Glucagon Gene 5′-Flanking Sequences Direct Expression of Simian Virus 40 Large T Antigen to the Intestine, Producing Carcinoma of the Large Bowel in Transgenic Mice*

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Glucagon and the glucagon-like peptides play important roles in the regulation of glucose homeostasis. Previous studies have demonstrated that ~1300 base pairs of rat glucagon gene 5′-flanking sequences direct transgene expression to the pancreas and brain, but not to the intestine, of transgenic mice. These observations suggested that different tissue-specific enhancer elements mediate activation of glucagon gene transcription in the pancreas and intestine. We have now generated mice that express SV40 large T antigen under the control of ~2000 base pairs of glucagon gene 5′-flanking sequences. Transgene expression was observed in the brain and pancreas in association with the development of pancreatic endocrine tumors. In contrast to the mice described previously, we also detected transgene expression throughout the gastrointestinal tract in endocrine cells of the stomach and small and large intestine. Focal areas of enteroendocrine cell hyperplasia in the large bowel invariably progressed to invasive and metastasizing plurihormonal endocrine carcinoma, which was clinically and pathologically evident by 4 weeks of age. In contrast, transgene expression in the small bowel and stomach was not associated with progression to either hyperplasia or carcinoma. The results of these studies provide functional evidence for the existence of an upstream cis-acting regulatory domain that directs glucagon gene transcription to the endocrine cells of the intestine in transgenic mice.

The glucagon gene is expressed in the A cells of the endocrine pancreas, in the L cells of the intestine, and in the central nervous system, predominantly in brainstem neurons (1, 2). Glucagon and the glucagon-like peptides are encoded within a common proglucagon precursor that undergoes tissue-specific post-translational processing to liberate a different profile of proglucagon-derived peptides in the pancreas, intestine, and brain (3). These peptides have important biological functions in vivo (4); glucagon secretion from the pancreas modulates hepatic gluconeogenesis and glycogenolysis, and glucagon-like peptide I (GLP-I)3 from the intestine mediates glucose-dependent insulin secretion from the pancreas to the B cell. The specific function(s) of the proglucagon-derived peptides in the brain have not yet been elucidated. The sequences of proglucagon cDNAs isolated from different tissues (1, 2, 5–7) and the results of ribonuclease protection studies (1, 2, 8, 9) have demonstrated that proglucagon mRNA transcripts are identical in the pancreas, intestine, and brain. A single promoter mediates transcription initiation from an identical transcription start site in the pancreas, intestine, and brain (10); and the results of gene transfer studies have identified specific cis-acting glucagon gene enhancer and promoter sequences that direct glucagon gene transcription preferentially in islet cell lines (11, 12). In contrast, little is known about the control of glucagon gene expression in the intestine. Fetal rat primary intestinal cell cultures have been utilized for the study of intestinal glucagon gene expression in vivo (1), but molecular factors or cis-acting domains that control glucagon gene transcription in the intestine have not been identified. Furthermore, intestinal glucagon-producing cell lines suitable for gene transfer studies have not yet been isolated.

Previous studies analyzing the expression of a glucagon-SV40 large T antigen transgene containing ~1300 bp of rat glucagon 5′-flanking sequences demonstrated expression in the brain and pancreas, but not in the gastrointestinal tract, of transgenic mice (13). These observations implied that DNA regulatory sequences mediating islet and neural specific glucagon gene expression were different from those necessary to direct transgene expression to the intestine. To understand the molecular basis for glucagon gene expression in the intestine, we have generated transgenic mice containing additional glucagon gene 5′-flanking sequences upstream of the SV40 large T antigen. These mice express the transgene in the small and large intestine and develop endocrine carcinoma of the large bowel. These observations provide important evidence for the localization of a tissue-specific element capable of directing glucagon gene expression to the intestine in vivo.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—For vector construction, a 2.1-kilobase rat glucagon gene EcoRI/Alcl fragment was ligated to aBeth/ EcoRI fragment of SV40 T antigen coding sequences to produce the GLUTag transgene. This fusion gene contains ~2.9 kilobases of rat glucagon gene sequences, including 5′-flanking sequences, and 58 bp of exon 1 (14) and contains ~700 additional nucleotides of 5′-flanking sequence upstream of the KpnI site that delineates the 5′-boundary of the GLUTag-SV40 transgene described previously (13). The KpnI site is located at approximately ~1300 bp (and not at ~850 bp as described (13)) according to analysis of the glucagon gene in our laboratory. Transgenic mice were generated on an outbred CD-1 background as previously described (15). DNA prepared from mouse tails was used for Southern blot analysis to identify transgenic progeny.

Expression Analysis—For immunohistochemical and RNA analyses,
a minimum of eight mice from each different age group were analyzed individually. RNA was isolated from tissues by the acid/phenol precipitation method (16), and Northern blot analysis was carried out as previously described (2). The polymerase chain reaction (PCR) was utilized for the identification of specific mRNA transcripts as previously described (17). The primers used to identify mouse glucagon mRNAs were 5'-TTCACCAGTGAATCCAGCACAAGT-3' and 5'-GGTT-TGAATCAGCCAGCAGTTGAT-3', which resulted in the generation of a 307-bp glucagon cDNA fragment. The primers used to generate a 219-bp SV40 T antigen cDNA fragment were 5'-AGAG- GAATCTTTGCAGCTAA and 5'-TGCATCCCAGAAGCCTCCAA.

RESULTS

Three different transgenic founders were obtained that expressed the GLUTag transgene. The three established lines demonstrated identical tissue-specific patterns of GLUTag gene expression as assessed by Northern blot, PCR, and immunohistochemical analyses. Transgene expression was detected in the brain, pancreas, and intestine of all three lines; the detailed analysis described below was carried out on the line designated GLUTag-Y. Transgenic GLUTag-Y mice were visibly distinguishable from their nontransgenic littermates by 2–3 weeks of age. The transgenic mice were smaller than their wild-type littermates by 3 weeks of age and weighed 40–50% less than the nontransgenic littermates by 4–6 weeks of age. The mice became gradually wasted and died 4–12 weeks after birth.

To determine the distribution and tissue specificity of transgene expression, RNA was prepared from various tissues and analyzed for expression of T antigen mRNA transcripts by Northern blotting. RNA prepared from day 1 mice contained T antigen mRNA transcripts in the pancreas and intestine, but not in the stomach, even with longer exposures of the same blot (Fig. 1, upper). In contrast, by day 10, T antigen mRNA transcripts were detectable in the stomach and small bowel; the relative amounts of T antigen mRNA transcripts were reduced in the pancreas and comparatively more abundant in the large bowel (Fig. 1, upper). Transgene expression in the brain was analyzed using reverse-transcribed RNA and PCR in view of the extremely low levels of glucagon (2) and glucagon-SV40 transgene (13) expression in the brain. T antigen mRNA transcripts were detected in RNA from the whole brain as well as in RNA from the brainstem and hypothalamus (Fig. 1, lower). Aliquots of the identical reverse-transcription reactions were also analyzed using glucagon-specific primers. In contrast to the widespread distribution of T antigen expression, glucagon mRNA transcripts were restricted in expression to the brainstem, in agreement with the results of previous studies (2).

Autopsies of the transgenic mice consistently revealed pathology in the large intestine. By 4–8 weeks of age, marked dilatation of the cecum was associated with a firm thickened segment of the large bowel extending from the cecum to the rectum (Fig. 2). To trace the temporal development of this lesion, mice were examined at different ages. In fetuses at day 19 of gestation, no obvious pathology was detected in any tissue with hematoxylin and eosin. The number of GLP-I-immunoreactive cells in the large bowel was slightly increased at day 19 (Fig. 3, upper and center). These cells were found individually or in very small groups and were confined to the epithelium of the mucosal crypts. In contrast, the pancreases and small bowels of fetal day 19 mice showed no histochemical evidence of hyperplasia of glucagon-containing cells using either the GLP-I or glucagon antisera (data not shown). The distribution and number of the other pancreatic endocrine cell types was entirely normal, with the exception of the pancreatic polypeptide cell, which was not consistently detected. Animals from 1 to 21 days following birth demonstrated progressive development of epithelial cell proliferation in the lamina propria of the large bowel. Immunohistochemistry demonstrated GLP-I-immunoreactive cells both within the mucosal crypts and infiltrating into the lamina propria. These cells exhibited strong nuclear staining for SV40 T antigen (Fig. 3, lower). In the stomach and small bowel, GLP-I- and SV40 T antigen-immunoreactive cells were detected, but no areas of cellular hyperplasia were observed (data not shown). Pancreases from animals 1–3 weeks of age contained a number of irregular islets with large cells harboring pleomorphic nuclei and clear cytoplasm at the periphery (Fig. 4, upper). The large pleomorphic cells had strong nuclear positivity for SV40 T antigen (Fig. 4, lower), and occasional cells were immunopositive for glucagon or GLP-I (data not shown). The central cytologically normal cells within these islets contained insulin and somatostatin in the usual distribution,
but no pancreatic polypeptide was detected in the islets of transgenic mice after birth.

In transgenic mice 4 weeks of age or older, the large bowel was increased in thickness, with nests and solid sheets of epithelial cells filling the lamina propria, infiltrating into and through the muscularis propria (Fig. 5) and beyond the serosa of the bowel. Occasional animals had lymph node metastases involving pericolonic and para-aortic lymph nodes. The tumor was composed of epithelial cells with pleomorphic nuclei often harboring multiple nucleoli and occasionally containing intranuclear eosinophilic inclusions; numerous tumor cells exhibited strong nuclear positivity for SV40 T antigen (Fig. 5). Tumor cells were positive for neuron-specific enolase and synaptophysin; stains for chromogranin were negative (data not shown). The tumor cells contained moderate cytoplasmic immunoreactivity for glucagon and were strongly positive for GLP-I and peptide YY. In a few animals, the tumors stained positively in a focal distribution for cholecystokinin, and scattered tumor cells were positive for the α-subunit of glycoprotein hormones. The tumors were entirely negative for insulin, somatostatin, pancreatic polypeptide, vasoactive intestinal peptide, serotonin, gastrin, calcitonin, bombesin,
ACTH, ß-endorphin, corticotropin-releasing hormone, growth hormone releasing hormone, and carinoembryonic antigen.

In the pancreas, by 4 weeks of age, many islets were large and displayed disrupted architecture (data not shown). The large islets contained two distinct cell populations. There were central compressed clusters of bland epithelial cells with vesicular nuclei and eosinophilic cytoplasm. The periphery of the islets was composed of numerous pleomorphic cells with hyperchromatic nuclei that often harbored intranuclear eosinophilic inclusions and stained for SV40 T antigen; their cytoplasm was abundant and chromophobic, and the cell borders were indistinct. Occasional large cells contained cytoplasmic GLP-I immunopositivity. The small clusters of residual islet cells contained insulin and somatostatin but no reactivity for SV40 T antigen, pancreatic polypeptide, glucagon, or GLP-I. Although in the largest islets the pleomorphic cells comprised the majority of the islet parenchyma, there was no evidence of infiltration into the surrounding exocrine pancreas.

**DISCUSSION**

The results of previous studies (13) have suggested that tissue-specific enhancer elements may regulate the expression of the glucagon gene in vivo. The observations described here provide evidence for a glucagon gene intestinal specific cis-acting element that resides between -1300 and -2000 bp relative to the start of transcription. This element directs transgene expression to endocrine cells in the stomach as well as to the small and large intestine of transgenic mice. In contrast, GLU2-Tag mice transgenic for a glucagon-SV40 transgene containing 1300 bp of rat glucagon gene 5' flanking sequences did not express the transgene in the stomach or intestine in any of the lines studied (13). The pancreatic pathology in our GLUTag-Y mice also differs significantly from that described previously in the GLU2-Tag transgenic line. GLUTag-Y transgenic mice contained two distinct populations of islet cells that were distinguishable by 3 weeks of age; large hyperplastic islets were consistently detected by 4 weeks, and progression to tumor formation was observed from 4 to 8 weeks of age. In contrast, proliferation of A cells in the GLU2-transgenic mice contained two distinct populations of islet cell lines that, when propagated in vitro, will be useful for the identification of the cis- and trans-acting factors important for the molecular control of glucagon gene expression in the intestine.

**REFERENCES**