THE ANATOMICAL RECORD 197:153-162 (1980)

Immunocytochemical Analysis of Prolactin Production by Monolayer Cultures of GH_3 Rat Anterior Pituitary Tumor Cells:

I. Long-Term Effects of Stimulation With Thyrotropin-Releasing Hormone (TRH)

RICHARD F. HOYT, JR. AND ARMEN H. TASHJIAN, JR.
Department of Anatomy, Boston University School of Medicine, Boston, Massachusetts 02118, and Laboratory of Toxicology, Harvard School of Public Health, and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Thyrotropin-releasing hormone (TRH) stimulates prolactin production in cultured GH_3 rat anterior pituitary tumor cells. For correlation of cell-by-cell prolactin distribution and intracellular hormone concentration, GH_3 cells were grown to plateau-phase density on glass coverslips in plastic dishes. Acetone-fixed, cell-bearing coverslips were stained for prolactin by an immunoglobulin-peroxidase bridge technique (Mason et al., '69); cells on the plastic dishes were assayed for prolactin (microcomplement fixation immunoassay, Tashjian, '73) and protein content. Intracellular prolactin, unaffected quantitatively by acetone fixation and choice of substratum, was localized immunocytochemically by a granular brown precipitate, abolished if anti-prolactin serum was preabsorbed with rat prolactin or omitted from the protocol. Intracellular prolactin was maximized with colchicine (5.0×10^{-6} M; final 3 hr of incubation) in control and TRH-treated (10 ng/ml; 48 hr) GH_3 cell cultures. A total of 8,500 cells were classified by light microscopy as unstained, heavily (H) or moderately (M) stained for prolactin. In controls, 35% of cells were prolactin-positive: 6% H and 29% M. After TRH, 45% were positive: 7% H and 38% M. Although prolactin-positive cells were unevenly distributed, comprising 25% to 46% of cells in individual microscopic fields in controls, TRH increased the proportion of M cells in all areas. TRH treatment raised prolactin levels to 450% of control, but mathematical analysis attributed less than 30% of the increase to new prolactin-positive cells. We conclude that TRH acts on GH_3 cultures principally by raising the mean hormone content of individual positive cells rather than by increasing the proportion of cells committed to prolactin production.

Cultured strains of functional tumor cells have been under study in our laboratory for more than a decade. One of these, the GH_3 clone of rat anterior pituitary cells, has proved useful as a model for the production of growth hormone and prolactin and is, in fact, the biological system in which thyrotropin-releasing hormone (TRH) first was recognized as a prolactin secretagogue (Tashjian et al., '71).

The GH_3 cells are one of a series of so-called "GH" clonal strains isolated in 1965 from a growth hormone-producing rat anterior pituitary tumor (Tashjian et al., '68). This particular clone has been studied more extensively than any of the other original strains, and its functional characteristics have been reviewed by Tashjian and Hoyt ('72), Hinkle and Tashjian ('74) and Martin and Tashjian ('77).

GH_3 cells synthesize and release both growth hormone and prolactin (Tashjian et al., '70). The hormones are indistinguishable from the native rat hormones on the basis of their biological and immunological properties. Under basal conditions, prolactin and growth hormone account for 1–3% of the total protein synthesized by GH_3 cells, and this may rise to as much as 10% under certain experimental circumstances.

The hormones are released continuously and spontaneously into the culture medium at a

Received August 28, 1978; accepted October 17, 1979.

0003-276X/80/1972-0153$02.00

rapid rate (2–30 μg of hormone/mg cell protein in 24 hr), and they are not stored in appreciable quantities. Intracellular concentrations are equivalent only to the amount secreted during 15–30 minutes in the case of growth hormone, and 1–2 hours in the case of prolactin. Nonetheless, the hormone release mechanism responds predictably to the administration of inorganic cations (Ca^{++}, Mg^{++}, K^{+}) and secretory blocking agents such as colchicine and ergot alkaloids (Gautvik et al., '73; Gautvik and Tashjian, '73; Tashjian et al., '78). Treatment of GH_3 cells with colchicine (5.0×10^{-6} M) for three hours causes a 2- to 3-fold increase in intracellular concentrations of prolactin and growth hormone, and during the third hour of incubation, accumulation of prolactin in the culture medium is depressed to less than 40% of control values.

Hormone production in GH_3 cells is sensitive to a variety of physiological and pharmacological regulatory signals, including steroid hormones and hypothalamic releasing factors (Tashjian et al., '70; Tashjian et al., '71), and similar responses have been confirmed qualitatively in intact or freshly explanted human and animal anterior pituitary glands (Bridson and Kohler, '70; Jacobs et al., '73). Hydrocortisone (10^{-7} to 10^{-6} M) causes a 4- to 8-fold increase in growth hormone production and a 30–80% decrease in prolactin production. The actions of hydrocortisone exhibit a 24–48 hour lag period and require about 100 hours to reach a maximum. The hypothalamic tripeptide, TRH (3×10^{-9} M), causes a 2- to 5-fold increase in prolactin synthesis by GH_3 cells and a 50–70% reduction in growth hormone production. Elevation of the rate of prolactin synthesis by the cells is observable after 3–4 hours of drug treatment (Dannies and Tashjian, '74) and reaches a maximum after 24–48 hours.

Microcomplement fixation, radioimmunoassay and other bulk, biochemical techniques used to study GH_3 cells have measured only the sum of changes in hormone production among many thousands of individual cells in unsynchronized cultures. They reveal nothing of possible functional heterogeneity within the clone and so do not, of themselves, furnish a complete picture of hormone synthesis and release. In the absence of information about individual cells, investigators have assumed that all functionally active cells in the clone produce both growth hormone and prolactin and that specific pharmacological agents alter the balance of hormone production in the individual cell.

The importance of this procedural limitation has been underscored by immunocytochemical experiments showing that the majority of GH_3 cells apparently do not contain prolactin (Hoyt and Tashjian, '73), and by other studies in which prolactin and growth hormone have been localized simultaneously by means of immunoperoxidase double staining methods. Mazurkiewicz ('73) has reported that the parent GH_3 clone consists of prolactin-containing cells, growth hormone-containing cells, and cells devoid of both hormones, and that no individual cell contains more than one hormone at any given time. These observations at once raise an important question: namely, do pharmacologically-induced changes in hormone production merely reflect metabolic fluctuations in GH_3 cells already committed to production of a specific hormone or do they represent changes in the actual proportion of cells committed to its synthesis? Even recent, sophisticated biochemical studies employing specific cDNA probes for prolactin and growth hormone mRNA's (Evans et al., '78) have not addressed this point directly. While these experiments have shown a progressive increase in prolactin mRNA in GH_4 cells over the course of 48 hours exposure to TRH, they have not been able to distinguish mRNA made in preexisting as opposed to "new" prolactin-producing cells.

In order to study the prolactin-positive subpopulation of GH_3 cells and its response to various regulatory agents, we have developed and tested a procedure which permits us to examine microscopically the cell-by-cell distribution of prolactin within a monolayer culture and to measure the overall intracellular concentration of the hormone by microcomplement fixation immunoassay. This method has been used in the present and subsequent (see the accompanying paper, Hoyt and Tashjian, '80) experiments to define the size and uniformity of the prolactin-containing subpopulation of GH_3 cells under control conditions, to determine changes in the subpopulation in response to treatment with TRH, and to relate such changes to fluctuations in prolactin production by the same cultures.

MATERIALS AND METHODS

Cell cultures

The cell cultures studied in these experiments are subclones of the GH_3 strain of rat anterior pituitary tumor cells first established in monolayer culture by Tashjian et al. ('68). They have been maintained continuously in culture, with serial subcloning, for over 10 years. While most are propagated routinely as monolayers, certain of the subclones have been

adapted to suspension culture (Bancroft and Tashjian, '71). Some experiments were performed with GH_4 subclones (Tashjian et al., '73) of GH_3 cells and with non-hormone-producing R_5 rat fibroblasts.

Methods of cell culture

Monolayer cultures of GH_3 and GH_4 subclones and of R_5 fibroblasts were grown on either glass or Falcon plastic culture dishes in Ham's F 10 medium supplemented with 15% horse serum and 2.5% fetal calf serum (complete medium) as described by Tashjian et al. ('68). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were recovered for subculture by incubation with 0.1% Viokase in phosphate-buffered saline for 2–8 minutes at 37°C (Yasumura et al., '66). Dislodged cells were centrifuged and washed twice by resuspension in normal saline. They were suspended in complete F 10 medium for replating. In each experiment, all subcultures were made at uniform density from a single, pooled stock cell suspension. Suspension cultures of GH_3 and GH_4 subclones were grown in serum-supplemented Eagle's MEM medium in standard spinner flasks at 37°C (Bancroft and Tashjian, '71).

Synthetic TRH, a gift of Abbott Laboratories, was used in these and subsequent experiments at final concentrations of 10–50 ng/ml of medium, all of which are on the plateau of the dose-response curve (Tashjian et al., '71).

Cell culture for correlated morphological and immunochemical analysis

Glass coverslips (thinness #1) were soaked in a dilute solution of 7X detergent (Curtin Matheson Scientific, Inc., Woburn, Mass.), washed thoroughly in large volumes of glass-distilled water, rinsed in three changes of absolute ethanol and flamed. Pairs of sterilized 22mm × 22mm coverslips were placed in the bottom of 60mm diameter Falcon plastic culture dishes. Stock suspensions were seeded into the dishes, where cells settled on glass and plastic surfaces alike. Subcultures were allowed to grow to plateau-phase density (Tashjian et al., '68), and TRH was administered by addition of a small volume of concentrated stock solution directly to the medium.

At the termination of an experiment, cell-bearing coverslips were removed from the dishes, rinsed thoroughly in 0.05M phosphate-buffered saline (PBS), pH. 7.2, soaked for 5 minutes in normal saline, drained and fixed in cold (4°C) acetone for 2 minutes. They then were frozen on dry ice and stored at −20°C in tightly covered Columbia jars for a maximum of 1 week prior to immunoperoxidase localization studies. Cells on the plastic surface of the culture dishes were rinsed three times with saline and stored at −20°C, pending assay for protein and prolactin content.

Measurement of prolactin and total protein

Frozen monolayers were thawed, scraped into distilled water, and ruptured by sonic oscillation (Tashjian et al., '68). Intracellular prolactin was measured by microcomplement fixation immunoassay (Wasserman and Levine, '61; Levine, '68; Tashjian, '73). Results are expressed as μg prolactin/ml test sample or μg prolactin/mg cell protein. The reproducibility of this technique is ± 15–20%, and the lower limits of detectability are 20–30 ng prolactin/assay tube. Cell sonicates were assayed for total protein by the method of Lowry et al. ('51), using bovine serum albumin as standard.

Immunocytochemical localization of prolactin

Prolactin was localized in whole-cell preparations by a slight modification of the immunoglobulin-enzyme bridge method of Mason et al. ('69).

1) Antisera: The following specific antisera were used in our studies. *Rabbit anti-rat prolactin,* designated Ra 410–C5, was prepared against rat prolactin as described by Tashjian et al. ('70). This antiserum is related closely to that used in our laboratory for microcomplement fixation immunoassay for prolactin; the two sera represent successive bleedings of a single immunized rabbit and do not cross-react with rat growth hormone. *Goat anti-rabbit immune globulin* was obtained from Clinical Assays (Cambridge, Mass.). *Rabbit anti-horseradish* peroxidase was kindly provided by Drs. Conrad Hsu and Earle Zimmerman of the Columbia University College of Physicians and Surgeons. The authors also are indebted to Drs. Hsu and Zimmerman for instruction in the use of the triple-antibody localization procedure.

2) Staining protocol: For staining, acetone-fixed cell cultures were thawed, rinsed in three 5-minute changes of PBS at room temperature, and incubated as follows: *rabbit anti-prolactin* (1:200) for 30 minutes; *goat anti-rabbit immune globulin* (1:50) for 30 minutes; *rabbit anti-horseradish peroxidase* (1:100) for 30 minutes; *horseradish peroxidase* (Sigma Type VI, Sigma

Chemical, St. Louis, Mo.), 0.5 mg/ml, for 20 minutes. All incubations were performed at room temperature; antisera and enzyme were diluted with PBS and were pipetted directly onto coverslips placed on moistened filter paper in covered Petri dishes. Between successive incubations, coverslips were removed from the staining dishes and rinsed thoroughly in PBS.

Peroxidase activity was demonstrated in the monolayers using the method of Graham and Karnovsky ('66). The reaction was carried out for 15 minutes at room temperature with gentle, intermittent agitation. Cultures were washed in PBS for 15 minutes, dehydrated rapidly in graded ethanols and cleared in xylene. They were mounted on glass slides with Permount for light microscopic examination. In some instances, cells were fixed for 10 minutes in cold 2% aqueous OsO_4 prior to dehydration.

3) Specificity of the staining reaction: A series of GH_4C_1 cultures were treated with TRH (20 ng/ml for 36 hr). One group was immunostained routinely for prolactin. For a second group, *anti-prolactin* was omitted from the reaction, and for a third, the *anti-prolactin* serum was first absorbed at antigen-antibody equivalence with rat prolactin. Histochemical activity of innate peroxidase was determined on a fourth group of immunologically unreacted cultures, and as an additional control, the complete localization was applied to monolayers of R_5 fibroblasts.

Evaluation of the experimental design

In these and subsequent studies (see the accompanying paper) monolayer cultures were exposed to TRH for varying periods of time (½–144 hr). Some were treated with colchicine and others were not. In all cases, however, cells examined morphologically were grown on glass, fixed in acetone, and air-dried before being frozen, while cells examined by quantitative immunoassay were grown on plastic and were neither fixed nor dried prior to freezing. The following experiments were designed to determine the effects, if any, of such basic procedural differences on the prolactin content of GH_3 cell monolayers.

1) Effects of different substrata on the prolactin content of GH_3 cell monolayers: Eight plastic and eight glass 100mm diameter culture dishes were plated at a uniform density from a single stock suspension of GH_3 cells. The medium was changed after 24 hours. Four plastic and four glass dishes were given TRH (20 ng/ml, in saline). The remaining glass and plastic dishes each received an aliquot (0.1 ml) of vehicle. After 72 hours of reincubation, media samples were collected and frozen at −20°C for microcomplement fixation assay for prolactin. All culture dishes were washed with saline and stored at −20°C for measurement of intracellular prolactin and total protein.

2) Effect of acetone fixation on the prolactin content of GH cells: Because exposure to acetone reduces the protein content of GH_3 cells (see Results), it is impossible to compare the prolactin concentration (μg hormone/mg *cell protein*) in fixed and unfixed cells. Instead, one must rely on absolute hormone content of perfectly matched fixed and unfixed samples derived from a single pool of cells. Such a pool can be obtained by chemical treatment or physical scraping of monolayers, but this might alter the response of cells to fixation. Therefore, we chose to use suspension cultures of GH cells which are virtually identical to monolayers in terms of hormone production (Bancroft and Tashjian, '71; Dannies and Tashjian, '76).

Suspension cultures of GH_3, GH_4C_1, and GH_4C_3 subclones were pooled to a total volume of 80 ml, thoroughly mixed, and divided into two 40 ml aliquots, I and II. In each, cells were collected by centrifugation and washed twice by resuspension in normal saline. Cell pellet I then was resuspended in 40 ml of saline, centrifuged, and frozen at −20°C. Pellet II was fixed by suspension in 40 ml of cold (4°C) acetone, swirled, centrifuged, dried under an air jet, and frozen at −20°C. Each pellet was thawed, suspended in 8 ml of distilled water, ruptured by sonic oscillation, and assayed for prolactin and total protein.

Effects of long-term TRH treatment on the amount and distribution of prolactin in GH_3 cell monolayers

A stock suspension of GH_3 cells was subcultured at a uniform density onto glass coverslips in plastic dishes. The cultures were allowed to grow to plateau-phase density and were divided into two treatment groups which received saline blank or TRH (10 ng/ml) for 48 hours. All cultures were given colchicine (5.0×10^{-6} M) for the final 3 hours of incubation to decrease the release of prolactin from the cells (Gautvik et al., '73). This was done in order: 1) to establish a closer relationship between staining intensity and the rate of hormone synthesis than that prevailing under normal circumstances, where intracellular hormone reflects the balance of synthesis and unrestrained secretion; and 2) to maximize intracellular prolactin to visualize cells which, because of low rates of synthesis, might ordinarily escape detection by the immunoperoxidase localization.

The culture dishes were rinsed in saline and frozen, pending determination of intracellular prolactin and total cell protein. Cell-bearing coverslips from duplicate control and TRH-treated dishes all were stained simultaneously for prolactin. Each monolayer was examined to determine the intensity of the overall staining reaction. Then, all cells in randomly selected microscopic fields were classified visually as unstained, moderately stained, or heavily stained. At least seven fields, a total of 1600–2600 cells, were counted per culture. The results for each staining category were expressed as per cent of cells in the total sample. This procedure was performed with the aid of a grid ocular, and frequent reference was made to reaction controls for help in distinguishing specific from nonspecific, background staining. Counts were analyzed statistically by the F- and student's T-tests.

RESULTS

Evaluation of the experimental design

1) Effects of plastic vs glass substrata: GH_3 cells grew and produced prolactin equally well on glass and plastic substrates. No significant differences were observed in total cell protein, intracellular prolactin content, or rate of hormone production.

2) Effects of acetone fixation: Acetone fixation had no effect on the absolute amount of intracellular prolactin in suspension cultures of GH cells. At equivalent dilutions, the microcomplement fixation curves of acetone-fixed and unfixed samples were superimposable. However, the concentration of prolactin was almost twice as high in acetone-fixed cells as in controls (3.0 vs 1.6 μg prolactin/mg protein, respectively), apparently because acetone treatment leached soluble amino acids and low molecular weight polypeptides which contributed to the measured total cell protein (0.23 and 0.45 mg/ml of sonicate for fixed and unfixed cells, respectively).

Nature and specificity of the immunoperoxidase localization of prolactin[1]

1) Character of the staining reaction: Prolactin was localized as granular deposits of dark brown precipitate against a nongranular gold toning of the cytoplasm and slightly deeper, but again nongranular, coloring of the nucleoli. As shown in Figure 4, the intensity of the immunoperoxidase reaction varied from cell to cell. Some cells were heavily stained throughout their cytoplasm, even to the point of obscuring the nucleus. Others were diffusely stained to a lesser degree, and still others had sharply localized reactions, most commonly in the form of a juxtanuclear "cap" or an intense spot immediately beneath the plasmalemma. Some cells were essentially unstained.

2) Specificity of the staining reaction: Omission (Fig. 5) or prior absorption (Fig. 6) of *anti-prolactin* serum abolished the dark brown granular staining ordinarily so prominent in GH_4C_1 cells (Fig. 4). The nongranular, golden toning of the cytoplasm and nucleoli persisted, however, indicating that this "background" was nonspecific and unrelated to the presence of prolactin in the cells. This conclusion was reinforced by the occurrence of similar cytoplasmic toning in R_5 rat fibroblasts (Fig. 7) which neither synthesize nor store nor secrete prolactin. Nucleolar staining was not as prominent in the fibroblasts as in the pituitary cells. Additional controls showed that GH cells have no intrinsic peroxidase activity.

The problem of nonspecific staining is compounded somewhat by the fact that the monolayers are whole-mount preparations rather than histological sections. The intensity of the background is a partial function of the depth of cytoplasm and, therefore, will vary considerably, being less noticeable in attenuated than in compact cells (Fig. 7).

Effects of long-term TRH treatment on the amount and distribution of prolactin in GH_3 cell monolayers

Microcomplement fixation and immunoperoxidase localization techniques were applied to control and TRH-stimulated (10 ng/ml for 48 hr) GH_3 monolayers treated with colchicine (5.0×10^{-6} M) for the final 3 hours of incubation.

1) Prolactin content: TRH-treated cultures contained 1.5 μg prolactin/mg cell protein, as compared to 0.33 μg/mg protein in the controls.

2) Microscopic appearance: Preliminary examination of the stained monolayers gave the impression that TRH treatment increased the fraction of prolactin-positive cells in addition to raising the hormone content of the average cell. Among the prolactin-positive subpopulation in control monolayers (Fig. 8) the immunoperoxidase reaction was heterogeneous. Certain cells were stained heavily throughout the cytoplasm, others were stained more moderately, and many others contained only a few positive

[1]Figures 4-9 are included in the color section on page 197, at the end of the accompanying article.

TABLE 1. Prolactin-positive cell subpopulations counted in control and TRH-treated GH_3 monolayers

Treatment[2]	Microscopic fields[3]	Cells counted (total)	Prolactin-positive cells[1] (% total population) Heavily stained (H)	Moderately stained (M)	Total stained (H+M)
Control	14	3,675	6 ± 4	29 ± 6	35 ± 6
TRH	16	4,816	7 ± 4	38 ± 6	45 ± 8
	Significance (t-test)		ns ($p > 0.50$)	$p < 0.001$	$p < 0.001$

[1] Mean ± s.d.
[2] Cultures from a single stock cell suspension were grown to plateau-phase density and given normal saline or TRH (10 ng/ml for 48 hr). Colchicine (5×10^{-6} M) was added to all cultures for the final 3 hr of incubation.
[3] All microscopic fields from duplicate cultures; F-test showed no significant difference between duplicates.

granules. In TRH-treated monolayers (Fig. 9) the majority of positive cells contained moderate deposits of reaction product in the peripheral cytoplasm, a few cells were heavily stained throughout, and very few showed barely discernible dark granules. The localization of prolactin as a marginal ring or crescent in the periphery of moderately stained cells in both control and TRH-treated cultures indicates a concentration of hormone beneath the plasma membrane, probably the result of inhibition of secretion by colchicine.

Although Figures 8 and 9 were selected to illustrate maximum local concentrations of stained cells, it is clear that significant numbers of cells in both control and TRH-treated cultures were free of specific reaction product.

3) Counting and classification of prolactin-positive cells: Changes in the prolactin-positive subpopulation were quantified by systematically evaluating the staining intensities of a minimum of 1600 cells/monolayer. A total of 8500 cells in duplicate control and TRH-treated cultures were grouped into: 1) unstained cells, lacking granular reaction product; 2) heavily stained (H) cells, containing intense deposits of reaction product throughout the cytoplasm; and 3) moderately stained (M) cells—all other cells containing specific reaction product.

Excellent agreement was obtained between cell assignments in duplicate TRH-treated and control monolayers. Application of the F-test revealed no significant differences between duplicates for H ($p > 0.10$), M ($p > 0.50$), and unstained ($p > 0.50$) cell categories. Therefore, results from all individual microscopic fields in duplicate cultures were combined (Table 1). In control cultures, 35% of cells were prolactin positive; 6% were heavily stained, and 29% moderately stained. In TRH-treated cultures, 45% of cells contained prolactin, an increase of 10 positive cells/100 over controls, which was accounted for principally by a rise in the proportion of moderately stained cells (from 29/100 in controls to 38/100 after TRH). The proportion of heavily stained cells increased insignificantly to 7/100.

4) Regional variation in the prolactin-positive subpopulation: The size of the prolactin-positive subpopulation varied from area to area within the monolayers (Fig. 1). The proportion of stained cells ranged from 25 to 46/100 in the microscopic fields counted from controls (median, 35/100) and from 31 to 59/100 in TRH-treated cultures (median, 46/100). If, for each microscopic field, heavily and moderately stained cells are plotted separately against total prolactin-positive cells (Fig. 2), it can be seen that moderately stained cells formed the bulk of the positive subpopulation in all fields examined, with the exception of a single control field where the M and H subgroups were equal.

Direct comparisons between the small, median, and large positive subpopulations from control and TRH-treated cultures (Fig. 3) show that the initial size of the total positive subpopulation did not affect the response to TRH. The overall rise of 10 positive cells/100 observed following TRH treatment reflected a substantial increase in the proportion of M cells in prolactin-positive subpopulations of all sizes. There was no significant effect on the proportion of H cells in any positive subpopulation, regardless of initial size.

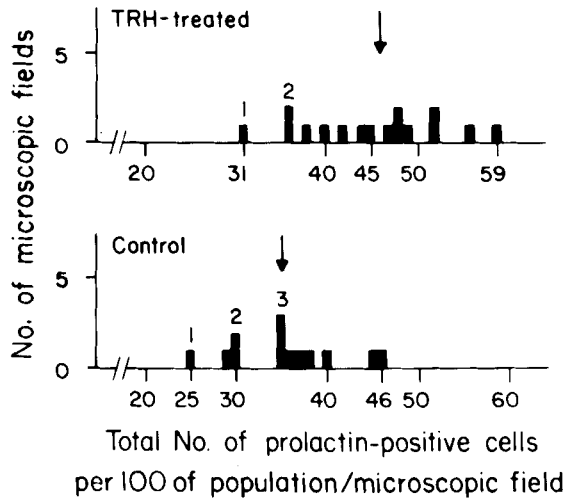

Fig. 1. Regional variation in the percentage of GH_3 cells containing immunocytochemically demonstrable prolactin. All cells in a total of 16 microscopic fields from duplicate TRH-treated (10 ng/ml for 48 hr) cultures were classified as unstained or heavily or moderately stained for prolactin. In control cultures, normal saline was substituted for TRH, and a total of 14 microscopic fields were examined. The median of each population distribution is indicated by an arrow.

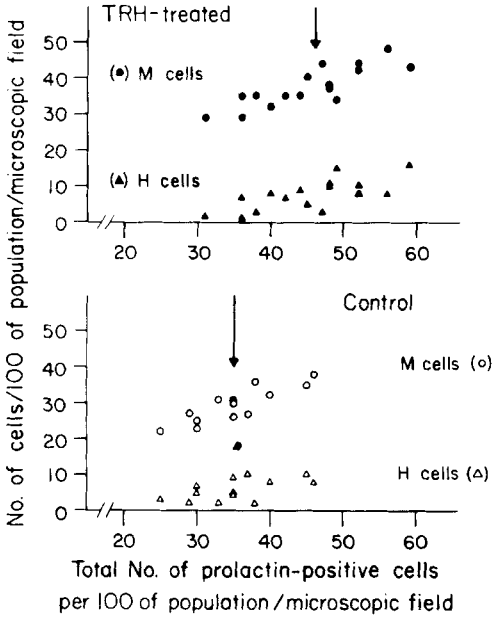

Fig. 2. Composition of the prolactin-positive cell subpopulations in the control and TRH-treated GH_3 monolayers shown in Figure 1. For each microscopic field, the percentage of heavily stained (H) and moderately stained (M) cells are plotted against that for total positive (H + M) cells. The median of each population distribution is indicated by an arrow.

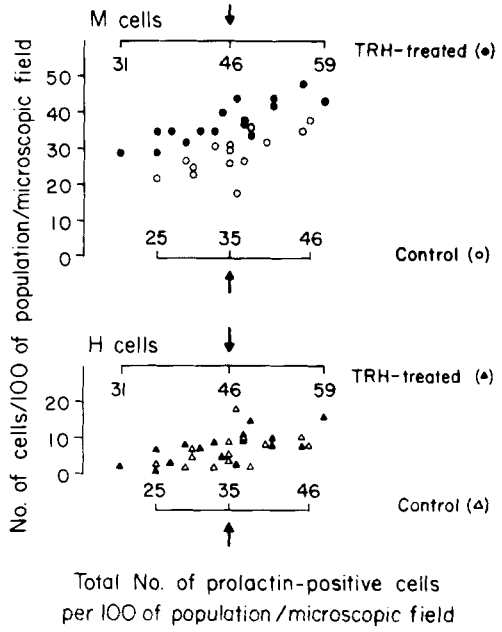

Fig. 3. Effects of TRH on the composition of the prolactin-positive GH_3 cell subpopulation. The data from Figure 2 have been replotted to permit direct comparison of the proportion of M cells and H cells at corresponding points on the population distributions in control and TRH-treated cultures. The median points (arrows) of the distributions have been aligned by shifting the distribution in TRH-treated specimens (upper scale) to the left.

DISCUSSION

Our aim was to determine whether TRH affects GH_3 cells by stimulating prolactin synthesis only in previously committed prolactin-producing cells or by recruiting previously noncommitted cells to prolactin production. We approached this problem by comparing the microscopic, cell-by-cell distribution of prolactin with the bulk intracellular hormone concentration under control conditions and after stimulation with TRH. In so doing, we were faced with three important technical questions, namely: 1) the validity of equating prolactin measured in plastic-grown, unfixed cells with that in glass-grown, acetone-fixed cells used in the immunoperoxidase studies; 2) the specificity of the prolactin localization procedure; and 3) the reliability of the method for quantitatively classifying cells according to their immunostaining characteristics.

Since the choice of glass vs plastic culture dishes had no significant effect on growth rate or prolactin production, and since acetone-fixation did not alter absolute, measurable intracellular prolactin in GH cells, we conclude that the hormone content of immunoperoxidase labeled GH_3 cell monolayers on glass coverslips was closely approximated by quantitative assay of similar cells growing on adjacent areas of the plastic culture dish.

Our confidence in the prolactin-specificity of the immunoperoxidase reaction is firmly based on the procedural and biological controls illustrated in Figures 4–7 and on the fact that: 1) the method has demonstrated similar patterns of localization in three closely related prolactin-producing cell strains (GH_3, GH_4C_1, GH_4C_3); 2) these localization patterns have proved consistent in an extended series of experiments (see the accompanying paper, Hoyt and Tashjian, '80); 3) the localizations in control and TRH-treated GH cell cultures (Hoyt and Tashjian, '73) have been confirmed by Gautvik and Fossum ('76) in GH_4C_1 cells using a different anti-prolactin serum with tetramethylrhodamine isothiocyanate as marker.

Microscopic examination of the stained cultures involved continuous refocusing in order to detect granular deposits of *specific* reaction product at all levels through the depth of the cell layer. Faint, nongranular background staining, compensated for by frequent reference to control preparations, posed no serious obstacle to optical assessment of the cultures. The cell classification procedure proved highly reliable: statistical analysis (F-test) revealed no significant differences in results obtained among 10 pairs of duplicate cultures (42,405 cells, total) counted in this and the accompanying study.

Heterogeneity of GH cells with respect to prolactin production

Immunoperoxidase localizations revealed that only a fraction of cells in GH_3 and GH_4C_1 cultures contained prolactin, even after stimulation with TRH. Moreover, intracellular prolactin varied widely in amount from one positive cell to another, and colchicine treatment for three hours failed to alter this heterogeneous pattern of distribution, despite the fact that inhibition of prolactin release resulted in substantial accumulations of hormone beneath the plasma membrane. These observations suggest that variation of prolactin content among individual cells reflects differences in the rate of hormone synthesis rather than release; they accord with biochemical measurements of prolactin synthesis in single-cell cultures of GH_4C_1 cells (Gautvik and Fossum, '76).

Regional variation in the proportion of prolactin-containing GH_3 cells

Cell counting of colchicine-treated cultures revealed that the relative size of the prolactin-positive subpopulation varied considerably from area to area within a given monolayer, reflecting differences in the proportion primarily of M cells and, to a much lesser degree, of H cells. For this there are three likely explanations. Firstly, the original clone, over successive generations in culture, may have spawned many subclones with unique proportions of prolactin-producing cells. Confirmation of this idea depends on immunostaining and cell counting in large numbers of subclones, because in the present experiments, subcultures were seeded at densities that precluded identification of the descendents of individual cells. Secondly, there may exist a cell cycle-dependent control of prolactin production, manifest as differing amounts of intracellular hormone among a nonsynchronous population of cells. Lastly, local cell-cell contacts may influence the amount of prolactin in any given cell at the time of harvest.

We wish to make it clear that we do not interpret our data to indicate that a large proportion of GH_3 or GH_4C_1 cells are permanently incapable of synthesizing prolactin. On the contrary, we have never obtained a prolactin-nonproducing clone (Hoyt and Tashjian, '80), even after as many as 40 serial, single-cell cloning operations (Tashjian, unpublished observations),

and Gautvik and Fossum ('76) have shown that while isolated, single GH_4C_1 cells may or may not synthesize prolactin, all such cells give rise to prolactin-producing cell colonies.

Effects of TRH on prolactin-positive GH_3 cell subpopulations

Regional differences in the proportion of prolactin-positive GH_3 cells had no effect on the nature of the response to TRH treatment, which was an increase in the percentage of M cells and an increase in their average staining intensity. There was no significant change in the proportion of H cells.

Recruitment of prolactin-nonproducing cells as a factor in the response to TRH treatment

The degree to which "new" positive cells might account for the measured increase in intracellular prolactin (PRL) can be estimated mathematically.

In expressing the number of prolactin-positive cells as per cent of the total, we have defined in each monolayer a typical, 100-cell population whose prolactin (PRL) content:

$$\#1 \quad (\mu g\ PRL/100\ cells) = (\mu g\ PRL/mg\ cell\ protein) \times (mg\ protein/cell) \times 100$$

where (μg prolactin/mg protein) is the intracellular prolactin concentration in the monolayer, measured by microcomplement fixation and Lowry protein assay.

Therefore, in TRH-treated ($_T$) and control ($_C$) monolayers:

$$\#2 \quad \left[\frac{(\mu g\ PRL/100\ cells)_T}{(\mu g\ PRL/100\ cells)_C}\right] = \left[\frac{(\mu g\ PRL/mg\ protein)_T \times (mg\ protein/cell)_T \times 100}{(\mu g\ PRL/mg\ protein)_C \times (mg\ protein/cell)_C \times 100}\right]$$

Because TRH does not significantly affect the mean protein content of GH_3 cells (Tashjian and Hoyt, '72; Hoyt and Tashjian, '80):

$$\#3 \quad \left[\frac{(\mu g\ PRL/100\ cells)_T}{(\mu g\ PRL/100\ cells)_C}\right] = \left[\frac{(\mu g\ PRL/mg\ protein)_T}{(\mu g\ PRL/mg\ protein)_C}\right]$$

= 4.5 in the present experiments, by direct measurement.

Having characterized the typical, 100-cell population by cell counting, one can compare the mean prolactin content of control and TRH-treated GH_3 cells. In control cultures, the mean prolactin content of each positive cell must be:

$$\#4 \quad 100\%\ PRL_C/35\ cells_C = 2.9\%\ PRL_C/cell_C$$

If newly identified positive cells were to account for the entire increase of prolactin measured after TRH, then each should contain:

$$\#5 \quad 350\%\ PRL_C/10\ new\ cells = 35\%\ PRL_C/new\ cell$$

This cannot be the case. Such cells, with 12 times the mean prolactin content of positive control cells (#4), should be highly immunoreactive, and yet no increase of heavily stained cells was observed.

Alternatively, if total hormone in TRH-treated cultures were distributed among the entire positive-cell subpopulation, then the mean prolactin content of each cell should be:

$$\#6 \quad 450\%\ PRL_C/45\ cells_T = 10\%\ PRL_C/cell_T$$

or roughly 3.5 times the corresponding control value (#4). This is consistent with the microscopic appearance of the stained monolayers.

For the simple reason that the new positive cells could not be identified as heavily stained, their mean hormone content cannot greatly exceed that among the entire positive subpopulation in TRH-treated cultures (#6). On this basis, the newly identified positive cells can contain no more than:

$$\#7 \quad 10\%\ PRL_C/cell_T \times 10\ new\ cells = 100\%\ PRL_C$$

or less than 30% of the total increase (350%$_C$) of prolactin measured after TRH treatment.

The preceeding analysis assumes that all biochemically measured hormone resides in stained cells, but this may not be totally accurate. Masur et al. ('74) have shown that certain GH cells contain growth hormone at concentrations too low to give an immunoreaction readily detectable by light microscopy. Such also may be the case with respect to prolactin in certain of our GH_3 cells. Nevertheless, the amount of prolactin sequestered in unstained cells must be low even in unstimulated controls. It is unlikely that as much as 50% of total hormone present in stained cells could be hidden from view in the unstained population, for even very faintly stained cells must contain far less prolactin than their intensely or moderately stained counterparts.

The presence of even moderate amounts of prolactin in unstained control cells would not alter significantly the outcome of our simple mathematical argument, except possibly to reduce still further the proportion of increased prolactin attributable to newly identified positive cells. In fact, extrapolation of the findings of Masur et al. ('74) to our cultures suggests that the appearance of "new" positive cells in

TRH-treated monolayers may reflect an increase of hormone synthesis in occult, prolactin-containing cells rather than a recruitment of naïve cells to prolactin production.

Thus, although the present experiments do not rule out the possibility that TRH might in some way influence the differentiation of cells newly formed through division of multicompetent "stem" cells, or induce prolactin synthesis in preexisting but uncommitted cells, it seems clear that such mechanisms were not major factors in the response of our GH_3 cell cultures to long-term stimulation with TRH.

ACKNOWLEDGMENTS

We are indebted to Yolanda Santo and Norma Barowsky Glazer for expert technical assistance; to Dr. Henry Feldman for statistical analysis; and to Dr. Priscilla Dannies for thoughtful discussion and helpful criticism.

This work was supported in part by Grant AM 11011 from the National Institute of Arthritis, Metabolism and Digestive Diseases and by US Public Health Service General Research Support, administered through the Boston University School of Medicine.

Dr. Hoyt was a Fellow in Pharmacology—Morphology of the Pharmaceutical Manufacturers Association Foundation. Reprint requests should be addressed to Dr. Hoyt at the Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118.

LITERATURE CITED

Bancroft, F.C., and A.H. Tashjian, Jr. (1971) Growth in suspension culture of rat pituitary cells which produce growth hormone and prolactin. Exp. Cell Res., *64:*125–128.

Bridson, W.E., and P.O. Kohler (1970) Cortisol stimulation of growth hormone production by human pituitary tissue in culture. J. Clin. Endocrinol. Metab., *30:*538–540.

Dannies, P.S., and A.H. Tashjian, Jr. (1974) Pyroglutamylhistidyl-prolineamide (TRH): a neurohormone which affects the release and synthesis of prolactin and thyrotropin. Israel J. Med. Sci., *10:*1294–1304.

Dannies, P.S., and A.H. Tashjian, Jr. (1976) Thyrotropin-releasing hormone increases prolactin mRNA activity in the cytoplasm of GH-cells as measured by translation in a wheat germ cell-free system. Biochem. Biophys. Res. Commun., *70:*1180–1189.

Evans, G.A., D.N. David, and M.G. Rosenfeld (1978) Regulation of prolactin and somatotropin mRNAs by thyroliberin. Proc. Natl. Acad. Sci. USA, *75:*1294–1298.

Gautvik, K.M., and S. Fossum (1976) Basal and thyroliberin-stimulated prolactin synthesis in single-cell cultures and in populations of rat pituitary cells. Biochem. J. (Cellular Aspects), *158:*119–125.

Gautvik, K.M., R.F. Hoyt, Jr., and A.H. Tashjian, Jr. (1973) Effects of colchicine and 2-Br-α-ergocryptine-methanesulfonate (CB 154) on the release of prolactin and growth hormone by functional pituitary tumor cells in culture. J. Cell. Physiol., *82:*401–410.

Gautvik, K.M., and A.H. Tashjian, Jr. (1973) Effects of Ca^{++} and Mg^{++} on secretion and synthesis of growth hormone and prolactin by clonal strains of tumor cells in culture. Endocrinology, *93:*573–583.

Graham, R.C., and M.J. Karnovsky (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem., *14:*291–302.

Hinkel, P.M., and A.H. Tashjian, Jr. (1974) Interaction of thyrotropin-releasing hormone with pituitary cells in culture. In: Hormones and Cancer. K. McKerns, ed. Academic Press, New York, pp. 203–227.

Hoyt, R.F., Jr., and A.H. Tashjian, Jr. (1973) Immunocytochemical localization of prolactin in clonal strains of rat anterior pituitary tumor cells in monolayer culture. Anat. Rec., *175:*347 (Abstract).

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: II. Variation in prolactin content of individual cell colonies, and dynamics of stimulation with thyrotropin-releasing hormone (TRH). Anat. Rec., *196:*163–181.

Jacobs, L.S., P.J. Snyder, R.D. Utiger, and W.H. Daughaday (1973) Prolactin response to thyrotropin-releasing hormone in normal subjects. J. Clin. Endocrinol. Metab., *36:*1069–1073.

Levine, L. (1968) Microcomplement fixation. In: Handbook of Experimental Immunology. D.M. Weir, ed. Blackwell Scientific Publications, Ltd., Oxford, pp. 707–719.

Lowry, O.H., N.F. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:*265–275.

Martin, T.F.J., and A.H. Tashjian, Jr. (1977) Cell culture studies of thyrotropin-releasing hormone action. In: Biochemical Action of Hormones. G. Litwack, ed. Academic Press, New York, Vol. 4, pp. 269–312.

Mason, T.E., R.F. Phifer, S.S. Spicer, R.A. Swallow, and R.B. Doeskin (1969) An immunoglobulin-enzyme bridge method for localizing tissue antigens. J. Histochem. Cytochem., *17:*563–569.

Masur, S.K., E. Holtzmann, and F.C. Bancroft (1974) Localization within cloned rat pituitary tumor cells of material that binds antigrowth hormone antibody. J. Histochem. Cytochem., *22:*385–394.

Mazurkiewicz, J.E. (1973) Differentiation of rat anterior pituitary tumor cells in tissue culture. Anat. Rec., *175:*387 (Abstract).

Tashjian, A.H., Jr. (1973) Micro-complement fixation. In: Peptide Hormones. S.A. Berson and R.S. Yalow, eds. North Holland Publishing Company, Amsterdam, pp. 141–153.

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. J. Cell Biol., *47:*61–70.

Tashjian, A.H., Jr., N.J. Barowsky, and D.K. Jensen (1971) Thyrotropin releasing hormone: direct evidence for stimulation of prolactin production by pituitary cells in culture. Biochem. Biophys. Res. Commun., *43:*516–523.

Tashjian, A.H., Jr., P.M. Hinkle, and P.S. Dannies (1973) Control of growth hormone and prolactin production by clonal strains of pituitary cells. In: Endocrinology. R.O. Scow, ed. Exerpta Medica, American Elsevier, Amsterdam, pp. 648–654.

Tashjian, A.H., Jr., and R.F. Hoyt, Jr. (1972) Transient controls of organ-specific functions in pituitary cells in culture. In: Molecular Genetics and Developmental Biology. M. Sussman, ed. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, pp. 353–387.

Tashjian, A.H., Jr., M.E. LoMedico, and D. Maina (1978) Role of calcium in the thyrotropin-releasing hormone-stimulated release of prolactin from pituitary cells in culture. Biochem. Biophys. Res. Commun., *81:*798–806.

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.

Wasserman, E., and L. Levin (1961) Quantitative microcomplement fixation and its use in the study of antigenic structure by specific antigen-antibody inhibition. J. Immunol., *87:*290–295.

Yasumura, Y., A.H. Tashjian, Jr., and G.H. Sato (1966) Establishment of four functional, clonal strains of animal cells in culture. Science, *154:*1186–1189.