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# Transforming Growth Factor-β, Transforming Growth Factor-β Receptor II, and p27$^{Kip1}$ Expression in Nontumorous and Neoplastic Human Pituitaries

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**Transforming growth factor (TGF)-β has been implicated in the regulation of normal and neoplastic anterior pituitary cell function. TGF-β regulates the expression of various proteins, including p27$^{kip1}$ (p27), a cell cycle inhibitory protein. We examined TGF-β, TGF-β type II receptor (TGF-β-RII), and p27 expression in normal pituitaries, pituitary adenomas, and carcinomas to analyze the possible roles of these proteins in pituitary tumorigenesis. Normal pituitary, pituitary adenomas, and pituitary carcinomas all expressed TGF-β and TGF-β-RII immunoreactivity. Reverse transcription polymerase chain reaction analysis showed TGF-β1, -β2, and -β3 isoforms and TGF-β-RII in normal pituitaries and pituitary adenomas. Pituitary adenoma cells cultured for 7 days in defined media showed a biphasic response to TGF-β with significant inhibition of follicle-stimulating hormone secretion at higher concentrations ($10^{-9}$ mol/L) and stimulation of follicle-stimulating hormone secretion at lower concentrations ($10^{-13}$ mol/L) of TGF-β1 in gonadotroph adenomas. Immunohistochemical analysis for p27 protein expression showed the highest levels in nontumorous pituitaries with decreased immunoreactivity in adenomas and carcinomas. When nontumorous pituitaries and various adenomas were analyzed for p27 and specific hormone production, growth hormone, luteinizing hormone, and thyroid-stimulating hormone cells and tumors had the highest percentages of cells expressing p27, whereas adrenocorticotrophic hormone cells and tumors had the lowest percentages. Immunoblotting analysis showed that adrenocorticotrophic hormone adenomas also had the lowest levels of p27 protein. Semiquantitative reverse transcription polymerase chain reaction and Northern hybridization analysis did not show significant differences in p27 mRNA expression in the various types of adenomas or in nontumorous pituitaries. *In situ* hybridization for p27 mRNA showed similar distributions of the gene product in nontumorous pituitaries, pituitary adenomas, and carcinomas. These results indicate that TGF-β and TGF-β-RII are widely expressed in nontumorous pituitaries and in pituitary neoplasms and that TGF-β1 regulates pituitary hormone secretion. The levels of the TGF-β-regulated protein p27 decreases in the progression of normal to neoplastic pituitaries. In contrast, the mRNA levels of p27 remained relatively constant in nontumorous pituitaries, pituitary adenomas, and carcinomas, indicating that p27 protein levels in adenomas and carcinomas are regulated by translational and post-translational mechanisms. *(Am J Pathol 1997, 151:509–519)***

Recent *in vitro* studies suggest that transforming growth factor (TGF)-β and related peptides may have important roles in pituitary cell proliferation and in hormone expression.[1–5] Various forms of TGF-β are expressed in rat[2–4] and human[6,7] pituitary cells. TGF-β receptors (TGF-β-R) use serine-threonine kinase signaling cascades for intracellular signal transduction. Various receptors for TGF-β have recently been cloned and sequenced.[8–10] Studies of tumorigenesis using cell culture models have shown that changes in TGF-β-RII may be important in tumor progression.[11–14] TGF-β-RII has been detected in human pituitary adenomas by immunostaining and by reverse transcription polymerase chain reaction (RT-PCR).[15,16] However, analysis of TGF-β or its receptors in pituitary carcinomas has not been reported. Because pituitary carcinomas represent metastatic disease at the final stage of pituitary tumor progression, analysis of TGF-β and TGF-β-R in these tissues might provide insight into the significance of this receptor in pituitary tumor development and progression.

TGF-β has an inhibitory effect on the cell cycle directed at the G1 to S phase transition, and this inhibition is reversible after removal of TGF-β.[17,18] Various studies

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have characterized some of the cell cycle inhibitory proteins regulated by TGF-β including $p27^{kip1}$ (p27).[19–22] p27 may function as a negative regulator of G1 cell cycle progression and may mediate TGF-β-induced G1 arrest. p27 protein levels are higher in quiescent than in proliferating cells, and it interacts with cyclin-cdk complexes such as cyclin E-cdk2 after stimulation by TGF-β.[19–22] We recently analyzed p27 expression in normal rat pituitary and $GH_3$ cells and observed decreased expression of p27 protein in $GH_3$ cells compared with normal pituitary cells.[5] Unlike the benign $GH_3$ cell line, human pituitary carcinomas represent a rare malignant neoplasm diagnosed by the presence of metastatic disease.[23,24] Recent studies of pituitary carcinomas showed a rapid proliferative rate, indicating an increased number of cells progressing through the cell cycle.[23] Because p27 inhibits G1 progression, analysis of p27 expression in pituitary adenomas and carcinomas should provide insight into the role of this protein in pituitary tumor growth. In the present study, the expression of TGF-β, TGF-β-RII, and p27 was analyzed in a series of pituitary adenomas and carcinomas to assess the regulatory role of this growth factor, its receptor, and cell cycle inhibition in pituitary tumor progression. In addition, the role of TGF-β in regulating the secretory function of cultured pituitary adenomas was also examined.

## Materials and Methods

### Study Groups

Formalin-fixed and paraffin-embedded tissues from 30 cases of surgically resected pituitary adenomas and 4 non-neoplastic autopsy pituitaries obtained within 6 hours postmortem were used for immunohistochemical (IHC) analysis. Eight cases of primary pituitary carcinomas with demonstrated metastases, including five prolactin (PRL)-producing and three adrenocorticotrophic hormone (ACTH)-producing carcinomas, were also analyzed. Seven of the latter tumors were included in a recent study of pituitary carcinomas.[24] Portions of tissue from 11 pituitary adenomas and 2 nontumorous autopsy pituitary tissues were used for RNA and protein extraction. Twelve pituitary adenomas (five gonadotroph (GTH), two null cell, one thyroid-stimulating hormone (TSH), one ACTH, one PRL, and two growth hormone (GH) adenomas) obtained from patients at the time of trans-sphenoidal surgery were used for cell culture study (Table 1). All tumors were classified by IHC staining of paraffin-embedded tissue sections as previously reported.[25,26]

### Cell Culture

Pituitary adenomas were dissociated with 0.25% trypsin and plated onto 35-mm dishes coated with extracellular matrix (Accurate, Hicksville, NY) at $0.2 \times 10^6$ to $1 \times 10^6$ cells/dish, as previously described.[25] The adenoma cells were cultured in serum-free medium for 7 days, and the medium was changed every other day. The aliquots of cells were treated with $10^{-13}$ mol/L, $10^{-11}$ mol/L, and $10^{-9}$ mol/L porcine TGF-β (R&D Systems, Minneapolis, MN) at 37°C in an atmosphere of 5% $CO_2$/95% air. One to four dishes were used for each treatment group, depending on the cell yield. After 7 days of culture, cells were harvested and used for IHC and other studies.

### Immunoassays

Cell culture medium was collected for immunoassays after 7 days of culture. Follicle-stimulating hormone (FSH) secretion was analyzed in GTH and null cell adenomas. The other hormones including PRL, GH, ACTH, and TSH were detected in the respective types of pituitary adenomas. Immunoassays were performed with kits supplied by manufacturers: FSH and PRL from Sanofi Labs, Chaska, MN; GH from ICN Biomedicals, Costa Mesa, CA; and TSH from Abbott Laboratories, Abbott Park, IL.[25] The ACTH assay was performed with a previously published method.[27]

### IHC

Paraffin sections from pituitary adenomas, pituitary carcinomas, and non-neoplastic pituitaries were used for IHC analysis, and the following antibodies were em-

**Table 1.** Characterization and Analysis of Cultured Pituitary Adenoma Cells

| Case | Diagnosis | IHC | IHC | | | ISH |
|---|---|---|---|---|---|---|
| | | | p27 | TGF-β | TGF-β-RII | TGF-β1 |
| 1 | GTH | LH, FSH | + | + | + | + |
| 2 | GTH | LH | + | + | + | + |
| 3 | GTH | LH, FSH | + | + | + | + |
| 4 | GTH | LH, FSH | + | + | + | + |
| 5 | GTH | LH, FSH | + | ND | ND | ND |
| 6 | NC | Negative | + | + (f) | + (f) | + |
| 7 | NC | LH (f) | + | + (f) | + (f) | + |
| 8 | TSH | TSH | + | + | + | + |
| 9 | ACTH | ACTH | + | − | + (f) | + |
| 10 | GH | GH, PRL (f) | + | + | + | + |
| 11 | GH | GH, PRL (f) | + | + (f) | − | − |
| 12 | PRL | PRL, GH (f) | + | + | + (f) | + |

NC, null cell adenomas; ND, not done; f, focal with less than 25% of tumor cells positive.

ployed: p27 (monoclonal, 1/1000) from Transduction, Lexington, KY; Ki-67 (monoclonal, 1/50) from AMAC, Westbrook, ME; TGF-$\beta^{pan}$ (polyclonal, 1/200) from R&D Systems; and TGF-β-RII (polyclonal, 1/250) from Santa Cruz Biotechnology, Santa Cruz, CA. Before incubation with primary antibodies, slides were microwaved for 15 minutes in 10 mmol/L citric acid (pH 6.0). The dispersed cells were placed on the slides by cytocentrifugation and fixed in 10% phosphate-buffered formalin overnight. The antibodies used for staining pituitary hormones included PRL (1/1000), GH (1/1000), luteinizing hormone (LH; 1/1000), FSH (1/500), and TSH (1/1000), the generous donation of the National Pituitary Agency (Baltimore, MD). ACTH antiserum (1/1000) was purchased from Dako (Carpinteria, CA). Chromogranin A (1/10) was produced in our laboratory.[28] IHC analysis with p27, TGF-β, and TGF-β-RII were performed on cytospin slides, with 5 minutes of microwave pretreatment for p27 immunostaining. All immunostaining was performed using the avidin-biotin-peroxidase method (Vector Kit, Vector Laboratories, Burlingame, CA). Positive control slides included normal rat pituitary cells for p27 and TGF-β[4,5] and Mv1Lu mink epithelial cells from the American Type Culture Collection (Rockville, MD) for TGF-β-RII. Negative control slides in which phosphate-buffered saline was substituted for the primary antibodies did not show any staining.

To distinguish specific pituitary cell types expressing p27, a double immunostaining method was employed. Pituitary sections were first stained with the p27 antibody using streptavidin-alkaline phosphatase (AP) conjugate and the nitroblue tetrazolium (NBT) and 5-bromo-4 chloro-3-indolyl phosphate (BCIP) detection system (Vector) followed by immunostaining for pituitary hormones, as described above.

## *In Situ Hybridization (ISH)*

The human TGF-β1 complementary DNA (cDNA) obtained from the American Type Culture Collection was cloned into PGEM-4Z plasmid and linearized with *Eco*RI or *Hin*dIII. The human p27 cDNA cloned into pBlue script II SK plasmid obtained from Dr. J. Massague, Sloan Kettering, NY, was linearized with *Pst*I or *Aa*TII. The *in vitro* transcription reaction and digoxigenin 11-UTP (Boehringer Mannheim, Indianapolis, IN) labeling were performed with either T7 (antisense) and SP6 (sense) RNA polymerase for TGF-β1 or T7 (antisense) and T3 (sense) RNA polymerase for p27, provided in the riboprobe labeling kit following the manufacturer's instruction (Promega, Madison, WI). The labeled probes were digested with deoxyribonuclease, extracted with phenol/chloroform, and precipitated with ethanol. ISH procedure was performed on 4% paraformaldehyde-fixed cytospin slides as previously described,[5,29] and positive ISH signals were detected by the anti-digoxigenin AP (1/200; Boehringer Mannheim) NBT/BCIP system.

## *Immunoblot Analysis*

One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with a ready-to-use 12% gel using the discontinuous buffer system of Laemmli (Bio-Rad Laboratories, Richmond, CA), as previously reported.[5] Mink Mv1Lu epithelial cells, which express TGF-β-RII, were used as a positive control. The electrophoresed proteins of pituitary adenomas and Mv1Lu cells were transferred to a polyvinylidene difluoride membrane and subjected to immunoblot analysis with $p27^{kip1}$ monoclonal antibody (1/250) or TGF-β-RII polyclonal antibody (1/1000). The reaction was detected with enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). To determine whether equal amounts of protein were added to the gels, the membranes were reblotted with a β-actin monoclonal antibody (1/2500; Sigma Chemical Co., St. Louis, MO) after washing.

## *RT-PCR*

Total RNA from pituitary adenomas was extracted with the TRIZOL reagent kit (Gibco BRL, Gaithersburg, MD) and used for analysis of mRNA of p27, TGF-β1, TGF-β2, TGF-β3, and TGF-β$pR^{II}$ by RT-PCR.

First-strand cDNA was prepared from total RNA by using a first-strand synthesis kit (Stratagene, La Jolla, CA), following the manufacturer's instruction. The RT reaction was performed in a final volume of 50 μl with 5 μg of total RNA, 100 ng of antisense primer (or 300 ng of oligo dT primers for p27) at 37°C for 60 minutes and then heated at 90°C for 5 minutes and immediately placed on ice. The sequences of primers and hybridization probes are shown in Table 2.

PCR amplification was performed in 100-μl final reaction volumes containing 10 μl of RT reaction product as template DNA, 1X PCR buffer, 1.5 mmol/L $MgCL_2$, 0.2 mmol/L each deoxynucleotide, 100 ng of each sense and antisense primer, and 2.5 U of *Taq* DNA polymerase (Promega). Programmable temperature cycling (Perkin-Elmer/Cetus 480, Norwalk, CT) was performed with the following cycle profile: 95°C for 4 minutes, followed by 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes. After the last cycle, the elongation step was extended by 10 minutes at 72°C. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. For semiquantitative analysis of p27 mRNA, GAPDH was amplified along with p27 in the same reaction using 300 and 50 ng of each sense and antisense primer for p27 and GAPDH, respectively.

A 20-μl aliquot of PCR product was analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were transferred to nylon membrane filters, and Southern hybridization was performed with $^{33}P$-labeled internal probes at 42°C for 18 hours. After washing with 6X SSC/0.1% SDS at 23°C for 20 minutes and at 42°C for 10 to 20 minutes, autoradiography was performed at −70°C with Kodak Omat-AR film (Eastman Kodak, Rochester, NY).

**Table 2.** Sequences of Primers and Internal Probes

| Gene | Sense | Antisense | Internal probes | Product size (bp) |
|---|---|---|---|---|
| TGF-β1 | GCCCTGGACACCAACTATTGCT 1-22 | AGGCTCCAAATGTAGGGGCAGG 140-161 | TCCTTGCGGAAGTCAATGTACAGCTGCCGC 51-80 | 161 |
| TGF-β2 | GACCCCACATCTCCTGCTAAT 1382-1402 | CGCAGCAAGGAGAAGC 1672-1692 | AAGCAATAGGCCCGATCCAAAGCACGCTCC 1451-1480 | 311 |
| TGF-β3 | ACACCTTTCAGCCCAATGGAG 951-971 | AGTCAATGTAGAGGGGGCGCA 1212-1232 | TTCCTCTGACCCCCCCTGGCC 1136-1155 | 282 |
| TGF-β-RII | TGTGTTCCTGTAGCTCTGATG 739-759 | AGATCTTGACTGCCACTGTCTC 1149-1170 | ACAGATATGGCAACTCCCAGTGGTGGCAGG 851-880 | 432 |
| p27 | GAGGGCAAGTACGAGTGGCAA 211-231 | CTGCGCATTGCTCCGCTAACC 428-448 | GGCGGCTCCCGCTGACATCCTGGCTCTCCT 311-340 | 238 |
| GAPDH | ATGGTGAAGGTCGGAGTCAACG 67-88 | GTTGTCATGGATGACCTTGGCC 540-561 | CTTGCCGTGGGTAGAGTCATACTGGAACAT 196-225 | 495 |

## *Northern Hybridization*

Total RNA from five pituitary adenomas and one nontumorous pituitary was used for Northern hybridization analysis for p27. Twenty micrograms of RNA was electrophoresed on a denatured formaldehyde 1% agarose gel and transferred to nylon filters. The riboprobe for p27 was labeled with $^{33}P$ with $T_7$ RNA polymerase (Promega) and $1 \times 10^6$ cpm/ml was used for Northern hybridization for 18 hours at 50°C. The filter was washed twice for 30 minute at 23°C and 80°C in 1X SSC/0.1% SDS, air dried, and then exposed for 3 days to Kodak X-omat film at −70°C with an intensifying screen.

## *Slide Analysis*

To quantify the immunostaining results for p27 and Ki-67, a minimum of 1000 cells from 10 fields of each slide were enumerated, and results were expressed as the percentage of positive cells. For p27 and pituitary hormone combined staining, 200 to 500 hormone-positive cells were enumerated, and p27-positive cells were expressed as a percentage of each type of hormone-producing cell. Student's *t*-test was used for statistical analysis, and the results are expressed as the mean ± SEM.

# *Results*

## *TGF-β and TGF-β-RII Expression*

IHC staining localized TGF-β and TGF-β-RII proteins in normal pituitaries and in most pituitary tumors (Table 3). The number of cells staining positive in each case was variable, but no distinct differences in immunoreactivity were noted between nontumorous pituitary adenomas and carcinomas. All subtypes of pituitary tumors expressed TGF-β and TGF-β-RII (Figure 1). Similar results were found in cultured pituitary adenoma cells (Table 1). ISH for TGF-β1 showed a positive hybridization signal for TGF-β1 mRNA present in all types of culture adenoma cells, confirming that the cells were producing TGF-β1 mRNA as well as TGF-β protein (Table 1; Figure 1). Immunoblotting for TGF-β-RII identified an ~70-kd band in normal pituitary and various pituitary adenomas (Figure 2). The Mv1Lu mink cells also had an ~70-kd band (data not shown). Analysis of TGF-β1, TGF-β2, TGF-β3, and TGF-β-RII by RT-PCR followed by Southern hybridization showed all three principal mammalian TGF-β isoforms as well as TGF-β-RII mRNAs expressed in normal pituitaries

**Table 3.** Immunohistochemical Analysis of TGF-β and TGF-β-RII in Normal and Neoplastic Pituitaries

| Diagnosis | n | Positive immunostaining | |
|---|---|---|---|
| | | TGF-β | TGF-β-RII |
| Nontumorous pituitary | 4 | 4 | 4 |
| Adenoma | 30 | 24 | 28 |
| Carcinoma | 8 | 8 | 8 |

n, number of cases.

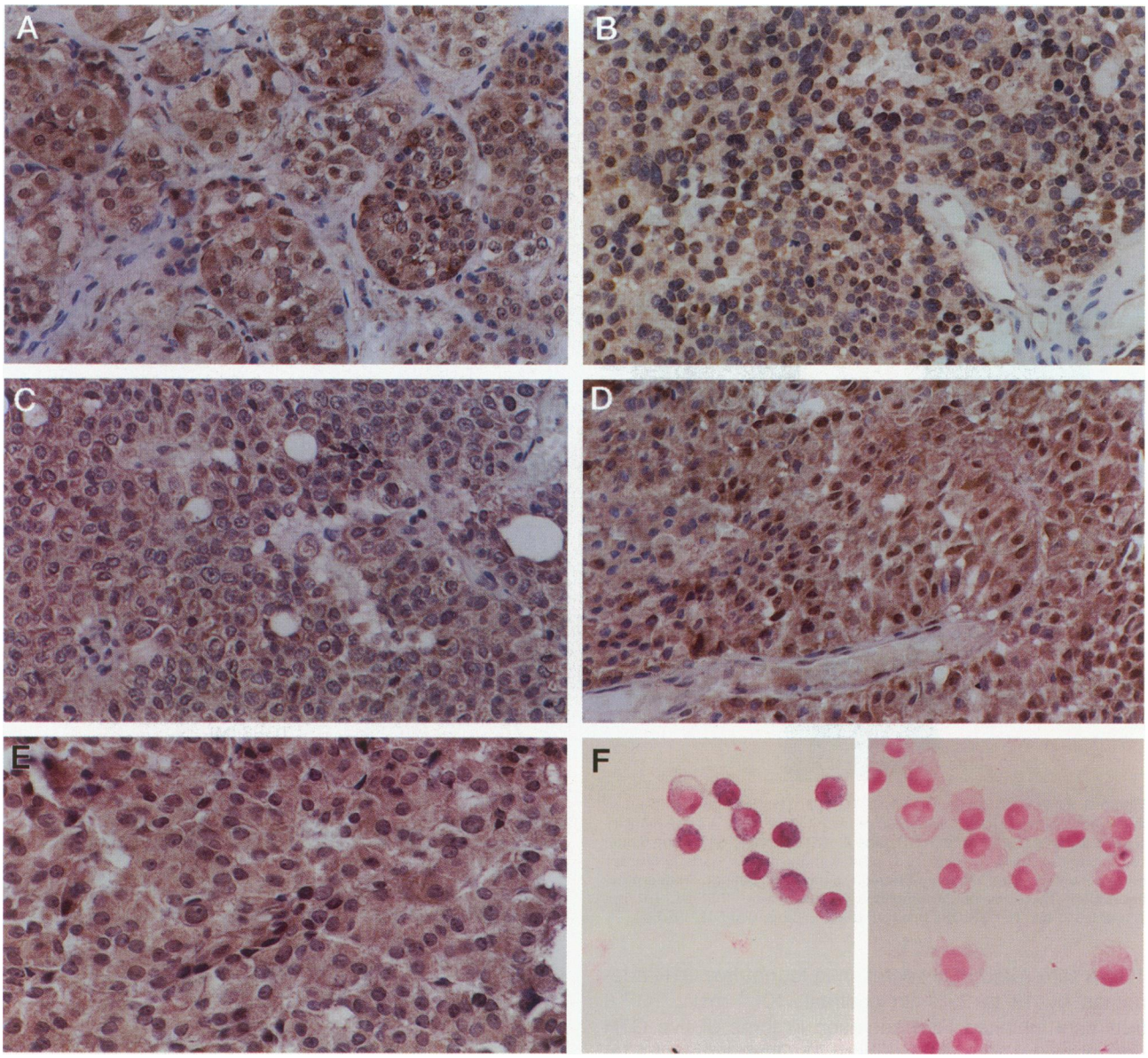


**Figure 1.** Immunohistochemical localization of TGF-β and TGF-β-RII in nontumorous and neoplastic pituitaries. TGF-β immunoreactivity was present in nontumorous pituitary (**A**), pituitary PRL adenoma (**B**), and pituitary PRL carcinomas (**C**). TGF-βR-II immunoreactivity was present in pituitary ACTH adenoma (**D**) and pituitary ACTH carcinoma (**E**). **F:** ISH with a TGF-β1 riboprobe showed positive signals in a GH adenoma (**left**) whereas the sense control (**right**) is negative. Magnification, ×250.

(2 cases) and all types of pituitary adenomas (10 cases). A representative RT-PCR result is shown in Figure 3. GAPDH was used as a control to check for the integrity of the mRNA.

## *Effects of TGF-β on Hormone Secretion in Pituitary Adenomas*

When the effects of TGF-β on hormone secretion by pituitary adenoma cells were analyzed *in vitro*, in one experiment with three replicate dishes using a GTH adenoma, there was significant stimulation and inhibition of FSH secretion at $10^{-13}$ mol/L and $10^{-9}$ mol/L TGF-β1, respectively (Figure 4). Treatment with $10^{-13}$ mol/L

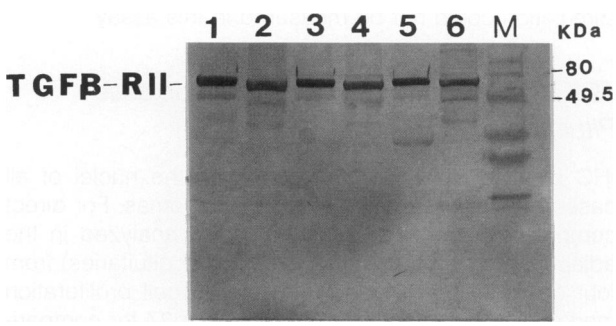


**Figure 2.** Immunoblot analysis of TGF-β-RII showed a ~70-kd band in normal and adenoma pituitaries. Fifty micrograms of protein was used for electrophoresis. **Lane 1**, nontumorous pituitary; **lanes 2** to **6**, PRL, GH, ACTH, GTH, and null cell adenomas, respectively.

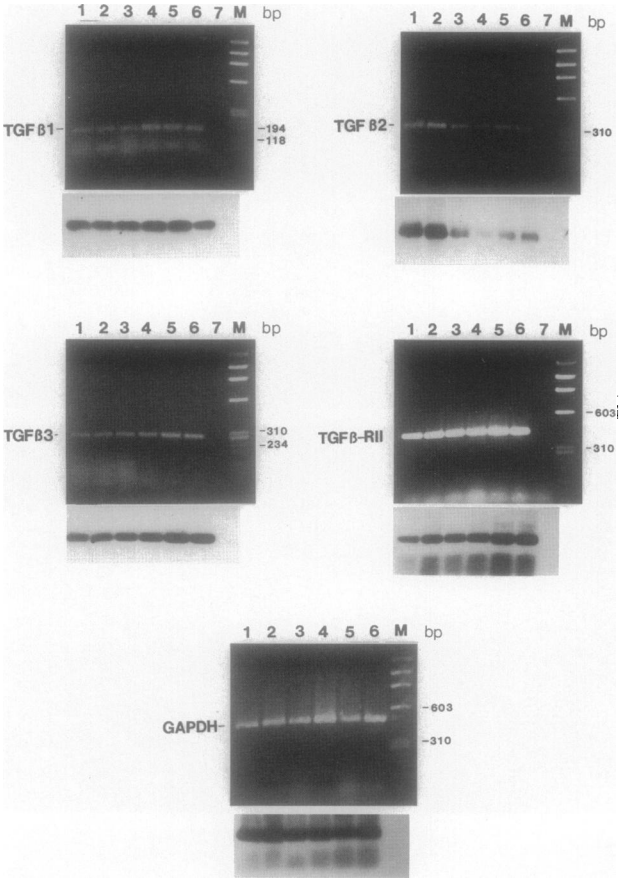


**Figure 3.** RT-PCR products of TGF-β1, TGF-β2, TGF-β3, and TGF-β-RII in the representative samples of nontumorous pituitary (**lane 1**) and pituitary adenomas (**lane 2**, PRL; **lane 3**, GH; **lane 4**, ACTH; **lane 5**, GTH; **lane 6**, null cell adenoma). **Lane 7**, negative control without reverse transcriptase for normal pituitary. **Top:** Ethidium-bromide-stained gel. **Bottom:** Southern hybridization. The sizes of the amplified products were: TGF-β1, 161 bp; TGF-β2, 311 bp; TGF-β3, 282 bp; TGF-β-RII, 432 bp; GAPDH, 495 bp.

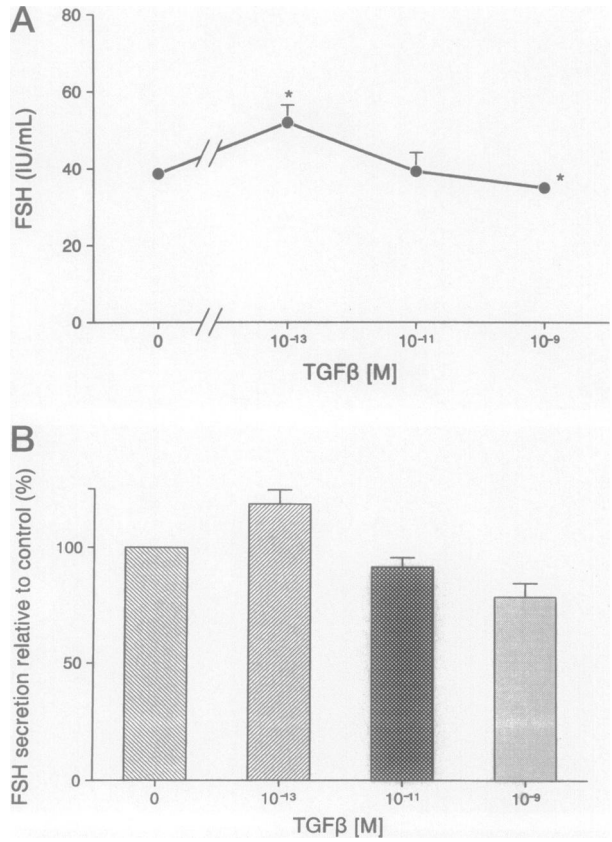


**Figure 4.** Effects of TGF-β concentration on FSH secretion in GTH adenomas. **A:** FSH secretion from a GTH adenoma. The values were presented as mean ± SEM for three culture dishes. $^*P < 0.05$, compared with control cells. **B:** FSH secretion is represented as relative percentage compared with control, and the results are mean ± SEM for five cases of GTH adenomas.

TGF-β stimulated FSH secretion in four of five GTH adenomas by 18 to 33%. In contrast, $10^{-9}$ mol/L TGF-β treatment inhibited FSH secretion in three of five GTH adenomas by 23 to 31% (Figure 4). Other types of adenomas showed similar responses to TGF-β with a slight stimulation and inhibition of hormone secretion with $10^{-13}$ mol/L and $10^{-9}$ mol/L TGF-β, respectively (data not shown). Null cell adenomas had no detectable hormone secretion by immunoassay, and the response to TGF-β1 stimulation could not be measured in this assay.

## *p27 Expression in Normal and Neoplastic Pituitaries*

IHC staining localized p27 protein in the nuclei of all cases of pituitary adenomas and carcinomas. For direct comparison, p27 expression was also analyzed in the adjacent non-neoplastic tissues (normal pituitaries) from four cases of pituitary adenomas. The cell proliferation marker Ki-67 was analyzed along with p27 for comparison in all cases. Eighty percent of normal pituitary cell nuclei were labeled for p27. There were significant decreases of p27 labeling index and immunostaining in both adenomas and carcinomas compared with the nontumorous pituitary (Figures 5 and 6, A–D). In contrast, Ki-67 was almost undetectable in nontumorous pituitary (0.2%) but was significantly increased in pituitary carcinomas (Figure 5).

A sequential staining method with two antibodies was used to analyze the relationship between p27 expression and specific pituitary cell types using autopsy pituitaries. The cell types that stained more frequently for p27 were LH and TSH cells, whereas ACTH cells expressed significantly less p27 (PRL, 23 ± 1.9%; GH, 19 ± 3.0%; ACTH, 2 ± 0.5%; TSH, 34 ± 1.4%; and LH, 35 ± 1.6%). Similarly, ACTH adenomas had the lowest levels of p27 protein-positive cells, which was twofold less than all other cell types (Table 4; Figure 6, E and F). Immunoblot analysis with monoclonal p27 antibody confirmed the low levels of p27 protein in the ACTH adenoma, whereas the GH adenoma had the highest level (Figure 7), which was also observed by immunostaining (Table 4).

Immunostaining for p27 protein in adenoma cells cultured for 1 week showed positive immunoreactivity in all tumors (Table 1). There was no detectable change in p27 protein by immunostaining after TGF-β1 ($10^{-9}$ mol/L) treatment.

When the levels of p27 mRNA were analyzed by semiquantitative RT-PCR, all types of pituitary adenomas and

nontumorous pituitaries expressed p27 mRNA. There were only slight differences between the various groups when the data were normalized with GAPDH (Figure 8).

Northern hybridization analysis showed a 2.5-kb p27 mRNA transcript in all adenomas and in the nontumorous pituitary. There was no significant difference between the tumors and the normal pituitary after densitometric analysis (data not shown).

ISH analyses for p27 localized the mRNA in most of the cells of nontumor pituitaries (n = 4), pituitary adenomas (n = 14), and pituitary carcinomas (n = 8; Figure 9), supporting the RT-PCR and Northern hybridization observations.

## Discussion

Our present studies show that TGF-β and TGF-β-RII are expressed in human normal pituitary cells and in pituitary adenomas as well as carcinomas. Loss of TGF-β-RII has been reported in various cell lines and occasionally in primary carcinomas such as esophageal carcinomas where high-density methylation of promotor sequences was observed in one of three cases with decreased TGF-β-RII expression.[30] In two previous studies of TGF-β-RII in pituitary adenomas, this receptor was detected in most cases by immunostaining or by RT-PCR.[15,16] Our analysis of eight pituitary carcinomas using formalin-fixed, paraffin-embedded blocks of these rare carcinomas showed expression of TGF-β-RII in all cases by immunostaining. These findings suggest that loss of TGF-β-RII may not be important for tumor progression in pituitary neoplasms. However, the functional status of TGF-β-RII or the presence of subtle genetic changes in the protein was not investigated, as only paraffin blocks were available. Our studies also showed that all three principal subtypes of TGF-β, including β1, β2, and β3, are expressed in normal pituitary and in pituitary adenomas by the RT-PCR method. To distinguish between expression of TGF-β isoforms by stromal cells or tumor cells, we performed *in situ* studies using IHC in tissue sections and ISH in cultured pituitary cells. Taken together, these data show that normal and neoplastic pituitary cells express TGF-β1, -β2, and -β3 as well as TGF-β-RII at the protein and mRNA levels.

The role of TGF-β in human pituitary function is uncertain. Our *in vitro* studies revealed a biphasic effect of TGF-β on gonadotropin secretion with $1 \times 10^{-9}$ mol/L having an inhibitory effect and $1 \times 10^{-13}$ mol/L having a stimulatory effect on FSH secretion in cultured cells. Our previous studies with rat pituitary cells also showed a biphasic effect of TGF-β on $GH_3$ cell proliferation with $10^{-9}$ mol/L inhibiting and $10^{-13}$ mol/L in stimulating cell proliferation.[5] These studies indicate that pituitary adenoma cells produce TGF-β, which has autocrine/paracrine effects on hormone secretion.

Analysis of p27 in nontumorous pituitaries and pituitary adenomas showed a significant decrease in the number of cells expressing p27 during progression from normal pituitary to pituitary adenomas and carcinomas. The opposite effect was seen with Ki-67, a marker of proliferation. These results support the concept that the highest levels of p27 are in quiescent cells and that, with increased proliferative activity, the levels of p27 decrease in tumor cells.[31–33] Studies with transgenic mice deficient in p27 has shown that this protein inhibits proliferation in some tissues such as the thymus, pituitary, and spleen leading to hyperplasias of these tissues and an increase in general body weight, despite normal levels of serum GH and insulin-like growth factor-1 when p27 was absent.[34–36] Interestingly, the pituitary of transgenic mice with p27 gene deletion showed ACTH cell hyperplasia from the intermediate lobe, whereas our studies in rats[5] and in human pituitaries have shown that ACTH cells in the anterior lobe have the lowest levels of p27 expression compared with other pituitary cell types. Thus, there may be inherent differences in p27 expression in intermediate and anterior lobe pro-opiomelanocorticotropin-producing pituitary cells.

The percentage of p27-positive cells was consistently higher in surgically excised nontumorous pituitary than in the autopsy pituitary, indicating that there was probably degradation of this cell cycle protein in postmortem specimens. This would also explain why the p27 protein levels detected by Western blotting were not higher in the autopsy-derived nontumorous pituitary than in surgically obtained adenomas. Although the levels of p27 protein decreased from surgically excised nontumorous pituitaries to neoplasms, there was a great deal of heterogeneity in p27 levels in various adenoma types and in different cell types in nontumorous pituitary cells. These differences might reflect variation in the cell cycle among different cell types or effects of different regulatory proteins such as growth factors on various cell types. Although we cannot completely exclude the possibility that the decreasing p27 levels seen in neoplastic pituitaries were the result of the clonal expansion of a population of

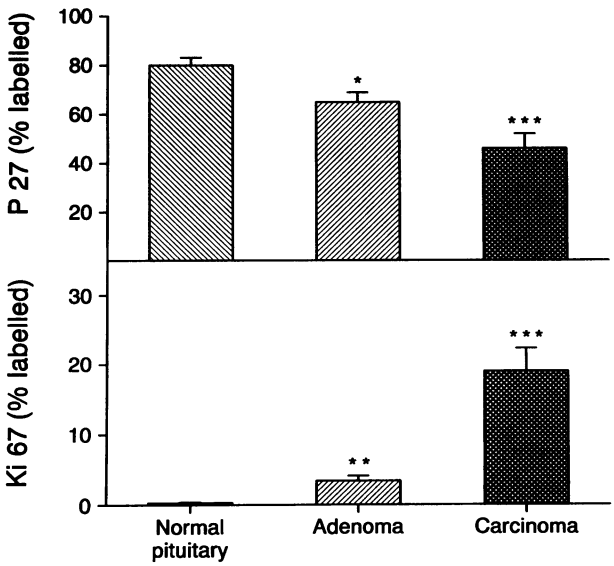


Figure 5. Immunohistochemical analysis of p27 and Ki-67 protein in nontumorous and neoplastic pituitaries. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with nontumorous pituitaries.

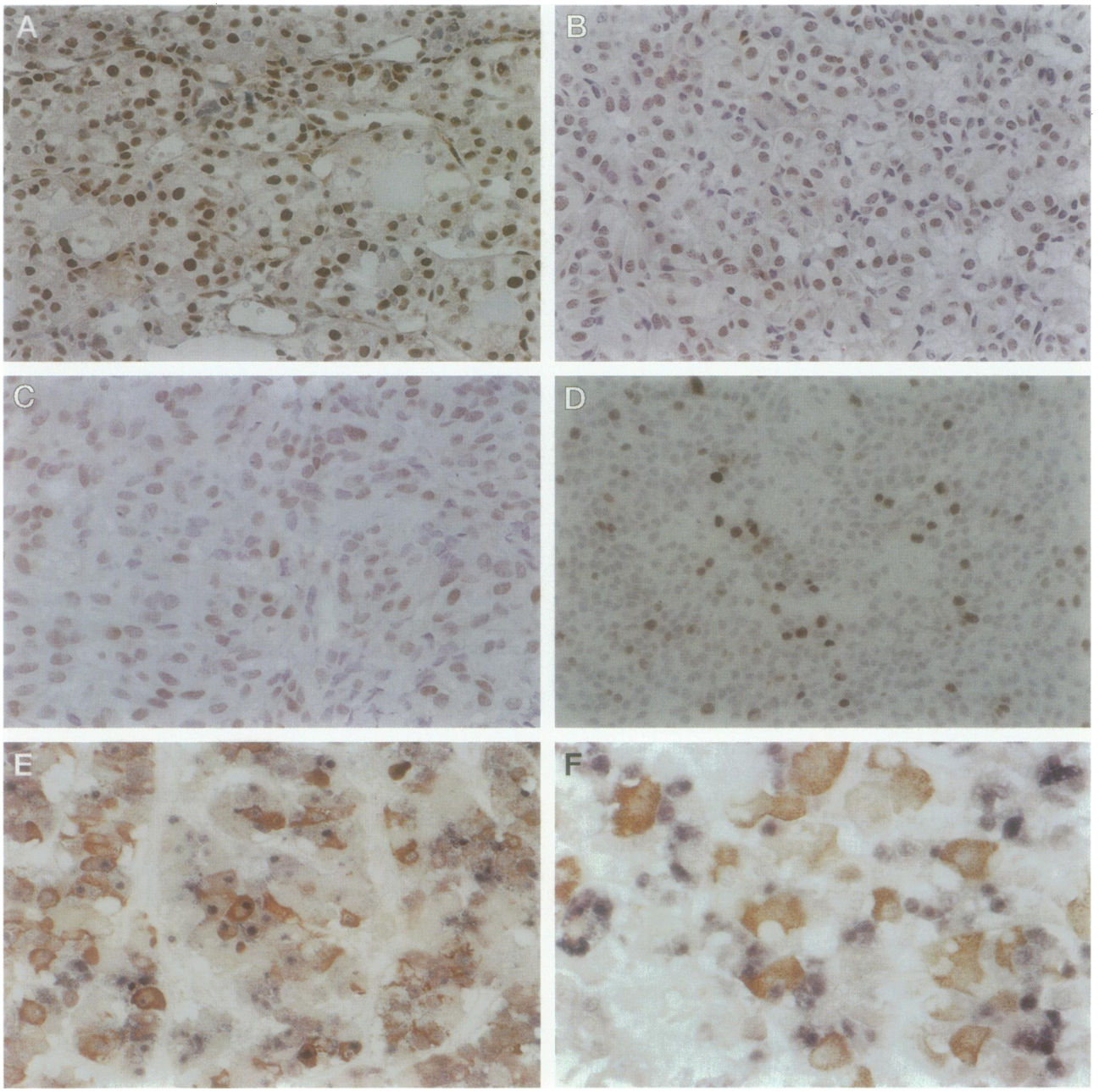


**Figure 6.** Immunostaining for p27 in nontumorous pituitary (**A**), pituitary adenoma (**B**), and pituitary carcinoma (**C**). **D:** Increased Ki-67 expression in a pituitary carcinoma. Magnification, ×250. **E:** Combined localization of p27 protein and pituitary hormones in TSH cells have brown cytoplasm, and many of these cells express p27 protein (blue cytoplasm). **F:** ACTH cells in nontumorous autopsy pituitaries with the brown cytoplasm have few p27 staining nuclei. Magnification, ×250.

a pituitary cell type that was normally low in p27, the differences in p27 in PRL adenomas (67 ± 6.3%) and PRL carcinomas (53 ± 5.8%) support the concept that, within homogeneous groups of neoplasms, p27 levels decrease during tumor progression.

Analysis of nontumorous and neoplastic pituitaries by RT-PCR, Northern hybridization, and ISH showed that the mRNA levels of p27, unlike the p27 protein, did not vary significantly between nontumorous and neoplastic pituitaries, indicating that control of p27 levels in the pituitary was by translational and post-translational mechanisms.[37]

The role of p27 as a possible tumor suppressor gene has been addressed by various investigators.[38–42] Although this gene is infrequently mutated in human neoplasms, Chen et al[42] reported that, when astrocytoma cell lines were transfected with p27, there was a decreased malignant potential of these cells in nude mice. This latter observation would be consistent with our observations of p27 regulation of cell proliferation with de-

**Table 4.** Immunohistochemical Analysis of p27 in Neoplastic Pituitaries

| Tumor type | Percent p27-positive cells | |
|---|---|---|
| | Adenoma | Carcinoma |
| PRL | 67 ± 6.3 (5)* | 53 ± 5.8 (5) |
| GH | 85 ± 2.1 (5)* | |
| ACTH | 26 ± 9.6 (5) | 34 ± 11.5 (3) |
| TSH | 72 ± 9.2 (3)* | |
| LH | 66 ± 4.4 (6)*† | |
| NC | 73 ± 4.7 (6)* | |

Percentages of positive cells were enumerated in pituitary tumors, as described in Materials and Methods. Numbers in parentheses indicate number of cases. NC, null cell adenomas.
*Significant difference compared with ACTH.
†Significant difference compared with GH.

creased expression during progression from pituitary adenomas to carcinomas in the present study.

The factors regulating p27 during cell cycle progression are being investigated. In the mink epithelial cell line Mv1Lv, cyclin D-cdk4 complexes bind and down-regulate p27 activity, which then reversed cdk2 inhibition enabling G1 progression.[31] Other studies have shown that *in vivo* TGF-β down-regulated p27 levels.[43,44] A recent study by Poon et al[44] showed that p27 was elevated in cells arrested in G0 by growth factor deprivation or contact inhibition. They noted that cyclin D1-cdk4 acted as a reservoir for p27 and that p27 was redistributed from cyclin D1-cdk4 to cyclin A-cdk 2 complexes during S phase or when cells were arrested by growth factor deprivation or ultraviolet irradiation.[44] However, the role of various factors, including TGF-β and contact inhibition, *in vivo* may not be directly mediated by p27, as p27 knockout mice still had intact cell cycle arrest mediated by TGF-β and contact inhibition. St. Croix et al[45] recently examined the role of p27 in contact inhibition of carcinoma cell lines. When these cells were transferred from monolayers to three-dimensional culture, there was a marked up-regulation of p27 protein. Differences in the ubiquitin-proteasome pathways may help to explain the

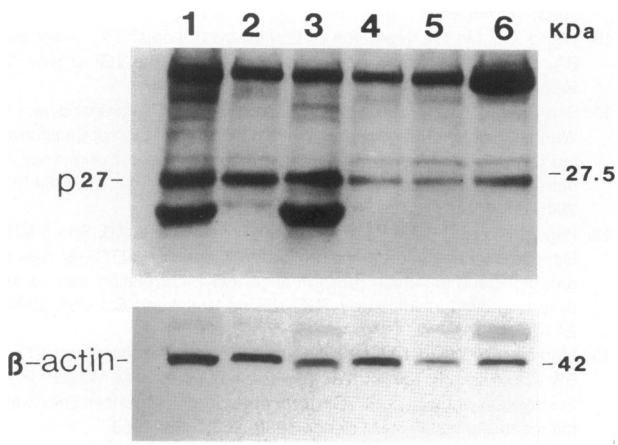


**Figure 7. Top:** Immunoblot detection of p27 protein in nontumorous pituitary and adenomatous pituitaries. **Lane 1**, nontumorous pituitary; **lane 2**, PRL; **lane 3**, GH; **lane 4**, ACTH; **lane 5**, GTH; **lane 6**, null cell adenoma. **Bottom:** Immunoblot for β-actin to check for equal protein loading on the gel.

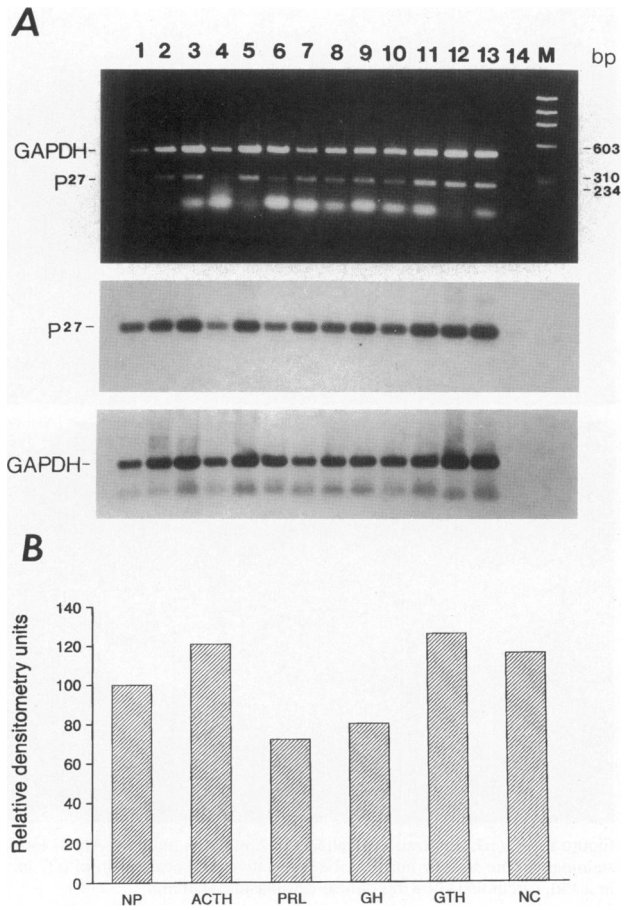


**Figure 8.** RT-PCR analysis of p27 mRNA. **A, top:** Ethidium-bromide-stained gel; Southern hybridization. A 238-bp band for p27 was found in all nontumorous pituitaries (**lanes 1** and **2**) and adenomas. **Lanes 3** and **4**, ACTH. **Lanes 5** and **6**, PRL; **lanes 7** and **8**, GH; **lanes 9** and **10**, GTH; **lanes 11** to **13**, null cell. **Lane 14**, negative control without reverse transcriptase of a nontumorous pituitary. **Middle:** Southern hybridization of p27 product. **Bottom:** Southern hybridization of GAPDH product. **B:** Densitometric analysis of bands normalized with GAPDH and expressed relative to nontumorous pituitary.

variable distribution of p27 protein in different pituitary cell types and tumors.[46] Ubiquitinating activity varied greatly between proliferating and quiescent cells, and this correlates with the half-life of p27 in these cells.[37] Our recent *in vitro* studies with normal rat pituitaries showed a down-regulation of p27 protein and mRNA by TGF-β1.[5] However, treatment of human pituitary adenoma cells with TGF-β1 for 7 days did not lead to significant changes in p27 protein levels as assessed by immunostaining. Because the immunostaining method was not very sensitive, small changes in p27 levels after TGF-β1 treatment may have been missed in these experiments.

Recent studies on breast and colon carcinomas have suggested that p27 protein expression has prognostic significance in these epithelial neoplasms.[47–49] Moreover, Loda et al[49] showed that colon carcinomas with low or absent p27 protein displayed enhanced proteolytic activity specific for p27, suggesting that low p27 expression can result from proteasome-mediated degradation rather than altered gene expression, which supports our observations in pituitary tumors.

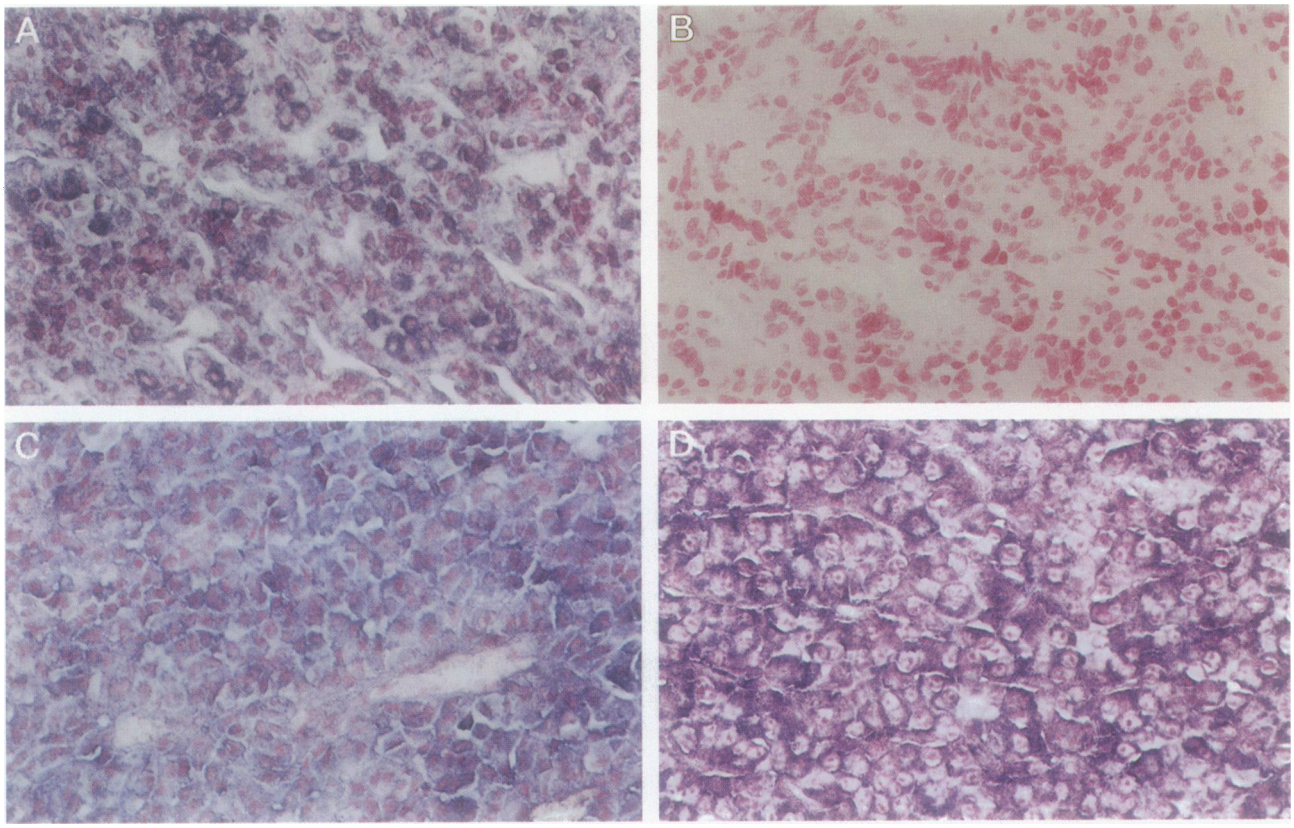


**Figure 9.** *In situ* hybridization localizing p27 mRNA in pituitary. **A:** p27 localization in a nontumorous pituitary with the antisense probe shows diffuse cytoplasmic staining. **B:** The sense control probe is negative. **C:** Localization of p27 in an ACTH adenoma showing diffuse staining in all tumor cells. **D:** Localization of p27 in a PRL carcinoma showing diffuse cytoplasmic staining.

In summary, nontumorous pituitaries, pituitary adenomas, and carcinomas all express TGF-β and TGF-β-RII, and TGF-β1 regulates pituitary hormone secretion. Although we observed decreased expression of the cell cycle inhibitor p27 in pituitary adenomas and carcinomas as compared with nontumorous pituitaries, p27 mRNA levels were not significantly different between nontumorous pituitaries and pituitary neoplasms. This indicates that the observed decrease in p27 proteins in adenomas and carcinomas is regulated by translational and post-translational mechanisms.

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