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HPAC, A NEW HUMAN GLUCOCORTICOID-SENSITIVE PANCREATIC DUCTAL ADENOCARCINOMA CELL LINE

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SUMMARY

A new human pancreatic cancer (HPAC) cell line was established from a nude mouse xenograft (CAP) of a primary human pancreatic ductal adenocarcinoma. In culture, HPAC cells form monolayers of morphologically heterogenous, polar epithelial cells, which synthesize carcinoembryonic antigen, CA 19–9, CA-125, cytokeratins, antigens for DU-PAN-2, HMFG1, and AUA1, but do not express chromogranin A or vimentin indicative of their pancreatic ductal epithelial cell character. In the presence of serum, HPAC cell DNA synthesis was stimulated by insulin, insulin growth factor-I, epidermal growth factor, and TGF-α but inhibited by physiologic concentrations of hydrocortisone and dexamethasone. Dose-dependent inhibition of DNA synthesis was limited to steroids with glucocorticoid activity. The inhibitory effect of dexamethasone was abolished by the glucocorticoid antagonist RU 38486. Binding of [^{3}H]dexamethasone to cytosolic proteins was specific and saturable at 4° C. Scatchard analysis of binding data demonstrated a single class of high-affinity binding sites (K_d = 3.8 ± 0.9 n*M*; B_{max} = 523 ± 128 fmol/mg protein). Western blot analysis revealed a major protein band that migrated at a M_r of 96 kDa. Northern blot analysis identified an mRNA of approximately 7 kilobases which hybridized with a specific glucocorticoid receptor complementary DNA probe (OB7). These findings support a role for glucocorticoids in the regulation of human malignant pancreatic cell function.

Key words: pancreatic cancer; glucocorticoids; growth inhibition; glucocorticoid receptor; dexamethasone.

INTRODUCTION

A variety of growth factors, gastrointestinal regulatory peptides, polypeptide, and steroid hormones have been implicated in the regulation of growth of human pancreatic cancer cells both in vivo and in vitro (28). Epidermal growth factor (EGF), transforming growth factor-α (TGF-α), insulinlike growth factor-I (IGF-I), and insulin have been found to stimulate the growth of several human pancreatic tumor cell lines (MIA CaPa-2, PANC-1, T_3M_4, ASPC-1) acting, at least in some instances, in an autocrine fashion (20,24,32,39). The EGF receptor, activated by both EGF and TGF-α, and the IGF-I receptor, activated by both IGF-I and insulin have been demonstrated in several established human pancreatic tumor cell lines (MIA CaPa-2, PANC-1, T_3M_4, COLO 357, UACC-462, ASPC-1) (20,32,39).

Evidence suggests that in some cases cancer of the exocrine pancreas may be steroid hormone sensitive (1,28). Sex steroid receptors, binding proteins, and enzymes found only in sex steroid-dependent tissue have been found in human ductal adenocarcinomas as well as in experimentally induced tumors in laboratory animals (1). Both steroidal and nonsteroidal endocrine agents influence the proliferation of human pancreatic tumor cells (4). Four human cell lines (MIA PaCa, Colo-357, RWP-1, and RWP-2) were found to contain specific estradiol-binding proteins with K_d values ranging from 1 to 9 n*M*. Estradiol inhibited the growth of all lines except MIA PaCa, which was stimulated. An antiestrogen, antiandrogen, progesterone, and various inhibitors of steroid-metabolizing enzymes suppressed the proliferation of all four cell lines. Glucocorticoids (dexamethasone, hydrocortisone) stimulated or had no significant effect on the proliferation of the four human lines. In contrast, glucocorticoids inhibited the growth of a rat pancreatic acinar cell line (AR42J) by as much as 50%. The AR42J cell line, originally derived from a hyperplastic nodule of the exocrine pancreas from a rat treated with azaserine, has been shown to express a functional glucocorticoid receptor (GR) (34). Glucocorticoids have been demonstrated to modulate not only AR42J cell proliferation, but also increase amylase, trypsinogen, and lipase gene expression, the number of secretory organelles, the level of amylase secretion, and cell surface components (15,26,42). These findings suggest that certain pancreatic tumors may be sensitive to glucocorticoids.

The occurrence of a functional glucocorticoid receptor (GR) in human pancreatic cancers is of interest because it has been demonstrated that glucocorticoids have antiproliferative effects on various tumors (10,25,47). Little is known about the potential role of glucocorticoids in pancreatic cancer. To the best of our knowledge there have been no reports of a glucocorticoid-sensitive human pancreatic

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tumor cell line that has been shown to express a functional GR. In this report we describe a new human pancreatic cell line which was derived from a ductal adenocarcinoma in which both DNA synthesis and cell proliferation are inhibited by dexamethasone and hydrocortisone. Evidence is presented that suggests this effect is mediated by a functional GR synthesized by these cells.

Materials and Methods

Clinical specimen. A representative portion of a primary adenocarcinoma removed from the head of the pancreas of a 64-yr-old Caucasian female was received after partial pancreatectomy in 1985. Histologically, the tumor was classified as a moderately well-differentiated adenocarcinoma of ductal origin.

Establishment of tumor line in athymic mice. Tumor tissue was cut into 2- to 3-mm cubes and implanted s.c. into both flanks of male BALB/c nu/nu mice (4 wk old). Animals were purchased from Life Sciences (St. Petersburg, FL) and maintained in a pathogen-free environment. Transplantation was performed under general anesthesia (0.05 mg/g sodium pentobarbital) using aseptic technique in an air-filtered laminar flow hood. Mice were determined to be free of murine hepatitis virus, reovirus type 3, pneumonia virus of mice, Sendai virus, minute virus, ectromelia virus, encephalomyelitis, polyoma, mouse adenovirus, lymphocytic choriomeningisis, and K-Virus (Microbiological Associates, Inc., Bethesda, MA). Tumor growth was observed in one mouse after 2 mo. Once the tumor reached an appreciable size (1.0 to 1.3 cm), the mouse was killed by cervical dislocation and the primary xenograft was removed for serial transplantation, histologic examination, and establishment of cell cultures. The human tumor line, designated CAP, has been continuously passed since its establishment in 1985, and both growth and histologic characteristics have remained unchanged.

Cell culture. Xenograft material was dispersed into single and aggregates of cells as described elsewhere (14). Cells were transferred to 25-cm^2 culture flasks in culture medium, designated DME/F12–4F10, which was a mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F12 nutrient medium (1:1) containing 1.2 g/liter $NaHCO_3$ and 15 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.3 (GIBCO BRL, Gaithersburg, MD), supplemented with four factors (2 μg/ml insulin, 5 μg/ml transferrin, 40 ng/ml hydrocortisone, 10 ng/ml EGF), 10% fetal bovine serum (FBS), and 1× antibiotic/antimycotic mixture. Cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2-enriched air. Fibroblastoid cells were removed from primary cultures by short incubations with 0.1% trypsin in Hanks' balanced salt solution (HBSS) for 2 min at 37° C. Cells from confluent cultures, free of fibroblastoid cells, were harvested by incubation with 0.25% trypsin:0.2% ethylenediaminetetraacetate (EDTA) in calcium- and magnesium-free HBSS. After several subcultures the FBS concentration in the culture or full-growth medium was gradually reduced to 5%. Human pancreatic cancer (HPAC) cells were routinely subcultured at a ratio of 1:5 in full growth medium, designated DME/F12–4F5. Stock cultures were stored in liquid nitrogen. Except where noted, studies were conducted on HPAC cells from Passage 90 to 120. Cultures were examined for Mycoplasma contamination by both broth-agar isolation (Hana Biologics, Inc., Berkeley, CA) and assay for the presence of Mycoplasma ribosomal RNA (Gen-Probe, San Diego, CA) and found to be negative.

Tumorigenicity. In vivo tumorigenicity was evaluated as described by Kyriazis et al. (21). Briefly, freshly trypsinized HPAC cells at Passages 5 and 90 (1×10^6 viable cells in 0.2 ml of HBSS) were injected s.c. into the anterior lateral thoracic wall of male nude mice. Tumor size was measured with calipers once a week. After the tumors reached an appreciable size (1.0 to 1.5 cm in diameter) the mice were killed by cervical dislocation, the tumor was excised, then processed for histologic and ultrastructural examination.

Cytogenetic analysis. Cultures were incubated in full growth medium containing colcemid (0.02 μg/ml) for 4 h. The cells were rinsed in HBSS, removed from the flasks by treatment with trypsin, exposed to a hypotonic solution for 20 min, then fixed. The chromosomes were spread and G-T banded by the method of Seabright (38).

Characterization of cell proliferation. HPAC cells were routinely plated at a density of 2.0×10^5 cells/25-cm^2 flask in DME/F12–4F5. After 24 h (Day 0), cells were washed twice in nutrient medium (DME/Ham's F12 nutrient medium [1:1] with 1.2 g/liter $NaHCO_3$ and 15 m*M* HEPES, pH 7.3) and the medium changed to the appropriate test or control medium. Except where noted, the culture medium used for evaluating the effects of various factors on the growth of HPAC cells was full-growth medium without the supplemental growth factors (insulin, EGF, hydrocortisone, transferrin) and containing only 3% FBS, designated DME/F12–0F3. Flasks were incubated at 37° C in a humidified atmosphere of 5% CO_2-enriched air, and medium was replaced every 2 days. At designated times the medium was removed, cells were collected by treatment with trypsin/EDTA, washed in DME/F12–0F3, then cells were counted by hemacytometer. Alternatively, because preparation of single cell suspensions of HPAC cells was difficult to achieve, cells were resuspended in 1.0 ml of 0.1 *M* sodium acetate buffer, pH 6.2, and stored at −20° C until assay for DNA. Total DNA content of each flask was determined by a fluorometic 4′,6-diamidino-2-phenylindole assay (18). Semilogarithmic plots of DNA/flask or cell number/flask vs. days had identical growth curves (data not shown). Cell viability was determined by trypan blue exclusion. For full growth curves,DNA content was determined in five flasks on Days 0, 1, 2, 3, 4, 5, and 6 for each culture medium formulation. Cell population doubling time was determined between 3 and 5 days after initial plating from a semilogarithmic plot of cell number vs. culture time. Plating efficiency and colony formation efficiency on plastic and in soft agar were determined according to Dexter et al. (9).

DNA synthesis. DNA synthesis was assayed by determining [^{3}H]-thymidine incorporation into DNA. HPAC cells were seeded (1×10^4 cells) in individual wells of a 24-well plate in 1.0 ml of DME/F12–4F5, and incubated at 37° C for 24 h. After this incubation, the growth medium was removed, the monolayer was rinsed with nutrient medium, then DME/F12–0F3 with or without test agents was added, and cells were incubated. Medium was changed after 48 h. Steroid hormones (11-deoxycorticosterone, 17β-estradiol, testosterone, progesterone, hydrocortisone, dexamethasone; Sigma, St. Louis, MO) and RU 38486 (Roussel-UCLAF, Romainville, France) were prepared as concentrated stock solutions in ethanol. Designated concentrations of steroids were prepared by dilution in DME/F12–0F3, and control medium contained equivalent amounts of ethanol. Cells were never exposed to more than 0.1% ethanol, which was determined to have no deleterious effect on HPAC cells. Human transferrin, insulin, IGF-I, EGF, and TGF-α (GIBCO BRL) were prepared and diluted in DME/F12–0F3. After 72 h, each culture was pulsed with 1 μCi/ml of [^{3}H]thymidine (60 Ci/mmol, ICN, Irvine, CA) for 60 min at 37° C, then the cells were rinsed 3 times with cold Dulbecco's phosphate-buffered NaCl solution, extracted with 10% cold trichloroacetic acid for 30 min, rinsed with buffer, then solubilized in 0.5 ml of 1% sodium dodecyl sulfate (SDS). The radioactivity in the extract was determined by liquid scintillation counting.

Glucocorticoid receptor binding assay. Specific binding of [^{3}H]-dexamethasone to glucocorticoid receptors was determined by the cell-free method essentially as described previously (40). Data were subjected to Scatchard analysis (37). Protein content was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as a standard.

Electrophoresis and Western blotting. Cytosol fractions were prepared from HPAC and HeLa cells cultured in the designated medium as described for the GR binding assay, except cells were lysed and homogenized in 25 m*M* Tris-HCl buffer, pH 7.4, containing 10 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* EDTA, 5 m*M* EGTA, 5 m*M* dithiothreitol, 100 μ*M* phenylmethylsulfonyl fluoride, 200 μ*M* leupeptin, 80 μ*M* aprotinin, and 0.1% Triton X-100. Western blot analysis of cyctosol fractions was performed according to Towbin et al. (46) using 7.5% SDS-polyacrylamide gels. Detection of GR protein was by a rabbit polyclonal anti-GR antiserum (Ahugr 150–175) (a gift from E. Brad Thompson, University of Texas Medical Branch, Galveston, TX) diluted 1:500. Nonspecific binding was detected by normal rabbit serum diluted 1:500. Incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG and development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were as described by the manufacturer of (Bio-Rad). Molecular weight determinations were derived from the positions of prestained molecular weight markers (Bio-Rad) included in adjacent lanes: myosin (M_r 205 000), β-galactosidase (M_r 116 500), bovine serum albumin (M_r 80 000), and ovalbumin (M_r 49 500).

RNA analysis. Total cellular RNA was prepared from CAP xenograft

tissue, HPAC, and HeLa cells using RNAzolB (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. Total RNA (20 μg/lane) was loaded onto a horizontal formaldehyde-0.8% agarose gel. RNA was transferred to a nylon membrane (Zeta-Probe, Bio-Rad) by capillary blotting. Hybridization, preparation of ^{32}P-labeled nick-translated DNA probe, blot washing, and autoradiography were as described previously (13,17). The probe for detecting the GR mRNA was the 4.2 kilobase *Bam*H1 fragment from the OB7 plasmid (17), a gift from E. Brad Thompson. To control for RNA loading and to determine efficiency of transfer, membranes were stained with ethidium bromide to visualize 28S and 18S rRNA.

Histochemical and immunochemical staining. Tumor tissue removed from nude mice was fixed in Bouin's fixative, then embedded and sectioned using standard histologic techniques. Hematoxylin:eosin and periodic acid-Schiff-diastase staining were performed using standard histochemical methods. Immunostaining for keratin, vimentin, chromogranin A, DU-PAN-2, HMFG1, and AUA1 in monolayer cultures of HPAC cells was by indirect immunofluorescence with anti-keratin antibodies AE1/AE3 (Hybritech, Inc., San Diego, CA), anti-vimentin antibodies V9 (ICN, Lisle, IL), anti-chromogranin A antibodies LK2H10 (Hybritech), DU-PAN-2 antibody (a gift from M. A. Hollingsworth, University of Nebraska, Omaha, NE), HMFG1 and AUA1 antibodies (a gift from A. Heal, J. A. Haley VA Hospital, Tampa, FL), and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Sigma) as described previously (14).

Biochemical and immunoradiochemical assays. HPAC cells were collected from 75-cm^2 culture flasks by scraping, and washed as described above. The final cell pellet was resuspended in 1 ml of cold Dulbecco's phosphate buffered NaCl solution and stored at −80° C. The cell suspension was thawed, rapidly frozen, and thawed 3 times in an acetone/dry ice bath, centrifuged (12 000 ×*g*) for 10 min, then the supernatant was removed and assayed for pancreatic enzymes, tumor-associated antigens, and protein. Amylase, trypsin, and chymotrypsin activities were determined by methods described in detail elsewhere (9). Tumor-associated antigens CA 125 and CA 19–9 were assayed by radioimmunoassay (Centocor, Malvern, PA) and carcinoembryonic antigen (CEA) was determined by immunoradiometric assay (Hybritech). All data are expressed per milligram of cellular protein.

Electron microscopy. Transmission electron microscopy of tumor tissue and cultured cells was performed as reported previously (14). For scanning electron microscopy HPAC cells in monolayer culture were fixed with 2% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.3, dehydrated in ethanol, then critical-point dried. Select areas of the monolayer were mounted on metal stubs, sputter coated, then examined in a Jeol JSM 35 scanning electron microscope.

Statistical analysis. Results are expressed as mean ± SEM. Differences between mean values were compared using Student's *t* test for unpaired data and were considered significant if $P < 0.05$.

Results

Histology and ultrastructure of heterotransplanted tumor. The histologic appearance of the first generation xenograft of the original tumor is shown in Fig. 1 *A*. The histology of this tumor recapitulates that of the patient's primary tumor and is consistent with the diagnosis of a moderate to well-differentiated adenocarcinoma (19). Solid-growing nests of tumor cells are interspersed with mucin-producing ductlike structures. Ductal structures exhibited a spectrum of differentiation, ranging from irregular to well-differentiated. The ultrastructural appearance of the heterotransplanted tumor revealed characteristic features of duct epithelium, including apical membranes consisting of well-developed microvilli and cytoplasm containing numerous secretory vesicles, rough endoplasmic reticulum, Golgi complexes, desmosomes, and tight junctions (Fig. 1 *B*). No zymogen granules were observed.

The HPAC cells grew as monolayers exhibiting a morphology typical of epithelial cells (Fig. 2 *A*). Cells were quite heterogeneous in size and shape with large nuclei and distinct nucleoli. Postconfluent monolayers formed domes of various sizes (Fig. 2 *B*). Ultrastructurally, HPAC cells were found to form a polarized epithelial sheet (Fig. 2 *C*). Tight junctions were observed linking the apical or medium surface. The apical surface had numerous microvilli that contained a dense core of microfilaments that extended into the cytoplasm. The basolateral portion of cell membranes lacked microvilli but had desmosomes and interdigitations. Scanning electron microscopy revealed abundant microvilli on the cell surfaces (Fig. 2 *D*). The morphologic appearance of HPAC cells has remained unchanged after 90 passages in vitro (data not shown).

Biological properties. The biological properties of HPAC cell line characterized from cells from an early passage (no. 5) and a late passage (no. 90) are summarized in Table 1. With the exception of colony formation on plastic, the biological properties of HPAC cells have remained relatively unchanged in 90 passages. An increase in colony forming efficiency on plastic, from an average of 46% to an average of 64% was observed with increasing passage of the cells. Although clonal growth of HPAC cells in soft agar was achieved, it occurred at a much lower efficiency than on plastic. Tumor nodules developed in athymic BALB/c nude mice at the site of inoculation within 6 wk. Histologically, the tumors that develop resemble the tumor line CAP (data not shown). Metastasis of the tumor was never observed. The epithelial nature of HPAC cells was confirmed by positive staining for cytokeratins. Negative staining for vimentin and chromogranin A indicates the lack of fibroblasts and endocrine cells in these cultures. Immunohistochemical staining revealed that HPAC cells express the pancreatic ductal epithelium marker DU-PAN-2 (5), as well as antigens recognized by the monoclonal antibodies HMFG1 and AUA1. These latter antigens are typically located on different membrane domains, i.e. apical (HMFG1) and basolateral (AUA1), of polarized neoplastic and non-neoplastic epithelial cells (2,45). Crude HPAC cell extracts contained significant concentrations of tumor-associated antigens CEA, CA 125, and CA 19–9, with CEA being produced at the highest concentration. The presence of pancreatic enzymes (amylase, trypsin, chymotrypsin) was not detected in crude cell extracts.

Karyotype. A study of 35 G-banded metaphases prepared from HPAC cells at Passage 90 revealed a modal number of 61 chromosomes. A narrow range of chromosome number was observed, with from 60 to 62 chromosomes appearing in 80% of the metaphases. The representative karyotype was 61,X,-msX,+1,+2,+3,+5,+7,+9,+11,−13,+15,+17,+19,; pl20,−21,+22,+der(8)t(5;8),+der(8)t(5;8),del(11)(p11),+der (12)t(12;?),+der(13)t(9;13),del(17)(p12),19p+,+mar[i(5p)?;c b,+mar[i(5p)?] (Fig. 3). The apparent isochromosome 5p was present in two copies in greater than 90% of the Passage 90 metaphases. The derivative chromosome 8 with the apparent 5p translocation was also very stable (>90%). Most karyotype variation consisted of random loss of normal autosomes.

Effect of supplemental factors and FBS on DNA synthesis. HPAC cells were initially established in monolayer culture in a serum-containing, hormone supplemented medium. To establish if the growth of HPAC cells was dependent on the presence of one or more of the medium supplements, the effects of the four individual supplemental factors and FBS on HPAC cell DNA synthesis were compared by means of a deletion study. Table 2 illustrates that omission of EGF, insulin, or FBS significantly decreased DNA synthesis relative to cells cultured in full growth medium. Exclusion of FBS resulted, not surprisingly, in the greatest inhibition (66 ± 5%), with EGF and insulin exclusion resulting in a 43 ± 4% and 28 ± 4% inhibition, respectively. Exclusion of transferrin or hydrocor-

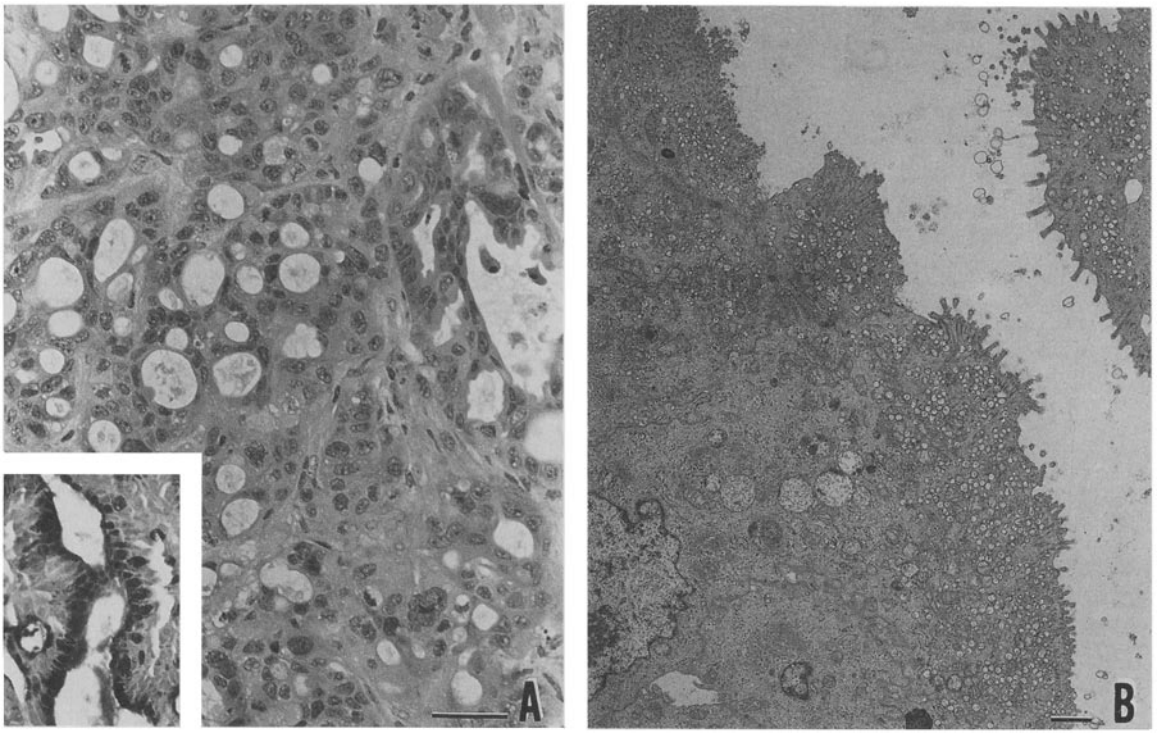

FIG. 1. Histologic and ultrastructural appearance of the first generation xenograft (CAP) grown in the nude mouse. *A*, tumor consists of nests of malignant epithelial cells many of which form glandular structures. Hematoxylin and eosin; *Inset*, cells surrounding the glandular structures contain periodic acid-Schiff (PAS) positive materials. PAS with diastase treatment. *Bar* = 50 μm. *B*, transmission electron photomicrograph of the same tumor in *A*. Cells are arranged around a lumen with basally located nuclei and apical plasma membranes have numerous microvilli extending into the lumen. *Bar* = 1 μm.

tisone had no significant effect on HPAC cell DNA synthesis. These studies suggest that in the presence of 5% FBS, EGF, and insulin are required for a maximal rate of DNA synthesis, whereas transferrin and hydrocortisone are not. Furthermore, in the absence of FBS the mixture of the four supplemental factors could not support maximal DNA synthesis in these cultures.

The effects of increasing concentrations of insulin, IGF-I, EGF, TGF-α, transferrin, and glucocorticoids on DNA synthesis after 72 h of treatment were studied in medium supplemented with 3% FBS (Table 3). As anticipated from the results of the deletion study, independent of the presence of the other factors, insulin and EGF individually elicited a dose-dependent stimulation of HPAC cell DNA synthesis. Insulin stimulation of DNA synthesis was maximal at 2.0 μg/ml (340 n*M*) and half-maximal (ED_{50}) at 0.5 μg/ml or 85 n*M*. These high insulin concentrations required to stimulate DNA synthesis suggested that insulin may be interacting with a receptor for IGF-I. Recombinant human IGF-I stimulated DNA synthesis in a dose-dependent manner with an ED_{50} of 5.1 ng/ml or 0.65 n*M*. In terms of ED_{50} value, IGF-I was significantly more potent than insulin in stimulating DNA synthesis; however, the maximal responses of the two peptides (~1.8-fold increase) were equivalent. EGF stimulation of DNA synthesis was maximal at the highest dose tested 20.0 ng/ml (3.3 n*M*) and half-maximal at 6.0 ng/ml (1.0 n*M*), which approximates the K_d values for EGF binding to four human pancreatic cancer cell lines (20). Recombinant human TGF-α, a homolog of EGF, was 3 times more potent that EGF in stimulating DNA synthesis (ED_{50} = 0.33 n*M*), which is in accord with the effects of these mitogens on other human pancreatic cancer cell lines (3). These results suggest that EGF and insulin exert their effects on HPAC DNA synthesis through interaction with specific EGF, insulin, and/or IGF-I receptors. In contrast, transferrin, at the concentrations studied (0.25 to 5.0 μg/ml or 3.2 to 64 n*M*), had no detectable effect on DNA synthesis. When HPAC cells were treated with hydrocortisone and the synthetic glucocorticoid dexamethasone, DNA synthesis was inhibited in a dose-dependent fashion. Dexamethasone was a more potent inhibitor than hydrocortisone, decreasing DNA synthesis half-maximally at 0.39 ng/ml (1.0 n*M*) compared to 7.5 ng/ml (21 n*M*) for hydrocortisone. The potency of hydrocortisone and dexamethasone to inhibit DNA synthesis is directly related to their affinity for the GR (35), suggesting that the effect of the glucocorticoids is mediated by a GR.

Specificity of glucocorticoid effect on DNA synthesis. To determine the specificity of the steroid effect on HPAC cell DNA synthesis, HPAC cells were incubated in the presence of various concentrations of steroids with different biological activities; after 72 h of treatment DNA synthesis was assayed by [^{3}H]thymidine incorporation. As shown in Fig. 4, of the six steroids tested only those with glucocorticoid biological activity inhibited HPAC cell DNA synthesis in a dose-dependent fashion. 17β-Estradiol, 11-deoxycorticosterone, testosterone, and progesterone were without an effect at physiologic concentrations. At the highest dose tested (1 μ*M*) both progesterone and testosterone reduced DNA synthesis, but to a much lesser degree than dexamethasone and hydrocortisone. To examine the effect of RU 38486, a potent glucocorticoid antagonist (29), on the dexamethasone-mediated inhibition of DNA synthesis, cells were exposed to the antagonist alone and in the presence of dexa-

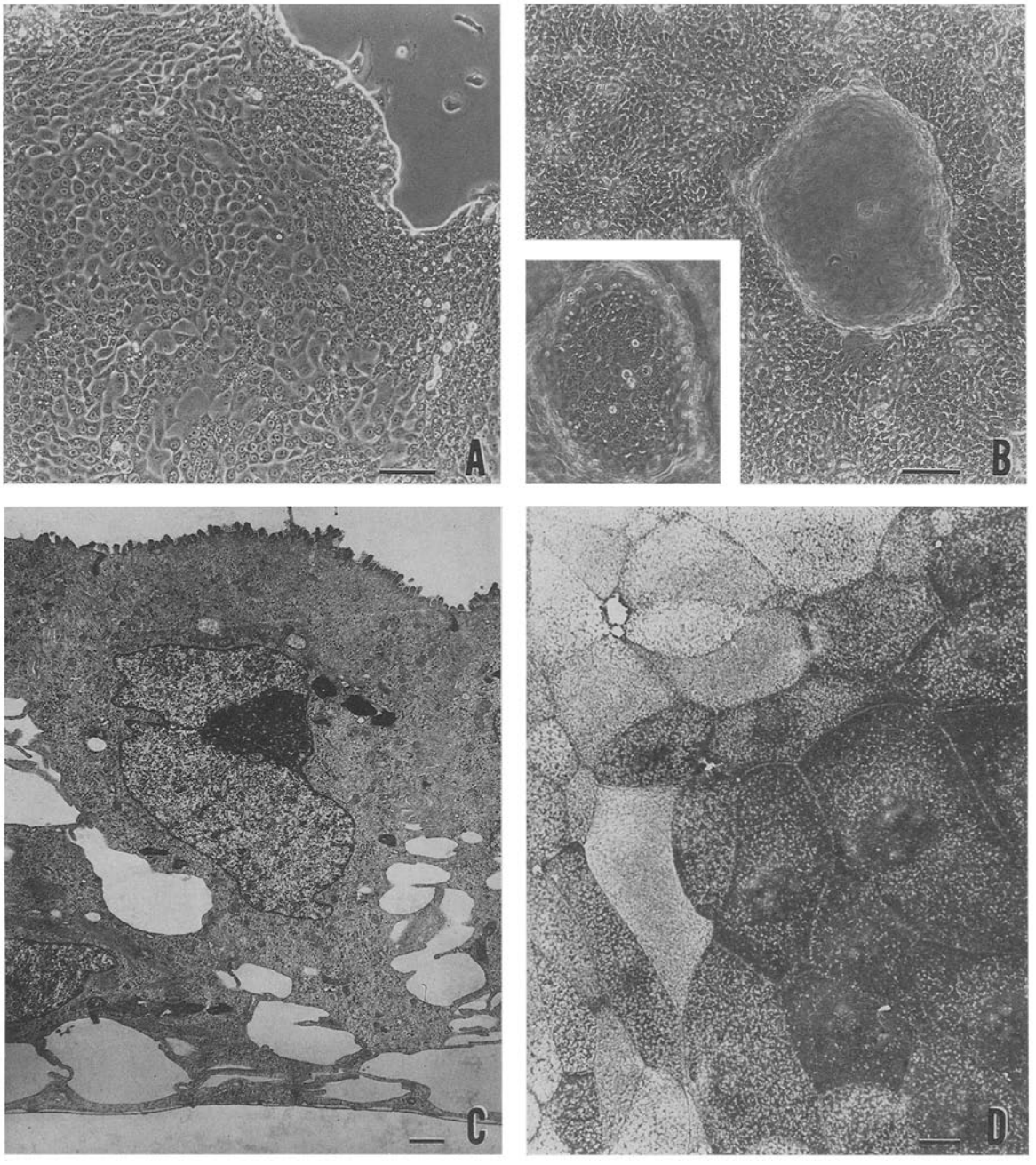

FIG. 2. Phase contrast and electron photomicrographs illustrating the morphology and ultrastructural appearance of HPAC cells in monolayer culture. Phase contrast photomicrographs illustrating the morphology of HPAC cells in pre-confluent (*A*) and postconfluent (*B*) cultures. *Bar* = 100 μm. *Inset*, focus is on the top of the cell dome. *Bar* = 100 μm. Transmission electron photomicrograph of HPAC cells in monolayer culture (*C*). Sections are cut perpendicular to the bottom of the culture flask; *Bar* = 1 μm. Scanning electron photomicrograph of HPAC cells in monolayer culture (*D*). *Bar* = 10 μm.

methasone. As shown in Table 4, exposure of cultures to a 10-fold excess of RU 38486 completely suppressed the dexamethasone-mediated inhibition of DNA synthesis in HPAC cells. Incubation of the cells with antagonist alone had no effect on DNA synthesis. These findings provide further support for the mediation of the growth suppression of HPAC cells by dexamethasone and hydrocortisone by a functional GR.

Effect of glucocorticoids on HPAC cell proliferation. To determine the effects of glucocorticoids on HPAC cell proliferation, cells were cultured in the presence or absence of 1 μ*M* of either hydrocortisone or dexamethasone, in medium containing 3% FBS with or without the mixture of supplemental factors (EGF, insulin, transferrin). After 6 days in the presence of 3% FBS but in the absence of any other growth factors, both steroids reduced total DNA content by an average of 63% compared to untreated cultures. This reflects an increase in the population doubling time from 80 to 112 and 120 h, respectively (Fig. 5 *A*). In the case of dexamethasone, growth suppression could be reversed by the removal of the steroid

TABLE 1

BIOLOGICAL PROPERTIES OF CELL LINE HPAC[a]

Passage No.	Doubling Time, h	Colony Formation: Plastic, %	Colony Formation: Agarose, %	Tumorigenicity[b]	Keratin[c]	Vimentin	Chromagranin A	DU-PAN2	HMFG 1	AUA 1	Tumor Markers[d]: CEA, ng/mg	Tumor Markers[d]: CA 19-9, U/mg	Tumor Markers[d]: CA 125, U/mg
5	38 ± 6	46 ± 7	2.2 ± 0.5	4/5	+	−	−	+	+	+	219 ± 22	1.5 ± 0.2	48 ± 25
90	41 ± 2	64 ± 5	3.2 ± 0.8	5/5	+	−	−	+	+	+	202 ± 26	1.0 ± 0.8	36 ± 20

[a] Except for tumorigenicity and immunostaining, data are expressed as mean ± SEM (n = 3).
[b] Data are number of growing tumors within 6 wk.
[c] Expression of keratin, vimentin, chromogranin A, and antigens recognized by antibodies DU-PAN-2, HMFG1, and AUA1 was determined by immunostaining of monolayer cultures.
[d] Data are nanogram or units of tumor-associated antigen per milligram protein in crude cell extracts.

in the medium. When cells were exposed to 1 μM of either glucocorticoid in the presence of the supplemental factors, no significant suppression of growth was observed (Fig. 5 *B*). Additionally, the population doubling times of both the untreated (47 h) and treated (hydrocortisone, 58 h; dexamethasone, 41 h) cells were decreased when compared to the cells cultured in the absence of the supplemental factors. These observations agree with the results from the DNA synthesis studies, demonstrating that the addition of exogenous insulin, EGF, and transferrin in combination totally blocks the growth suppression of HPAC cells by glucocorticoids, and enhances the proliferation of HPAC cells.

Glucocorticoid receptor in HPAC cells. To establish whether HPAC cells synthesized a functional GR, competition equilibrium binding assays were performed. Figure 6 shows a representative result of binding data when cytosol fractions were incubated with varying concentrations of [^{3}H]dexamethasone at 4° C for 2 h, a time determined to be sufficient for maximal binding to occur at all concentrations evaluated. Binding was saturable and demonstrated high affinity. Scatchard analysis revealed a single class of high-affinity binding sites. The dissociation constant (K_d) was 3.8 ± 0.9 nM with a ligand binding site concentration (B_{max}) of 523 ± 128 fmol/mg of cytosol protein. The K_d value for binding of [^{3}H]-

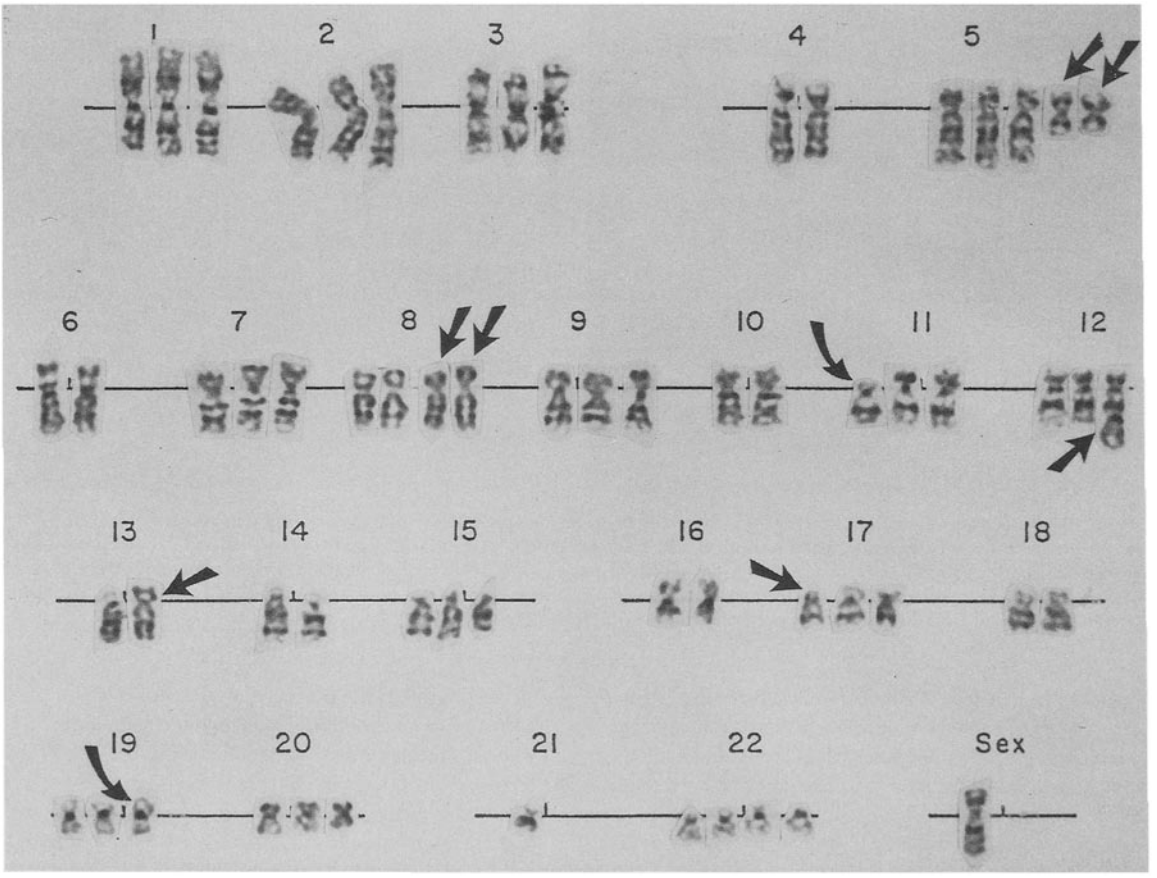

FIG. 3. Representative G-banded karyotype of HPAC cells at Passage 90. *Arrows*, abnormal chromosomes.

TABLE 2

EFFECT OF EXCLUDING INDIVIDUAL MEDIUM SUPPLEMENTAL FACTORS ON DNA SYNTHESIS BY HPAC CELLS

Culture Medium	Factor Excluded	Percent Control[a]
DME/F12-4F5[b]	—	100
	EGF	57 ± 6[c]
	Transferrin	97 ± 6
	Insulin	72 ± 6[c]
	Hydrocortisone	93 ± 4
	FBS	34 ± 4[c]

[a] Values are representative of two experiments (Passages 95 and 96) and are expressed as the mean percentage of control ± SEM of triplicate cultures for [^{3}H]thymidine incorporation per well. Cells (1×10^4 cells/well) were cultured in the presence of DME/F12-4F5 (*control*) or DME/F12-4F5 lacking a single supplemental factor. After 72 h incubation, DNA synthesis was measured as described in Materials and Methods. DNA synthesis for control wells was 14 840 ± 740 cpm of [^{3}H]thymidine.

[b] DME/F12-4F5 contains four supplemental factors (EGF [10 ng/ml], transferrin [5 μg/ml], insulin [2 μg/ml], hydrocortisone [40 mg/ml]) and 5% FBS.

[c] $P < 0.05$ vs. DME/F12-4F5.

dexamethasone is very similar to the concentration of dexamethasone at which half-maximal inhibition (IC_{50}) of DNA synthesis occurs (Table 3). In agreement with previous reports (8), the cytosol fraction from HeLa cells demonstrated a single class of high-affinity binding sites with an average K_d of 2.5 ± 0.5 n*M* and the number of binding sites equal to 1453 ± 134 fmol/mg cytosol protein.

To identify the size of the functional GR protein in HPAC cells, cytoplasmic extracts were subjected to Western blot analysis using the anti-GR antiserum Ahugr 150–175 GR. (Fig. 7). A major band of approximately M_r 96 000 was identified in the cytosol of HPAC

TABLE 3

EFFECT OF VARIOUS HORMONES AND GROWTH FACTORS ON DNA SYNTHESIS BY HPAC CELLS

Factor Included (Concentration Range)	ED_{50} or IC_{50}	Maximum Effective Concentration	Percent Control[a]
Control[b]	—	—	100 ± 4
Insulin (0.25–5.0 μg/ml)	0.5 μg/ml	2.0 μg/ml	188 ± 16[c]
IGF-I (0.5–50 ng/ml)	5.1 ng/ml	10.0 ng/ml	178 ± 12[c]
EGF (0.5–20.0 ng/ml)	6.0 ng/ml	20.0 ng/ml	167 ± 16[c]
TGF-α (0.5–50 ng/ml)	2.0 ng/ml	10.0 ng/ml	209 ± 12[c]
Transferrin (0.25–5.0 μg/ml)	ND[d]	ND	—
Hydrocortisone (3.6 pg/ml-3.6 μg/ml)	7.5 ng/ml	0.36 μg/ml	36 ± 8[c]
Dexamethasone (3.9 pg/ml-3.9 μg/ml)	0.39 ng/ml	39.0 ng/ml	33 ± 6[c]

[a] Values are representative of two experiments (Passages 96 and 98) and are expressed as the mean percentage of control ± SEM of triplicate cultures for [^{3}H]thymidine incorporation per well at the maximum effective concentration. Cells (1×10^4 cells/well) were cultured in the presence of DME/F12-0F3 alone (*control*) or with varying concentrations of insulin, EGF, transferrin, hydrocortisone and dexamethasone. After 72 h incubation, DNA synthesis was measured as described in Materials and Methods. DNA synthesis for control wells was 9970 ± 441 cpm of [^{3}H]thymidine.

[b] Control is DME/F12-0F3 which contains no supplemental factors and 3% FBS.

[c] $P < 0.05$ vs. control; [d] ND = none detected.

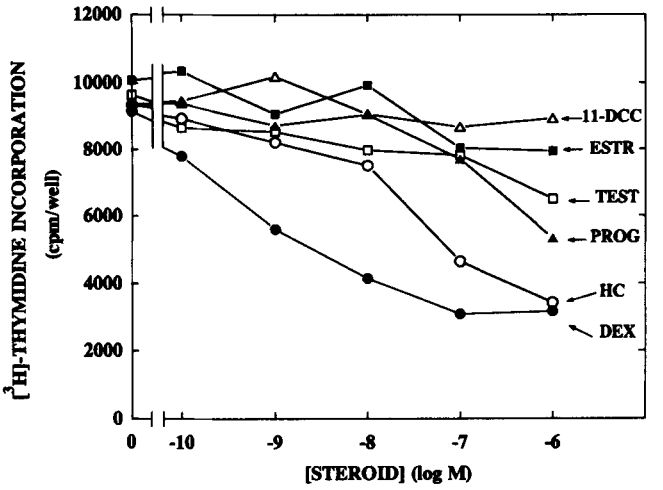

FIG. 4. Effect of various steroid hormones on HPAC cell DNA synthesis. HPAC cells (1×10^4 cells/well) were cultured in complete growth medium (DME/F12–4F5) for 24 h. DME/F12-4F5 was replaced with DME/F12–0F3 containing the indicated concentrations of 11-deoxycorticosterone (*11-DCC*), 17β-estradiol (*ESTR*), testosterone (*TEST*), progesterone (*PROG*), hydrocortisone (*HC*), and dexamethasone (*DEX*). After 72 h incubation, DNA synthesis was measured by [^{3}H]thymidine incorporation as described in Materials and Methods. *Each point* represents the mean of triplicate cultures.

(*lane 1*). An identical band was observed in the cytosol fraction prepared from HeLa cells (*lane 3*). These findings are in agreement with previous reports on the size of the GR protein expressed in HeLa cells (7) as well as other human tumor cell lines (16). A number of minor bands of immunoreactivity were detected in both HPAC and HeLa cytosol fractions, some of which co-migrated with nonspecific signals (*lanes 2* and *4*). A second band representing a protein of lower molecular weight (M_r 56 000) was consistently observed in the HPAC cytosol fraction. Inasmuch as no corresponding band was observed in the blots probed with normal rabbit serum, it may be the product of proteolytic modification of the GR.

To determine the size of the GR mRNA, a Northern blot analysis was performed with total RNA from the CAP xenograft, HPAC, and HeLa cells, using the *Bam*HI fragment of the OB7 plasmid as the

TABLE 4

EFFECT OF DEXAMETHASONE AND THE GLUCOCORTICOID ANTAGONIST RU 38486 ON DNA SYNTHESIS IN HPAC CELLS

Treatment	DNA Synthesis, cpm/well[a]	Percent Control
Control	9164 ± 689	100 ± 7
RU 38486	9785 ± 346	106 ± 4
Dexamethasone	3087 ± 220	34 ± 2[b]
Dexamethasone + RU 38486	9659 ± 539	105 ± 5

[a] Values are representative of two experiments (Passages 95 and 96) and are expressed as the mean ± SEM of cpm of [^{3}H]thymidine incorporated per well from triplicate cultures. Cells (1×10^4 cells/well) were cultured in the presence of DME/F12-0F3 alone (control) or with RU 38486 (1 μ*M*), dexamethasone (0.1 μ*M*), or a combination of both. After 72 h incubation, DNA synthesis was measured as described in Materials and Methods.

[b] $P < 0.05$ vs. control.

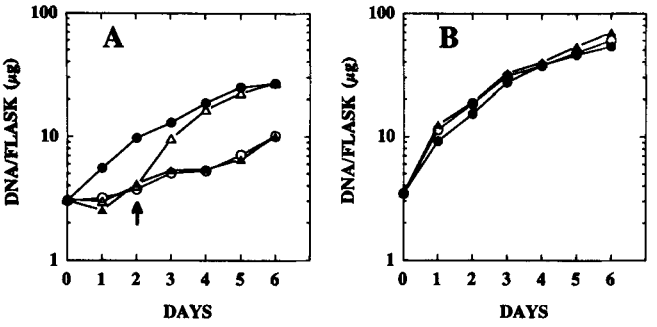

FIG. 5. Effect of glucocorticoids on HPAC cell proliferation in the absence or presence of supplemental growth factors (insulin, transferrin, EGF). HPAC cells (2×10^5 cells/flask) were cultured in DME/F12–4F5 for 24 h, then DME/F12–4F5 was replaced with either (*A*) DME/F12–0F3 (no supplemental growth factors) or (*B*) DME/F12–3F3 (with supplemental growth factors [insulin, transferrin, EGF]) containing 1 μM dexamethasone (*open circles*), 1 μM hydrocortisone (*solid triangles*), or without any additive (*solid circles*) for 6 days. At 2 days (*arrow*) in parallel cultures containing DME/F12–0F3 and 1 μM dexamethasone the dexamethasone medium was replaced with DME/F12–0F3 lacking any steroid (*open triangles*). Culture medium was changed every 2 days and at the indicated times DNA content was determined. *Each point* represents the mean of five flasks.

probe. Both the CAP xenograft and HPAC total RNA extracts contained an identical single hybridizing band with a size of approximately 7 kilobases (Fig. 8). HPAC cells cultured in DME/F12–0F3 medium with or without the supplemental factors exhibited similar hybridization bands. An identical single band was found in the RNA extract from HeLa cells, which is in agreement with previous reports (17).

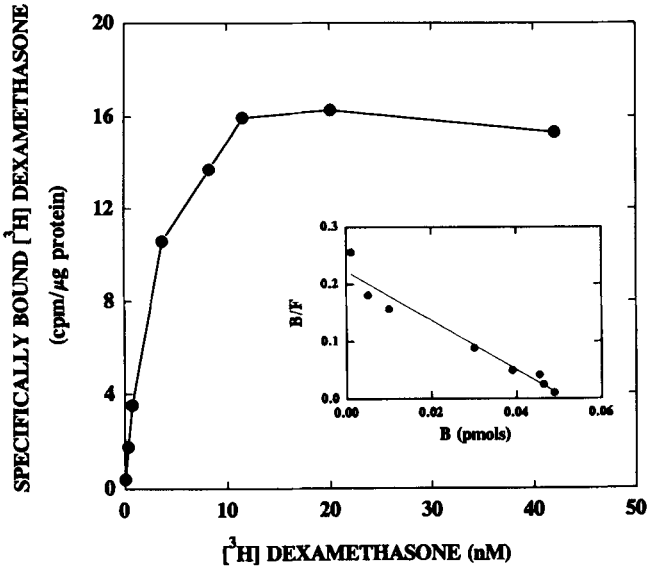

FIG. 6. Competition equilibrium radioligand binding analysis of [^{3}H]-dexamethasone binding to cytosol fraction prepared from HPAC cells. HPAC cells were grown to confluence in growth medium lacking supplemental growth factors, then a cytosol fraction was prepared and specific binding of [^{3}H]dexamethasone as described in Materials and Methods. Saturable specific binding is plotted as a function of ligand concentration. *Inset*, Scatchard analysis of the binding data.

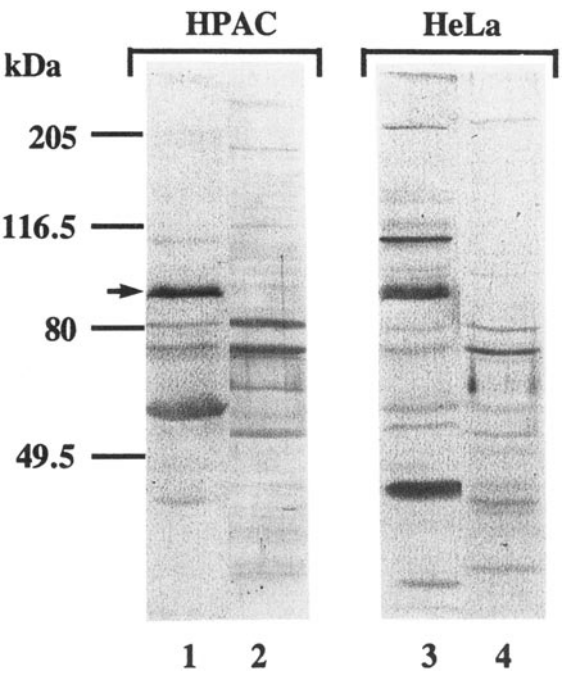

FIG. 7. Immunoblot analysis of HPAC and HeLa cell cytosol fractions. Cytosol proteins (80 μg) from HPAC cells (*lanes 1* and *2*), and HeLa cells (*lanes 3* and *4*) were separated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane. Immunoblots were reacted with polyclonal rabbit anti-human GR (*lanes 1* and *3*) or normal rabbit serum (*lanes 2* and *4*) and detected colorimetrically after exposure to goat anti-rabbit IgG coupled to alkaline phosphatase. Migration of prestained molecular weight standards and their apparent molecular weight (kDa = $M_r \times 10^{-3}$) are shown to the *left* of *lane 1*. *Arrow*, marks the position of a major immunoreactive band at a M_r = 96 kDa.

DISCUSSION

Although a number of human pancreatic carcinoma cell lines have been established, none has been shown to express a functional GR and/or to have its growth inhibited by glucocorticoids. The results reported here demonstrate that physiologic concentrations of glucocorticoids can directly and reversibly inhibit the proliferation of HPAC cells, a new human pancreatic cancer cell line. Growth of HPAC cells could be inhibited in vitro up to 63% by dexamethasone, and inhibition of DNA synthesis could be prevented by the presence of a mixture of exogenous growth factors (EGF, insulin, transferrin) and RU 38486. The dose-response curves of the hydrocortisone and dexamethasone effects on DNA synthesis, the antagonism of RU 38486 toward this effect, and the lack of action of other steroid hormones at physiologic doses support the hypothesis that the GR is the mediator of glucocorticoid inhibition of HPAC cell growth. This assumption is strengthened by our demonstration that HPAC cell synthesize a functional GR protein.

With the single exception of the glucocorticoid-sensitive feature of these cells, HPAC cells exhibit biological properties which are similar to those exhibited by other human pancreatic cancer cell lines that were derived from moderate to well-differentiated ductal adenocarcinomas, e.g., RWP-1, RWP-2, Capan-1, Capan-2, SW-1990, BxPC-3, and MDAPanc-3 (9,12,21–23,44). The in vitro growth characteristics of HPAC are similar to those previously described for other pancreatic carcinoma cell lines (12,22,23,44).

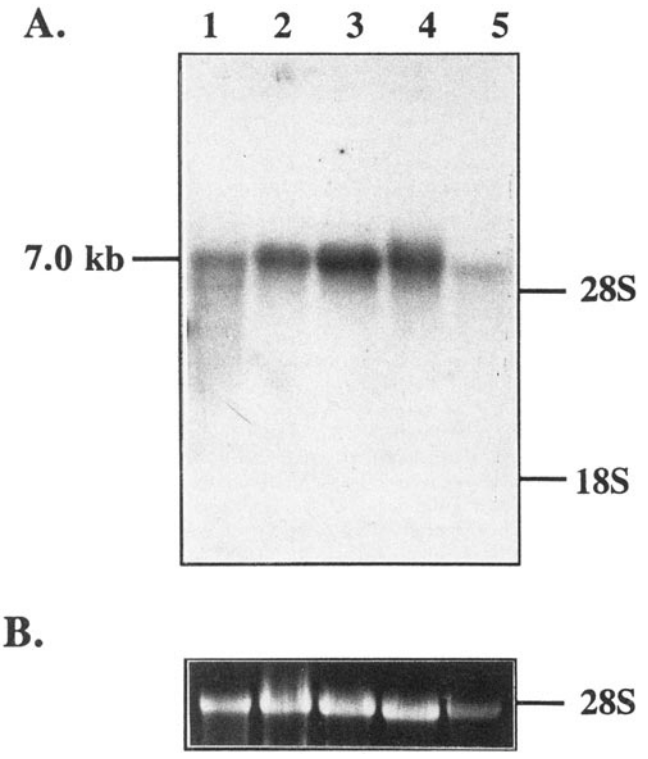

FIG. 8. Northern blot analysis of GR mRNA. Total cell RNA (20 μg/lane) prepared from CAP tumor tissue (*lane 1*), HPAC cells cultured in DME/F12–0F3 (*lane 2*), DME/F12–0F3 containing the supplemental factors insulin (2 μg/ml), EGF (10 ng/ml), transferrin (5 μg/ml) but no hydrocortisone (*lane 3*), the same as in lane 3 except with the addition of hydrocortisone (40 ng/ml) (*lane 4*) and HeLa cells (*lane 5*) was fractionated and transferred to nylon membranes as described in Materials and Methods. *A*, membrane was probed with a [^{32}P]-labeled GR cDNA; *B*, same membrane was stained with ethidium bromide.

The average doubling time of approximately 40 h (range, 38 to 41 h) for HPAC cells is well within the range of times reported for cell lines of similar differentiation, 30 to 64 h (9,12,21,23,44). Our findings that EGF, TGF-α, insulin, and IGF-I stimulated significant increases in thymidine incorporation in a dose-dependent fashion with ED_{50} values approximating the respective receptor K_d values, suggest that HPAC cells possess specific receptors for EGF, insulin, and/or IGF-I. However, since these results were achieved in serum-containing medium, it is possible that the ED_{50} values for these peptides may have been overestimated due to degradation of the mitogens in the culture medium. Thus, from the present results we cannot determine whether insulin's effect is mediated by the interaction with IGF-I or with its homologous receptor. Further studies will be required to determine the nature and number of growth factor receptors expressed in HPAC cells.

Glucocorticoids have an important regulatory role in the growth and development of a variety of tissues including the pancreas (43,48). The actions of glucocorticoids on the pancreas are mediated by a GR (41). Evidence from functional studies in vivo, as well as those performed in vitro that show glucocorticoids directly modulate the growth and function of pancreatic acinar and islet cells, support the view that these cells express a GR in vivo (6,15,27). Until the present report there had been no evidence to suggest that pancreatic ductal cells express a GR. Inasmuch as there is some controversy as to the cell of origin for pancreatic ductal adenocarcinoma, it is not certain what cell type the HPAC cell line originated from (36). Further studies will be required to ascertain whether HPAC cells are genetically ductal or represent a progenitor or intermediate cell type with features of acinar/islet and duct cells. Clearly, studies aimed at identifying the cell types that synthesize the GR in the human pancreas are needed. Nevertheless, the regulation of neoplastic pancreas cell growth by glucocorticoids is most certainly a modified remnant of normal mechanisms for pancreatic cell proliferation and differentiation.

Several mechanisms may be responsible for glucocorticoid-induced HPAC cell growth inhibition. Ligand-bound glucocorticoid receptors enhance or decrease the rate of gene transcription by binding as a homodimer to short sequences of DNA termed glucocorticoid response elements (GRE) (11). Unlike the lytic effect observed in lymphoid-derived cells (13), the effect of dexamethasone on HPAC cells is reversible, suggesting that the cells are arrested at some point in the cell cycle. Similar cytostatic effects induced by glucocorticoids have been observed in a variety of human and rodent tumor cells (10,33,47). Likely mechanisms of glucocorticoid action on HPAC cell proliferation will involve the stimulation or inhibition of transcription of genes coding for autocrine growth factors and/or their cognate receptors, inhibitors of cell proliferation, or a combination of both. We have identified four mitogens for HPAC cells in the present report, insulin, IGF-I, EGF and TGF-α. Because the cell's response to mitogens can be modulated by both the levels of the mitogen and its receptor, glucocorticoid regulation of insulin, IGF-I, and EGF receptors may be potentially important in glucocorticoid-mediated growth suppression of HPAC cells. Indeed, glucocorticoids have been reported to modulate both the receptor concentration and affinity for ligand of EGF and insulin receptors (30,31). However, our findings that the combination of EGF/insulin/transferrin prevents the suppression of DNA synthesis and cell proliferation suggest that the receptor population is unaffected by the steroid hormones. Alternatively, because EGF has been shown to down-regulate the GR in HBL 100 breast cancer cells associated with phosphorylation of GR, this could explain the supplemental factors' attenuation of glucocorticoid-induced inhibition of HPAC cell growth (33). Interestingly, both EGF and insulin have been shown to suppress the growth inhibitory effects of dexamethasone on AR42J cells (15).

These data are compatible with the autocrine hypothesis of HPAC cell replication in that if glucocorticoids inhibited HPAC cell cycle progression by inhibiting autocrine growth factor production and action, then the effect would be expected to be attenuated by addition of exogenous growth factor, which is what we observed. Alternatively, other mechanisms, specifically those involving growth factor modulation of steroid sensitivity through changes in receptors and vice versa, cannot be ruled out. Ultimately, determination of the mechanism underlying the glucocorticoid growth suppression of HPAC cells will require the identification of those hormone-regulated genes that regulate the proliferative response in these cells.

Inasmuch as HPAC cells display many characteristics of pancreatic carcinoma cells in culture, future experiments examining the expression of the GR in other pancreatic carcinomas may implicate a role for glucocorticoids in the regulation of malignant pancreatic cell function. HPAC cells will be useful to study the effects of glucocorticoid action at the cellular level and its interrelationship with

growth promoters, e.g., EGF, TGF-α, insulin, and IGF-I, knowledge of which may be important for therapy of pancreatic adenocarcinoma and may provide information pertaining to the etiology, progression, and differentiation of this type of neoplasm. Identification of HPAC cell subpopulations, resistant to the effects of glucocorticoids, combined with sensitive cells should provide a versatile model in which to study the molecular basis for growth inhibition by glucocorticoids of human pancreatic tumor cells.

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