Table 3.

In a set of control experiments we also isolated 10 clones of cells from the mutagenized JZ parental population. All 10 of the clones induce TyrATase normally, and 9 of them exhibit induced levels of gp52 comparable to or greater than the gp52 level in JZ (data not shown). One clone displayed a partial induction of gp52; we have not yet characterized this cell line in any detail. We surmise that the selection procedure in the FACS is quite powerful because, of 14 JZN3 clones analyzed, 13 are poor inducers of gp52.

Glucocorticoid Receptors in Variant Clones. Variant clones with subnormal inductions of both TyrATase and gp52 presumably contain defects in a common component required for hormone responsiveness. In the case of the MSN5 and MSN6 variants previously described (10), the lack of gp52 and TyrATase induction could be correlated with the loss of glucocorticoid receptors. We concluded that the same receptor was therefore responsible for mediating the induction of these two genes. In contrast, cells displaying normal induction of TyrATase and altered induction of gp52 would not be expected to exhibit reduced levels of receptor. That this is indeed the case is shown in Table 4. Representative clones with the phenotypes TyrATase (I), gp52 (P) contain amounts of receptor essentially equivalent to the amount in JZ. In JZN3.6, which exhibits coordinate loss of TyrATase and gp52 inducibility, there is a marked decrease in receptor content.

Analysis of Viral RNA. It is conceivable that the defects giving rise to the reduced levels of gp52 in the JZN3 variants are associated with structural mutations in the glycoprotein. Alternatively, these variants could be defective in a regulatory region or in the promoter for viral RNA synthesis. To distinguish

Table 4. Properties of glucocorticoid receptor in parental and variant cells

Cells	K_d, nM	Receptors × 10^{-3} per cell	n
JZ	13.5 ± 2.8	121 ± 26.6	7
JZN3.4	10.8 ± 1.8	113 ± 8.5	3
JZN3.5	16.2 ± 8.1	129 ± 41.5	2
JZN3.6	11.7 ± 1.9	58.6 ± 14.4	3
JZN3.14	16.9 ± 7.4	118 ± 48	3

The content of glucocorticoid receptor and the affinity of that receptor for [^{3}H]dexamethasone were determined in intact cells as described (10). The results are expressed as mean and SEM for the indicated number of determinations (n).

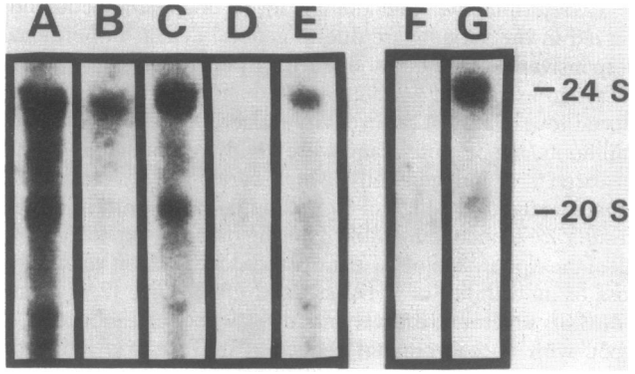

FIG. 2. Analysis of MMTV RNA. For each lane, 15 μg of poly(A)-containing cytoplasmic RNA was separated by electrophoresis, transferred and covalently bound to DPT-paper, and probed with ^{32}P-labeled MMTV DNA. Lanes: A, JZ; B, JZN3.2; C, JZN3.5; D, JZN3.7; and E, JZN3.14. In a separate experiment, JZN3.6 (F) and JZ (G) were analyzed together.

among these possibilities, we have analyzed the viral RNA in several of the JZN3 variants. The poly(A)-containing fraction of cytoplasmic RNA was collected from cells treated with dexamethasone for 48 hr. RNA was separated by electrophoresis, transferred to DPT-paper, and hybridized with ^{32}P-labeled viral DNA as described in *Materials and Methods*. The major viral RNA species observed in wild-type cells migrated as a band corresponding in size to a molecule of approximately 24 S; this represents the mRNA for the envelope glycoprotein, gp52 (26). Lower levels of 20S and 13S viral RNA are also present, but full-length viral transcripts of 35S are scarce. The sizes of the viral RNAs correspond to those seen by other investigators (27, 28). RNA preparations from variants exhibiting reduced or undetectable levels of cell surface gp52 contain viral RNA of the same size classes but at correspondingly reduced levels (Fig. 2). Partial inducers of gp52 (JZN3.4 and JZN3.5) show slightly reduced levels of MMTV RNA, while noninducers or very poor inducers show greatly decreased (JZN3.2 and JZN3.14) or undetectable (JZN3.6 and JZN3.7) levels of viral RNA. We have been unable to reliably detect viral RNA in uninduced JZ cells; previous measurements indicate that there is on average less than one MMTV RNA molecule per 10 J2.17 cells grown in the absence of dexamethasone (16).

Deletions or other chromosomal rearrangements of the proviral DNA could be responsible for the inability of such clones as JZN3.6 and JZN3.7 to produce viral RNA. This possibility was tested by the analysis of restriction endonuclease-digested cell DNA, according to the method of Southern (29). The MMTV provirus in each of these clones (as well as in all the other JZN3 clones) appears to have suffered no alterations detectable by this analysis (data not shown).

DISCUSSION

We have described the isolation and preliminary biochemical characterization of MMTV-infected HTC cells with altered responses to glucocorticoids. Those variants exhibiting coordinately altered TyrATase and gp52 induction presumably contain a defect in a component common to the induction of both markers. We have previously shown that cells of this phenotype derived from M1.19, a clone of HTC cells containing multiple copies of MMTV DNA, are in fact defective in the glucocorticoid receptor (10). These studies and others have provided genetic evidence for the requirement of the receptor in eliciting a hormonal response (8, 9, 30, 31).

HTC cells have been used by us and by others as a model system for studying specific gene regulation in response to glucocorticoids. Thompson *et al.* (32) have isolated HTC variants with low TyrATase inducibility. Interestingly, these variants contain a receptor that binds dexamethasone and translocates to the nucleus; whether the receptor is defective in a subtle fashion has not been determined because studies of other inducible markers are lacking. Seifert and Gelehrter (33) have described HTC variants with a selective loss of the inhibiting effect of dexamethasone on plasminogen activator. These variants display TyrATase inductions indistinguishable from those of wild-type cells, suggesting a specific defect in the regulation of one response. Because the effect of the hormone on plasminogen activator appears to be indirect, the possibility that these clones might harbor defects in the structural gene for an inhibitor of plasminogen activator has not been excluded.

The fact that J2.17 cells contain a single MMTV provirus (i.e., are functionally haploid for the gene used in our selection procedure) has been particularly advantageous to us in attempting to select regulatory mutants. The set of variants we have described, which induce TyrATase normally but fail to induce gp52 and its mRNA, appears to represent a unique class of glucocorticoid-unresponsive cells. The presence of inducible TyrATase demonstrates that these cells contain functional receptor, while the observation that the inductions of gp52 and viral RNA are altered coordinately strongly suggests that these cells contain a defect specific to the transcription of the MMTV provirus.

Although steroid–receptor complexes bind to DNA (34, 35), there is at present no evidence for high-affinity binding to specific sequences or regions of DNA. Nevertheless, in analogy with prokaryotic regulatory proteins, it may be that such specific interactions mediate the effects of the hormone–receptor complex on gene activity. Suggestive evidence in *Drosophila* indicates that ecdysone–receptor complexes bind to regions of polytene chromosomes that are hormonally regulated (36). Whether the binding is a result of receptor–DNA or receptor–protein interactions is not known. In the JZN3 variants described here, we do not know whether the defects in production of MMTV RNA are associated with the viral DNA. Alterations in the promoter or in a putative hormonal regulatory sequence would manifest themselves as cells defective in glucocorticoid-regulated production of viral gene products. Alternatively, the defect leading to decreased production of viral RNA could, for example, be associated with a chromosomal protein that interacts with the MMTV genome. Somatic cell genetic studies may allow us to determine whether the defects in the JZN3 variants are *cis*-acting lesions (i.e., associated with the viral DNA) or not. It would of course be of interest to identify diffusible factors other than the glucocorticoid receptor that affect the transcription of MMTV DNA.

It is noteworthy that the selection procedure we have used does not rely on large differences in expression of gp52. Separation of cells in the FACS has allowed us to identify cells exhibiting subtle changes in production of MMTV RNA. This is unlike most other procedures used for selection of variants in tissue culture, which rely on a selective growth advantage or disadvantage of the mutant phenotype. These methods generally select for structural gene defects or for large changes in the level of gene expression. As an extreme example, a 50-fold reduction in the production of dihydrofolate reductase mRNA relative to wild-type levels does not alter the ability of cells to grow in a medium that selects against cells lacking dihydrofolate reductase enzymatic activity (unpublished). Even in prokaryotes, the isolation of regulatory mutants has been facilitated by studies of carefully balanced systems such as lysogeny by bacteriophage λ in which small changes in gene expression have

dramatic biological consequences. It is clear that similar systems must be developed and assays that are capable of detecting small changes in gene expression must be employed in order for the successful isolation of large numbers of eukaryotic regulatory mutants for future studies.

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