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The Level of Expression of the Rat Growth Hormone Gene in Liver Tumor Cells Is at Least Eight Orders of Magnitude Less Than That in Anterior Pituitary Cells

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Rat liver hepatoma cells (HTC) which express liver-specific gene products were assayed for the expression of the rat growth hormone (rGH) gene, which is normally expressed in anterior pituitary somatotrophs. The combination of immunoprecipitation and two-dimensional gel electrophoresis provided a highly sensitive assay for rGH synthesis at levels as low as one part in 10^9 of cell protein synthesis (or four molecules of rGH per cell). No rGH expression was detected at this level. The lack of expression in HTC cells did not derive from a deletion of the rGH gene, as shown by Southern hybridization analysis of genomic DNA. Because the gene is expressed at >30% of anterior pituitary protein synthesis, differentiation regulated rGH expression by over 10^8-fold between the two cell types. Additionally, DNA-excess solution hybridization was used to measure the level of rGH mRNA sequences. A novel and general method for preparing single-strand probes from recombinant plasmids was developed. Hybridization analyses with a sensitivity of detection of 1 part in 10^8 failed to detect any rGH RNA sequences in either the nucleus or cytoplasm of HTC cells. It is concluded, therefore, that the restriction in rGH expression in the liver tumor cells is likely to occur at the level of the transcription of the gene, and that for all practical purposes, the rGH gene is completely shut off in the hepatoma cells.

Known mechanisms of gene repression in microbial systems are subject to some leakage as expected from the equilibrium of the binding between repressors and operators. A well-studied example is the lactose operon of *Escherichia coli*. Although one of the most efficiently repressed operons, basal expression in the fully repressed state allows a β-galactosidase level about one-thousandth of the fully induced level (3). Any regulatory systems based on a similar mechanism might be expected at most to function across a comparable range.

In multicellular organisms, developmental regulation of gene expression can result in a heritable switch in the activity of a specific gene, either to a state of inactivity characterized by exceedingly low levels of basal expression or to a state of activity characterized by substantially high levels of expression (reviewed in references 5 and 33). As a consequence, different patterns of gene expression are established in various cell types of the organism, and these have been extensively characterized in the analysis of developmental regulation. Although previous studies (10–13, 21, 23, 32, 36) have shown that gene activity is very efficiently repressed in cell types in which expression of that gene would be inappropriate, the degree to which a differentially expressed gene can be held inactive is still uncertain.

In this report, we determined the extent to which expression of the differentially expressed rat growth hormone (rGH) gene could be inactivated by the cell type-specific mode of control. Our analysis is notable because of its unusually high sensitivity, allowing detection at a level two orders of magnitude below the limit assays used in earlier studies. By combining immunoprecipitation (14, 15) and two-dimensional protein separation (20, 25, 26, 28), we showed that the level of expression of the rGH gene in a rat liver cell line (HTC) is less than 1 part in 10^9 of cell protein synthesis. This represents about four rGH polypeptides per cell or, assuming normal processing and translation, about 0.01 to 0.001 mRNA molecule for rGH per cell. The difference between the level of expression of the gene in HTC cells and that in anterior pituitary cells, which are the normal site of rGH production,

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exceeds 10^8-fold, a value five orders of magnitude above that achieved in the lactose operon.

MATERIALS AND METHODS

Cell lines and culture conditions. HTC_4 and GH_3D_6 cells used in this analysis are clonal derivatives of the original HTC and GH_3 lines (14, 16, 20). They were grown as monolayers in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum at 37°C in 95% humidity and 10% CO_2 (14); for large-scale RNA preparations, HTC cells were also grown as suspension cultures in Swimm 77 medium containing 10% calf serum (20).

Preparation of labeled cell extracts. HTC_4 cells growing as monolayers in 100-mm plastic petri dishes (Falcon Plastics) were treated overnight with 10^{-6} M dexamethasone and labeled with 500 μCi of [^{35}S]methionine (900 to 1,000 Ci/mmol; Amersham Corp.) per ml (2.5 ml per dish) in methionine-free Dulbecco modified Eagle medium (14). Cells were rinsed three times with ice-cold phosphate-buffered saline (0.025 M potassium phosphate [pH 7.4], 0.1 M sodium chloride) and lysed in buffer (1.5 ml per plate) containing 0.5% Nonidet P-40 (14, 15). Bovine serum albumin was added to 5 mg/ml, and nuclei were removed by centrifugation at low speed; 100,000 × *g* supernatants (S100) were prepared by centrifugation for 10 min at 30 lb/in^2 in a Beckman airfuge. Acid-insoluble radioactivity was assayed by trichloroacetic acid precipitation (20).

Immunoprecipitation. Labeled HTC_4 cell extracts (1.5 ml each) were reacted with 1 μl of nonimmune or anti-rGH serum from rhesus monkeys (14). Immune complexes were collected by adsorption to heat-inactivated, Formalin-treated *Staphylococcus aureus* (15).

[^{35}S]methionine-labeled rGH. GH_3D_6 cells were induced for 2 days with 10^{-8} M triiodothyronine and 10^{-6} M dexamethasone in a 60-mm petri dish and labeled for 3 h with 100 μCi of [^{35}S]methionine per ml. Medium was removed and cleared of cells and debris by low-speed centrifugation. Labeled rGH was then isolated and quantitated by specific precipitation with anti-rGH serum (15).

rGH synthesis in anterior pituitary cells. Pituitaries were excised from four female Sprague-Dawley rats, and the anterior lobes were labeled for 3 h with 600 μCi of [^{35}S]methionine per ml as described previously (17). Cells were washed three times in ice-cold phosphate-buffered saline by low-speed centrifugation and lysed, and an S100 supernatant was prepared as described above. Acid-insoluble radioactivity was determined, and labeled rGH was quantitated by specific immunoprecipitation with anti-rGH serum.

Two-dimensional gel electrophoresis. Proteins solubilized in lysis buffer (25) were electrophoresed in two dimensions with nonequilibrium pH gel electrophoresis (NEPHGE) for the first dimension (26) and 10 to 16% exponential acrylamide gradient slab gels for the second dimension (14, 20). Destained gels were processed for fluorography as described by Bonner and Laskey (4).

mRNA titration. Cytoplasmic and nuclear RNAs were extracted from GH_3 and HTC_4 cells preinduced for 2 days with triiodothyronine (10^{-8} M) and dexamethasone (10^{-6} M). Cells were harvested and lysed by homogenization in 10 mM Tris-hydrochloride (pH 7.6)–10 mM NaCl–3 mM $MgCl_2$–0.5% Nonidet P-40 (19). Nuclei were isolated by centrifugation at 2,000 × *g* at 2°C. RNA was extracted from each fraction by 6 M guanidinium thiocyanate solubilization and sedimentation of the solubilized RNA through CsCl density gradients (37). Hybridization reactions contained the indicated amounts of RNA (see the legend to Fig. 4) and 10 pg of ^{32}P-labeled rGH probe (100 cpm/pg; see Fig. 3) in 10 μl of 0.4 M NaCl–9.5 mM Tris-hydrochloride (pH 7.5)–1.85 mM EDTA. Hybridization proceeded at 70°C for 215 h, after which reactions were diluted into S1 buffer (0.3 M NaCl, 30 mM sodium acetate (pH 4.5), 3 mM $ZnCl_2$) and digested with S1 nuclease (100 U, Sigma Chemical Co.) at 37°C for 1 h with denatured salmon sperm DNA (0.6 μg) as the carrier. Resistant counts per minute were measured by binding to DEAE-81 filters, which were rinsed extensively with 5% Na_2HPO_4 before drying and counting.

Southern hybridization. Nuclei were isolated from GH_3, HTC, rat liver, and anterior pituitary cells after homogenization in hypotonic buffer containing Nonidet P-40 (see above). Nuclei were digested overnight at 37°C with 200 μg of protease K per ml in 0.1% sodium dodecyl sulfate, and DNA was extracted with buffer-saturated redistilled phenol and chloroform. After precipitation with 70% ethanol and collection by spooling, DNA was dissolved in 10 mM Tris (pH 8.0)–10 mM sodium chloride and digested with RNase A (20 μg/ml) for 2 h at 37°C. Samples were then extracted with phenol and chloroform, precipitated with ethanol, and finally dissolved in Tris-hydrochloride (pH 8.0)–0.1 mM EDTA. DNAs were digested with various restriction enzymes, and fragments were separated on agarose gels and transferred to nitrocellulose filter paper (Schleicher & Schuell Co.) (35, 38). Filters were hybridized with 10^6 cpm of ^{32}P-labeled cDNA to rGH mRNA (34). The probe was labeled by nick translation (31) by using [α-^{32}P]dATP and [α-^{32}P]dCTP as labeled substrates; probe specific activity averaged 10^8 cpm/μg. Conditions for hybridization of the DNA blots were those described by Wahl et al. (38) as modified by Thomas (35). Hybridized counts were visualized by autoradiography at −70°C with intensifying screens (Lightning-Plus; Du Pont Co.).

RESULTS

We first asked whether HTC cells, which express liver-specific tyrosine aminotransferase (20), also express, at any level, the gene for rGH normally produced by anterior pituitary somatotrophs (24). In the assay, an extract of HTC_4 cells heavily labeled with [^{35}S]methionine was reacted with a monospecific antiserum against rGH (14), and the immune complexes were absorbed to *S. aureus* protein A (15). Precipitated proteins were then separated in two dimensions to complete the purification of the rGH polypeptide. Because as little as 0.3 cpm of [^{35}S]methionine-labeled rGH can be detected fluorographically after a 50-day exposure (unpublished data), the combined methods of immunoprecipitation and two-dimensional electrophoresis provide an extraordinarily sensitive assay for the expression of the gene.

Figure 1 illustrates the results of one experi-

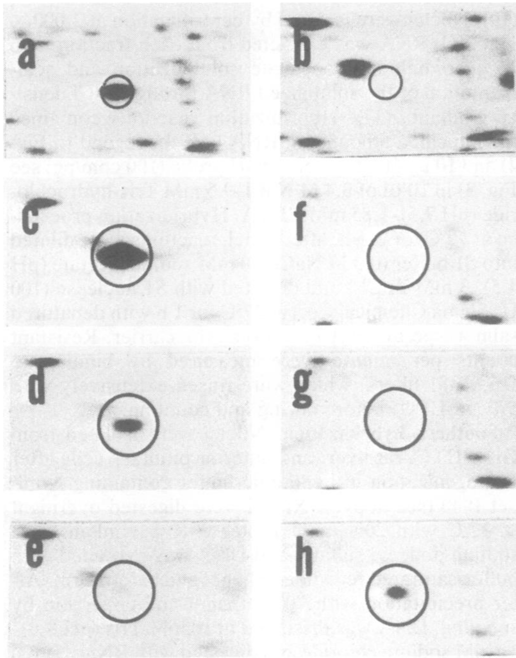

FIG. 1. rGH polypeptides in HTC_4 cells constitute less than 1 part in 10^9 of total protein synthesis. Extracts of HTC_4 cells containing 8.7×10^8 cpm of incorporated [^{35}S]methionine (acid-insoluble) were subjected to immune precipitation by anti-rGH serum (f) or nonimmune serum (g), and precipitated proteins were separated in two dimensions. Reference standards (c, d, and e), whole-cell extracts (a, b), and the reconstruction control (h) were also electrophoresed (see below). Two-dimensional gel electrophoresis of label proteins used nonequilibrium pH gel electrophoresis (NEPGHE) as the first dimension; the acidic end of the gel is on the right. (a) Whole-cell extract from GH_3 cells induced for 2 days with 10^{-8} M triidothyronine and 10^{-6} M dexamethasone and labeled for 30 min with 200 μCi of [^{35}S]methionine per ml (10^6 cpm electrophoresed); (b) whole-cell extract from induced HTC cells labeled for 6 h as described above (10^6 cpm electrophoresed); (c, d, and e) gels contained 870, 8.7, and 0.87 cpm, respectively, of [^{35}S]methionine-labeled rGH as reference standards; (f) anti-rGH immunoprecipitated proteins from an HTC cell extract containing 8.7×10^8 acid-insoluble cpm; (g) same as (f) except that nonimmune serum was used in place of anti-rGH serum; (h) same as (f) except that 20,000 acid-insoluble cpm of a GH_3 cell extract was added before precipitation with anti-rGH serum. In (a) and (b) gels were exposed to X-ray film for 10 days; the remainder of the gels were exposed for 30 days.

ment which indicated that inappropriate synthesis of rGH in HTC cells, if it occurs, must represent less than one part in 10^{-9} of total cell protein synthesis. Only the region of the fluorograms containing rGH is shown, and the position of the spot corresponding to rGH is indicated. Spot intensities from 870 (Fig. 1c), 8.7 (d), and 0.87 (e) cpm of [^{35}S]methionine-labeled rGH provide reference standards for sensitivity of detection. The rGH spot can be seen readily in the fluorogram of a whole-cell extract from GH_3 rat pituitary tumor cells (Fig. 1a), which express rGH at about 1% of cell protein synthesis when hormonally stimulated (14, 18). In contrast, the HTC whole-cell extract (Fig. 1b) did not contain a polypeptide detectably migrating as rGH. Furthermore, immunoprecipitating an HTC cell extract containing 8.7×10^8 cpm with anti-rGH serum still failed to reveal an rGH spot (Fig. 1f). All proteins in the anti-rGH precipitate were also seen in the nonimmune control precipitate (Fig. 1g), indicating that they were nonspecifically precipitated proteins.

Two control experiments were undertaken to ensure that the method would detect trace amounts of rGH. First, a reconstruction experiment was performed in which a small amount of a labeled extract from uninduced GH_3 cells (20,000 cpm, roughly corresponding to 10 cpm of rGH) was added to the HTC cell extract (8.7×10^8 cpm) and subjected to immunoprecipitation and two-dimensional separation (Fig. 1h). Clearly, rGH was precipitable from the heavily labeled HTC cell extract and assayable by subsequent two-dimensional gel electrophoresis. Second, to ensure that immunoprecipitation was quantitative under the experimental conditions, a known amount of labeled rGH was diluted over a 1,000-fold range and immunoprecipitated in the presence or absence of 1.5 mg of unlabeled HTC cell protein per ml. rGH was quantitatively precipitated under these conditions even after a 1,000-fold dilution, and the HTC cell extract did not inhibit precipitation (Fig. 2). It was concluded, therefore, that rGH polypeptides are quantitatively precipitable from HTC cell extracts under the conditions of the assay. Thus, based on the reference standards, the failure to detect an rGH spot in HTC cells indicated that <0.87 cpm of rGH was present in the HTC extract containing 8.7×10^8 cpm of labeled proteins.

Since rGH is processed from a larger precursor protein (21 kilodaltons) (1), it was possible that in HTC cells the protein might be expressed as the preform. The position of pre-rGH has been identified on two-dimensional gels by immunoprecipitation of cell-free translations of GH_3 cell cytoplasmic RNA (6; unpublished data). However, no pre-rGH spot could be seen in the complete fluorogram of the immunoprecipitated proteins from the HTC cell extract of Fig. 1. We also tested to determine whether rGH was secreted from the cells during the labeling period. Medium from HTC cells labeled in the experiment in Fig. 1 was assayed for secreted

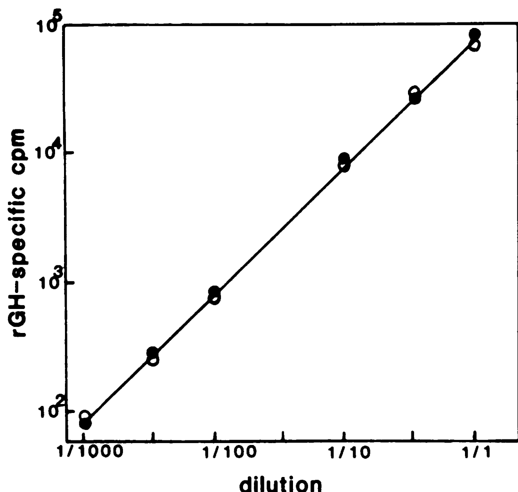

FIG. 2. Quantitative immunoprecipitation of [^{35}S]methionine-labeled rGH from HTC cell extracts. Medium from GH_3 cells containing a known amount of [^{35}S]methionine-labeled rGH (see text) was diluted over a 1,000-fold range in 1.5 ml of buffer containing 5 mg of bovine serum albumin per ml with (●) or without (○) 1.5 mg of unlabeled HTC cell protein per ml. Each sample was immunoprecipitated with 0.5 μl of anti-rGH serum, and the amount of precipitated radioactivity was determined (14). Background radioactivity was measured in immunoprecipitates with nonimmune serum and subtracted from the values shown.

rGH by immunoprecipitation with anti-rGH serum and electrophoresis. No detectable rGH was found to be secreted from the cells.

Because of the extremely high sensitivity of the assay, rare variants of HTC cells expressing high levels of rGH might be detectable in a large population. For example, a variant HTC cell producing rGH at a level characteristic of induced GH_3 cells (1% of cellular protein synthesis) would be detectable if it were present in the HTC cell population at a frequency of $>10^{-7}$ (e.g., variant frequency, 10^{-7}, multiplied by the level of expression, 10^{-2}, yields rGH at 10^{-9} of total protein synthesis in the population). Since 10^7 cells were assayed in each immunoprecipitation (Fig. 1), variants occurring at frequencies as low as the spontaneous mutation frequency of many genes in cultured cells should be detectable. Reasoning that there might be many ways to "relax" the stringent rGH control, either by mutation or by DNA modification (16, 17, 19, 30), we treated 10^7 HTC cells with the chemical mutagen ethyl methane sulfonate and assayed survivors (ca. 20%) for rGH expression 6 days later. At a sensitivity of 10^{-9}, we were unable to detect any rGH (data not shown). Thus, if mutations capable of activating rGH gene expression can be induced by ethyl methane sulfonate, they must be extremely rare events.

Since expression of the mature protein could be blocked at a number of steps, we measured the level of rGH mRNA sequences to obtain a more proximal measure of the gene's activity. A novel procedure, outlined in Fig. 3, was used to make a single-stranded ^{32}P-labeled probe com-

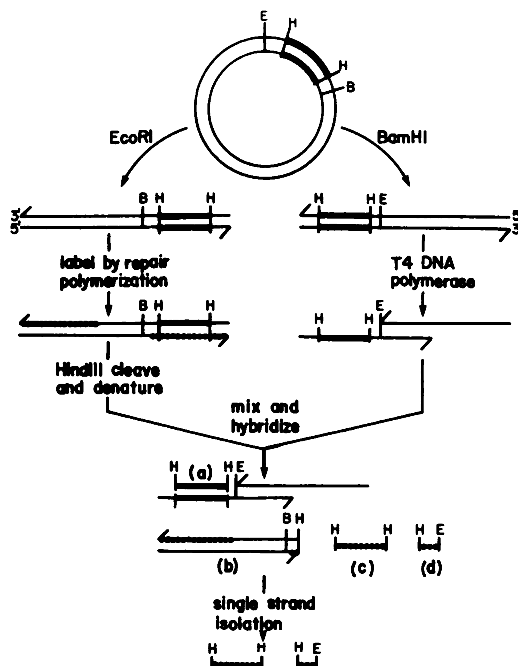

FIG. 3. Preparation of single-stranded hybridization probe. The pRGH plasmid, which carries an 811-bp cDNA copy of rGH mRNA (bold lines) in the *Hin*dIII site of pBR322 (34), was cut at the *Eco*RI site, labeled with [α-^{32}P]dATP by repair polymerization (27), and cut with *Hin*dIII. A second sample of the plasmid was cut at the plasmid *Bam*HI site, and 3′ to 5′ exonuclease activity of T4 DNA polymerase was used to partially degrade the linearized molecule so that, in the resected product, one strand of the insert region was degraded. Denaturation of the labeled plasmid and renaturation in the presence of the resected plasmid left some of the labeled insert single stranded because of the unequal stoichiometry between the strands. Single strands were isolated by hydroxylapatite chromatography. Purified single strands (c) were complementary to the mRNA and contaminated with only a small 33-bp *Eco*RI-*Hin*dIII fragment (d) from pBR322 that copurified through the procedure. Structure (a) contains the hybridized unlabeled cDNA strand, and structure (b) is the reannealed labeled vector. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. In a reciprocal procedure the antisense strand was labeled and purified. In this instance the *Bam*HI-cut plasmid was labeled by repair polymerization, and a resected plasmid was prepared from *Eco*RI-cut plasmid.

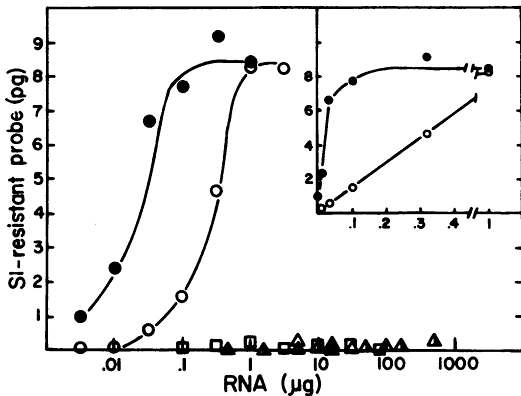

FIG. 4. Titration of the rGH probe with RNA from GH_3 and HTC cells. Nuclear and cytoplasmic RNAs from preinduced HTC_4 and GH_3D_6 cells were hybridized to a sense-strand ^{32}P-labeled rGH probe (10^3 cpm per reaction) prepared as described in the legend to Fig. 3. The main figure shows the amount of probe driven into hybrid by cytoplasmic RNA from induced GH_3 cells (●), cytoplasmic RNA from uninduced GH_3 cells (○), cytoplasmic RNA from induced HTC cells (□), nuclear RNA from induced HTC cells (△), and nuclear RNA from uninduced HTC cells (▲). To demonstrate linearity and saturation, the inset reproduces the data for induced and uninduced GH_3 cytoplasmic RNAs, using a linear scale for the amount of added RNA.

plementary to rGH mRNA. Having a probe that was single-stranded, we could use a highly sensitive and direct method for quantitating rGH mRNA sequences by titration (13, 41). The probe was titrated by hybridization with different amounts of total cytoplasmic RNA extracted from GH_3 cells which had been induced with dexamethasone and triiodothyronine (Fig. 4). Because the rate of rGH synthesis can be accounted for by the levels of rGH mRNA in GH_3 cells (22), rGH mRNA should represent 1% of cellular mRNA or about 0.03% of the total cytoplasmic RNA, assuming that this fraction contains 3% message sequences. A 10-ng amount of GH_3 cell cytoplasmic RNA protected 2.4 pg of the probe (Fig. 4). Thus, the amount of rGH RNA sequences detected (2.4×10^{-12} g/10×10^{-9} g = 0.024%) was about the expected level. Furthermore, as anticipated by earlier characterization of hormonal effects (14, 18, 22), cytoplasmic RNA from uninduced GH_3 cells titrated the probe 19 times less efficiently. In contrast, HTC cell RNA was unable to titrate the probe, and the maximum level of rGH RNA sequences was estimated (Table 1) to be present at less than 2×10^{-9} of total nuclear HTC RNA. Furthermore, rGH-specific transcripts were not detected in either nuclear or cytoplasmic RNA preparations from HTC cells, regardless of hormonal treatment (Table 1).

It was possible that antisense transcripts or related sequences might have hybridized to and masked the presence of rare rGH transcripts. We therefore made an antisense probe to test for their presence (Fig. 3). Antisense sequences could not be detected in either HTC nuclear RNA (sensitivity, 10^{-8}) or in GH_3 nuclear RNA (sensitivity, 5×10^{-7}).

The majority of sequences in both nuclear and cytoplasmic RNA preparations is noncoding RNA (>90%), including rRNA and RNA, as well as unprocessed intervening sequences of primary transcripts in nuclear RNA. When this factor is taken into account, the sensitivity of the hybridization experiments would have been adequate to detect rGH sequences present at $>6 \times 10^{-8}$ of all coding sequences. Although almost

TABLE 1. Estimates of rGH mRNA levels in GH_3 and HTC cells

Type of cells	rGH mRNA levels			
	Fraction in nucleus complementary to rGH probe	Molecules of rGH mRNA per cell nucleus[a]	Fraction in cytoplasm complementary to rGH probe	Molecules of rGH mRNA per cell cytoplasm[a]
GH_3				
Uninduced	1.7×10^{-6}	42	15×10^{-6}	188
Induced	2.5×10^{-6}	625	280×10^{-6}	3,500
HTC				
Uninduced	$<2 \times 10^{-9}$[b]	<0.005	$<1.2 \times 10^{-8}$[b]	<0.15
Induced	$<2 \times 10^{-9}$	<0.005	$<1.0 \times 10^{-8}$	<0.125

[a] The total amount of RNA in the cell nucleus and cytoplasm was taken to be 1 and 5.2 pg, respectively; the number of copies of rGH sequences are calculated as: total bases of RNA in cell compartment/800 bases of protected probe for each transcript × fraction of RNA complementary to the probe.

[b] Limits of sensitivity were calculated on the basis that 100 cpm of probe (10% of the total) driven into hybrid was readily detected. Because this represents 1 pg of probe, the limiting sensitivity of reactions showing no hybridization was taken as: 10^{12} g/gram of RNA used in the hybridization reaction.

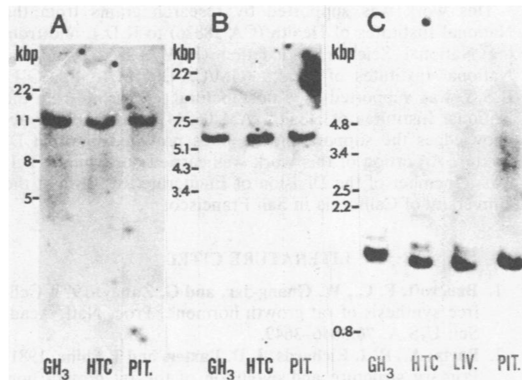

FIG. 5. Analysis of the rGH gene in genomic DNA from GH_3, HTC, rat liver (LIV.), and anterior pituitary (PIT.) cells by Southern hybridization. DNAs were digested with *Eco*RI (A), *Hin*dIII (B), or *Pvu*II (C), and 15 μg of each digest was electrophoresed on agarose gels (0.8% in A, 1.2% in B, and 1.5% in C). DNA fragments were blotted onto nitrocellulose filter paper and hybridized with ^{32}P-labeled cDNA to rGH mRNA (see text). Gels were calibrated for molecular weight by using *Eco*RI-digested DNA of pBR322 (4.3 kbp), pRGH (5.1 kbp; 34), and bacteriophage λ. Faint, higher-molecular-weight bands in C are probably incomplete digestion products since they were absent from another *Pvu*II blot of GH_3 and HTC cell DNA; the bands also exhibit the 100-bp length polymorphism (see text).

two orders of magnitude less sensitive than the protein analysis, the hybridization data do indicate that cell type-specific control alters levels of rGH RNA sequences.

A trivial explanation for the failure to detect expression of the rGH gene is that heteroploid HTC cells had lost the gene itself. To test this possibility, genomic DNA from GH_3, HTC, and rat anterior pituitary cells was assayed for the presence of rGH coding sequences by hybridization to an rGH cDNA probe by Southern blotting. The rGH gene is 2.5 kilobase pairs (kbp) in length, with a coding region interrupted by four intervening sequences (2, 6, 29). The gene is flanked by two *Eco*RI sites 11 kbp apart and by two *Hin*dIII sites 6 kbp apart. HTC cells contained both the 11-kbp *Eco*RI fragment (Fig. 5A) and the 6-kbp *Hin*dIII fragment (Fig. 5B), as did GH_3 and anterior pituitary cells. At this level of resolution, therefore, HTC cells had not suffered deletion of the rGH gene which would have accounted for its lack of expression.

We also used enzymes that cut at sites within the 2.5-kbp rGH gene including *Pvu*II, which yields an internal gene fragment of about 1.6 kbp, and *Hae*III, which yields two internal fragments of about 0.6 and 0.8 kbp. A restriction fragment polymorphism was detected in genomic DNA from GH_3 and HTC cells. An example of the *Pvu*II data is illustrated in Fig. 5C, in which the internal *Pvu*II fragment has a length of 1.6 kbp in HTC cells and 1.7 kbp in GH_3 cells. However, only the 1.6-kbp fragment was detected in genomic DNA from liver and anterior pituitary cells of Buffalo rats (Fig. 5C), the strain from which HTC cells were derived. Thus, the polymorphism is unrelated to expression and is likely to refelct a strain difference, inasmuch as GH_3 cells were derived from a Wistar-Furth rat. The rGH gene has also been cloned from two different strains (Sprague-Dawley and Hooded), and the two genes differ by a length polymorphism of 105 base pairs (bp) in intron B (2, 29). All of our restriction enzyme results, including *Hae*III (unpublished data) and *Pst*I (B. Johnson, unpublished data), are consistent with the idea that Wistar-Furth rats and GH_3 cells contain the long form of the rGH gene, whereas Buffalo rats and HTC cells have the short form.

DISCUSSION

rGH synthesis constitutes over 30% of total protein synthesis in anterior pituitary cells (Table 2) and must be even higher in somatotrophs, which constitute about 50% of anterior pituitary cells (24). This contrasts with a level of synthesis at $<10^{-9}$ in HTC cells as shown in this study. The difference in expression between the cell types is, therefore, greater than 10^8-fold. This ratio vastly exceeds changes in expression effected by known mechanisms of regulation in simpler systems, such as the 10^3-fold change in the expression of the *lac* operon after derepression (3). The remarkable effectiveness of cell type-specific regulation suggests that an unusual mechanism(s) is involved.

In general, observations in other systems support the conclusions reported here. These stud-

TABLE 2. Growth hormone is synthesized at greater than 30% of protein synthesis in rat anterior pituitary cells

Anti-rGH serum[a] (μl)	% rGH specifically immunoprecipitated[b]
10	7.2
20	13.8
40	29.1
50	32.5
60	32.8

[a] S100 extracts containing 115,000 cpm of incorporated [^{35}S]-methionine were reacted with indicated amounts of anti-rGH (or nonimmune) serum in a volume of 100 μl.

[b] Counts per minute in anti-rGH precipitates were corrected for nonspecific precipitation by subtracting counts per minute in the nonimmune serum precipitates; these values were then expressed as a percentage of the counts per minute in the cell extract subjected to immunoprecipitation.

ies (9, 21, 23) also failed to detect inappropriate gene expression, albeit at much lower sensitivities of detection.

Although some reports have described the detection of rare mRNAs for tissue-specific proteins in nonexpressing tissues (9, 10, 32, 36), only in one was evidence presented indicating that the mRNA was functional and translated into protein (36). Ovalbumin mRNAs were present in several nontarget chick tissues at 0.3 to 0.7 molecules per cell (roughly equivalent to 1 part in 10^6 of total mRNA). They were associated with polyribosomes and were detected only after treating chicks with estrogen. As shown immunocytochemically, low-level expression in liver apparently derives from rare cells producing ovalbumin polypeptides at high levels (36). Thus, some reports of inappropriate gene expression might be attributable to developmentally errant cells and leave intact the conclusion that gene inactivation is remarkably stringent in most cells.

There are, however, other reports of basal expression which cannot be explained in the same way and which imply that cell type-specific control can be relaxed in some cases. Expression of adult globin was activated in chicken embryo fibroblasts after viral transformation (9, 10) and has been correlated with changes in the chromatin structure of the globin genes (H. Weintraub, personal communication). Perhaps this unusual transition is related to normal differentiative events. Relaxed control has also been found in the course of differentiation of pancreatic acinar cells to the protodifferentiated state (reviewed in reference 33).

Chromatin structure (7, 8, 39, 40) and DNA modification (8, 16, 17, 19, 30) are both thought to specify the state of activity of a gene during development. Either might provide the basis for control mechanisms that could account for the wide range of the observed regulation. In regard to the role of chromatin structure, it is notable that inactivity of the rGH gene was measured in dividing cells in which chromatin must cycle through a variety of structures as a result of replication and segregation. Our results indicate that "leaky" expression from all states must be low.

Because the effectiveness of any biological mechanism is unlikely to exhibit 100% fidelity, we think that cell type-specific regulation must have some basal level of expression. However, our results indicate that this mode of regulation is especially potent and, for practical purposes, inactivates expression of specific genes completely.

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