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Glucocorticoid Receptor-Dependent Inhibition of Cellular Proliferation in Dexamethasone-Resistant and Hypersensitive Rat Hepatoma Cell Variants

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Exposure of the Fu5 rat hepatoma cell line to glucocorticoids, such as dexamethasone and hydrocortisone, suppressed the growth rate and final density of cells grown in the presence of serum. This hormonal effect was proportional to receptor occupancy and affinity and, in addition, the glucocorticoid antagonist RU38486 prevented this response. Two classes of dexamethasone-resistant variants that failed to be growth inhibited were recovered from ethyl methylsulfonate-mutagenized populations by continuous culture in the presence of 1 μM dexamethasone. The first class, represented by the EDR3 subclone, was completely glucocorticoid unresponsive and failed to express receptor transcripts. The second class, represented by the EDR1, EDR5, and EDR7 subclones, possessed significant levels of glucocorticoid receptor but were only partially glucocorticoid responsive when stimulated with saturating levels of hormone. Introduction of functional glucocorticoid receptor genes into both classes of dexamethasone-resistant variants by a recombinant retrovirus expression vector restored glucocorticoid responsiveness and suppression of cell growth. A hypersensitive variant (BDS1), recovered by bromodeoxyuridine selection, was fully glucocorticoid responsive, and its inhibition of proliferation was more acutely regulated by dexamethasone. Taken together, our results established that the inhibition of proliferation in Fu5 rat hepatoma cells represents a new glucocorticoid response that requires the expression of a functional glucocorticoid receptor.

The regulation of normal and tumor cell growth is mediated by a dynamic balance between both positive and negative acting stimuli. For example, cellular escape from regulated growth inhibition or induction of growth-promoting processes are thought to be components of the complex series of events which occur during neoplastic transformation (19). Moreover, the hormone-regulated proliferation of certain tumor cell types can be correlated with changes in expression of combinations of growth inhibitors and stimulators (10, 26). The role of extracellular growth factors in stimulating cellular proliferation is a well-documented phenomenon; however, relatively less is known about inhibitory factors or their mechanisms of action. Growth-inhibiting factors include a wide range of extracellular hormone and hormone-like signalling molecules such as steroids, transforming growth factor type beta, tumor necrosis factor, lymphotoxin, and interferons as well as intracellular activities assigned to putative tumor-suppressing genes (12, 33). Evidence also suggests that some growth inhibitors exert their effects by blocking the action of stimulatory growth factors or their receptors (5, 40, 50). Many specific growth-inhibitory substances have been identified, but their modes of action have not been clearly delineated.

The in vivo administration of glucocorticoids to immature animals causes a dramatic suppression of somatic growth (55). In addition, certain tumors have been shown to be sensitive to the inhibitory effects of glucocorticoids (24, 37, 47). Moreover, many different cell types, both normal and transformed, have been shown to be sensitive to the growth-inhibitory effects of glucocorticoid in vitro (2, 11, 12). The effects of glucocorticoids on cellular proliferation appear to be particularly pronounced in hepatic tissue. For example, low doses of cortisone given to weanling rats results in a rapid and reversible cessation of DNA synthesis in growing normal liver tissue independent of the effects of glucocorticoids on growth hormone levels (21, 22). Interestingly, regenerating liver was shown to be less sensitive to the inhibitory effects of glucocorticoids and to display a concomitant reduction in cytosolic glucocorticoid receptor levels during the regeneration process itself (6, 31). In addition, normal adult rat hepatocytes as well as several hepatoma cell lines have been shown to be growth inhibited by glucocorticoids (2, 30, 44, 49). One recent study suggests that glucocorticoids can stimulate the production of a factor from rat fetal hepatocytes capable of blocking the mitogenic stimulation of insulinlike growth factor (43). However, little is known about the cellular requirements or molecular events responsible for the glucocorticoid inhibition of cellular proliferation in liver and liver-derived cells.

The Fu5 subclone of the H35 Reuber rat hepatoma has been previously reported to be growth inhibited by physiological doses of glucocorticoids (49). These cells are a relatively differentiated hepatoma line and are highly glucocorticoid responsive (9). In the following study we utilized the Fu5 cell line as a target for genetic selections to obtain subclones which were either resistant or hypersensitive to the growth-inhibitory actions of glucocorticoids. Our results demonstrated that resistance to glucocorticoid-mediated growth inhibition resulted from the total or partial loss of glucocorticoid receptor function and that the inhibition of cellular proliferation represents a new glucocorticoid receptor-mediated response.

MATERIALS AND METHODS

Materials. Dexamethasone, hydrocortisone, bromodeoxyuridine, and ethane methylsulfonic acid were all purchased

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from Sigma Chemical Co., St. Louis, Mo. 3,5-Diaminobenzoic acid dihydrochloride was procured from Aldrich Chemical Co., Milwaukee, Wis. [^{3}H]thymidine (5 Ci/mmol), [^{3}H]dexamethasone (85 Ci/mmol), [^{35}S]methionine (1,000 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and nick translation kits were purchased from Amersham Corp., Arlington Heights, Ill. Culture-grade epidermal growth factor and highly purified basic fibroblast growth factor were both obtained from Collaborative Research, Bedford, Mass. Bovine insulin (25 U/mg) was purchased from GIBCO Laboratories, Santa Clara, Calif. Pansorbin was obtained from Calbiochem-Behring, LaJolla, Calif., and Kodak X-ray film was purchased from Merry X-Ray Chemical Corp., Burlingame, Calif. RU38486 was generously supplied by Roussel-UCLAF, Romainville, France. The anti-rat albumin antisera was obtained from Organon Teknika, Malvern, Pa. The anti-plasminogen activator-inhibitor antiserum (7, 17) was a generous gift from Thomas D. Gelehrter, University of Michigan Medical School, Ann Arbor, and the anti-alpha$_1$-acid glycoprotein antibodies (42) was a generous gift John Taylor, Gladstone Foundation Laboratories, San Francisco, Calif. All other reagents used were of the highest possible purity.

Cells and method of culture. The Fu5 cell line was a kind gift from Jerry A. Peterson, John Muir Research Institute, Walnut Creek, Calif.; the origin of this cell line has been previously described (9). The Fu5 cell line and its derived subclones (BDS1, EDR1, EDR3, EDR5, and EDR7) were routinely grown in Dulbecco modified Eagle medium (DME)/F12 (50:50) supplemented with 10% fetal calf serum (Hyclone Laboratories) and nonessential amino acids. For serum-free experiments, all cells were cultured in a serum-free base consisting of DME/F12 (50:50) supplemented with nonessential amino acids, trace elements, and linoleic acid (84 ng/ml). The VDG12ψ_2 cell line was a generous gift from Paul J. Godowski and Keith R. Yamamoto, University of California, San Francisco. These cells carry a packaging-defective helper virus and externalize a recombinant retrovirus containing RNA sequences for the rat glucocorticoid receptor gene linked to the neomycin resistance gene in the DOL$^-$ vector (29). BALB/c 3T3 cells (clone A31) were obtained from the American Type Culture Collection, Rockville, Md. The VDG12ψ_2 and BALB/c 3T3 cell lines were cultured in DME supplemented with 10% calf serum. All cells were grown on Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.) at 37°C in a humidified atmosphere of air-CO_2 (95:5%).

Selection of subclones hypersensitive to dexamethasone-induced growth inhibition. Fu5 cells were passaged at 10,000 cells per cm^2 on 100-mm tissue culture plates in serum-supplemented media. After 48 h of culture, the cells were exposed to 10^{-6} M dexamethasone for 24 h, bromodeoxyuridine was added to a final concentration of 5×10^{-6} M, and the cells were cultured under dark conditions for an additional 48 h. The cell culture medium was then replaced with 9 ml of phosphate-buffered saline, and the cells were exposed to fluorescent light for 90 min from two 15-W cool white fluorescent bulbs set 3 to 4 cm from the surface of the cells. After exposure to the light source, >99% of the cells died within 48 h. Surviving colonies were cultured and specific subclones were harvested from the plate. Fifteen subclones were subsequently screened for hypersensitivity to dexamethasone-induced growth inhibition, and the most sensitive subclone was designated BDS1.

In vitro mutagenesis and selection of dexamethasone-resistant cell lines. Fu5 cell populations were mutagenized with ethyl methylsulfonic acid, and dexamethasone-resistant variants that failed to be growth inhibited in the presence of glucocorticoids (denoted EDR) were recovered out as previously described (15). Briefly, mutagenized cell populations were cultured continuously for 11 months in 1 μM dexamethasone and single cell-derived lines were recovered by dilution subcloning.

Recovery of conditioned media. Hormone-induced and uninduced EDR3 hepatoma cells were plated at 3×10^6 cells per 100-mm plate and subsequently cultured for 48 h in serum-containing medium. The cells were then washed two times with DME and cultured in 15 ml of serum-free base in the presence or absence of 10^{-6} M dexamethasone. When appropriate, dexamethasone was added 8 h prior to the washing of cells. After 72 h, the conditioned media were removed and clarified of floating cells by centrifugation for 5 min at $600 \times g$. Dexamethasone (1 μM) was then added to the media not previously exposed to hormone. This compensated for the weak progression activity of glucocorticoids on BALB/c 3T3 cells. The media were then tested for ability to induce DNA synthesis in quiescent monolayers of BALB/c 3T3 cells as described below.

DNA synthesis assays. Total incorporation of [^{3}H]thymidine was determined from a modified protocol previously described (48). Briefly, Fu5 cells grown in 24-multiwell plates (as described under proliferation assays) were exposed for 72 h to various concentrations of dexamethasone in serum-supplemented medium. The medium was then removed and replaced with serum-supplemented medium containing dexamethasone and [^{3}H]thymidine (10 μCi/ml). After 1 h, the cells were washed two times with cold 10% trichloroacetic acid and subsequently exposed to 300 μl of 0.2 M NaOH containing 40 μg of salmon sperm DNA per ml. After shaking for 10 min, the radioactivity in 100-μl samples was analyzed by liquid scintillation. The incorporated [^{3}H]thymidine was normalized for the total cell number per well as determined by DNA content.

BALB/c 3T3 cells were routinely passaged at 25,000 cells per well and grown to confluence in medium supplemented with 5% calf serum. After density-induced cell cycle arrest was attained, the cells were washed two times with DME and exposed to 0.2 ml of serum-free base containing basic fibroblast growth factor (2 ng/ml). After 7-h exposure to basic fibroblast growth factor, the competent cells were exposed to 0.5 ml of conditioned media or defined experimental media containing various growth factors (10 ng of epidermal growth factor or 10 μg of insulin per ml or both) in the presence of 5 μCi of [^{3}H]thymidine per ml. After 24 h the total incorporation of [^{3}H]thymidine was determined as described above.

Cell proliferation assays. Fu5 and its derived subclones were routinely passaged at a cell density of 10,000 or 20,000 (serum-free experiments) cells per cm^2 onto Falcon 24-Multiwell plates in the presence of serum-supplemented medium. After 48 h the cell medium was changed to the indicated experimental conditions. For serum-free experiments, the cells were washed two times with DME prior to exposure to experimental conditions; at designated times during the course of an experiment, the medium was aspirated from each well, and cells were subsequently fixed in 70% ethanol. Cell number in each well was calculated by determining total DNA content as previously described (14, 23).

Assay for tyrosine aminotransferase and glucocorticoid receptor transcript. Detection of specific RNA transcripts was accomplished via a cytoblot technique previously described

(54). Cells were cultured under appropriate experimental conditions on 60-mm plates and harvested for the cytoblot assay after reaching confluence (approximately 5×10^6 cells). Cytosols were prepared and immediately frozen at −80°C for storage. After thawing, the cytosolic fractions were blotted onto nitrocellulose under suction with a minimanifold at 40 μg of protein per blot, air dried, baked at 80°C in vacuo for 2 h, preannealed, and hybridized with [α-^{32}P]dCTP-labeled cDNA probes (generated from a nick translation kit) for tyrosine aminotransferase and rat glucocorticoid receptor as previously described (13, 16). Filters were then washed twice in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min per wash and then two more times in the same buffer at 50°C for 1 h. The dried filters were then exposed to X-omat AR X-ray film for 24 to 96 h at −80°C. The level of expressed transcripts was quantitated by soft laser densitometry of the autoradiographs.

A plasmid (pcTAT19) containing rat TAT genomic sequences (45, 46) was a kind gift from Stoney Simons, Jr., (Steroid Hormones Section, Laboratory of Analytical Chemistry, National Institute of Health). Digestion of this plasmid with *SalI* yielded a 3.7-kilobase fragment (corresponding to the first intron and most of the first exon) and was used as a hybridization probe. Plasmid pRdN93 containing rat glucocorticoid receptor cDNA (36) was a generous gift from Keith R. Yamamoto, University of California, San Francisco. Digestion of this plasmid with *XbaI* yielded a 2.7-kilobase cDNA fragment which was used as a receptor-specific hybridization probe.

Glutamine synthetase assay. Glutamine synthetase activity was assayed in confluent cultures of dexamethasone-induced and uninduced cells by the method of Thorndike and Reif-Lehrer (53). The specific enzymatic activity was calculated by normalizing for protein content determined by the method of Lowry et al. (32).

Glucocorticoid binding assay. Specific binding of [^{3}H] dexamethasone to high-affinity glucocorticoid receptors was carried out by a glass-fiber filter assay as previously described (8, 18).

Metabolic radiolabeling. Cells were treated in the presence or absence of 1 μM dexamethasone for 18 h, washed once with phosphate-buffered saline; and overlaid with 4 ml of methionine-free DME containing 0.5% dialyzed fetal calf serum; dexamethasone was added to the appropriate cultures. [^{35}S]methionine was then added to each dish to a final concentration of 50 μCi/ml. Cells were then incubated for 6 h, washed two times in phosphate-buffered saline, and harvested by centrifugation at 600 × *g* for 10 min. Extracellular fractions were collected and clarified by centrifugation. Cellular and extracellular fractions were routinely stored at −80°C. Cellular fractions were sonicated in immunoprecipitation buffer, and incorporation of radiolabel into macromolecular material was determined by precipitation with 10% trichloroacetic acid. The volume of culture media to be immunoprecipitated was adjusted in proportion to the level of [^{35}S]methionine incorporated into each cellular fraction.

Immunoprecipitation and electrophoretic analysis. Immunoprecipitations of the extracellular fractions were carried out by using a highly sensitive staphylococcus A-immunoadsorption procedure that detects proteins in the virtual absence of nonspecific background (25, 41). Immunoprecipitated proteins bound to final staphylococcus A pellets were electrophoretically fractionated in SDS-polyacrylamide gels and analyzed by fluorography as previously described (13).

Infection of cell lines with a retroviral vector containing the rat glucocorticoid receptor gene. A 3-ml portion of VDG12ψ_2 cell conditioned medium, containing recombinant virions carrying RNA for the intact rat glucocorticoid receptor gene linked to the bacterial neomycin resistance gene, was filtered and placed onto 50% confluent 100-mm dishes of various Fu5-derived cell lines. Polybrene was added to each dish at a final concentration of 8 μg/ml, the cells were incubated for 2 h, and the medium was replaced with normal culture medium. After 24 h the cells were incubated with medium containing G418 (900 μg/ml) for 10 days to select for neomycin-resistant cells. Under these conditions approximately 1 to 5% of the infected population acquired resistance to G418. A stable population of G418-resistant cells was recovered and cultured in the absence of selective medium for 1 week before use.

RESULTS

Glucocorticoid inhibition of Fu5 proliferation. To assess the effects of glucocorticoids on Fu5 cellular proliferation, steroid-treated and untreated cells were cultured in medium supplemented with 10% fetal calf serum and cell number was monitored as a function of total DNA content over time. When plated at 10% confluency, uninduced Fu5 cells initially proliferated with a doubling time of approximately 18 to 24 h, and as the cells reached confluency, their growth rate slowed until quiescence was attained after 5 days in culture (Fig. 1). [^{3}H]thymidine autoradiography confirmed the quiescent state of these cells (data not shown). In contrast, exposure to 1 μM dexamethasone, a synthetic glucocorticoid, or 5 μM hydrocortisone, a naturally occurring glucocorticoid, resulted in an approximately 30% inhibition of growth rate (Fig. 1). Glucocorticoid-treated cells attained a final cell

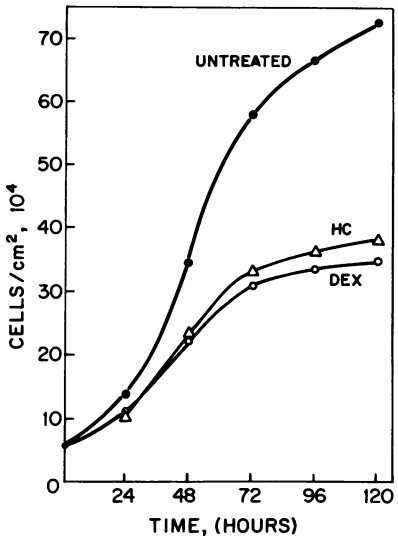

FIG. 1. Glucocorticoid-mediated suppression of Fu5 cell proliferation. Fu5 cells were plated in medium containing 10% fetal calf serum at a density of 10,000 cells per cm^2. After 48 h, cells were cultured in media without added hormones (●), 5 μM hydrocortisone (HC) (△), or 1 μM dexamethasone (DEX) (○). At the indicated times cells were fixed in ethanol and DNA content was monitored by a fluorometric 3,5-diaminobenzoic acid dihydrochloride assay as described in the text. Cell number was calculated from DNA content, using predetermined numbers of cells as standards, and plotted as cell density versus time in culture. Each value represents the average of triplicate cultures.

density one-half that of untreated cells, well below the normal level of confluency. Glucocorticoids, therefore, affect two proliferative properties of Fu5; both the growth rate and final cell density attained were significantly reduced. Importantly, under our culture conditions, hormone treatment did not impair global RNA or protein synthesis, nor does it cause a loss of cell viability (data not shown).

Glucocorticoid inhibition of Fu5 proliferation requires glucocorticoid receptor occupancy and function. To determine the relationship between glucocorticoid receptor occupancy and the hormone-induced inhibition of growth rate, Fu5 cells were exposed to various concentrations of dexamethasone for 72 h. Final cell density and DNA synthesis (monitored by [^{3}H]thymidine incorporation) were determined and compared with the level of [^{3}H]dexamethasone specifically bound to high-affinity glucocorticoid receptors (monitored by a glass-fiber filter assay) over an identical range of dexamethasone concentrations. The dose response for growth inhibition is shifted to concentrations of hormone lower than that required for comparable levels of hormone binding (Fig. 2a). For example, 20% receptor occupancy (2.5 nM) elicited a half-maximal inhibition of cellular proliferation. A similar hypersensitive response to receptor occupancy has been observed for other Fu5 glucocorticoid-inducible genes (35). Importantly, the dose-response curves for inhibition of [^{3}H]thymidine incorporation and final cell density were nearly identical, indicating that changes in cell number accurately reflect the changes in the rate of de novo DNA synthesis. Moreover, these results demonstrated that low physiological concentrations of glucocorticoids can effectively suppress Fu5 cell proliferation in in vitro cultures.

In a complementary experiment, Fu5 cells were exposed to various concentrations of RU38486, a potent antagonist of glucocorticoid receptor action (38), in the presence of 20 nM unlabeled dexamethasone. Total cell number was determined after 4 days of exposure to RU38486, and the final cell density was calculated as the percent maximal cell density in the absence of added steroid. In parallel, an identical dose range of RU38486 was tested for competition with 20 mM [^{3}H]dexamethasone for Fu5 cytosolic glucocorticoid receptors; steroid binding was monitored by a glass-fiber filter assay. The amount of [^{3}H]dexamethasone specifically bound at each competing concentration of RU38486 was then calculated as the percent maximal bound hormone. Direct comparisons of each curve revealed that RU38486 antagonized the dexamethasone-mediated inhibition of Fu5 cell proliferation in proportion to the occupancy of glucocorticoid receptors by dexamethasone (Fig. 2b). Taken together, our results indicated that functional glucocorticoid receptors are required for hormonal inhibition of growth. Consistent with this notion, the ability of different steroids to inhibit Fu5 cellular proliferation strongly correlated with their known glucocorticoid agonist properties in this cell line. For example, aldosterone acted as an strong agonist, testosterone showed weak antagonistic effects, progesterone displayed both antagonistic action in the presence of dexamethasone and weak agonistic effects when added alone, while vitamin D_3 and 17-β-estradiol had no effect (data not shown).

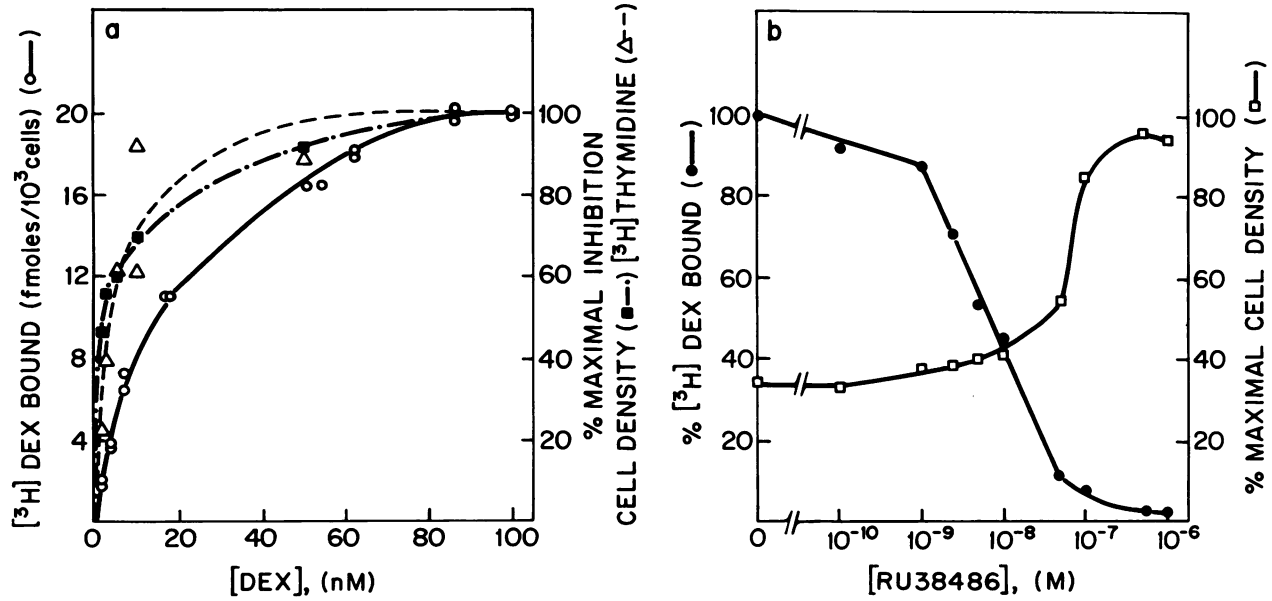

FIG. 2. Effects of glucocorticoid receptor occupancy on Fu5 DNA synthesis and proliferation. (a) Fu5 cells were plated as described for Fig. 1 and incubated with the indicated concentrations of dexamethasone (DEX) for 3 days. Cell density was determined by assaying total DNA content (Fig. 1), while the amount of [^{3}H]thymidine incorporated into trichloroacetic acid-precipitable material in 1 h was used to measure rate of DNA synthesis as described in the text. All values represent the average of triplicate cultures. The differences in cell density or [^{3}H]thymidine incorporation between uninduced cells and cells treated with 1 μM dexamethasone represented the maximal inhibition, and all experimental values with the indicated steroid concentrations were compared to this value from which the percent maximal inhibition was calculated. Glucocorticoid receptor binding in extracts from subconfluent cells was monitored by a glass-fiber filter assay, using the indicated concentrations of [^{3}H]dexamethasone. Each value presents one determination. (b) Fu5 cells were plated as described in the legend to Fig. 1 and incubated with 20 nM dexamethasone at the indicated concentrations of RU38486 for 4 days. Final cell density was monitored at each concentration of RU38486 by determining total DNA content, and the final cell density obtained in the absence of any added steroid was used as the 100% value to calculate percent maximal cell density. The inhibition of 20 nM [^{3}H]dexamethasone binding to glucocorticoid receptors at the indicated concentrations of RU38486 in extracts from subconfluent Fu5 cells was analyzed by a glass-fiber filter assay, and the percent binding was calculated relative to the amount bound in the absence of RU38486. All values are an average of triplicate samples.

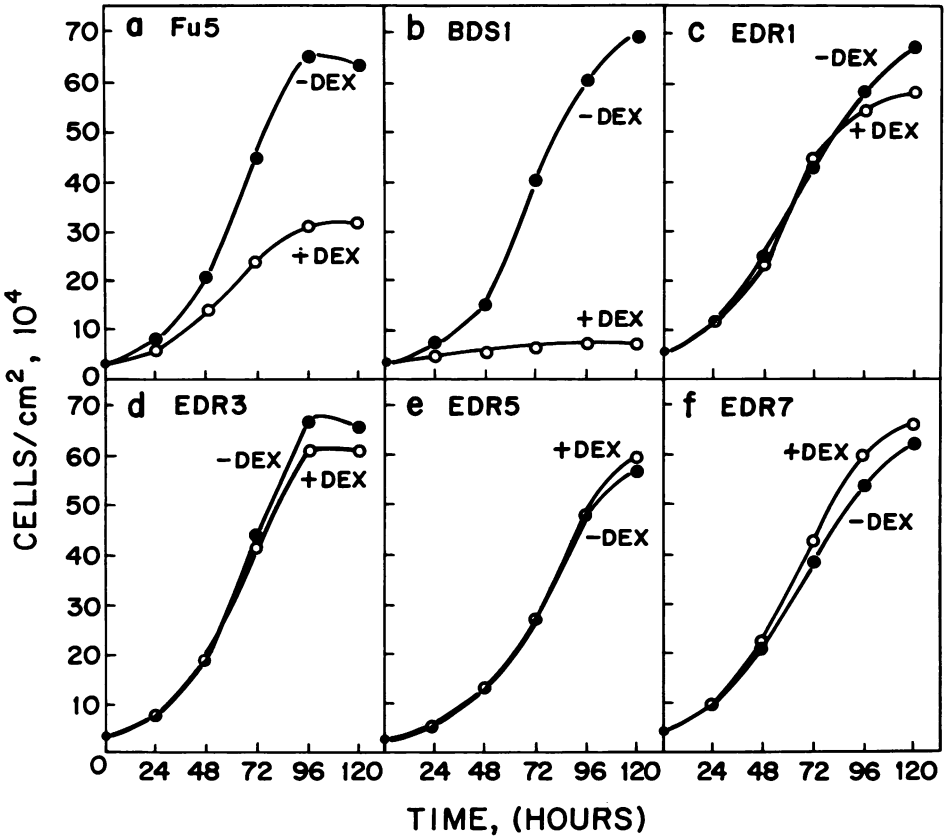

FIG. 3. Effect of dexamethasone (DEX) on proliferation of Fu5 and variant subclones. Cells were plated as described in the legend to Fig. 1, and at time zero the cells were cultured in the presence (●) or absence (○) of 1 μM dexamethasone. At the indicated times, total cell number was calculated by determining the total DNA content of each well. Each point represents the average of triplicate samples.

Selection and recovery of glucocorticoid-hypersensitive and -resistant proliferation variants. To understand potential relationships between expression of glucocorticoid-regulated gene products to the hormonal inhibition of Fu5 cell proliferation, it was crucial to genetically dissect this system. Fu5-derived variants unable to respond to glucocorticoids were recovered from ethyl methylsulfonic acid-mutagenized populations by continuous culturing in the presence of 1 μM dexamethasone (15). These dexamethasone-resistant subclones are designated EDR1, EDR3, EDR5, and EDR7. In a complementary selection process, variants of Fu5 hypersensitive to the growth-inhibitory effects of glucocorticoids were selected from glucocorticoid-treated cell populations by a bromodeoxyuridine selection against cycling cells (see Materials and Methods). Briefly, proliferating cells incorporate bromodeoxyuridine into replicating DNA and are rendered more sensitive to near-ultraviolet light-induced DNA damage. One particularly hypersensitive variant was recovered and designated BDS1. The proliferation of parental Fu5 and its derived variants was assayed in the presence or absence of 1 μM dexamethasone. BDS1 cells appear hypersensitive to the inhibitory actions of dexamethasone, eliciting slightly greater than one doubling in 5 days of culture (Fig. 3a versus 3b). In contrast, EDR1, EDR3, EDR5, and EDR7 cells display a glucocorticoid-resistant phenotype and are not growth inhibited in the presence of hormone (Fig. 3c to f). Thus, these resistant variants are likely to be deficient in one or more glucocorticoid-induced events that mediate Fu5 growth inhibition.

Glucocorticoid responsiveness of Fu5 and its derived variant subclones. To assess the glucocorticoid responsiveness of the growth variants, the dexamethasone-mediated induction of tyrosine aminotransferase (TAT) RNA transcripts was examined by using a cytoblot assay. BDS1, EDR1, EDR5, and EDR7 competently induced TAT RNA after exposure to dexamethasone, while EDR3 failed to express TAT transcripts in a hormone-regulated manner (Table 1). A second cytoplasmic glucocorticoid-inducible gene product, glutamine synthetase activity, was also tested. Only Fu5 and the

TABLE 1. Glucocorticoid responsiveness of Fu5 and variant subclones

Cell line	Fold induction of TAT RNA[a]	Fold induction of glutamine synthetase[b]
Fu5	5.3	19.2
BDS1	7.5	18.7
EDR1	5.4	0.8
EDR3	0.8	NS[c]
EDR5	4.6	2.2
EDR7	3.4	1.3

[a] Fu5 and variant subclones were incubated in the presence or absence of 1 μM dexamethasone for 24 h, and the level of TAT transcripts was measured by a cytoblot assay as described in the text. The fold induction was calculated as the ratio of detected transcripts expressed in hormone-induced cells to the level of expression in uninduced cells.

[b] Cells were treated in the presence or absence of 1 μM dexamethasone for 24 h. Glutamine synthestase specific activity was determined in triplicate, and the average values were used to calculate the fold induction as described in the text.

[c] NS, No significant activity detected.

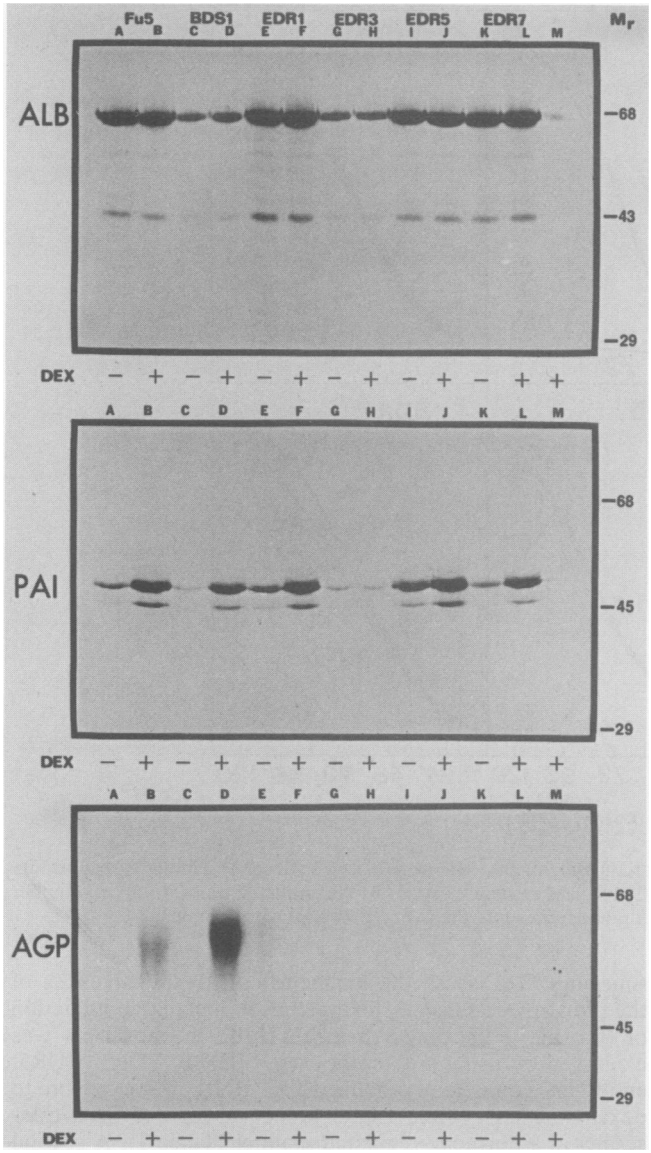

FIG. 4. Expression of glucocorticoid-regulated and constitutive secreted proteins. Fu5 and its derived subclones were cultured in the absence (lanes A, C, E, G, I, and K) or presence (lanes B, D, F, H, J, L, and M) of 1 μM dexamethasone for 24 h and radiolabeled with [^{35}S]methionine during the last 6 h. The culture media were harvested, and the expression of albumin (ALB; top panel), PAI (middle panel) and AGP (bottom panel) was analyzed by immunoprecipitation. Immunoprecipitated materials were fractionated in SDS-polyacrylamide gels, and radioactive proteins were visualized by fluorography. Lane M represents immunoprecipitations with nonimmune serum. Molecular weight markers are bovine serum albumin (68,000 M_r), ovalbumin (45,000 M_r), and carbonic anhydrase (29,000 M_r).

hypersensitive variant BDS1 were able to elicit a significant dexamethasone-induced induction of glutamine synthetase activity (Table 1). All other clones (EDR1 to EDR7) display little or no induction of glutamine synthetase, although EDR5 did demonstrate a small (2.2-fold) hormone-dependent induction.

The regulated expression of specific extracellular gene products was also monitored in each variant. The culture media from dexamethasone-induced and uninduced cells radiolabeled with [^{35}S]methionine were analyzed for the known glucocorticoid-regulated gene products plasminogen activator-inhibitor (PAI) (7, 17) and alpha$_1$-acid glycoprotein (AGP) (3) as well as constitutively expressed albumin. SDS electrophoretic analysis of radiolabeled extracellular proteins revealed that all tested cell lines competently expressed albumin in the presence or absence of dexamethasone (Fig. 4). Moreover, in a manner similar to TAT, all of the variants except EDR3 (PAI panel, lane G versus H) expressed elevated levels of PAI in a dexamethasone-dependent fashion (Fig. 4). Immunoprecipitation of the same culture media revealed that only parental Fu5 and variant BDS1 cells expressed AGP in a glucocorticoid-dependent manner. Interestingly, the hormone-induced levels of AGP in this hypersensitive variant were significant elevated relative to wild-type Fu5 (AGP panel, lane D versus B).

Taken together, our results demonstrated that two distinct phenotypic classes of glucocorticoid-resistant growth variants were recovered. One class, represented by EDR3, failed to regulate any of the tested glucocorticoid-responsive gene products, including the hormone-dependent ones. The second class, represented by EDR1, EDR5, and EDR7, is partially responsive to glucocorticoids in that TAT and PAI were induced normally while the expression of AGP and glutamine synthetase failed to be regulated by hormone treatment.

Expression of glucocorticoid receptors in hormone-resistant variants. To analyze the expression of glucocorticoid receptors in each of the selected variants, the levels of hormone binding and receptor transcripts were determined. All of the cell lines, with the exception of EDR3, bind significant levels of [^{3}H]dexamethasone to varying degrees (Table 2). For example, EDR5 expressed 118% the Fu5 level and EDR7 contained approximately 26% the level of Fu5 [^{3}H]dexamethasone binding activity. Furthermore, a Scatchard plot analysis of [^{3}H]dexamethasone binding revealed no alterations in the steroid binding affinities expressed in these variants (data not shown). The relative amount of glucocorticoid receptor transcripts expressed in each variant was determined by cytoblot analysis, using a ^{32}P-labeled cDNA probe to the rat glucocorticoid receptor and quantitated by soft laser densitometry. Relative values are expressed as the percentage of parental Fu5 levels (Table 2). Although BDS1, EDR1, EDR5, and EDR7 cells expressed levels of receptor transcripts greater than the parental levels, the amount of specific bound hormone did not always precisely correlate with the amount of expressed receptor transcripts. Only EDR3 failed to show significant levels of glucocorticoid

TABLE 2. Expression of glucocorticoid receptors in Fu5 and variant subclones

Cell line	[^{3}H]dexamethasone bound (% Fu5)[a]	Glucocorticoid receptor transcripts (% Fu5)[b]
Fu5	100	100
BDS1	45	219
EDR1	54	108
EDR3	2	4
EDR5	118	120
EDR7	26	107

[a] Specific [^{3}H]dexamethasone (15 nM) binding activity was determined in Fu5 and variant subclones by a glass-fiber filter assay. Values were calculated as the percent level detected in wild-type Fu5 cells.

[b] Expression of glucocorticoid receptor transcripts was analyzed by a cytoblot assay as described in the text. The percent receptor transcripts expressed were calculated relative to the transcript levels present in wild-type Fu5.

receptor transcripts and is therefore a true glucocorticoid receptor-"minus" variant. This is in agreement with earlier data which indicated that EDR3 possessed little or no high-affinity binding for glucocorticoids (15).

Introduction of functional glucocorticoid receptor into a receptor-minus cell line confers hormonally induced growth inhibition and negative regulation of secreted mitogenic activity. To demonstrate that glucocorticoid receptor function is required for the inhibition of cellular proliferation, we introduced a functional rat glucocorticoid receptor gene into EDR3 cells. This was accomplished by infection with a retroviral vector containing RNA for the rat glucocorticoid receptor gene linked to the bacterial neomycin resistance gene. The virally infected G418-resistant cell population (E3G) as well as EDR3 and Fu5 cells were analyzed for expression of glucocorticoid receptor transcripts. Wild-type Fu5 cells display significant levels of glucocorticoid receptor RNA (Fig. 5a), while transcript levels in EDR3 are at or near background. The infected E3G cell population expressed levels of receptor RNA nearly equal to those observed for Fu5. Introduction of the glucocorticoid receptor into variant EDR3 restored specific binding of 15 nM [^{3}H]dexamethasone (Fig. 5b) to 60% the level observed in Fu5. Importantly, introduction of the receptor gene restored the glucocorticoid-mediated growth inhibition. After 4 days in culture, EDR3 displayed no sensitivity to glucocorticoid-mediated growth inhibition, while E3G shows significant glucocorticoid-dependent growth inhibition (Fig. 5c). Interestingly, in E3G cell populations dexamethasone inhibited the activity of extracellular factors mitogenic for BALB/c 3T3 cells not observed in EDR3 (Fig. 5d). This glucocorticoid-suppressible extracellular mitogenic activity is also present in Fu5 and BDS1 and appears to be a thermal and acid-stable protein(s) with cotransforming activity (P. W. Cook, C. P. Edwards, and G. L. Firestone, manuscript in preparation). Thus, introduction of a functional glucocorticoid receptor into the receptor-minus EDR3 subclone confers dexamethasone binding activity and glucocorticoid inhibition of both cellular proliferation and the activity of secreted factors mitogenic for quiescent fibroblasts. These results unambiguously demonstrate that the glucocorticoid inhibition of cellular proliferation and extracellular growth factor activity required receptor function.

Introduction of functional glucocorticoid receptors into partially responsive clones restores hormone responsiveness. The partial glucocorticoid responsiveness of EDR1, EDR5, and EDR7 suggests that a common factor may be required for the normal induction of hormone-regulated products that fail to

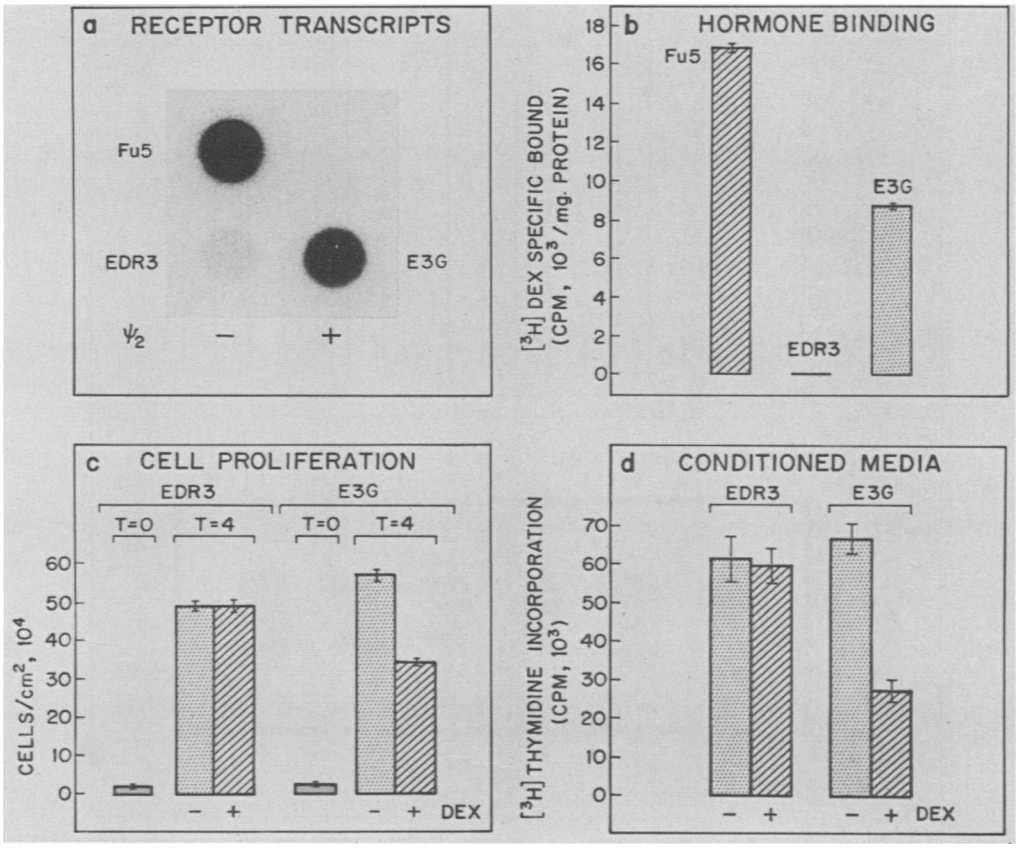

FIG. 5. Restoration of normal hormone-regulated cell proliferation in EDR3 cells by introduction of functional glucocorticoid receptor genes. The EDR3 receptor-negative subclone was infected with recombinant retrovirus (VDG12ψ_2) containing a functional rat glucocorticoid receptor gene linked to the neomycin resistance gene. (a) Expression of glucocorticoid receptor transcripts was monitored in the G418-resistant population of EDR3 cells (E3G) as well as wild-type Fu5 and variant EDR3 by a cytoblot assay as described in Materials and Methods. (b) Specifically bound 15 nM [^{3}H]dexamethasone (DEX) was measured in Fu5, EDR3, and E3G cells as described in the text. (c) EDR3 and E3G were cultured in the presence or absence of 1 μM dexamethasone for 4 days and assayed for cell proliferation as described in the legend to Fig. 1. (d) Conditioned media from EDR3 and E3G cultured in the presence or absence of 1 μM dexamethasone serum-free were harvested and added to quiescent BALB/c 3T3 cells. DNA synthesis was monitored by the incorporation of [^{3}H]thymidine as described in the text.

be expressed in these variants (AGP, glutamine synthetase, growth-inhibitory products). Infection of EDR1, EDR5, and EDR7 with the recombinant retrovirus containing RNA for a functional glucocorticoid receptor gene was used to determine whether the defect resulting in partial hormone responsiveness was due to subtle mutations in the receptor itself. Infected variant cell populations denoted (E1G, E5G, and E7G, as well as E3G cells described earlier) were cultured in the presence or absence of dexamethasone for 4 days and total cell number per well was determined by assaying total DNA. E1G, E5G, and E7G now show glucocorticoid-induced growth inhibition in a manner similar to that observed for wild-type Fu5 (Fig. 6a). As a control, receptors were also introduced into the hypersensitive variant (B1G), resulting in a slightly greater degree of growth inhibition than observed for uninfected BDS1.

To test for the restoration of other glucocorticoid-regulated products, infected variants were cultured in the presence or absence of dexamethasone for 24 h and radiolabeled with [^{35}S]methionine for the final 6 h of the hormonal exposure, and the radiolabeled culture media were then immunoprecipitated for AGP. The hormonal induction of secreted AGP was restored (Fig. 6b) in all infected variants that had not previously displayed this response (Fig. 4). Collectively, our results demonstrated that the hormonal regulation of cell proliferation as well as general hormone responsiveness can be directly restored in two classes of resistant variants by the introduction of a functional glucocorticoid receptor gene.

DISCUSSION

Glucocorticoid hormones act via specific intracellular receptor proteins to elicit a broad spectrum of characteristic responses in many types of target cells. Several lines of evidence demonstrated that one such receptor-dependent response in Fu5 rat hepatoma cells is a suppression of cellular proliferation. First, dexamethasone, a synthetic glu-

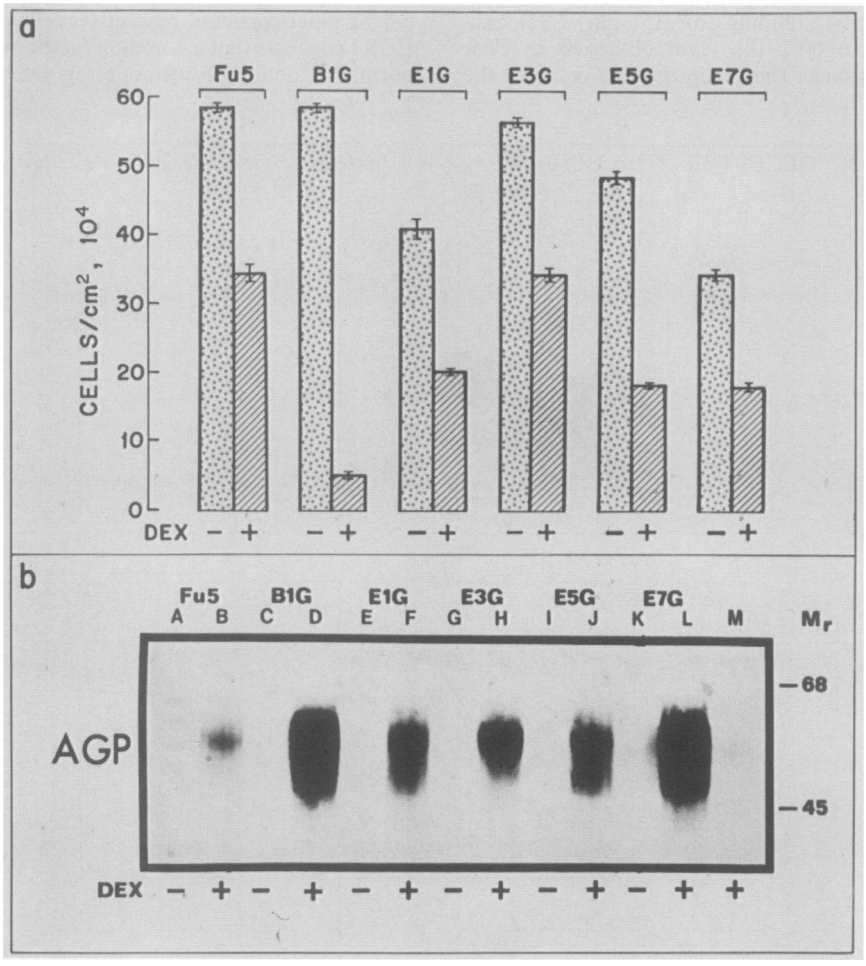

FIG. 6. Glucocorticoid responsiveness of partially responsive, receptor-positive and nonresponsive, receptor-negative subclones after introduction of functional glucocorticoid receptor genes. (a) Functional rat glucocorticoid receptor genes were stably introduced into BSD1, EDR1, EDR3, EDR5, and EDR7 variant subclones (denoted B1G, E1G, E3G, E5G, and E7G, respectively) as described in the legend to Fig. 5. G418-resistant cell populations, as well as wild-type Fu5, were incubated in the presence or absence of 1 μM dexamethasone (DEX) for 4 days, and cell proliferation was determined as a function of total DNA content as described in the legend to Fig. 1. All values are an average of triplicate samples. (b) Fu5 and indicated G418-resistant cell populations were cultured in the absence (lanes A, C, E, G, I, and K) or presence (lanes B, D, F, H, J, L, and M) of 1 μM dexamethasone for 24 h and radiolabeled with [^{35}S]methionine during the last 6 h of incubation. Radiolabeled media were immunoprecipitated with anti-AGP (lanes A to L) or nonimmune serum (lane M), and immunoprecipitated material was fractionated in SDS-polyacrylamide gels. Radioactive proteins were visualized by fluorography; molecular weight markers are described in the legend to Fig. 4.

cocorticoid, and hydrocortisone inhibited Fu5 growth in a manner proportional to their occupancy and affinity for glucocorticoid receptors, while RU38486, a powerful antagonist of glucocorticoid hormone action, blocked the dexamethasone effect on cellular proliferation. Second, genetic selection of two classes of glucocorticoid-resistant variants derived from Fu5 resulted in the recovery of subclones that proliferated constitutively in the presence of dexamethasone. One such variant, EDR3, failed to express glucocorticoid receptor transcripts and displayed a concomitant loss of several tested hormone responses, including the inhibition of proliferation. Introduction of functional glucocorticoid receptor genes back into EDR3 restored the expression of receptor RNA, dexamethasone binding activity, the induction of all tested glucocorticoid responses, and importantly, the hormone-dependent inhibition of cell growth. Thus, the addition of one gene, a functional glucocorticoid receptor, restored EDR3 cells to a wild-type phenotype and demonstrated that the proliferative response is dependent upon expression of a functional glucocorticoid receptor.

As we and others have reported for HTC hepatoma cell variants (16, 20, 51, 52), the class of Fu5-derived variants represented by EDR1, EDR5, and EDR7 displayed partial pleiotropy with respect to their steroid-responsive domain. Certain combinations of glucocorticoid-regulated genes failed to be induced, whereas the remaining set of responses were regulated normally. For example, in EDR1, EDR5, and EDR7 cells, the dexamethasone-regulated inhibition of cellular proliferation and induction of glutamine synthetase and AGP were not observed, while the expression of PAI and TAT were efficiently induced at saturating levels of dexamethasone. Surprisingly, introduction of functional glucocorticoid receptors also restored the wild-type phenotype in these variants, suggesting that their glucocorticoid receptors may have been defective in a subtle manner that allowed a selective expression of certain glucocorticoid-mediated responses. Alternatively, these cells may require higher concentrations of receptor for complete hormone responsiveness. In either case, that proliferation and a select number of certain other glucocorticoid-inducible genes failed to respond to hormone treatment suggests that the gene expression events that mediate growth inhibition may be linked in part to the expression of other steroid-inducible gene products.

Given current concepts of glucocorticoid hormone action, which has demonstrated a transcriptional mechanism of action (1, 56), likely mechanisms by which dexamethasone can directly inhibit Fu5 cell proliferation include the stimulated synthesis of growth inhibitors, the reduced synthesis of specific growth-promoting factors, or a combination of both effects. For example, in cultured human mammary tumor cells (10, 26), estrogen simultaneously inhibits the production of secreted transforming growth factor type beta (a proliferation inhibitor) and stimulates the release of mitogenic transforming growth factor type alpha. Conceivably, glucocorticoid-induced factors may also regulate the functional expression of cellular components or receptors that respond to extracellular mitogenic activities. Indeed, glucocorticoids have been shown to down-regulate insulin-binding activity (4), while insulin, acting through the insulin receptor, has been reported to be a potent mitogen for the H35 and H4-EII-C3 hepatoma cells (27, 28, 34, 39) as well as for Fu5 cells (P. W. Cook and G. L. Firestone, unpublished data). We have also obtained data which suggest that glucocorticoids may be regulating the activity of mitogenic polypeptide factors secreted by both Fu5 and BDS1 cells. Potential disruptions of the growth-stimulating autocrine pathways by glucocorticoids are being explored in these rat hepatoma cell lines.

It will be interesting to determine whether the ability of glucocorticoids to regulate the proliferation of hepatoma cells can be correlated to a specific differentiation or tumorigenic state of liver-derived cells and whether this response is expressed in a tissue-specific manner. Indeed, we are beginning to test this notion in other tumor cells with specific hormone-responsive and unresponsive proliferative properties such as cultured rat mammary tumor cells. The selection of glucocorticoid-resistant variants was crucial to unambiguously establish the role of glucocorticoid receptors in the inhibition of Fu5 hepatoma cell proliferation. This genetic approach is being continued to recover variants that express fully functional glucocorticoid receptors but fail to be growth inhibited in the presence of hormone. The response of these variants to glucocorticoids at the molecular level will be used to genetically identify the key glucocorticoid-regulated hepatoma gene products responsible for mediating growth inhibition in the hypersensitive BDS1 subclone of Fu5.

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