MOLECULAR AND CELLULAR BIOLOGY, Feb. 1982, p. 179–189
0270-7306/82/020179-11$02.00/0

Vol. 2, No. 2

Prolactin-Deficient Variants of GH_3 Rat Pituitary Tumor Cells: Linked Expression of Prolactin and Another Hormonally Responsive Protein in GH_3 Cells

ROBERT D. IVARIE,[1]* JULIE A. MORRIS,[1] AND JOSEPH A. MARTIAL[2]

Department of Molecular and Population Genetics, University of Georgia, Athens, Georgia 30602,[1] and Laboratoire de Génie Genetique, Université de Liège, B-4000 Sart-Tilman, Belgium[2]

Received 6 July 1981/Accepted 8 September 1981

GH_3 cells normally synthesize and secrete two pituitary polypeptide hormones, prolactin and growth hormone. From an ethyl methane sulfonate-mutagenized population, prolactin low-producing variants have been isolated at a frequency near 20%. Intracellular prolactin synthesis in the variants was reduced 40- to 100-fold compared to wild-type cells while growth hormone synthesis varied less than 2-fold. This decrease was paralleled by a decrease in intracellular preprolactin mRNA. Although reduced, prolactin synthesis was still repressible by glucocorticoids. There was a coordinate loss of expression of p21, a thyroid and glucocorticoid hormone-regulated protein, in GH_3 cells, whereas the synthesis and regulation of other hormonally responsive proteins were unimpaired in the variants. Since p21 expression was coordinately regained in a high-producing prolactin revertant cell, expression of the two proteins is tightly coupled in GH_3 cells. The stability of the low-producing phenotype differed among variants. One (B_2) gave rise to revertants at about 20% frequency even after two rounds of subcloning, whereas another (B_3) was more stable in that only 1 weak revertant was found in 47 subclones. The reversion frequency of B_3 cells was also measured at less than 0.5%. Unmutagenized GH_3 cells were phenotypically stable in that no prolactin-deficient variant was found among 57 subclones. Since variants were only found after ethyl methane sulfonate mutagenesis, the DNA alkylating agent appears to have promoted an epigenetic change in pituitary gene expression.

GH_3 cells are an established tissue culture line derived from a rat anterior pituitary tumor (31, 37). They maintain pituitary-specific gene expression in culture by synthesizing and secreting two pituitary polypeptide hormones, prolactin and growth hormone (30, 31). Thus, they exhibit both a lactotrophic and somatotrophic phenotype, unlike their anterior pituitary cell counterparts which synthesize only one or the other peptide hormone (19). The cells have been a model system for analyzing hormone action because the synthesis of the two proteins is under the control of several hormones including thyroid and glucocorticoid hormones (8, 12, 16, 27, 28). One property of the cells, however, that has received little attention is an instability in the expression of the peptide hormones. For instance, two variant lines have arisen "spontaneously" during continuous culture of GH_3 cells. One (GH_4) synthesizes "normal" levels of prolactin but very low levels of growth hormone (4), whereas the other (GC) does not detectably synthesize prolactin but does express growth hormone at a level severalfold higher than the parent line (2, 9). Although both variants arose in the absence of known selection pressure, their phenotypes have apparently been stable in subsequent cell culture. However, no systematic cloning analysis of either variant or the parent GH_3 line has been reported to our knowledge, nor have any revertant lines been isolated.

In this report, we describe the isolation and biochemical analysis of phenotypically stable and unstable variants of GH_3 cells deficient in prolactin synthesis. Such variants were found at high frequency (ca. 20%) in the course of screening for growth hormone and prolactin production by randomly picked clones from an ethyl methane sulfonate (EMS)-mutagenized cell population. Since they represent cells in which the lactotrophic phenotype has been lost while the somatotrophic character has been retained, they have been studied in considerable detail. We show here that the change in phenotype also

involves a coordinate loss in the expression of one other hormonally responsive protein, termed p21, in GH_3 cells.

MATERIALS AND METHODS

Cells and culture conditions. The parent cell line (GH_3D_6) used in this study was a subclone isolated in 1976 from the GH_3 line originally established in culture by Tashjian et al. (31). When originally isolated, the cells synthesized nearly equal amounts of prolactin and growth hormone under standard culture conditions. However, production of both proteins is differentially sensitive to cell density, so this is only a relative estimate (5, 10). Cells were routinely grown as monolayers in Dulbecco modified Eagle medium with 10% fetal calf serum in a 5% CO_2, humidified incubator at 37°C. Under these conditions, the cells divide every 41 h.

Mutagenesis. The cells were treated for 24 h with 300 μg of EMS (Sigma) per ml, washed free of EMS, and grown for 4 days. Survival of GH_3 cells was 20 to 30% under these conditions.

Cloning GH_3 cells. To isolate clones in a single step, cells from monolayer cultures were removed from the dish with trypsin or EDTA (2 mM) in Dulbecco phosphate-buffered saline and washed two times by low-speed centrifugation and resuspension in culture medium. From 100 to 200 cells in 0.25 ml were then spread over the surface of 0.6% agar (Difco, Noble Agar) in culture medium. After excess medium was absorbed by the agar at room temperature, dishes were incubated cell-side-up at 37°C until visible colonies appeared (ca. 3 weeks). Cloning efficiency averaged 75%. Individual colonies were transferred by sterile Pasteur pipettes to wells of a 24-well culture dish (Costar) and grown to mass culture.

Assay for growth hormone and prolactin production. Individual clones were labeled with 250 μCi of [^{35}S]methionine per ml for 2 to 3 h in 24-well culture dishes in 200 μl of methionine-free Dulbecco modified Eagle medium containing 10% serum substitute (3) supplemented with 10^{-8} M triiodothyronine, 10^{-6} M dexamethasone, and 10 μg of insulin per ml. The medium was removed and mixed 1:1 with 2× sodium dodecyl sulfate (SDS) sample buffer (20), and samples were electrophoresed on 12.5% SDS-polyacrylamide slab gels. No more that 5 μl of original medium was loaded per lane. Visualization of the secreted proteins on the gels was by autoradiography using Kodak X-Omat R X-ray film. In some experiments, autoradiographic enhancers were used before autoradiography (En^{3}Hance, New England Nuclear Corp.).

RNA extraction and analysis. Total cytoplasmic RNA was extracted from wild-type and variant cells by using an SDS-phenol-chloroform extraction procedure as described (16). Samples of the RNA preparations (20 μg) were translated in a cell-free, micrococcal nuclease-treated lysate of rabbit reticulocytes as described, and labeled preprolactin was assayed by immunoprecipitation and electrophoresis on 12.5% SDS-polyacrylamide slab gels as described (13). To assay for hybridizable preprolactin mRNA, samples (3 μl) of each RNA were bound to nitrocellulose filters in high salt and assayed by hybridization to 2×10^6 cpm of ^{32}P-labeled recombinant plasmid containing a cDNA to rat preprolactin mRNA (7). Conditions for the Dot blots were those described by Thomas (32). Nick translation of plasmid DNA was performed under the conditions described by Rigby et al. (24), using [^{32}P]dATP as the labeled substrate; probe specific activity averaged 5×10^7 cpm/μg. Hybridized counts were visualized by autoradiography at −70°C with intensifying screens (Lightning Plus, Dupont).

Colony hybridization. Colonies from wild-type and variant cells were transferred from agar plates to paper disks by overlaying each plate with a sterile disk of Whatman no. 54 paper (36; D. A. Peterson and K. R. Yamamoto, manuscript in preparation), then wetted with about 0.5 ml of culture medium and incubating at 37°C for 60 min to permit colonies to attach to the paper. Disks were then removed and blotted free of medium, and attached colonies were fixed for 10 min with ethanol-acetic acid (3:1) by laying disks on filter paper saturated with fixative. The disks were then washed several times with 95% ethanol, air dried, and hybridized with 10^6 cpm of ^{32}P-labeled, nick-translated prolactin cDNA plasmid in annealing buffer (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 500 μg of herring sperm DNA per ml, and 50% formamide). After hybridization for 20 h at 42°C, disks were washed five times for 45 min with 5× SSC containing 0.1% SDS at 68°C and then twice with 2× SSC–0.1% SDS for 30 min at room temperature. Hybridized counts were visualized by autoradiography for 1 to 5 days at −70°C with intensifying screens (DuPont Lightning Plus). After autoradiography, colonies on disks were stained with 0.2% Coomassie Brilliant Blue R (Sigma) in 50% methanol and 7% acetic acid and destained in the same solvent.

RESULTS

Initial detection of prolactin-deficient variants. In the initial experiments, GH_3 cells were mutagenized with EMS for 24 h, and after 4 days of growth the cells were cloned on agar plates. One or two colonies from each plate were picked and assayed for secretion of growth hormone and prolactin by labeling cells with [^{35}S]methionine and electrophoresing a sample of the medium on one-dimensional SDS-polyacrylamide slab gels. Figure 1 shows regions of the autoradiograms containing the bands corresponding to prolactin (22.5×10^3 daltons) and growth hormone (19.5×10^3 daltons), which have been identified by specific immunoprecipitation (12, 16, 17). Of 12 clones analyzed in this way, three (4_1, 6, and 16) were producing little or no prolactin, whereas growth hormone production was readily detectable in all clones. Since at least 25 generations had passed by the time each clone was assayed, the variants exhibited some degree of phenotypic stability. Moreover, growth hormone production provided an internal control; its expression was stable in all clones and argued against any physiological effect produced by the cloning method and subsequent growth to mass culture.

Since only secretion of prolactin was assayed in Fig. 1, it was possible that the low-producing

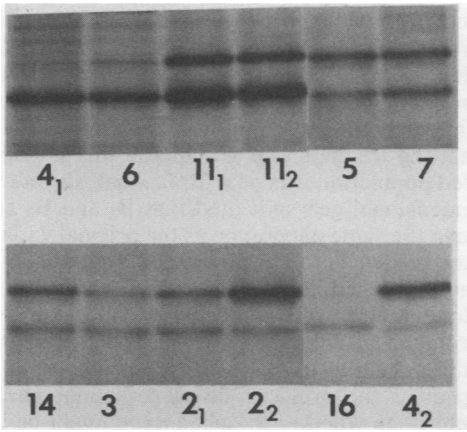

FIG. 1. Initial finding of low-producing prolactin variants in EMS-mutagenized GH$_3$ cells. After mutagenesis, GH$_3$ cells were grown for 4 days and cloned on agar plates. One or two colonies (subscripts) were picked from individual plates (numbered), grown to mass cultures, and labeled for 3 h with [^{35}S]-methionine in methionine-free culture medium. In this experiment, individual colonies were labeled at different times. Samples (3 μl) of the medium from each clone were then electrophoresed on a 12.5% SDS-polyacrylamide slab gel, and labeled prolactin and growth hormone were detected by autoradiography. Only the regions of the gels containing the two peptide hormones are shown since there were no detectable differences in other proteins found in the medium. The upper band is prolactin (22.5 × 10^3 daltons), and the lower band is growth hormone (19.5 × 10^3 daltons).

cells may have been able to synthesize prolactin normally but unable to secrete it. To test this and to measure the specificity of the effect, the three variant clones, wild-type GH$_3$ cells, and wild-type clone 11$_1$ were treated with and without dexamethasone, pulse-labeled with [^{35}S]methionine, and analyzed by two-dimensional gel electrophoresis. GH$_3$ cells contain several glucocorticoid-responsive proteins whose synthesis can be induced or repressed by the steroid, and prolactin is a member of the steroid-repressible group (12, 14). Figure 2 shows regions of the resulting two-dimensional gel autoradiograms containing prolactin, one steroid-inducible protein (i-10), and one steroid-repressible protein (p21). Clearly, the rate of prolactin synthesis in the variants was substantially reduced compared to wild-type cells and was still repressible by dexamethasone. Furthermore, basal synthesis of i-10 was unimpaired and remained inducible by the steroid in the variants. Although not shown in Fig. 2, growth hormone inducibility was intact as well. However, the synthesis of steroid-repressible p21 was not detectable in the variants, whereas it was expressed and repressed by dexamethasone in wild type GH$_3$ cells and clone 11$_1$.

Table 1 provides quantitative data on the level of prolactin and growth hormone synthesis in the variants compared to wild-type cells. The data were obtained by microdensitometry of the gels shown in Fig. 2. Prolactin was synthesized at only 1.0 to 2.5% the rate seen in wild-type cells, whereas the synthesis of growth hormone varied less than twofold among the cells assayed.

From these data, it is concluded that the synthesis of prolactin, not its secretion, was

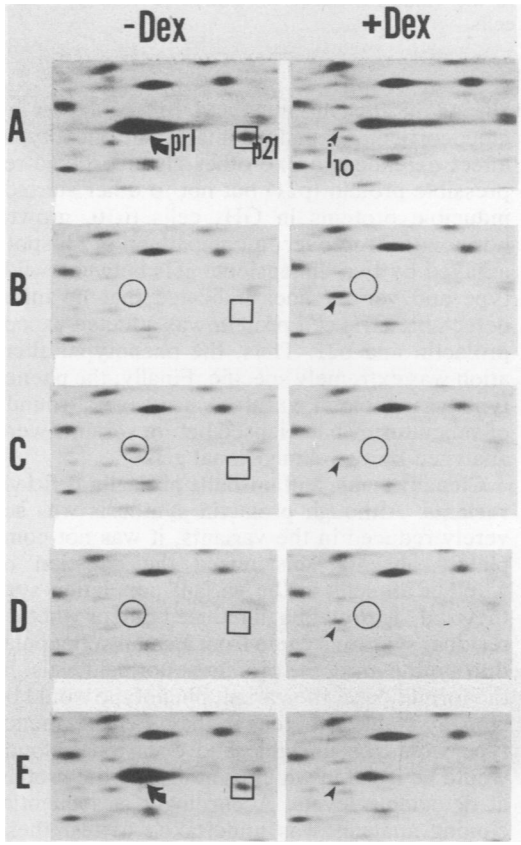

FIG. 2. Two-dimensional gel analysis of prolactin-deficient clones and wild-type GH$_3$ cells. Wild-type GH$_3$ cells and clones 4$_1$, 6, 16, and 11$_1$ (seen in Fig. 1) were treated with and without 10^{-6} M dexamethasone for 2 days and then labeled for 30 min with [^{35}S]-methionine. Samples of each whole-cell lysate were then subjected to two-dimensional separation, and radioactively labeled proteins were detected by autoradiography. Samples of 500 kcpm were electrophoresed for wild-type GH$_3$ cells (A) and wild-type clone 11$_1$ (E), and 200-kcpm samples were electrophoresed for clones 4$_1$ (B), 6 (C), and 16 (D). Gels were exposed to film for 10 days. The positions of prolactin (prl) and p21, both steroid repressible, are marked, as is the position of steroid-inducible protein i-10.

TABLE 1. Relative rate of prolactin synthesis in wild-type and variant GH_3 cells

Cells	Relative rate of synthesis[a]	
	Prolactin	Growth hormone
Wild-type GH_3	1.00	1.00
Clone 11_1	1.36	0.989
Clone 4_1	0.008	1.065
Clone 6	0.022	0.922
Clone 16	0.014	0.492

[a] Expressed as a fraction of the wild-type GH_3 rate. Weights of the areas under peaks obtained by scanning in a Joyce-Loebl microdensitometer were normalized to the value measured for untreated wild-type GH_3 cells.

altered in the variants, and its regulation by glucocorticoids remained intact. The phenotypic effect extended to one other glucocorticoid-repressible protein (p21) but not to other steroid-inducible proteins in GH_3 cells (i-10, growth hormone). Moreover, a comparison of all spots assayed by two-dimensional gels between wild-type and variant lines indicated that no other detectable GH_3 cell protein was affected except prolactin and p21. Thus, the phenotypic alteration was extremely specific. Finally, the phenotype was stable in culture, since several rounds of subculturing had elapsed before variants were analyzed by two-dimensional gels.

Clonally stable and unstable prolactin-deficient variants. Although prolactin synthesis was severely reduced in the variants, it was not completely absent. This raised the question of whether all cells in the variant population synthesized the protein at a reduced rate or whether residual synthesis came from a small subpopulation synthesizing the protein at normal levels. In the former case, the variant phenotype would be clonally stable, whereas in the latter the phenotype would be unstable and occasional clones would be isolated which synthesized the protein at detectable levels. Accordingly, a sequential cloning analysis was undertaken to test these possibilities. Unfortunately, the three variants initially isolated were lost in an incubator accident, so it was necessary to reisolate them. The same population of EMS-mutagenized GH_3 cells were cloned on agar, and nine clones were assayed for prolactin expression as before (Fig. 3A). Note that in this and subsequent experiments, the precaution was taken that samples of the medium to be electrophoresed were loaded proportional to the level of radioactivity incorporated by each clone. Since clones were not at the same cell density at the time of assay, this partially corrected for density-dependent variation in growth hormone and prolactin production known to occur in GH_3 cells (5, 10; Morris and Ivarie, unpublished data). Figure 3A shows that clones 2 and 3 were prolactin deficient, and these have been termed B_2 and B_3, respectively. This experiment confirmed the initial finding that the frequency of variants in this mutagenized population was near 20%. Analysis on two-dimensional gels indicated that B_2 and B_3 cells have the same phenotype as the original variants (see below, Fig. 8 and 9). Both B_2 and B_3 were then recloned. Figure 3B and C illustrate that clones 1 and 6 of B_2 and clone 9 of B_3 expressed prolactin at detectable levels. Thus, both variants appeared to be clonally unstable with respect to the prolactin-deficient phenotype. To determine whether the phenotype might be stabilized by a second round of cloning, prolactin-deficient clone 4 of B_2 (designated $B_{2\text{-}4}$) was recloned on agar. Figure 3D shows that clones 1 and 8 of $B_{2\text{-}4}$ were producing detectable levels of prolactin. In all cases, growth hormone production provided an internal control and was stable throughout the cloning experiments.

Several conclusions can be drawn from these results. First, low-expressing lines contained cells synthesizing prolactin at detectable levels at high frequency (ca. 10 to 20%). Thus, part of the residual prolactin synthesis seen in the variant population reflected a subpopulation of cells expressing the protein at detectable levels, implying that low-producing variants can revert to producing lines at high frequency. However, revertants do not all regain prolactin synthesis at the wild-type level, as judged qualitatively by the ratio of prolactin and growth hormone levels among revertant clones in Fig. 3. This appears to account qualitatively for the lack of correlation between the high reversion frequency (ca. 20%) and low-level synthesis in the variant population (ca. 1 to 2 %).

One revertant of B_3 cells (clone 9) was detected; however, it produced only a trace level of prolactin detectable upon prolonged film exposure. We reassayed the stability of B_3 cells by several rounds of cloning, and Fig. 3E shows the results for one B_3 subclone ($B_{3\text{-}14}$). Clearly, no wild-type revertants were found among 20 subclones of the $B_{3\text{-}14}$ population, and except for $B_{3\text{-}9}$, no revertants have been found among 47 clones and subclones of B_3. Thus, by these criteria, B_3 is phenotypically more stable than the B_2 line.

As an additional test of the stability of B_3, a large number of clones were assayed for prolactin mRNA content by replica plating and "colony hybridization" (36; Peterson and Yamamoto, in preparation). Colonies of B_2, B_3, and wild-type GH_3 cells were replica-plated from agar plates to filter paper disks, fixed, and then hybridized with a ^{32}P-labeled nick-translated

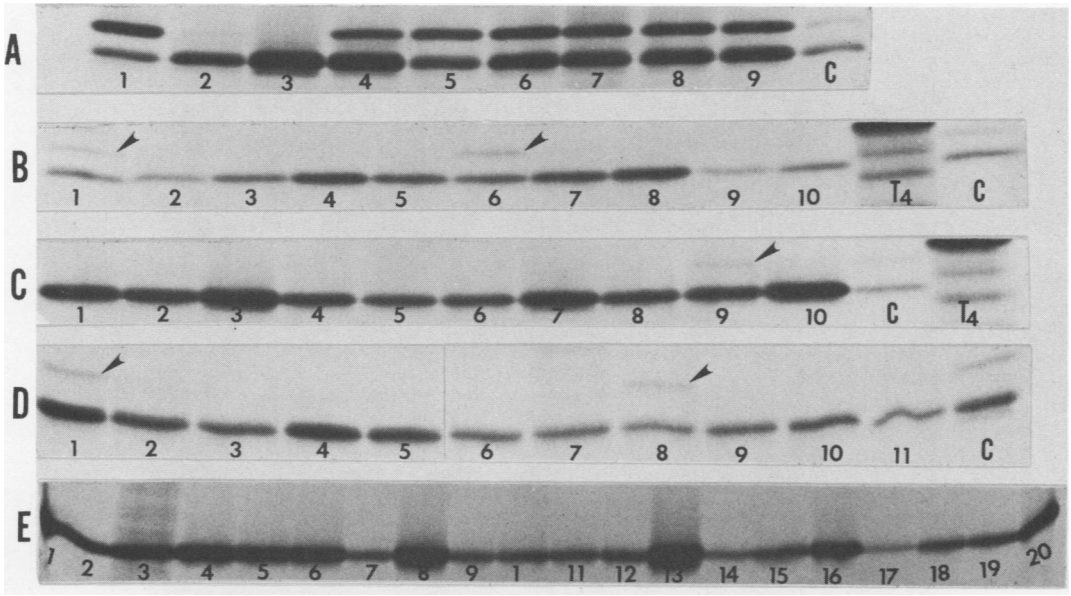

FIG. 3. Serial cloning analysis of prolactin-deficient variants. The same EMS-mutagenized population used in Fig. 1 was subjected to cloning on agar plates, and nine clones were picked and assayed for prolactin and growth hormone as described in Fig. 1. To correct for differences in cell density at the time of labeling, the amount of radioactivity incorporated by each cloned line was determined and used to normalize the loading volume of each medium sample to be electrophoresed. From the initial cloning experiment (A), clones 2 and 3 (referred to as B_2 and B_3) were picked as prolactin deficient and subjected to another round of cloning and assaying for prolactin and growth hormone production. The recloning of B_2 and B_3 is shown in (B) and (C), respectively. Subclone 4 of B_2 was also recloned (D), as was a subclone of B_3 (termed $B_{3\text{-}14}$; E). Arrowheads mark revertant clones expressing prolactin. T_4 stands for T_4 bacteriophage marker proteins labeled with [^{14}C]leucine (13), and C indicates medium from thyroid hormone-induced GH_3 cells after labeling for 6 h with [^{14}C]leucine.

recombinant plasmid containing an 850-base pair DNA complementary to rat preprolactin mRNA (7). Figure 4 shows that the labeled probe hybridized strongly to wild-type colonies, but not at all or very weakly to B_3 colonies. About 250 B_3 colonies were assayed in this experiment, indicating a reversion frequency of 0.5% or less. B_2 colonies gave a wide spectrum of hybridization signals consistent with its known population heterogeneity. These results support the conclusions drawn from the previous experiments on the clonal stabilities of B_2 and B_3; they also suggest that the drop in prolactin synthesis in variants is accompanied by a parallel drop in the level of mRNA for prolactin.

B_2 and B_3 variants contain a reduced level of preprolactin mRNA. Two assays have been performed supporting the conclusion that the reduction in prolactin synthesis in B_2 and B_3 cells is caused by parallel reduction in prolactin cytoplasmic mRNA. First, total cytoplasmic RNA was extracted from B_2, B_3, and wild-type cells and translated in a lysate from rabbit reticulocytes in the presence of [^{35}S]methionine; preprolactin synthesis was assayed by immunoprecipitation and one-dimensional SDS-polyacrylamide slab gel electrophoresis (13). Figure 5 shows that cytoplasmic RNA from wild-type clone 11_1 directed the synthesis of an immunoprecipitable polypeptide with a molecular weight of 28×10^3 as expected for preprolactin (6, 9). This protein was not detected in translations of RNA from variants B_2 and B_3. Therefore, the cytoplasmic levels of translatable preprolactin mRNA were decreased in the deficient cells.

The extent of this decrease has been estimated by using a semiquantitative RNA-cDNA hybridization assay (32). Total cytoplasmic RNA from B_2, B_3, and wild-type cells was spotted in a series of threefold dilutions onto nitrocellulose filters and hybridized with ^{32}P-labeled prolactin-cDNA plasmid. Figure 6 demonstrates that the probe hybridized strongly to RNA spotted from wild-type cells and that the signal was proportional to the amount of RNA bound to the filter and substantially reduced in RNA from glucocorticoid-treated wild-type cells, consistent with known effects of the steroid on prolactin synthesis in these cells. The extent of inhibition by the hormone was about ninefold since the signal intensity for any given amount of RNA from steroid-treated cells was about the same as ninefold dilutions of the same amounts of RNA from untreated cells. The probe hybridized weakly to

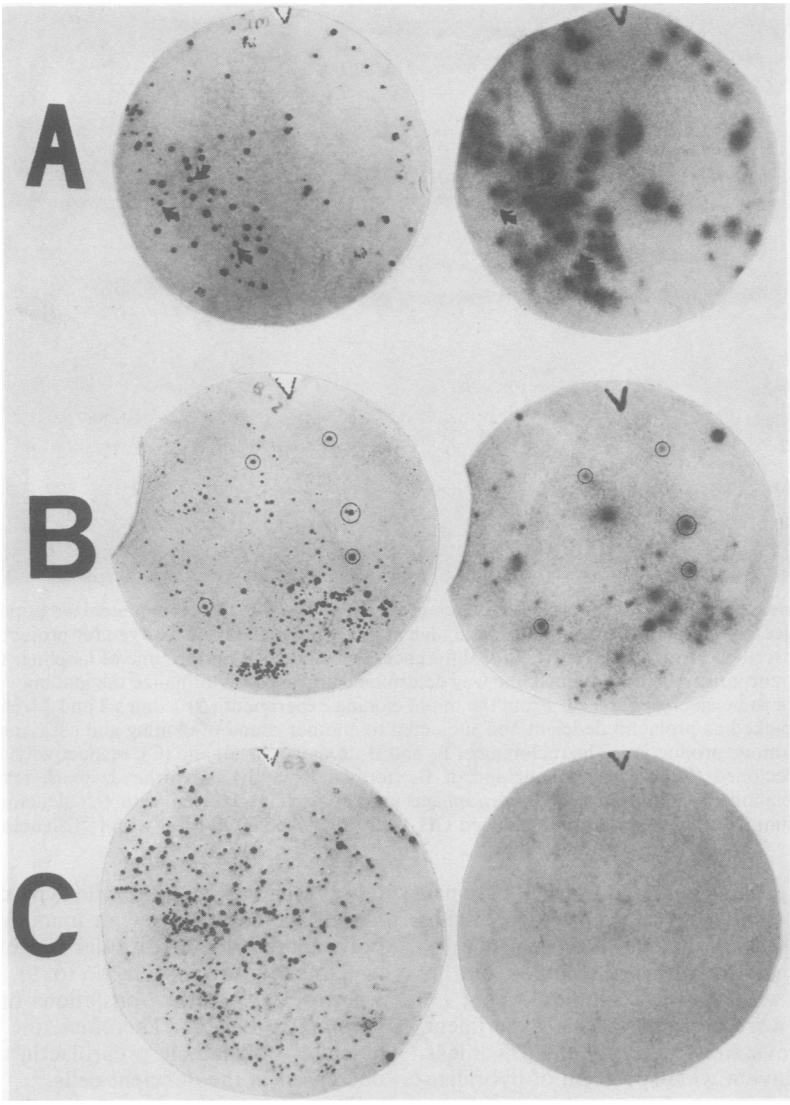

FIG. 4. Colony hybridization of replica-plated colonies of B_2, B_3, and unmutagenized GH_3 cells. B_2, B_3, and unmutagenized GH_3 cells were spread on agar plates. When colonies were visible, they were replica-plated to Whatman no. 54 filter paper, fixed, and hybridized to a nick-translated ^{32}P-labeled recombinant plasmid DNA containing a cDNA to preprolactin mRNA. On the left are the Coomassie blue-stained colonies, and on the right are the resulting autoradiograms. (A) Wild-type GH_3 cells; (B) B_2 cells; (C) B_3 cells. Arrowheads in (A) mark colonies giving a weak hybridization signal; another replica of GH_3 cells (not shown) contained 1 weakly hybridizing colony among 70 strongly hybridizing ones. It is likely that these weak signals came from occasional dead colonies on the plates. Invariably, 1 to 3% of the colonies grown on agar surfaces were nonviable in our procedure: they did not attach to dishes and failed to grow to mass culture. The cause of this is not known. Some colonies in (B) are circled to aid in aligning the autoradiograph with the stained replica.

RNA from untreated B_2 and B_3 cells. By comparison with RNA from untreated wild-type cells, the reduction in preprolactin mRNA in the variants was about 27-fold for B_2 and near 81-fold for B_3 cells.

Prolactin expression is clonally stable in unmutagenized GH_3 cells. Initially, the high frequency of prolactin-deficient cells in the EMS-treated population was thought to reflect a general property of the GH_3 line since it was found in a GH_3 cell population which, although mutagenized, had been cloned only 4 days after mutagenesis. This was clearly insufficient time for mutants to have accumulated in such high proportion and they must, therefore, have preexisted in the unmutagenized population. However, this con-

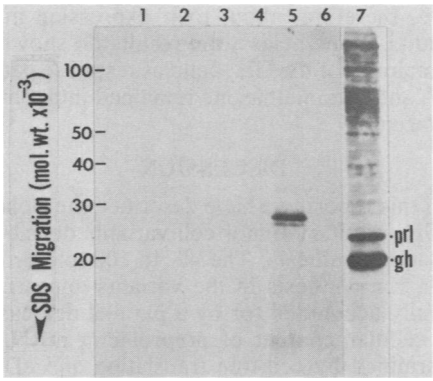

FIG. 5. Cell-free translation of total cytoplasmic RNA from B_2, B_3, and wild-type clone 11_1. Total cytoplasmic RNA (20 μg in each sample) from prolactin-deficient clones B_2 and B_3 and wild-type clone 11_1 was translated in a lysate from rabbit reticulocytes in the presence of [^{35}S]methionine. After translation, reactions were treated with 2 μl of antiprolactin serum (lanes 1, 3, 5) or nonimmune serum (lanes 2, 4, 6), and the precipitated proteins were assayed by electrophoresis on 12.5% SDS-polyacrylamide slab gels. Lanes 1 and 2 are from B_2 cells, lanes 3 and 4 are from B_3 cells, and lanes 5 and 6 are from wild-type clone 11_1.

clusion proved to be unwarranted since no prolactin-deficient clones have yet been found in 57 clones of *unmutagenized* GH_3 cells when assayed by [^{35}S]methionine labeling and SDS-polyacrylamide slab gel electrophoresis of the labeled medium. Figure 7 illustrates the results

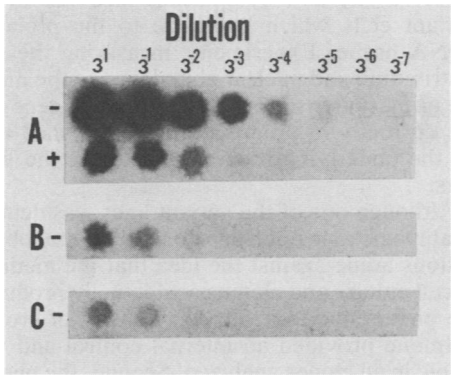

FIG. 6. RNA dot hydridization assays of total cytoplasmic RNA from B_2, B_3, and wild-type cells. Total cytoplasmic RNA was extracted from wild-type cells (A) treated with (+) and without (−) 10^{-6} M dexamethsone for 2 days and from untreated clones B_2 (C) and B_3 (D). Samples of each were then spotted in a volume of 3 μl onto nitrocellulose filters in high salts in a series of threefold dilutions. The initial amount of spotted RNA was 3 μg for each sample. Filters were then hybridized to nick-translated ^{32}P-labeled preprolactin cDNA plasmid as in Fig. 4, and hybridized counts were detected by autoradiography.

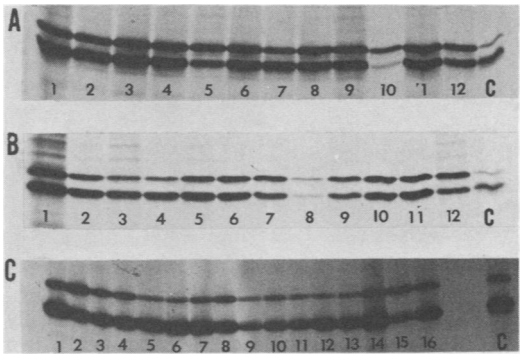

FIG. 7. Phenotypic stability of unmutagenized GH_3 cells. The original stock of the GH_3 cell line (termed GH_3D_6; see the text) was prepared in 1976 (A), and two subsequent stocks were prepared from this line in 1978 (B) and 1979 (C). These stocks bracket the time during which the EMS-mutagenized culture was screened for the variants described here. Note that the electrophoresed samples were loaded proportional to the level of [^{35}S]methionine incorporation of each clone except for clone 10 of (A) and clone 8 of (B), where 5 μl of medium was electrophoresed owing to a low cell density and incorporation level. (C) contains [^{14}C]leucine-labeled medium from T_3-induced GH_3 cells.

of these cloning experiments on stocks of GH_3 cells; including the original starter stock of GH_3 cells, which had been subcloned in 1976 from an original ATCC source, as well as two descendant stocks frozen and stored in 1978 and 1979. The 1979 stock was frozen just after the time that the EMS mutagenesis took place and the deficient variants were discovered. Clearly, these data substantiate the conclusion that the unmutagenized cell population contains few if any prolactin-deficient cells. They also imply that EMS may have "induced" the variants.

Prolactin and p21 corevert. In five variants, the reduction in prolactin synthesis was accompanied by a loss in p21 synthesis as well (Fig. 2). This result implied that the expression of the two proteins was coupled in some way. We therefore wanted to know whether lines having reverted to prolactin expression also regained p21 expression. Accordingly, a revertant line was isolated from the unstable B_2 line after two rounds of subcloning for high prolactin expression. The revertant was treated for 2 days with and without dexamethasone and then assayed for prolactin and p21 synthesis by two-dimensional gel electrophoresis as before. Figure 8 illustrates that both prolactin and p21 expression occurred at wild-type levels in the revertant line and both were repressible by dexamethasone. The expression of the two proteins is, therefore, tightly coupled in GH_3 cells.

Besides being repressed by glucocorticoid

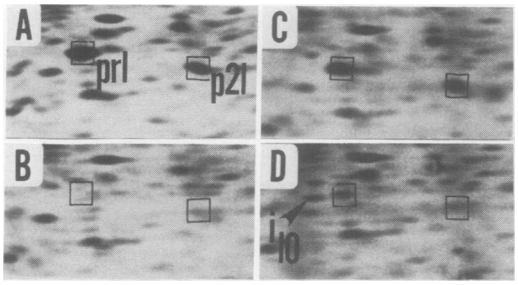

FIG. 8. Two-dimensional gel assay for p21 expression in a prolactin revertant cell line. From the clonally unstable B_2 line a revertant cell was isolated by two rounds of subcloning for high prolactin expression. The revertant line was treated for 2 days with (D) and without (C) 10^{-6} M dexamethasone and assayed for prolactin and p21 expression by two-dimensional gel electrophoresis as described in the legend to Fig. 2. For comparison, the same region of the gels from wild-type GH_3 cells and prolactin-deficient B_3 cells are shown in (A) and (B), respectively. Note that GH_3 and B_3 cells were not treated with dexamethasone and that they were labeled and electrophoresed in a separate experiment from that shown for the revertant line.

treatment, p21 synthesis is also induced by triiodothyronine treatment (12, 14). This raised the possibility that other thyroid hormone-sensitive gene products might be affected in the variants. However, in the experiments shown in Fig. 2 and 8, thyroid hormone responsiveness was not measured since cells were grown and labeled in medium supplemented with calf serum which contains inducing levels of thyroid hormone (26, 28). This also prevented assaying two other dexamethasone-repressible proteins, r-4 and r-5, whose synthesis is also repressible by thyroid hormone (12). Accordingly, B_2 and B_3 cells were treated with and without T_3 and dexamethasone, singly or together, in medium supplemented with 10% serum substitute (3). Figure 9 shows the resulting two-dimensional autoradiograms only from B_3 cells. The conclusions drawn from the data for B_3 cells also apply to B_2 cells as well. It can be seen that except for p21 and prolactin, the basal synthesis and hormonal responsiveness of all T_3- and dexamethasone-sensitive proteins detected in this experiment were unaltered in the B_3 variant, including basal synthesis and regulation of r-4 and r-5 by T_3 and dexamethasone. Furthermore, the induction of growth hormone and the protein p16 by T_3 and the synergistic interaction between T_3 and dexamethasone in inducing both proteins was unimpaired in the variant. Note that these conclusions apply only to the responsive proteins detected in this single experiment, since as shown previously in GH_3 and hepatoma cells (12, 15), not all hormonally responsive proteins are detected in any one experiment. Thus, six of the T_3-regulated proteins were not assayed in this experiment, and no conclusions can be made, therefore, about their expression in the variants. Nonetheless, the results do show that the majority of the GH_3 cellular response pattern to T_3 and dexamethasone remained intact in the B_3 variant.

DISCUSSION

In this report, we have described the isolation of GH_3 pituitary tumor cell variants deficient in prolactin synthesis. The 40- to 100-fold drop in prolactin synthesis in the variants appeared to be fully accounted for by a parallel decrease in the cellular content of preprolactin mRNA as determined by cell-free translation and cDNA-RNA hybridization assays. Thus, the process-(es) affected in the prolactin-deficient variants must involve some step before the cytoplasmic appearance of preprolactin mRNA, such as transcription of the gene or posttranscriptional processing and transport of the primary transcript. In this regard, Rosenfeld et al. (25) have recently shown that lines of medullary thyroid carcinoma tumor cells producing low levels of calcitonin express a cytoplasmic mRNA for calcitonin that is demonstrably larger than the message produced in high-producing tumor cell lines. Furthermore, they detected major changes in the abundance of nuclear transcripts in the low-producing cells, suggesting that changes in the structure of the calcitonin gene or in the processing of the primary transcript may have occurred in the generation of the low-producing phenotype. In preliminary experiments, we have not detected any new cytoplasmic mRNA's in the variant cells which hybridize to the prolactin cDNA probe. Experiments measuring the size distribution and nuclear abundance of the multiple primary transcripts of the prolactin gene (18) are currently in progress and should shed light on the underlying process affected in the variants.

Although one of the variant lines was demonstrably unstable upon subcloning, several observations argue against the idea that the methods of cell culture and cloning artifactually produced the phenotypic trait. First, synthesis of growth hormone provided an internal control and was stable in all clones analyzed. Second, the phenotypic effect was highly specific, limited to prolactin and to the steroid- and thyroid hormone-responsive protein p21 among the nearly 1,000 individual GH_3 cell proteins assayed by two-dimensional gel electrophoresis. Third, one variant (B_3) was phenotypically stable when assayed by serial subcloning and colony hybridization. Fourth, prolactin expression was clonally stable in unmutagenized GH_3 cells inasmuch as no prolactin-deficient cell was found among 57 clones analyzed. These cloning experiments on unmutagenized cells were conducted under identical culture conditions as were used for

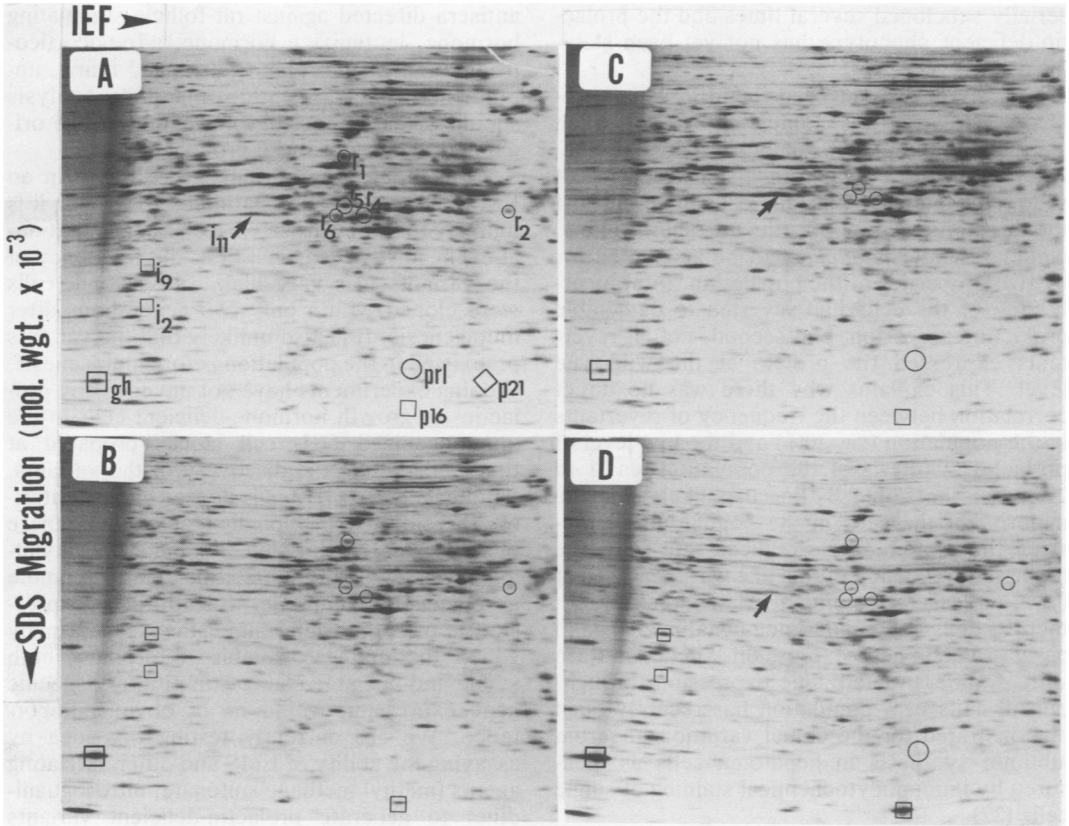

FIG. 9. Two-dimensional gel analysis of the response of prolactin-deficient B_3 cells to triiodothyronine and dexamethasone. B_3 cells were incubated for 2 days in Dulbecco modified Eagle medium supplemented with 10% serum substitute which lacked dexamethasone and T_3 but contained 10 μg of insulin per ml. Then 10^{-8}M T_3 and 10^{-6} M dexamethasone were added singly or together for an additional 2 days before pulse-labeling for 30 min with 250 μCi of [^{35}S]methionine per ml in methionine-free medium (12). Whole-cell lysates were electrophoresed in two dimensions using isoelectric focusing as the first dimension. The basic end of the gel is on the left and the acidic end is on the right. Inducible proteins are enclosed in squares, and repressible proteins are enclosed in circles. An identical sample (500,000 cpm) was electrophoresed for each experiment. (A) Control; (B) T_3 treated; (C) dexamethasone treated; (D) T_3 and dexamethasone treated.

cloning the mutagenized GH_3 cell population in which the variants were discovered. Hoyt and Tashjian (11) have also found that the GH_3 line is clonally stable in spite of the fact that the content of prolactin varied considerably among cells in the population when assayed immunocytochemically.

The production of both growth hormone and prolactin is known to be differentially affected by cell density (5, 10). High density stimulates growth hormone but inhibits prolactin production. Therefore, if the effect were large enough, some clones might have been scored incorrectly as "prolactin deficient" since it was difficult to ensure that all clones were at equal density at the time of assay. However, we tested whether highly dense cultures of wild-type cells could in fact be scored as prolactin deficient in the slab gel assay, and found that both prolactin and growth hormone were readily detectable at all cell densities, even at very high ones where cells were acidifying the medium and detaching from the dish (Ivarie and Morris, unpublished data). Thus, the cell density effect was not sufficiently large to introduce scoring errors in this analysis. Although cell density effects were minimized by normalizing the electrophoresis samples to the amount of labeled methionine incorporated by each clone, there was still considerable clonal variation in the production of both prolactin and growth hormone (Fig. 1, 3, and 7). Such variation has been reported for other cultured cell types (1, 21, 22) and has been utilized in one instance to isolate noninducible variants of rat hepatoma cells simply by serially subcloning HTC cells for low steroid inducibility of tyrosine aminotransferase (33, 34). Although this suggests that serial subcloning for a given phenotype may eventually generate stable cultured cell variants, the unstable B_2 line has been

serially subcloned several times and the prolactin-deficient phenotype has not yet been stabilized.

No variant has yet been found in which prolactin synthesis was completely absent when assayed by two-dimensional gels or by extremely long exposures of one-dimensional slab gels of secreted proteins. Thus, the cells have not lost the prolactin gene. For the unstable B_2 line, residual synthesis appears to be accounted for by two properties of the population. First, nearly 20% of the cells had reverted to detectable prolactin expression, and second, not all revertants expressed the protein at the wild-type level. This explains why there was no direct correlation between the frequency of revertants in the population (ca. 20%) and the low level of prolactin synthesis of the population (ca. 1 to 2%). For the stable B_3 line it is likely that the majority of the cells in the population synthesized the protein at the reduced rate since the reversion frequency of this line was measured at 0.5% or less by colony hybridization. If true, then B_3 cells have undergone a stable change in basal prolactin gene expression. Such a uniform shift in differentiated gene expression throughout all cells of a population has recently been demonstrated for the clonal variation in serum albumin synthesis in hepatoma cells as measured by immunocytochemical staining of single cells (22).

Although the variants were isolated solely on the basis of their lack of prolactin production, two-dimensional gel analysis revealed that the variants were also defective in synthesis of the steroid- and thyroid hormone-responsive protein p21. The finding that a revertant line expressing a high level of prolactin also expressed p21 at a high level confirms that the expression of the two proteins is linked in GH_3 cells. The two proteins share another similarity in that both are repressible by glucocorticoids. However, p21, but not prolactin, is inducible by thyroid hormone in GH_3 cells, implying that p21 is encoded by a distinct gene. Two other observations support this conclusion: p21 was not secreted from GH_3 cells (Fig. 1, 3, and 7), nor was it immunoreactive with antiprolactin serum (12). GH_3 cells also contain another protein termed p16 (12, 14), whose expression appears to be tightly coupled to the expression of growth hormone. For example, p16 has the same complex response pattern to thyroid hormone, glucocorticoids, and insulin as growth hormone, and its synthesis was absent from the growth hormone-deficient GH_4 line of GH_3 cells (Ivarie and Martial, unpublished data). Like p21, p16 is not secreted from GH_3 cells, nor is it reactive with anti-growth hormone serum. Although the identity of both proteins is unknown, neither appears to be a known pituitary hormone inasmuch as neither reacted with antisera directed against rat follicle stimulating hormone, leutenizing hormone, adrenocorticotropin, and β-endorphin (Morris and Ivarie, unpublished data). Peptide mapping analysis should reveal important information on the origin and identity of these proteins.

Even though the variants were found in an EMS-mutagenized population of GH_3 cells, it is unlikely that they arose via a classical mutational event for two reasons: first, the frequency of the variants was very high, and second, cells were cloned within only two generations after mutagenesis. It is also unlikely that the variants preexisted in the population before mutagenesis. Cloning experiments have not unveiled any prolactin- or growth hormone-deficient cells in the unmutagenized GH_3 cell stocks prepared at times bracketing the discovery of the variants. Unmutagenized GH_3 cells appear by this criterion, therefore, to be clonally stable. Given these observations, it is possible that the DNA alkylating agent EMS may in itself be able to promote an epigenetic change in prolactin gene expression. If true, then determining the mechanism by which the mutagen is able to promote both stable and unstable changes in pituitary-specific gene expression would be of obvious importance. We are currently testing this idea by assaying the ability of EMS and other alkylating agents (methyl methane sulfonate, nitrosoguanidine) to generate prolactin-deficient variants from the wild-type population. One possible mechanism is that EMS destabilizes expression of the prolactin gene by alkylating guanine and adenine residues within or flanking the gene. Such base modifications might promote methylation of nearby cytosine residues, thereby reducing prolactin gene transcription (reviewed in reference 23), or they might alter the ability of the gene to remain in an open, transcriptionally active configuration (29, 35). If p21 is encoded by a tightly linked gene, such effects might also extend to the p21 gene and account for the coexpression of the two proteins. However, a variety of other mechanisms can account for our data, including changes in the structure of the gene, in the processing of its primary transcript and eventual transport to the cytoplasm, or in the expression of an outside gene specifying a regulator of the expression of both prolactin and p21.

ACKNOWLEDGMENTS

We especially thank Dave Peterson and Keith Yamamoto for communicating the colony hybridization method prior to publication and Bob Lansman for aid with plasmid purification. Rabbit antiserum to rat prolactin was generously supplied by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases Rat Pituitary Hormone Distribution Program and by A. F. Parlow. Part of this work was performed at the Division of Endocrine Research, Department of Medicine, University of California, San Francisco.

This work was supported by Public Health Service grants CA-23046 and CA-28826 from the National Cancer Institute.

ADDENDUM IN PROOF

Recent experiments (Ivarie and Morris, Proc. Natl. Acad. Sci. U.S.A., in press) have shown that UV irradiation of GH_3 cells does not generate prolactin-deficient variants at high frequency. Moreover, the EMS effect appears to occur at the level of DNA methylation since the stable B_3 variant was reverted to wild type at 50% frequency by treating cells for one generation with 10 μM 5-azacytidine, an inhibitor of DNA methylation.

LITERATURE CITED

1. **Aviv, D., and E. B. Thompson.** 1972. Variation in tyrosine aminotransferase induction in HTC cell clones. Science **177**:1201–1203.
2. **Bancroft, F. C.** 1973. Measurement of growth hormone synthesis by rat pituitary cells in culture. Endocrinology **92**:1014–1021.
3. **Bauer, R. F., L. O. Arthur, and D. L. Fine.** 1976. Propagation of mouse mammary tumor cell lines and production of mouse mammary tumor virus in a serum-free medium. In Vitro **12**:558–563.
4. **Biswas, D. K., J. Lyons, and A. H. Tashjian, Jr.** 1977. Induction of prolactin synthesis in rat pituitary tumor cells by 5-bromodeoxyuridine. Cell **11**:431–439.
5. **Bolton, W. E., and A. E. Boyd, III.** 1980. Evaluation of growth hormone production from GH cells in vitro: effect of culture media and time in culture. In Vitro **16**:330–336.
6. **Chien, Y.-H., and E. B. Thompson.** 1980. Genomic organization of rat prolactin and growth hormone genes. Proc. Natl. Acad. Sci. U.S.A. **77**:4583–4587.
7. **Cooke, N. E., D. Coit, R. I. Weiner, J. D. Baxter, and J. A. Martial.** 1980. Structure of cloned DNA complementary to rat prolactin messinger RNA. J. Biol. Chem. **255**:6502–6510.
8. **Dannies, P. S., and A. H. Tashjian, Jr.** 1973. Effects of thyrotropin releasing hormone and hydrocortisone on synthesis and degradation of prolactin in a rat pituitary cell strain. J. Biol. Chem. **248**:6174–6179.
9. **Evans, G. A., and M. G. Rosenfeld.** 1979. Regulation of prolactin mRNA analyzed using a specific cDNA probe. J. Biol. Chem. **254**:8023–8030.
10. **Hang, E., J. Tjernshaugen, and K. M. Gautvik.** 1976. Variation in prolactin and growth hormone production during cellular growth in clonal strains of rat pituitary cells. J. Cell Physiol. **91**:15–30.
11. **Hoyt, R. F., Jr. and A. H. Tashjian, Jr.** 1980. Immunocytochemical analysis of prolactin production by monolayer cultures of GH_3 rat anterior pituitary tumor cells: variation in prolactin content of individual cell colonies and dynamics of stimulation with thyrotropin-releasing hormone. Anat. Rec. **197**:163–181.
12. **Ivarie, R. D., J. D. Baxter, and J. A. Morris.** 1981. Interaction of thyroid and glucocorticoid hormones in rat pituitary tumor cells: specificity and diversity of the responses analyzed by two-dimensional gel electrophoresis. J. Biol. Chem. **256**:4520–4528.
13. **Ivarie, R. D., and P. P. Jones.** 1979. A rapid sensitive assay for specific protein synthesis in cells and in cell-free translations: use of *Staphylococcus aureus* as an absorbent for immune complexes. Anal. Biochem. **97**:24–35.
14. **Ivarie, R. D., J. A. Morris, and N. L. Eberhardt.** 1980. Hormonal domains of response: actions of glucocorticoid and thyroid hormones in regulating pleiotropic responses in cultured cells. Rec. Prog. Horm. Res. **36**:195–239.
15. **Ivarie, R. D., and P. H. O'Farrell.** 1978. The glucocorticoid domain: steroid-mediated changes in the rate of synthesis of rat hepatoma proteins. Cell **13**:41–55.
16. **Martial, J. A., J. D. Baxter, H. M. Goodman, and P. H. Seeburg.** 1977. Regulation of growth hormone messenger RNA by thyroid and glucocorticoid hormones. Proc. Natl. Acad. Sci. U.S.A. **74**:1816–1820.
17. **Martial, J. A., P. H. Seeburg, D. Guenzi, H. M. Goodman, and J. D. Baxter.** 1977. Regulation of growth hormone gene expression: synergistic effects of thyroid and glucocorticoid hormones. Proc. Natl. Acad. Sci. U.S.A. **74**:4293–4295.
18. **Maurer, R. A., E. J. Gubbins, C. R. Erwin, and J. E. Donelson.** 1980. Comparison of potential nuclear precursors for prolactin and growth hormone messenger RNA. J. Biol. Chem. **255**:2243–2246.
19. **Nakane, P. K.** 1970. Classifications of anterior pituitary cell types with immunoenzyme histochemistry. J. Histochem. Cytochem. **18**:9–20.
20. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
21. **Peterson, J. A.** 1976. Clonal variation in albumin messenger RNA activity in hepatoma cells. Proc. Natl. Acad. Sci. U.S.A. **73**:2056–2060.
22. **Peterson, J. A.** 1979. Analysis of discontinuous variation in albumin production by hepatoma cells at the cellular level. Somatic Cell Genet. **5**:641–651.
23. **Razin, A., and A. D. Riggs.** 1980. DNA methylation and gene function. Science **210**:604–610.
24. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
25. **Rosenfeld, M. G., S. G. Amara, B. A. Roos, E. S. Ong, and R. M. Evans.** 1981. Altered expression of the calcitonin gene associated with RNA polymorphism. Nature (London) **290**:63–65.
26. **Samuels, H. H., Z. D. Horwitz, F. Stanley, J. Casanova, and L. E Shapiro.** 1977. Thyroid hormone controls glucocorticoid action in cultured GH_1 cells. Nature (London) **268**:254–257.
27. **Samuels, H. H., and L. E. Shapiro.** 1976. Thyroid hormone stimulates de novo growth hormone synthesis in cultured GH_1 cells: evidence for the accumulation of a rate limiting RNA species in the induction process. Proc. Natl. Acad. Sci. U.S.A. **73**:3369–3373.
28. **Samuels, H. H., J. S. Tsai, and R. Cintron.** 1973. Thyroid hormone action: a cell-culture system responsive to physiological concentrations of thyroid hormones. Science **181**:1253–1256.
29. **Stalder, J., A. Larsen, J. D. Engel, M. Dolan, M. Groudine, and H. Weintraub.** 1980. Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNAse I. Cell **20**:451–460.
30. **Tashjian, A. H., Jr., F. C. Bancroft, and L. Levine.** 1970. Production of both prolactin and growth hormone by clonal strains of rat pituitary tumor cells: differential effects of hydrocortisone and tissue extracts. J. Cell Biol. **47**:61–70.
31. **Tashjian, A. H., Jr., Y. Yasumura, L. Levine, G. H. Sato, and M. L. Parker.** 1968. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinology **82**:342–352.
32. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
33. **Thompson, E. B., D. Aviv, and M. E. Lippman.** 1977. Variants of HTC cells with low tyrosine aminotransferase inducibility and apparently normal glucocorticoid receptors. Endocrinology **100**:406–419.
34. **Thompson, E. B., D. K. Granner, T. Gelehrter, J. Erickson, and G. L. Hager.** 1979. Unlinked control of multiple glucocorticoid-induced processes in HTC cells. Mol. Cell. Endocrinol. **15**:135–150.
35. **Weisbrod, S., M. Groudine, and H. Weintraub.** 1980. Interaction of HMG 14 and 17 with actively transcribed gene. Cell **19**:289–301.
36. **Yamamoto, K. R., V. L. Chandler, S. R. Ross, D. S. Vicker, J. C. Ring, and S. C. Feinstein.** 1980. Integration and activity of mammary tumor virus genes: regulation by hormone receptors and chromosomal position. Cold Spring Harbor Symp. Quant. Biol. **45**:687–697.
37. **Yasumura, Y., A. H. Tashjian, Jr. and G. Sato.** 1966. Establishment of four functional clonal strains of animal cells in culture. Science **154**:1186–1188.