Expression of Neurotensin Messenger RNA in a Human Carcinoid Tumor

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Neurotensin (NT), a distal gut peptide, has important regulatory and trophic effects throughout the gut; however the intracellular mechanisms that regulate the gene expression and release of human NT are not known. The purpose of this endeavor was to study a functioning human pancreatic carcinoid cell line (called BON) *in vitro* that expresses the NT gene, and to study the effect of the cyclic adenosine monophosphate (cAMP) signal-transduction pathway on the expression and release of human NT. RNA was prepared from BON cell line (which has been established in this laboratory); the RNA was analyzed for NT mRNA expression by Northern hybridization with a complementary DNA probe. RNA blot analysis demonstrated that the NT gene is expressed in BON and is transcribed to two mRNAs of 1.0- and 1.5-kb sizes. In the second part of this study, BON cells were treated with either forskolin (FSK), which increases intracellular levels of cAMP, or with serotonin (5-HT), which reduces cAMP in BON cells. Forskolin produced a dose-dependent increase in NT peptide release and, furthermore, FSK (10^{-6} mol/L) rapidly increased NT mRNA abundance 1 hour after addition; conversely, 5-HT (10^{-5} mol/L) decreased NT mRNA at 1 hour. Neurotensin mRNA levels returned to control values by 3 hours after either FSK or 5-HT, which suggests that the transcript half-life for NT is relatively short. These findings show that the expression and peptide release of human NT is mediated, in part, by the cAMP signal-transduction pathway. Our human carcinoid cell line will provide a useful model to study the *in vitro* regulation of NT gene expression and peptide release.

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NEUROTENSIN (NT) IS A tridecapeptide with greatest abundance in the central nervous system and gut (particularly distal small intestine).[1-4] Physiologic actions of NT in the gut include stimulation of pancreatic secretion and colonic motility[5,6] and inhibition of gastric acid secretion and small bowel motility.[7,8] Recently studies have shown that NT stimulates the growth of the normal small bowel,[9] gastric antrum,[10] and pancreas.[10,11] We have found that NT can prevent the small bowel mucosal atrophy associated with feeding rats an elemental diet[12] and that this effect appears to be mediated in part by a direct systemic effect.[13] In addition to its trophic effects on normal gut tissues, NT has been identified in human small cell lung cancers[14] and stimulates the growth and intracellular calcium mobilization in a number of these cancer lines *in vitro*.[15,16] Collectively these studies suggest a possible role for NT in the autocrine or paracrine growth of both normal and malignant tissues. Studies that examine the molecular mechanisms of NT gene expression and release will facilitate better understanding of the intracellular mechanisms responsible for these trophic effects; efforts to date have been hampered by the lack of an established human cell line that expresses the NT gene.

We have established and characterized a human foregut carcinoid tumor line (BON) in athymic nude mice[17] and in tissue culture. The BON line possesses receptors for gastrin and somatostatin, and produces and secretes serotonin (5-hydroxytryptamine [5-HT]),[18] pancreastatin,[19] and chromogranin A.[20] To our knowledge no other long-term functioning cell line from human carcinoid tumors has been described. BON has proven to be a useful model to study carcinoid tumor biology[18,21] and to examine the effects of various chemotherapeutic agents on the inhibition of carcinoid tumor growth.[22,23] BON produces a variety of hormones and amines; however the presence of the NT gene in BON has not been determined previously.

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The purpose of this study was to determine whether BON cells express the gene for NT and, if so, to begin to define the molecular mechanisms responsible for the gene regulation and release of human NT.

Materials and Methods

Maintenance of BON in Tissue Culture

BON cells are maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) and F12K (Gibco) in a 1:1 ratio supplemented with 5% (vol/vol) fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) in a humidified atmosphere of 95% air and 5% CO_2 at 37 C. Stock cultures are passed at a 1:2 ratio when cells reach 80% confluence. Cell cultures are routinely monitored for mycoplasma contamination, and no mycoplasma growth has been detected.

In the present study, we have used BON cells in passages 9 through 12.

Phase Contrast and Electron Microscopy

For phase contrast microscopy, BON cells were grown for 4 days in DMEM/F12K + 5% FCS and photographed by means of a Nikon (Diaphot-TMD) inverted microscope.

For electron microscopy BON cells (1×10^5) were grown on Millicell-CM (Millipore) filters in DMEM/F12K + 5% FCS for 4 to 7 days. Cells (grown as monolayers) on the filters then were fixed in half-strength Karnovsky's fixative for 4 to 6 hours; they were processed routinely and embedded in epoxy resin (Epon). Sections (70 nm) were stained with uranyl acetate and lead citrate and examined with a Phillips 410 electron microscope (Philips Medical Systems Inc., Shelton, CT).

Neurotensin Radioimmunoassay

Measurement of NT levels in incubation media and sonicated cells was carried out using a specific radioimmunoassay (RIA) established in our laboratory and previously described in detail.[3]

RNA Preparation, Northern Blot Analysis, and Labeling

Total RNA was prepared using the acid guanidinium isothiocyanate-phenol-chloroform procedure.[24] Polyadenylated (Poly $[A]^+$) RNA was selected by oligo(dT) cellulose (Collaborative Research Inc., Bedford, MA) column chromatography. The final RNA concentration was quantified by measuring its absorbance at 260 nm.

For Northern blot analyses, either 20 μg total RNA or 5 μg poly $(A)^+$ RNA was separated on a 1.2% agarose-formaldehyde gel. Size-fractionated RNA then was transferred to nitrocellulose filters by capillary action. After transfer filters were baked for 2 hours in a vacuum oven at 80 C.

^{32}P-CTP (New England Nuclear Research Products, Boston, MA)–labeled antisense (cRNA) probes were synthesized.[4] The canine NT/neuromedin N cRNA probe was synthesized from a 729-bp cDNA insert (NT-8) subcloned into the *Eco*RI site of pGEM 4 vector.[25] Complementary RNA probes corresponding to the cyclophilin gene (1B15) were synthesized from a 680-bp cDNA insert in SP65-1B15.[26] This gene is constitutively expressed in BON and was used to control for RNA loading.

Hybridization and posthybridization washes were performed as described previously,[4] and after washes were completed, filters were blotted dry and exposed to XAR-5 x-ray film (Eastman Kodak, Rochester, NY) in the presence of intensifying screens at −70 C.

The hybridization signals on the blots were analyzed quantitatively using a Bio Image Visage 60 densitometer (Bio Image, Ann Arbor, MI).

NT Release Experiments

BON cells (5×10^5) were harvested by trypsin (1:250, Gibco) and plated into 35-mm tissue culture dishes in DMEM/F12K + 5% FCS. After a 2-day incubation, cells were washed twice with 2 mL oxygenated Krebs-Ringer bicarbonate buffer (KRBB, pH 7.4) containing HEPES (10 mmol/L), bovine serum albumin (BSA) (0.1%), glucose (2.5 mmol/L), and incubated for a further 30 minutes in fresh KRBB containing forskolin (FSK, 10^{-8} to 10^{-6} mol/L) (Sigma Chemical Co., St. Louis, MO), prepared in 100% ethanol. Control cells received the same amount of ethanol, and the final concentration of ethanol in the medium did not exceed 0.1%. After this incubation period, media were saved for measurement of NT levels by RIA. Cells were scraped and sonicated for 30 seconds using a sonic dismembranator (Model 300, Fisher Sonicator) for measurement of intracellular NT levels. All experiments were performed three times using six dishes/treatment; representative data are shown.

Results are calculated as percent fractional release (amount of NT secreted into the medium × [amount of NT secreted into the medium + amount of NT present in the cell extract]$^{-1}$ × 100) and are expressed as percent of control.

Neurotensin mRNA Induction Experiments

BON cells (5×10^6) were grown in monolayer culture in DMEM/F12K medium that was supplemented with 5% FCS in T150 culture flasks for 48 hours. After this incubation period, cells were approximately 70% confluent; the medium was changed to DMEM/F12K with 0.5% FCS for 24 hours, at which time FSK (10^{-6} mol/L) or 5-HT (10^{-5} mol/L) was added to the culture medium.

At the indicated times after addition, the cells were washed with phosphate-buffered saline (PBS) and lysed with 4 mol/L guanidinium isothiocyanate. The cell lysate was frozen at −70 C until RNA extraction.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM) and analyzed using Student's unpaired t test at the 0.05 level of significance.

Results

Microscopic Studies

Phase-contrast microscopy. BON cells in culture are composed of polygonal cells growing in a cobblestone fashion. The cells are found to extend long cytoplasmic processes resembling dendrites, similar to other cells of neuroendocrine differentiation (Fig. 1).

Electron microscopy. BON cells grow in monolayers and bilayers. They exhibit abundant cytoplasm with a variety of organelles such as mitochondria, profiles of endoplasmic reticulum, pinocytotic vesicles, and numerous membrane-bound secretory granules. The granules are of varying sizes and concentrated at the poles of cytoplasmic processes. The nuclei exhibit irregular contours with one or two inconspicuous nucleoli. The cell-attachment junctions are poorly developed, and the apical surfaces of the cells exhibit numerous microvilli. Production of basement membrane is not apparent (Fig. 2).

Northern Blot Analysis of NT mRNA

Figure 3 shows the Northern blot analysis of NT mRNA detected in BON cells and normal ileal mucosa obtained from a patient at operation. Neurotensin mRNA transcripts of approximately 1.0- and 1.5-kb sizes were demonstrated in both samples after high-stringency washes.

Neurotensin Peptide Release After Addition of Forskolin

To determine whether cyclic adenosine monophosphate (cAMP) affects the release of NT from BON, FSK, which increases intracellular cAMP, was added to the incubation medium. Forskolin stimulated NT release from BON cells in a dose-dependent fashion (Fig. 4). Forskolin (10^{-6} mol/L) produced a maximal 51% increase in NT fractional release compared with control values.

Northern Blot Analysis of NT mRNA After Addition of FSK or 5-HT

We next investigated changes of NT mRNA levels in response to FSK or 5-HT in BON cells. Forskolin (10^{-6}

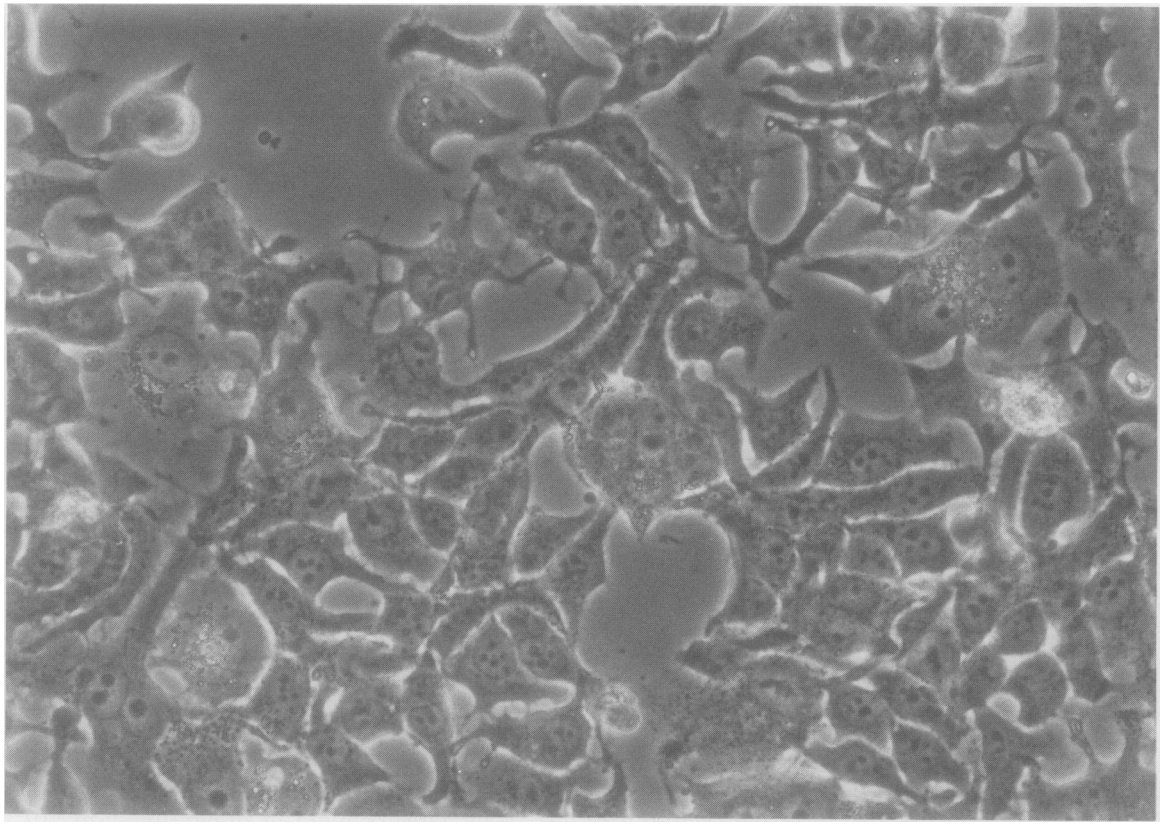

FIG. 1. Phase contrast photomicrograph of BON cells showing long cytoplasmic processes resembling dendrites (original magnification, ×200).

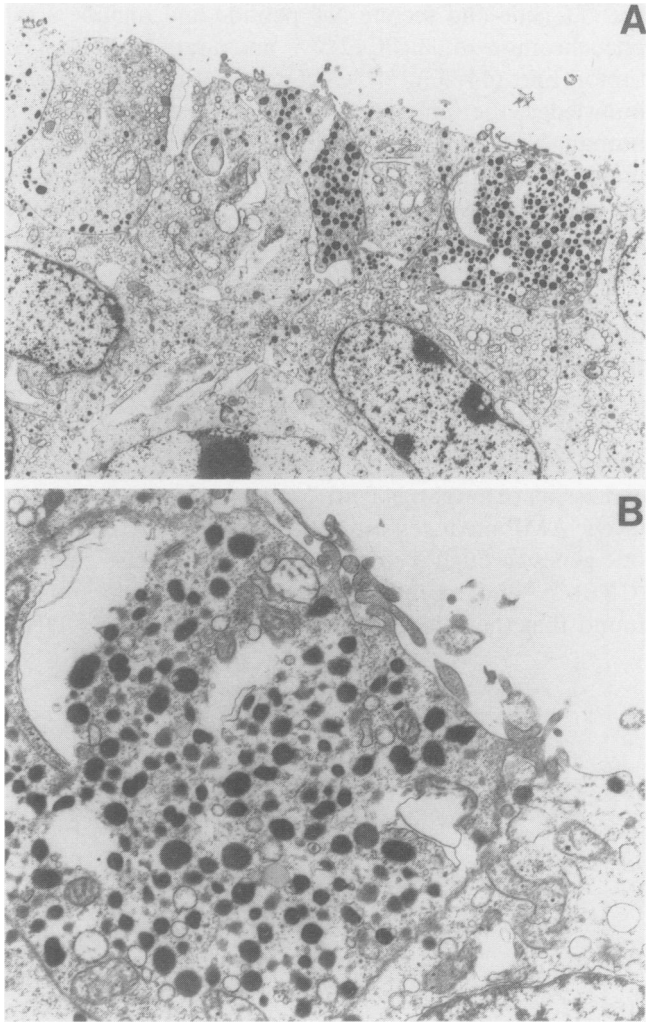

FIG. 2. Electron micrograph of BON cells showing abundant dense secretory granules. (A, original magnification, ×7000) (B, original magnification, ×25,000).

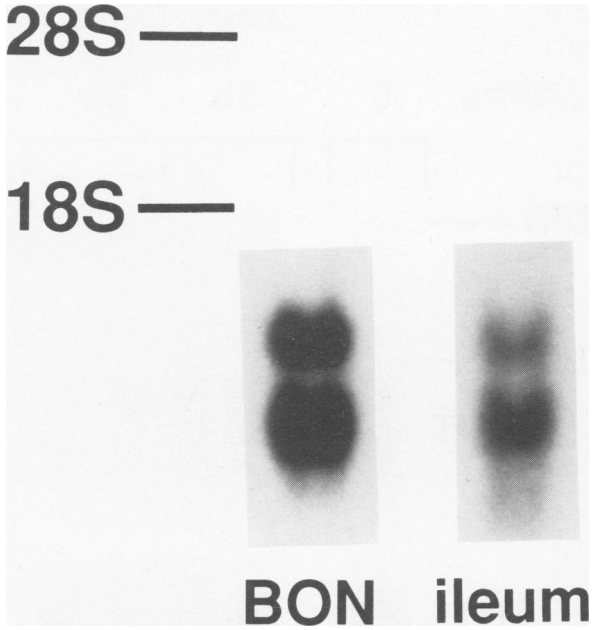

FIG. 3. Expression of NT mRNA in BON and human ileum. Five micrograms of poly (A) + RNA from BON cells and ileal mucosa was analyzed by the Northern blot method. The blots were probed with a canine NT cRNA and were washed at high stringency.

mol/L) was added to the medium, and after 1 and 3 hours, total RNA was extracted and subjected to Northern analysis. Figure 5 shows the basal and cAMP-stimulated NT mRNA levels in BON cells. One hour after addition, there was a 2.4-fold increase in NT mRNA abundance; however at 3 hours, NT mRNA levels had returned to control values. Conversely 5-HT (10^{-5} mol/L), which decreases intracellular cAMP in BON, decreased NT mRNA at 1 hour (Fig. 6). Values rapidly returned to control values by 3 hours (data not shown).

Discussion

In this study we report, for the first time, the long-term establishment of a human carcinoid cell line (BON) *in vitro* that expresses the gene for neurotensin at a relatively high level. Increasing cAMP by adding FSK increases NT gene expression and fractional release. Conversely inhibiting cAMP in BON cells by adding 5-HT decreases NT mRNA expression.

The BON tumor line was established 4 years ago in our laboratory from a peripancreatic lymph node metastasis of a pancreatic carcinoid tumor. BON has remained histologically identical to the original patient tumor and has also retained membrane receptors for both gastrin and somatostatin.[17] Characteristic of the foregut carcinoid, BON cells are multihormonal; previously we have described the production and release of chromogranin A

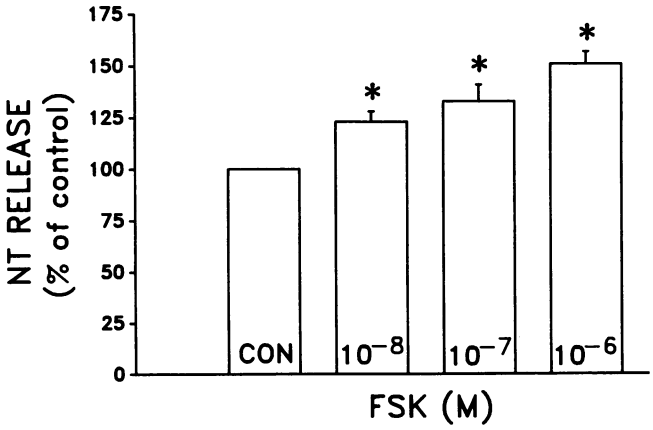

FIG. 4. Effect of FSK (10^{-8} to 10^{-6} M) on NT release from BON cells. Error bars indicate SEM of six plates, and data represent results from three separate experiments. NT fractional release was determined (as stated in Materials and Methods) and was expressed as percent of control. *$p < 0.05$ *versus* control.

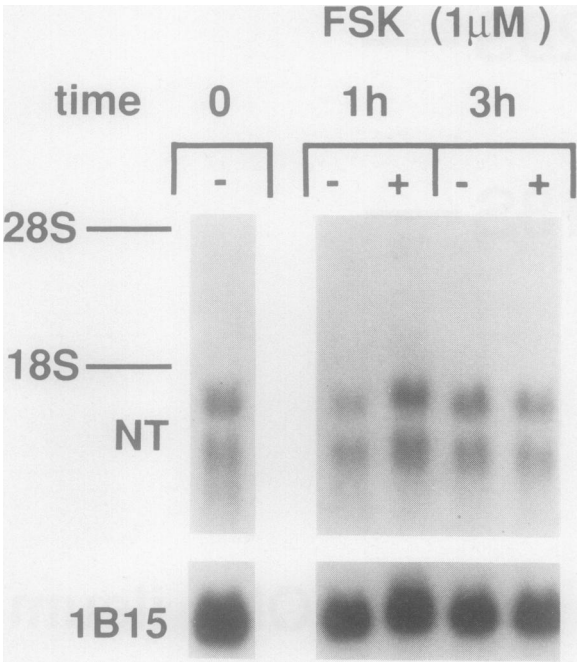

FIG. 5. Expression of NT mRNA in BON cells after addition of FSK (1 μM). BON cells were harvested at the indicated times, and total RNA (20 μg/lane) was extracted. Blots were probed with the canine NT cRNA and then stripped and reprobed with 1B15 to control for loading.

and pancreastatin from BON.[19,20] In addition BON produces and secretes 5-HT both basally[18,21] and in response to gastrin[18]; furthermore we have found that 5-HT is an autocrine growth stimulator for BON cells in culture.[21] We have also used this tumor line for *in vivo* studies. BON cells, when injected into athymic nude mice, will produce discrete tumors[17,22,23]; these tumors have been a useful model to study the effects of various novel chemotherapeutic agents.[22,23]

The gene for NT/neuromedin N is highly conserved in mammalian species and contains four exons separated by three introns.[25,27] The coding region for NT and neuromedin N is located in tandem position on exon 4.[27] Using high-stringency conditions, we have used a canine cDNA probe, cloned by Dobner and colleagues,[25] to probe RNA samples from BON cells and human ileum, which has the highest abundance of gut NT.[3,4] We found that BON expresses the gene for NT and, similar to the findings that we[4] and others[25,27,28] have reported, NT transcribes to two mRNA species of approximately 1.0- and 1.5-kb sizes with the 1.0-kb the predominant transcript. Similar transcript sizes were noted in the ileum.

By means of a variety of techniques, including Northern hybridization, RIA, and high-pressure liquid chromatography, NT has been found in operative specimens of endocrine tumors of the gastrointestinal tract[28,29] and lung.[14] In addition other cell lines, derived from rat endocrine tumors, have been established. These cell lines express the NT gene and secrete NT peptide and include a rat pheochromocytoma (PC12)[30–32] and a rat C-cell–derived tumor line (44-2C).[33,34] Our present study is, to our knowledge, the first to report of the establishment of a human NT-producing carcinoid cell line. Using this unique tumor line as an *in vitro* model, we hope to expand on our previous *in vivo* studies and begin to define the intracellular mechanisms responsible for the regulation of NT expression and peptide secretion.

The transcription of eukaryotic genes is mediated through the activation of intracellular second-messenger pathways that in turn activate specific proteins that bind to promoter elements located in the 5′-flanking region of the gene.[35–38] In our present study, we have examined the effect of the cAMP signal transduction pathway on the expression of human NT mRNA and NT peptide release. Cyclic AMP mediates stimulation of a variety of eukaryotic genes through a conserved cAMP response element (CRE),[36,39] and, recently, Kislauskis and colleagues[27] have found that the rat NT-gene promoter contains a DNA

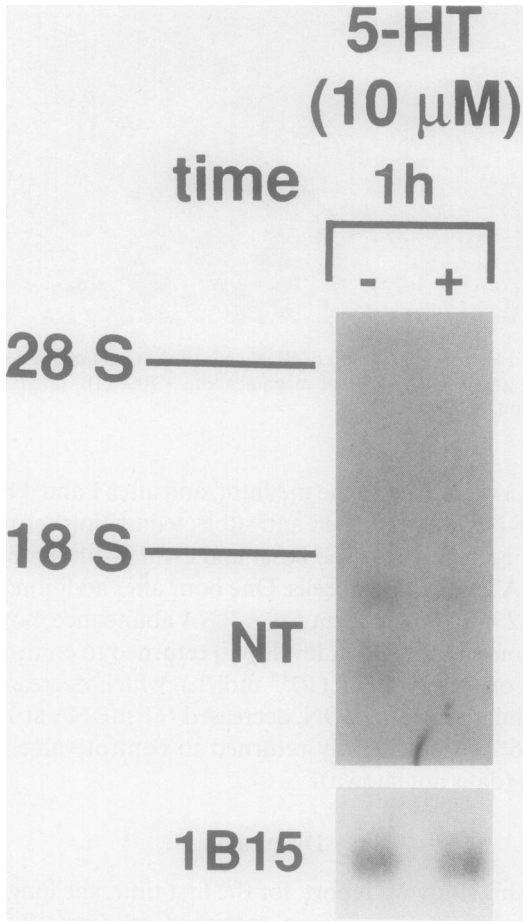

FIG. 6. Expression of NT mRNA in BON cells after addition of 5-HT (10 μM). BON cells were harvested at 1 hour, and total RNA (20 μg/lane) was extracted. Blots were probed with the canine NT cRNA and then stripped and reprobed with 1B15 to control for loading.

sequence that matches seven of eight nucleotides of the CRE octamer motif present in cAMP-responsive genes, such as the rat somatostatin gene.[39] Forskolin, a diterpene that increases intracellular cAMP by stimulation of adenylate-cyclase activity, increases the fractional release of NT peptide and increases NT mRNA abundance at 1 hour after addition to BON cells. Conversely the addition of 5-HT, which we have shown decreases cAMP levels in BON,[21] decreases NT mRNA at 1 hour. At 3 hours after adding either FSK or 5-HT, NT mRNA levels had returned to control values. These findings suggest that the control of human NT gene expression and peptide secretion are mediated in part by activation of the cAMP signal-transduction pathway.

The rapid turnover in steady-state levels of NT mRNA found in this study suggests that the half-life of the NT mRNA transcript is relatively short. Similar transcript lability has been noted with certain proto-oncogenes (for example c-*myc* and c-*fos*) that are important in cellular proliferation,[40,41] and in other cAMP-regulated genes.[36,42]

We have found definite, albeit modest, cAMP-mediated effects on the regulation of NT expression in BON cells. Others[31,32] have reported little change in rat NT mRNA levels in PC12 cells after single addition of the inducing-agents FSK, dexamethasone, and nerve-growth factor; when these agents were added in combination, however, there was a significant synergistic induction in the expression of NT. This suggests that multiple environmental stimuli are necessary to produce a concerted transcriptional response of the NT gene. We currently are studying the effects of the addition of multiple inducing agents to BON cells to determine whether the transcriptional response of human NT can be enhanced to a degree similar to that found with the rat NT gene.

In conclusion we have identified a human carcinoid cell line that expresses the human NT gene and secretes NT peptide, both in the basal (uninduced) state and after induction with FSK. Our unique human carcinoid cell line, BON, will be a useful model to further define intracellular mechanisms involved in the transcriptional regulation and release of human NT.

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Discussion

DR. HAILE T. DEBAS (San Francisco, California): I believe Drs. Evers, Townsend, and Thompson asked me to discuss this paper in the hope, rather than the belief, they would nudge me into the new era of molecular biology. I have to assure them that they failed in that effort.

This group is to be congratulated for its success in developing this unique human carcinoid cell line. Having shown that there is cyclic and mediated transcription of regulation of neurotensin, they are now poised to ask the important questions of what happens proximal and distal to the forskolin step. Specifically is the initiation of the intracellular process purely dependent on adenylate cyclase activation or are other regulatory membrane proteins involved?

And more importantly, how is the signal transferred to the nucleus for transcription to occur? If there is autocrine growth regulation by neurotensin, is this mediated by a different pathway; specifically are protooncogenes involved?

I have two questions for Dr. Evers. First you have shown increased message in response to forskolin, but the important issue is showing translation. Specifically how well did the increase in neurotensin messenger RNA correlate, both temporarily and in the dose–response relationship, to the secretion of neurotensin as measured by RIA?

Second you have shown that neurotensin is transcribed in two messenger RNAs. Do you think this is specific splicing that might have functional significance? If so could you hypothesize on the significance of the two messenger RNAs?

Thank you.

DR. DANA K. ANDERSEN (Chicago, Illinois): This laboratory has established a tradition at this meeting of providing state-of-the-art information on the biology and surgical significance of gastrointestinal endocrinology, and this paper certainly extends this tradition.

Perhaps the real significance of this work is the totality of the accomplishment. A portion of a metastatic tumor was transferred to a stable *in vivo* system, which allows the examination of mechanisms responsible for tumor function. The expression of specific genes, such as the neurotensin-neuromedin-C gene, has been quantified, and specific agents that promote or suppress the expression of these genes can be identified. This is an outstanding accomplishment.

Gene expression, or the transcription of mRNA from the gene, is a vital step in our understanding of the function of the tumor cell because the mere measurement of the final secretory product, in this case neurotensin, fails to provide a complete picture of the function of the cell. For example this study shows that serotonin suppresses the expression of the neurotensin gene. But this fact would go unnoticed if we relied on the measurement of neurotensin alone, as little neurotensin secretion was observed under basal circumstances.

That being the case, my first question is whether the authors could detect a reduction in cellular neurotensin content coincident with the suppression of neurotensin mRNA, and if not, whether they can provide any corroborative evidence that this suppression has any functional outcome on the secretory or growth activities of this tumor?

Because the tumor is a foregut carcinoid that produces serotinin, my second question is whether the tumor tonically suppresses neurotensin gene expression because of its own endogenous serotonin synthesis? Are the levels of serotonin administered to the cell culture similar to the levels of serotonin one might expect in the intracellular or extracellular compartments of the tumor? Does ongoing synthesis and secretion of serotonin by the tumor result in permanent suppression of neurotensin gene expression and synthesis of neurotensin by the tumor? If so one wonders why or how neurotensin is produced at all by this tumor.

This study corroborates the finding that expression of the neurotensin gene is related to a cyclic AMP–dependent element of the gene itself. One wonders, however, whether the expression of a whole host of genes is not stimulated by raising endogenous cyclic AMP levels by the addition of forskolin. Is there some specificity for synthetic processes related to secretory as opposed to growth responses of the tumor?

Finally although the data show recovery of the mRNA transcription stimulated by forskolin back to basal levels, and the authors conclude that induced transcription therefore is time limited, I would ask also whether this recovery might not simply be due to decreasing availability of cyclic AMP produced by forskolin?

DR. JOHN NIEDERHUBER (Baltimore, Maryland): The task before this particular investigator and his group is to use a specific cell line to look at signal transduction pathways and to ask specific questions about a given gene that they have identified as potentially involved in this process. Their goal is to study the expression of this gene and to characterize the resultant peptide product of the gene.

I think you have gathered from the comments of the previous two discussants that these are complex and difficult questions.

The obvious cautions with such a model, of course, need to be noted. Their model involves the use of a transformed cell line. As the investigators learn more about the expression of their specific gene, they will have to make constant comparisons between the abnormal cell and appropriate normal cells. They will need to prove that BON cells use the same mechanism of triggering NT gene expression as are used by normal cells and that the promoter region of the BON NT gene is unaltered. This laboratory group will obviously do that.

A question I would like to raise relates to forskolin and serotonin used in their experiments. These agonists tend to be fairly pleomorphic in their effects on the cell, a fact alluded to by the other discussants. Perhaps Dr. Evers could comment on why these two agents were selected instead of perhaps other agonists; for example dibuteryl cyclic AMP or phosphodiesterase inhibitors such as theophylin—reagents that have a more direct effect on cyclic AMP.

Finally the authors have implied that because the rat NT-gene promoter contains a DNA sequence that matches seven of eight nucleotides known