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SELECTION OF GLUCOCORTICOID-RESISTANT MUTATIONS FROM AN AtT-20 CELL LINE CONTAINING A GLUCOCORTICOID-REGULATED SELECTABLE TRANSGENE+

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SUMMARY: AtT-20/IDG8 cells contain the stably transfected, selectable gene, neomycin phosphotransferase, under negative glucocorticoid regulation. Thus, when cultured in the simultaneous presence of the neomycin analogue, G418, and dexamethasone, AtT-20/IDG8 cells fail to grow. Our hypothesis was that mutated AtT-20/IDG8 cells capable of growth in such medium would have a defect in the glucocorticoid-mediated regulation of the neo^r gene. AtT-20/IDG8 cells were chemically mutagenized using ethyl-methane sulfonate and cloned in the presence of G418 and dexamethasone. Fourteen clones were obtained and loss of glucocorticoid control of neo^r expression was confirmed in them all. The naturally occurring gene, pro-opiomelanocortin, which is down-regulated by glucocorticoids in parent AtT-20/IDG8 cells, was down-regulated by dexamethasone in ten of the mutant lines, indicating that in those cells the receptor was functional in spite of aberrant regulation of neo^r. In the other four lines, pro-opiomelanocortin regulation was lost,also suggesting that a general transcription factor, such as the receptor, had been altered. These results indicate that multiple factors are involved in glucocorticoid-mediated gene regulation and that new, informative mutations can be produced after insertion of a regulated, selectable gene into a previously non-selectable cell line.

Glucocorticoids affect target cells through the glucocorticoid receptor, a gene regulatory protein (1). Although the receptor is known to interact with various intracellular proteins such as heat shock protein (2,3) and other transcription factors (4-8), the exact nature of these interactions and their effects on gene regulation is unclear. Although genetic mutational analysis should be useful in dissecting this complex, regulatory mechanism, almost all hormone-resistant cell lines produced thus far have been found to have absent or

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Nonconventional abbreviations used: neo^r, neomycin phosphotransferase; EMS1-14, AtT-20/EMS1-14 cell clones.

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structurally abnormal receptors (9). In general, cells lines that are killed or lysed by glucocorticoid treatment have been used to isolate resistant mutants. The rat lymphoma cell line S49, the mouse thymoma line WEHI7, and the human leukemia cell line CEM-7 have been used most often (10, 11). Biochemical analysis of the resistent clones produced have shown that the vast majority reflect some abnormality of the receptor. Absence of detectable hormone binding and abnormal nuclear translocation or DNA binding of activated receptor are seen as the basis of glucocorticoid resistance in almost all mutant lines tested (10, 12). Although study of these cell lines clarified many aspects of receptor function, these approaches failed to reliably produce steroid-resistant phenotypes with functional receptors so that other associated trans-acting proteins could be identified and studied. Moreover, these approaches were limited to cell types that were normally growth inhibited by glucocorticoids. The limited spectrum of mutations is in contrast to in vivo observations where glucocorticoid-resistant leukemia and lymphoma cells may have normal receptor levels (13, 14). We speculated that introduction of a selectable gene into a cell not normally killed by glucocorticoids might result in selection of a wider range of mutations.

The AtT-20 cell line is a mouse pituitary tumor cell line that synthesizes pro-opiomelanocortin under the negative transcriptional control of glucocorticoids (15, 17). These cells have been stably transfected with the neomycin phosphotransferase gene (neo^r) and a cell line isolated in which a single copy of this gene is under negative glucocorticoid transcriptional control (8, 9). Thus, growth of this cell line, designated AtT-20/IDG8, is unaffected by dexamethasone or G418 individually but growth is inhibited in the simultaneous presence of both agents. This paper reports the isolation of mutants of AtT-20/IDG8 cells that are unresponsive to glucocorticoids and appear to have functional receptors.

MATERIALS AND METHODS

Plasmid DNA and other reagents: Construction of the plasmids used is described elsewhere: pRBME1 is a full-length cDNA clone of mouse POMC (20), and pSVOneo contains the neo^r structural gene driven by an enhancerless SV40 promoter (21). Restriction enzymes and DNA-labeling reagents were obtained from BRL and used according to the vendor's instructions.

Cell culture: AtT-20/D1 cells were originally obtained from the American Type Culture Collection. Their growth and cloning in soft agar has been previously described (22). Cell numbers were enumerated using a Coulter counter.

RNA electrophoresis and Northern blot analysis: Total RNA prepared by the guanidinium acid-phenol method (23) was run on 1.5% agarose gels containing 2% formaldehyde. The sample buffer contained 40 μg/ml ethidium bromide, and the 28S and 18S ribosomal RNA bands were used as size markers of 6,333 and 2,366 bp respectively. The gels were

transferred by blotting onto GeneScreen Plus, using 20 x SSC. Hybridizations were performed for 16-18 hours at 42°C in 30 mM Tris (ph 7.5), 50% formamide, 1% SDS, 1 m NaCl, 10% Dextran Sulfate with DNA probes labeled using random primers (24) and washed in 2 x SCC, 1% SDS and 0.2 x SSC, 1% SDS at 65°C.

RESULTS AND DISCUSSION

Selectable AtT-20 cells, mutagenesis and isolation of resistant clones: The AtT-20/IDG8 cell line was produced by stable transfection of AtT-20/D1 cells with the neomycin phosphotransferase gene (neo^r) driven by a minimal SV40 promoter which required an enhancer for effective expression (18-19). Thus, only when the transfected DNA inserted near an endogenous enhancer would a cell have sufficient neo^r expression to be capable of growth in medium containing G418. The AtT-20/IDG8 clonal line was chosen for these studies because its resistance to G418 is diminished by co-incubation with dexamethasone, indicating that the endogenous enhancer affecting the SV40 promoter is one that is negatively regulated by glucocorticoids (18-19). Thus AtT-20/IDG8 cells have the phenotype of growth inhibition in the presence of glucocorticoids and G418.

The reduction in G418 resistance by glucocorticoids is due to transcriptional repression of the neo^r gene (19). Thus, cells capable of growth in medium containing glucocorticoids and G418 would have a defective response mechanism. AtT-20/IDG8 cells were mutagenized and cells capable of growth in medium containing dexamethasone and G418 were cloned. These clones represent cells with a deficient glucocorticoid response mechanism. Mutagenesis was done by culturing 10^8 cells in 200 μg/ml ethyl methane sulfonate for three days. Cells were cultured an additional two weeks in 2.0 mg/ml G418 and 10^{-6}M dexamethasone to allow differential enrichment of glucocorticoid-resistant cells. Cells (50,000/90mm dish) were then cloned in soft agarose containing G418 and dexamethasone. After two weeks, 25 to 50 colonies/plate were visible. Typical fields consisted of numerous single cells and an occasional large colony. In a similar experiment in which only 10^7 cells were mutagenized, no colonies were seen. Nigrosin staining of the plates showed that the single AtT-20/IDG8 cells were viable, indicating that they were growth-inhibited by G418 and dexamethasone but not killed. Lastly, both isolated single cells and large colonies recovered from the plates proliferated when cultured in medium containing G418 but not dexamethasone. Thus, mutations which prevented glucocorticoid-mediated reduction in G418 sensitivity were easily detected by formation of large colonies. Several large colonies were recovered, designated AtT-20/EMS1-14 (EMS1-14) and expanded for further study.

Growth characteristics of parent and mutant cells: Figure 1a is a comparison of parental (AtT-20/IDG8) and mutant cell growth over six days. AtT-20/IDG8 (parental) and EMS1

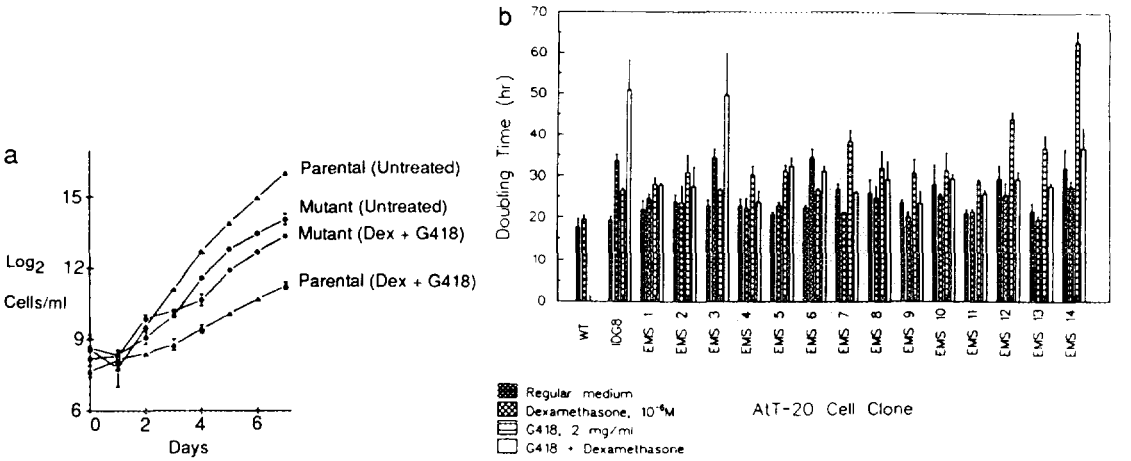

Figure 1. Effect of dexamethasone on parental and mutant cell growth.
a. Cells were grown under 10% CO_2 in DME/F12 medium containing 10% newborn calf serum (Regular) and 10^{-6}M dexamethasone or 2 mg/ml G418 at 200 cells/ml. Results are typical of several experiments. G418 concentrations in this and subsequent figures are active compound. Samples from three wells were counted. Bars indicate S.E.M.
b. Cells were grown for 6 days as described above. Growth curve slopes were determined by regression analysis (gamma coefficient 0.85-0.99) and used to calculate the doubling time. Growth media used were regular, Regular + 10^{-6}M dexamethasone, Regular + 2 mg/ml G418 and Regular + 2 mg/ml G418 and 10^{-6}M dexamethasone. Each point represents cell counts from three wells. Bars indicate S.E.M.

(mutant) cells grew at similar rates in medium containing neither dexamethasone or G418 (untreated). The growth rate of EMS3 cells was undiminished in medium containing dexamethasone and G418 whereas AtT-20/IDG8 cells were inhibited. Growth results for all cell lines were obtained and calculated as doubling times (Figure 1b). AtT-20 cell growth (WT) was unaffected by dexamethasone and abolished by G418. AtT-20/IDG8 cell growth (IDG8) was minimally affected by dexamethasone or G418 but doubling time was markedly increased by dexamethasone and G418 together. With the exception of EMS3 (G418 + dexamethasone treatment) and EMS14 (dexamethasone treatment), treatments did not affect the doubling times. The basis of the exception is unclear since similar effects have not been seen at other times and EMS3 has been subsequently recloned in the presence of dexamethasone and G418 to confirm that it is phenotypically glucocorticoid resistant.

Dexamethasone effects on neo^r mRNA in parent and mutant cells: We reasoned that, since the growth arrest of AtT-20/IDG8 cells in G418 + dexamethasone was due to transcriptional down-regulation of neo^r (16), dexamethasone treatment should reduce neo^r mRNA levels in AtT-20/IDG8 cells but not in EMS cells. This is illustrated by a typical Northern blot analysis (Figure 2a) which compares the levels of neo^r mRNA in

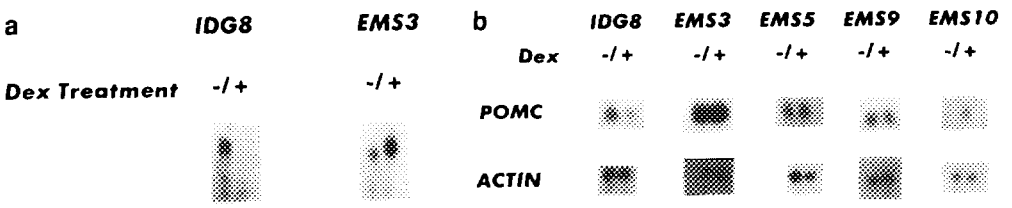

Figure 2. Dexamethasone regulation of neo^r and pro-opiomelanocortin mRNA. In several individual experiments, cells were grown in regular medium +/- 10^{-6}M dexamethasone for 24 h. After washing in ice-cold Tris-buffered saline by suspension and centrifugation, Total RNA was prepared and analyzed by Northern blotting. Presented results are from a typical experiment.
a. Northern blot of parental (IDG8) and mutant (EMS3) RNA probed with neo^r cDNA.
b. Northern blots of parental (IDG8) and mutant (EMS 3, 5, 9, and 10) RNA probed with pRBME1 cDNA (upper panels labeled pro-opiomelanocortin) dehybridized and reprobed with ß-actin cDNA (lower panel).

AtT-20/IDG8 and EMS3 in response to dexamethasone treatment for 24h. Whereas, dexamethasone treatment resulted in a clear reduction of AtT-20/IGD8 neo^r mRNA, EMS3 neo^r mRNA levels were unaffected or even increased. Although there is no clear explanation for this phenomenon, several possibilities will be explored based on the work of others. For example, dexamethasone regulation of the proliferin gene can be switched from positive to negative when fos/jun levels are altered (7). Neo^r mRNA was unaffected or even increased by dexamethasone treatment in all fourteen EMS lines confirming that transcriptional down-regulation of neo^r had been abolished by the mutations.

Identification of mutant cell lines with "globally" defective regulation: Pro-opiomelanocortin is actively synthesized in AtT-20/Dl cells and is normally down-regulated by glucocorticoids such as dexamethasone (25-26). Thus, assessment of pro-opiomelanocortin mRNA levels was a logical way to test whether the defect in dexamethasone regulation of neo^r was a general one or limited to neo^r. Dexamethasone treatment reduced pro-opiomelanocortin levels in several EMS lines (not shown) suggesting that failure to regulate neo^r mRNA was not due to an abnormal receptor since that would be a global defect that would abolish the response of all genes. However, pro-opiomelanocortin mRNA was not reduced by dexamethasone in four lines (Figure 2b). These results, which included sequential hybridization with a ß-actin probe to insure equal amounts of RNA per lane show that dexamethasone had no effect on pro-opiomelanocortin mRNA levels in EMS3, EMS5 or EMS9 and levels apparently increased slightly after dexamethasone treatment of EMS10.

These results are compatible with the concept that glucocorticoid regulation of transcriptional events is complex and multi-factorial. Several mutant cell lines contained functional receptor

since negative POMC regulation by dexamethasone was retained. Interestingly, four mutant lines exhibited defective regulation of neo^r and POMC. The exact nature of the defects in these lines remains to be determined and further study may well reveal the presence of a receptor lesion. However, the results contained in this paper suggest that non-receptor cellular components thought to participate in the glucocorticoid response may be accessible to identification and study by this genetic approach. Finally, it is noteworthy that insertion of a selectable and regulated gene is a feasible approach to the mutational analysis of any regulatory system and thus promises to be a flexible strategy of great future utility.

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