Cloned Cell Lines from a Transplantable Islet Cell Tumor Are Heterogeneous and Express Cholecystokinin In Addition To Islet Hormones

O. D. Madsen,* L.-I. Larsson,# J. F. Rehfeld,§ T. W. Schwartz,§ A. Lernmark,* A. D. Labrecque,‖ D. F. Steiner‖

*Hagedorn Research Laboratory, World Health Organization Collaborating Center for Research and Training on the Pathogenesis of Diabetes Mellitus, Gentofte, Denmark; †Unit of Histochemistry, University Institute of Pathology, Copenhagen, Denmark; ‖University Department of Clinical Chemistry, and Laboratory of Molecular Endocrinology, Rigshospitalet, Copenhagen, Denmark; ‖New England College of Optometry, Boston, Massachusetts; and †Department of Biochemistry, University of Chicago, Chicago, Illinois

Abstract. A liver metastasis (MSL) with a remarkable in vitro proliferation potential has been identified in an NEDH rat carrying a transplantable x-ray-induced islet cell tumor. Two insulin-secreting cell lines, MSL-G and MSL-H, with doubling times of 3–5 d were established by repeated limiting dilution cloning. In vivo inoculation of MSL-G cells induced severe hypoglycemia caused by a small but highly heterogeneous tumor as revealed by immunocytochemistry. Whereas most cells stained for the islet hormones, insulin, glucagon, and somatostatin, clustered cells were discovered to contain cholecystokinin (CCK). Additional in vitro-limiting dilution cloning, followed by immunocytochemical characterization, clearly demonstrated the capacity of single cell clones to simultaneously express the same four hormones. Radioimmunoassays with a panel of site-specific antisera of culture supernatants and purified cell extracts showed the MSL-G2 cells to produce, store, and secrete readily detectable amounts of processed and unprocessed CCK. Gastrin was not detected while coexpression of glucagon and CCK were demonstrated. Mutant clones selected for resistance to 6-thioguanine (frequency, $2 \times 10^{-7}$) and checked for HAT (hypoxanthine, aminopterin, thymidine) sensitivity retained the capacity for multi-hormone expression.

We propose that the MSL tumor contains pluripotent endocrine stem cells. The MSL tumor and the MSL-G2 cells in particular will allow studies of not only CCK biosynthesis and processing but also of mechanisms involved in tumor and islet cell differentiation.

SLET cell tumors frequently consist of mixed cell populations producing multiple hormones, although insulin is the major product (2, 16, 22). Whether such tumors are induced by a simultaneous transformation of different mature endocrine cell types or by a single transformation of a pluripotent islet cell is not known. The high frequency of occurrence of mixed multihormonal tumors (16, 22) supports the latter hypothesis, assuming that the transformed stem cell has retained the potential to differentiate into various hormone-secreting cells. It also has been speculated that each tumor cell may produce several hormones simultaneously (27, 28). Support for this idea has come from the establishment of clonal endocrine cell lines from an x-ray-induced transplantable islet cell tumor (2), some of which, although initially producing somatostatin, were capable of also expressing insulin (7, 26). In the mature islets of Lang- erhans in most vertebrates, however, the four major hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) are found in cytologically distinct B, A, D, and PP cells, respectively. Immunocytochemical staining experiments and electron microscopic cell identification have not provided support for the coexistence of any of these hormones within a single cell (16). Two other hormones—thyrotropin-releasing hormone (6) and gastrin—may be detected by biochemical or immunocytochemical techniques in the islets of Lang- erhans. Gastrin, however, is found only in the fetal and neonatal stage (23). Interestingly, gastrin cells are often detected in insulinomas and other pancreatic endocrine tumors, without clinical signs of hypergastrinemia (16). Additionally, the production of hormones such as growth (hormone)-releasing factor (9, 34, 40), ACTH (16, 22), and calcitonin (38), has been noted in pancreatic endocrine tumors. So far, cholecystokinin (CCK)–producing pancreatic endocrine tumors have not been described.

The identification of a malignant variant of an x-ray-in-
duced islet cell tumor (2) with an unusually high in vitro proliferation potential as well as a broad hormonal repertoire has allowed us to carry out a long-term study on the nature of multiple hormone-producing tumors. Wild-type and drug-resistant cells from the variant were cloned in vitro and subsequently used for formation of hormone-producing tumors in vivo.

In this report we have characterized these cell lines with respect to their growth properties and potential for gastro-entero-pancreatic hormone expression in vitro and in vivo.

**Materials and Methods**

**Transplantation**

Tumors were maintained in vivo by subcutaneous inoculation between the shoulder blades or by intraperitoneal injection in young NEDH rats (80-120 g) of small tumor pieces (1-3 mm³) from severely hypoglycemic (glucose concentration below 1.5 mM) donors carrying small tumors (0.5-1 g). Similarly, tumors were induced from cloned cell lines by subcutaneous injection of 100 μl serum-free tissue culture medium containing 5-10 × 10⁶ cells. After tumor excision, all donors were autopsied for detection of metastases. Blood glucose was measured using Hypocount (Hypoguard Ltd., Woodbridge, UK).

**Cell Culture**

Primary cultures were prepared by gently mincing freshly excised tumors with forceps and a scalpel in complete tissue culture medium (CTM) composed of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 5-10% fetal calf serum (KC Biological Inc., Lenexa, KS), 5-10% donor horse serum (KC Biological Inc.), and 2 mM L-glutamine and Pen-Strep (100 U/ml) from Gibco. The cell preparation was resuspended followed by sedimentation for 1 min to allow the removal of larger, mostly fibrous, tissue clumps. The supernatant was collected and centrifuged for 5 min at 250 g. The pellet was resuspended in CTM and primary cultures were prepared by serial dilution in 96-well (200 μl), 24-well (1 ml), 12-well (2.5 ml), or 6-well (5 ml) plates from Costar (Cambridge, MA). The culture dilutions spanned the cell concentrations of 0 to 500,000 cells/ml to ensure optimal conditions in at least a fraction of the cultures with respect to initial tumor cell concentrations and low fibroblast contamination. Plates were incubated at 37°C in 5% CO₂ in air for 2-4 wk without change of medium. Fibroblast-contaminated cultures could be identified by the color change of medium after 1-2 wk in culture. Residual wells were routinely checked for tumor cell proliferation. Medium was replaced approximately every 3 wk.

Proliferating colonies were isolated in 5 μl using a Pipetman P-20 (Gilson Co., Worthington, OH) equipped with a sterile tip and transferred to 0.5 ml medium in 24-well plates. If colonies attached and spread out during 3-4 wk, they were mechanically disrupted into smaller clumps to allow new colonies to form. A colony density of at least 10 per well was required before conventional trypsinization procedures could be used for further propagation. Established cultures were maintained by bi-weekly medium change and trypsinization every 2-4 wk with a split ratio of 1:2 to 1:4.

**Cell Cloning**

Clonal cell lines were established by the limiting dilution technique. Cell suspensions containing 5-8 cells/ml were distributed into microtiter plates (100 μl/well). Single cell proliferation was supported by 50% (vol/vol) conditioned medium obtained from 1-2-d-old medium from dense primary cultures. Plates were sealed with parafilm strips to allow free CO₂/O₂ exchange and reduce evaporation, and then left in the incubator for up to 1 mo before proliferation was directly visible. Selected clones were expanded as described above.

**Trypsinization**

Cells were prewashed in Hank’s balanced salt solution (HBSS) without Ca⁺⁺ and Mg⁺⁺ (Gibco) and incubated for 5 min at 37°C with trypsin, EDTA (Gibco). After extensive pipetting, the reaction was stopped by the addition of 1 vol of CTM. Cells were centrifuged and resuspended at the desired density. Several of the MSL cells, especially MSL-G2, attached very firmly.
Insulin assays were performed as described (8, 25) with the following modifications. The use of Sarstedt tubes (no 73.1055) and 96-tube racks (no. 95.1046; Sarstedt, Inc., Princeton, NJ) allowed a semiautomation at the microtiter scale with the use of microtiter plate carriers (Beckman Instruments, Inc., Palo Alto, CA), multichannel pipettes (Flow Laboratories, Inc., McLean, VA), and manifolds (Wheaton Instruments Div., Wheaton Industries, Millville, NJ).

In the assay, 100 µl of moniodinated insulin (5,000 cpm/tube, obtained from Dr. Bruce H. Frank, Eli Lilly Research Laboratories, Indianapolis, IN) was added to 50 µl tissue culture sample, and 100 µl of guinea pig anti-insulin diluted 1:100,000 (Dako Corp., Santa Barbara, CA; Accurate Chemical & Scientific Corp., Westbury, NY). Bovine γ-globulin (0.22 %; Sigma Chemical Co., St. Louis, MO) was present as carrier during the incubation. Carbowax 8000 (12.5 %; Fischer Chemical Co., Fair Lawn, NJ) was used to separate bound from free antigen.

Cloning for Drug Resistance

Titration studies were performed using serial dilutions of 6-thioguanine (1 ng/ml to 100 µg/ml; Sigma Chemical Co.) in CTM to optimize selection conditions for particular cell lines. Mass cultures (2–3 × 10^7 cells/75-cm² flask) were grown in 25 ml of medium in the presence of the desired concentration of 6-thioguanine with or without ethylmethanesulphonate (150 µg/ml; Sigma Chemical Co.) as mutagen. After ∼2 wk, 80% of medium with dead cells was replaced with 10 ml fresh selective medium. Cultures were maintained by a 50% medium change every 4–5 wk until proliferating clones could be observed. Colonies were picked out of bottles using bent Pasteur pipettes and propagated according to the method previously mentioned. Surviving clones were tested for HAT (hypoxanthine, aminopterin, thymidine) sensitivity.

Immunocytochemistry

Cell cultures growing on coverslips (9 × 9-mm, Belco Glass, Inc., Vineland, NJ) were fixed in 1% paraformaldehyde in 0.05 M sodium phosphate buffer (pH 7.4) (18) and could be stored for prolonged times in fixative without loss of immunoreactivity. The cells were permeabilized as described (18). In addition, solid tumors were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for at least 24 h, soaked in 20% (wt/vol) sucrose in the same buffer overnight, and then quenched in melting Freon-22 as described (18). Cryostat sections were cut at 8 µm at −20°C. Permeabilized coverslips and cryostat sections were stained by previously detailed procedures (18), using the following: (a) rabbit gastrin/cholecystokinin antiserum No. 2717, recognizing the COOH-terminal tetrapeptide amide common to gastrin (Fig. 1) and CCK (20); (b) rabbit CCK-antiserum 1561, recognizing the NH₂-terminal 15–20 region of CCK-33 and devoid of cross-reactivity to gastrin (Fig. 1) (32, 33); (c) rabbit CCK-antiserum 4698, recognizing the NH₂-terminal tetrapeptide of CCK-8 and devoid of cross-reactivity to gastrin (Fig. 1) as detailed elsewhere (20, 29); (d) rabbit gastrin-34-specific antiserum, specifically recognizing the NH₂-terminal sequence of gastrin-34 (Fig. 1), kindly donated by Dr. N. Yanaihara, Shizuoka, Japan, and described in detail elsewhere (13); (e) rabbit anti-glucagon sera Nos. 4304 and 4316, kindly donated by Dr. Jens Holst, Dept. of Physiology, Panum Institute, Copenhagen. Antiserum 4304 reacts with both pancreatic glucagon and with larger glucagon precursor forms in-
including glicentin, whereas serum 4316 reacts exclusively with pancreatic-type glucagon (19); (f) rabbit anti-somatostatin antiserum R213/3, previously described in detail (21); (g) rabbit anti-human pancreatic polypeptide serum No. 4488, raised against CCK-33 and reacting as described (29); (h) guinea pig anti-motilin serum, a kind gift of Professor N. Yanaihara; or (k) rabbit anti-human growth hormone serum kindly donated by Dr. K. Hanssen, Department of Medicine, Akers Sykehus, Norway, and previously characterized (17). All antisera were applied at their optimal dilutions for 24 h at 4°C as described (18). The site of antigen-antibody reaction was revealed either by the peroxidase-antiperoxidase procedure of Sternberger (37) or by indirect immunofluorescence using fluorescein isothiocyanate-(FITC-) or tetramethylrhodamine isothiocyanate-(TRITC-) labeled antibodies (18). Immunofluorescence preparations were examined in a Leitz Orthoplan epifluorescence microscope with selective interference filters and mercury/xenon burners for excitation of FITC and TRITC, at 490 nm or 546 nm, respectively.

Controls included conventional staining controls as recommended (37) as well as specificity controls using antisera preabsorbed against synthetic human gastrin I (ICL), synthetic somatostatin-14 (Peninsula Laboratories, Inc., Belmont, CA) purified porcine glucagon (NOVO, Bagsvaerd, Denmark), synthetic tetragastrin and gastric inhibitory polypeptide (Peninsula Laboratories, Inc.), 99% pure porcine CCK-33 (a kind gift from Professor V. Mutt, Department of Chemistry, Karolinska Institute, Stockholm, Sweden), and insulin (Nordisk Gentofte A/S, Gentofte, Denmark). All stainings were specific according to these criteria and could be abolished only by preabsorption against the appropriate but not unrelated peptides.

Indirect immunofluorescence was used in double-staining experiments to test for the joint presence of two hormones in the same cell. Permeabilized X-ray induced insulinoma

(W. Chick et al. 1977)

Successive transplantation

Gen 12:
A liver metastasis (MSL) was observed in addition to the main tumor (MP)

MP

Gen 13
(n=2)

Gen 14
(n=9)

Gen 15
(n=11)

Gen 13

Gen 1

In vitro cultures

MSL-A (insulin neg.)

MSL-B (insulin neg.)

Gen 1

Gen 2

Gen 3

Gen 4

(n=1)

(n=5)

(n=9)

(n=11)

MSL-C

MSL-D

MSL-E

MSL-F

MSL-G

MSL-H

Insulin producing
sub-clones

tall insulin pos.

Gen 2

Gen 3

Gen 4

(n=5)

(n=9)

(n=11)

Figure 3. Subsequent transplantations and tissue cultures derived from the animal with the observed metastasis. The corresponding generations are indicated for MP and MSL (n is number of animals per generation).
Results

During routine propagation of the x-ray–induced transplantable islet cell tumor (2) in NEDH rats, involving selective transplantation of small tumors from severely hypoglycemic animals, we observed a tumor nodule in the liver which was assumed to represent a metastasis (Fig. 2). In this single rat, which had received the tumor tissue sample intraperitoneally, the main tumor mass was attached to the pancreas. The metastasis was clearly embedded within the liver tissue.

Separate propagation in vivo of the metastasis (MSL) as well as of the main tumor (MP) from the same animal by subcutaneous transplantation to the back resulted in a series of insulin-producing tumors (Fig. 3). However, in successive generations of the MSL tumor, the insulin expression was unstable as indicated by gradually increasing tumor size, where large cystic tumors developed in the fourth generation with only a single animal becoming hypoglycemic (Table I). In contrast, transplants from the main tumor consistently produced small-sized tumors associated with severe hypoglycemia in the subsequent generations. Interestingly, no metastases were observed in either MSL or MP recipients at the time of tumor removal (n=26 and 22, respectively). The MSL tumor displayed a remarkably high in vitro proliferation potential as compared to the original x-ray tumor and the MP-variant (Table I). The relationship of MSL cultures and the RIN cell lines (7) of similar origin is shown in Fig. 2.

Primary Cultures

All tumor cell preparations from the MSL tumor yielded proliferating primary cultures (n = 5), whereas none of the attempts using identical procedures with the MP tumor were successful (n = 20) (Table I). It was observed that fibroblast contamination strongly reduced attachment of the primary tumor cells, thus inhibiting subsequent proliferation. Our serial dilution culture procedure ensured, however, that a certain fraction of primary microcultures from a particular tumor would be devoid of fibroblasts.

Establishment of Insulin-producing Cell Cultures

The simple screening assay for immunoreactive insulin allowed large-scale screening to identify insulin-secreting microcultures. A second generation MSL tumor causing severe hypoglycemia was used to start more than 200 secondary cultures, each containing a proliferating colony, which was isolated from insulin-positive primary cultures. Four secondary cultures with insulin concentrations greater than 20 ng/ml after 1 wk in 1-ml cultures were selected for expansion and cryopreservation (MSL-C, -D, -E, and -F; Fig. 3). One of these cultures (MSL-F) was subjected to limiting dilution cloning twice, to establish two insulin-secreting cell lines; MSL-G and MSL-H. The doubling times of the various cultures varied between 3 and 6 d.

Multihormone Expression In Vivo by the Clonal Culture MSL-G

Subcutaneous inoculation of MSL-G cells in NEDH rats induced small, solid tumors (200–500 mg) associated with severe hypoglycemia. Immunocytochemical characterization of MSL-G tumor sections showed that, in addition to numerous insulin cells, discrete small areas in the tumor also contained glucagon, somatostatin, and CCK immunoreactive cells (Fig. 4, somatostatin staining not shown).

The Cholecystokinin/Glucagon–producing MSL-G2 Variant

After ~8 mo of continuous culture of the MSL-G cells, a morphologically distinct and slightly faster growing variant with a doubling time of ~4 d was observed (Fig. 6). Isolation and propagation of this variant (MSL-G2) resulted in a continuously constant expression of this particular phenotype for ~9 mo. The MSL-G2 culture gradually ceased to secrete detectable amounts of insulin into the medium. Immunocytochemical analysis of monolayer cultures of MSL-G2 (Fig. 7, A–C) as well as of its subclone, CI–3 (Fig. 7, D–F) revealed numerous glucagon and COOH-terminal gastrin/CCK-like immunoreactive cells (Fig. 1). The use of non–cross-reacting antibodies towards gastrin and CCK (Fig. 1) demonstrated that the immunoreactivity was due to CCK, whereas gastrin-specific staining could not be detected. These results were confirmed by radioimmunoassay on supernatants (Table II) and purified extracts (Fig. 8) of MSL-G2 cells. Larger forms of presumably non-amidated CCK accumulate in the medium under normal culture conditions (Table II) whereas smaller amidated forms are found in cell extracts thus assumed to be the major stored products (Fig. 8). This was also reflected in supernatants of cultures stimulated to secrete stored immunoreactivity (Table II). The reaction of Ab 1561 (Fig. 1) to the CI–3 cells is shown in Fig. 7 F. The fractions of MSL-G2 cells positive for glucagon or CCK were of the same size and varied from 50 to 80% throughout a 6-mo cul-

Table I. Comparison of MP and MSL Tumor Cells

| Tumor generation | 1 MSL | MP | 2 MSL | MP | 3 MSL | MP | 4 MSL |
|------------------|-------|----|-------|----|-------|----|-------|
| Animals per generation | 1    | 2  | 5     | 9  | 9     | 11 | 11    |
| Hypoglycemic animals (%) | 1    | 2  | 5     | 9  | 5     | 11 | 1     |
| Continuous in vitro growth/culture attempt | 1/1  | 0/2 | 2/2  | 0/9 | 2/2  | 0/11 | –     |
| Tumor size (g) | <1   | <1 | 4–6   | <1 | >10   | <1 | >10   |

The size, the ability to induce hypoglycemia in vivo, and the proliferation potential in vitro is shown for MSL and MP tumors in subsequent generations. Large tumors were cystic.

Madsen et al. Multihormonal Clones of Endocrine Cell Cultures
ture period (Table III). Somatostatin- and insulin-positive cells were present throughout the culture period but in much lower numbers (Table III). The distribution of the expression of the two hormones was rather remarkable since groups of typically 2–4 strongly stained cells were scattered in areas of negative cells (as shown in Fig. 7 C). A similar profile of hormone expression was found in Cl–3. Double-staining immunofluorescence analyses with glucagon and CCK antisera raised in a rabbit and a guinea pig, respectively, showed that the majority of the positive cells contained both types of immunoreactivity. However, a minority of cells in the same culture stained for only either glucagon or CCK (Fig. 5). MSL-G2 and Cl–3 were negative for the other hormones tested including motilin, growth hormone, and pancreatic polypeptide.

**Other Multihormone-producing Cell Lines**

The immunocytochemical analysis of the in vitro insulin-producing clone MSL-H revealed a mixed hormone expression including the same four hormones as above (Table III). Since the limiting dilution technique does not provide the ultimate guarantee of monoclonal origin, we performed drug selection experiments to confirm our data. Selection by 6-thioguanine was used to obtain HAT (hypoxanthine, aminopterin, thymidine)-sensitive cultures and allowed us to isolate a series of insulin- and non-insulin-secreting clones. The frequency of proliferating 6-thioguanine resistant clones in the MSL-H culture was extremely low (2.5 × 10⁻⁸) and independent of the presence of ethylmethanesulphonate. One insulin-secreting clone (MSL-R7,2E) tested so far retained the capacity for multihormone expression, although the fraction of positive cells for a particular hormone was very low (Table III). However, the intensity of staining of the few positive cells with glucagon, CCK, and somatostatin, respectively, was strong (Fig. 7, G, H, and F). Among the non-insulin-secreting cell lines tested so far, including MSL-A, (Table III) all expressed glucagon and CCK.

**Discussion**

We have identified a liver metastasis (MSL) of an x-ray-induced pancreatic islet cell tumor (2). The metastatic cells expressed an enhanced potential for in vitro proliferation. This is in accordance with the observation that malignant cells can be cultured more readily in vitro as compared to their normal and benign counterparts (11, 36). To our knowledge, metastatic spreads have not previously been reported for this tumor. Interestingly, no metastases were identified in any of the rats carrying the MSL tumor (n = 26). However, two MSL cell lines (MSL-A and MSL-G) have occasionally produced...
Immunocytochemical staining of monolayer cell cultures. MSL-G2 (A–C), MSL-C13 (D–F), and MSL-R7,2E (G–I) were stained for glucagon (A, D, and G), somatostatin (I), insulin (C), COOH-terminal gastrin/CCK (Ab 2717) (B, E, and H), and CCK (Ab 1561) (F). Bar, 10 μm.

Figure 7. Immunocytochemical staining of monolayer cell cultures. MSL-G2 (A–C), MSL-C13 (D–F), and MSL-R7,2E (G–I) were stained for glucagon (A, D, and G), somatostatin (I), insulin (C), COOH-terminal gastrin/CCK (Ab 2717) (B, E, and H), and CCK (Ab 1561) (F). Bar, 10 μm.

metastases when grown subcutaneously in vivo. In two rats carrying MSL-A tumors, multiple small cystic tumors were observed in the liver. One rat carrying an MSL-G tumor developed two small solid insulin-producing metastases, one in each lung (Madsen, O. D., unpublished observations).

All the clonal MSL-cell lines studied show a multihormonal expression. Interestingly, RIN cell clones of same origin (Fig. 2) were also found to express insulin and somatostatin (7, 26). These results strongly suggest that the original tumor arose from a transformed pluripotent islet stem cell, or that transformation lead to the dedifferentiation of a mature cell to a pluripotent stage. The fact that most human pancreatic islet cell tumors are multihormonal (16, 22) is consistent with either of the above hypotheses regarding the mechanism of islet tumor genesis. The MSL cells thus allow studies of in vitro differentiation processes associated to the expression of particular hormones.

The demonstration of the presence of CCK and not gastrin reveals a hitherto undescribed hormone expression by transformed pancreatic islet cells. The MSL-G2 cells in particular have been shown to produce and secrete various forms of processed and unprocessed CCK. The hormone regulates gall bladder contraction (12), secretion of pancreatic enzymes (10, 14), and has neurotransmitter activity (5; see 31 for review). As with other regulatory peptides, preproCCK undergoes a series of posttranslational modifications leading to a heterogeneous group of CCK peptides with different biological activities (30, 31). The posttranslational modi-
terminal-directed CCK-antiserum 278/9, which binds CCK-4, -8, -12, and -33. 1-ml extract (10⁶ cells) was applied to a calibrated Sephadex G-50 superfine column (10 × 1,000 mm) eluted at 4°C in 0.2 M sodium veronal (pH 8.4) containing 0.1% bovine serum albumin at a flow rate of 5 ml/h. 1-ml fractions were collected and measured by cholecystokinin radioimmunoassays as described (29, 32). The measurements shown here were performed with the COOH-terminal-directed CCK-antiserum 278/9, which binds CCK-4, -8, -12, and -33 with equimolar potency.

The establishment of HAT-sensitive pluripotent pancreatic endocrine cells such as MSL-R7,2E provides a vehicle by which novel hormones may be expressed using cell fusion techniques.

The authors gratefully acknowledge the technical assistance by Benedikte Traasdal, Patrick Isacss, Carol Caro, John Michael, Mette Simons, and Tove Funder and the invaluable secretarial help of Mrs. Lisbeth Brandt-Møller and Ms. Myrna Duckett.

The work was supported by the United States Public Health Service Grants AM-12994, AM 13941, AM 20595, AM 2690. The Unit of Histochemistry is sponsored by the Danish Medical Research Council and Cancer Society.

Received for publication 1 November 1985, and in revised form 26 June 1986.

References

1. Boel, E., J. Vuust, F. Norris, K. Norris, A. Wind, J. F. Rehfeld, and K. A. Markser. 1983. Molecular cloning of human gastrin cDNA: evidence for evolution of gastrin by gene duplication. Proc. Natl. Acad. Sci. USA. 80: 2866-2869.

2. Chich, W. L., S. Warren, R. N. Chute, A. A. Like, V. Lauris, and K. C. Kitchen. 1977. A transplantable insulinoma in the rat. Proc. Natl. Acad. Sci. USA. 74:628-632.

3. Deschesnes, R. J., R. S. Haun, C. L. Function, and J. E. Dixon. 1985. A gene encoding rat cholecystokinin. J. Biol. Chem. 260:1280-1286.

4. Deschesnes, R. J., J. L. Lorenz, R. A. Haun, B. A. Roos, R. J. Collier, and J. E. Dixon. 1984. Cloning and sequence analysis of a cDNA encoding rat preprocholecystokinin. Proc. Natl. Acad. Sci. USA. 81:726-730.

5. Dockray, G. J., R. G. Williams, C. Vaillant, and M. Schultzberg. 1982.
amino acid sequence determined from the nucleotide sequence of the mRNA. Peptides. 3:693-696.
36. Sporn, M. B., and A. B. Roberts. 1985. Autocrine growth factors and cancer. Nature (Lond.). 313:745-747.
37. Sternberger, L. A. 1979. Immunocytochemistry, 2nd ed. John Wiley and Sons Inc. New York. 1-169.
38. Tobler, P. H., M. A. Dambacher, W. Born, P. U. Heitz, R. Maier, and J. A. Fischer. 1983. Ectopically produced human calcitonin in a pancreatic tumor: a new form characterized by reverse phase high performance liquid chromatography. Cancer Res. 43:3793-3799.
39. Wiborg, O., L. Berglund, E. Boel, F. Norris, K. Norris, J. F. Rehfeld, K. A. Marcker, and J. Vuust. 1984. Structure of a human gastrin gene. Proc. Natl. Acad. Sci. USA. 81:1067-1069.
40. Wilson, D. M., G. P. Ceda, D. G. Bostwick, R. J. Webber, J. R. Minkoff, A. Pont, R. L. Hintz, K. G. Bensch, F. B. Kraemer, R. G. Rosenfeld, and A. R. Hoffman. 1984. Acromegaly and Zollinger-Ellison syndrome secondary to an islet cell tumor: characterization and quantification of plasma and tumor human growth hormone-releasing factor. J. Clin. Endocrinol. Metab. 59:1002-1005.