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Glucagonomas of transgenic mice express a wide range of general neuroendocrine markers and bioactive peptides

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Summary. Pancreatic tumours of transgenic mice carrying a glucagon-promoted simian virus 40 (SV40) T antigen oncogene have been analysed by histological, histochemical, ultrastructural and radioimmunological means. Seven transgenic mice were examined revealing dysplastic and neoplastic lesions in the endocrine pancreas. Four tumours were identified, one of which metastasized to periadrenal spaces and paravertebral lymph nodes. Benign tumours were composed of argyrophilic, endocrine cells reactive to a range of antibodies against neuroendocrine markers (neuron-specific enolase, protein gene product 9.5, chromogranin A, synaptophysin and protein 7B2) and different fragments of the proglucagon molecule (glucagon, glicentin, glucagon-like polypeptides 1 and 2). A few tumour cells expressed pancreatic polypeptide, somatostatin or insulin. Conventional ultrastructural analysis and immunogold labelling revealed typical glucagon-immunoreactive alpha granules which co-stored glicentin and glucagon-like polypeptides 1 and 2. The malignant primary tumour and its metastases were composed mainly of cells which did not show immunoreactivity for neuroendocrine markers or peptides. Atypical, glucagon-immunogold labelled granules were detected at electron microscopy in differentiated tumour cells and C-type retroviral particles in the largest tumour population of degranulated cells. The transgene-encoded oncoprotein SV40 large T-antigen was detected in the nuclei of well-differentiated tumour cells and in alpha cells of some dysplastic islets. All tumour-bearing mice showed high levels of circulating glucagon-like immunoreactivity. Transgenic mice harbouring the glucagon-promoted SV40 T antigen oncogene may provide a model for human glucagonoma.

Key words: Transgenic mice – Glucagonomas – Immunohistochemistry – Electron microscopy – Radioimmunoassay

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Introduction

Endocrine tumours of the human pancreas are a rare entity (Klöppel and Heitz 1988) capable of producing a variety of bioactive peptides (Heitz et al. 1982) and are often associated with characteristic clinical syndromes (Ch'ng et al. 1985). Glucagon expression has been reported in a number of these neoplasms (Heitz et al. 1982), although the clinical syndrome related to hypersecretion of glucagon, and possibly other products, is associated only with glucagon-producing tumours which still retain releasing properties (Mallinson et al. 1974; Bloom and Polak 1987). In this case, the tumours are defined as glucagonomas.

Recently, gene transfer technology (Gordon et al. 1980; Hogan et al. 1986; Murphy and Hanson 1988) allowed the production of functioning endocrine tumours in transgenic mice (Hanahan 1985, 1988; Murphy et al. 1987; Efrat et al. 1988; Rindi et al. 1990). In brief, a hybrid oncogene is constructed by fusing the regulatory region from a hormone gene linked to the coding region of an oncogene. This hybrid gene is then introduced into the mouse germ line via microinjection of fertilized eggs. Resulting transgenic mice are expected to express the oncogene in the cell type that normally expresses the hormone. Following this strategy, Efrat et al. (1988) constructed a hybrid oncogene made up of the regulatory region of the rat proglucagon gene (Glu2) linked to the potent oncogene simian virus 40 (SV40) T antigen (Tag) (Tooze 1982; Rigby and Lane 1983). Four Glu2-Tag transgenic mice were obtained and used to found separate lineages. SV40 Tag immunoreactivity was reported in pancreatic A cells and derived tumours that heritably develop in these mice. Tumour RNA was also shown to contain high levels of Tag transcripts. In addition, the Glu2-Tag mice expressed Tag in several groups of neurons in the brain stem, without any discernible consequence. No expression of Tag was detected in the intestinal mucosa, where the endogenous glucagon gene is normally expressed.

The main aim of this study was to establish whether Glu2-Tag transgenic mice tumours are a possible model

for human glucagonomas. Thus conventional histology, histochemistry, immunohistochemistry and electron microscopy were applied in order to assess the morphological features and the expression pattern of neuroendocrine markers (Bishop et al. 1988) and peptide hormones in tumours of Glu2-Tag transgenic mice. Sera from corresponding transgenic mice were also assayed for hormones to determine the releasing properties of the histologically analysed tumours.

Materials and methods

Twelve mice were examined in detail. Seven transgenic mice representative of the established Glu2-Tag strain (lineages Glu2-Tag3, 5 and 6) (Efrat et al. 1988) of different ages (6–18 months old) and 5 wild-type (C57B1/6J) adult (6 months or approx.) controls were deeply anaesthetized by ether inhalation, bled by cardiac puncture and sacrificed by cervical dislocation. Blood was centrifuged at room temperature and plasma was kept at −20° C until assayed for peptide hormones.

A complete autopsy was performed and all visceral organs were removed, fixed by immersion in Bouin's fluid for 4–6 h at room temperature and processed into paraffin wax. Serial sections (3–5 μm) were stained with haematoxylin and eosin for conventional histology and Grimelius' silver impregnation method for endocrine cells (Grimelius 1968). Immunohistochemical tests for large Tag and a range of endocrine cell products (Table 1) were performed using the peroxidase anti-peroxidase (PAP) method (Sternberger et al. 1970), or the avidin-biotinylated perixodase complex (ABC; Vector, Burlington, Calif., USA) method (Hsu et al. 1981). Before immunostaining for large Tag, sections were treated with the proteolytic enzyme subtilisin (protease type XXIV; Sigma, Poole, UK) (0.003% w/v in 0.01 M phosphate-buffered 0.15 M saline, PBS, pH 7.4) for 5 to 15 min at room temperature to unmask antigenic sites (Fiocca et al. 1978). Specificity tests for the immunostains consisted of absorption of each antiserum with its homolgous antigen (10 nmol/ml of diluted antiserum), omission of the first layer and use of control tissue with or without the pertinent antigen (Van Noorden 1986).

Table 1. Antisera used in this study

Antiserum to	Dilution	Source
Large T-antigen	1:2000	S. Alpert, USA
Chromogranin A	1:2000	H. Winkler, Austria
Synaptophysin	1:1500	R. Jahn, FRG
7B2	1:2000	Hammersmith Hospital, UK
Neuron-specific enolase	Kit dilution	Zymed, USA
Pancreatic glucagon P 9.5	1:2000	Hammersmith Hospital, UK
Insulin	1:5000	Sorin Biomedica, Italy
Glucagon	1:5000	Hammersmith Hospital, UK
Pancreatic polypeptide	1:16000	R. Chance, USA
Somatostatin	1:10000	RIA UK, UK
Glicentin	1:1000	A. Moody, USA
GLP 1	1:2000	Hammersmith Hospital, UK
GLP 2	1:2000	Hammersmith Hospital, UK
Total glucagon[a]	1:15000	Hammersmith Hospital, UK
Pancreatic glucagon[a]	1:320000	Hammersmith Hospital, UK
GLP 1[a]	1:375000	Hammersmith Hospital, UK
Insulin[a]	1:1000000	Hammersmith Hospital, UK

All antisera raised in rabbit except for anti-insulin, which was raised in guinea pig

[a] Antisera used in radioimmunoassay tests only

For ultrastructural analysis, small samples from pancreatic tumours ($n=3$) and from large retroperitoneal ($n=1$) and mediastinal ($n=1$) lymph-node metastases were fixed by immersion in glutaraldehyde (2.5% v/v in 0.1 M phosphate buffer, pH 7.4) for 2–4 h at 4° C. Part of the tissue was further postfixed in osmium tetroxide (1% v/v, 0.1 M phosphate buffer, pH 7.4) for 1 h at 4° C and then processed into Araldite. Semithin sections (0.5–1 μm) were cut from non-osmicated and osmicated tissue, stained with toluidine blue (1% v/v in 3% aqueous borax) and immunostained with PAP or ABC methods in order to assess the preservation of proglucagon fragments after resin embedding. Areas of interest were trimmed and then sectioned (60–100 nm) with a Reichert Ultracut E ultramicrotome. Ultrathin sections were collected on uncoated nickel 300 mesh grids, desiccated, counterstained with uranyl acetate and Reynold's lead citrate and then observed in a Zeiss 10 CR transmission electron microscope. Single immunogold staining was performed using gold labelled protein A or IgG (Bio Clin, Biochemical Services, Sheffield, UK) methods (Roth et al. 1978; Varndell et al. 1982). For co-localization studies, the double-face immunogold technique was adopted (Bendayan 1982; Varndell and Polak 1984).

Hormone measurements were carried out using previously described radioimmunoassays for insulin (Albano et al. 1977), glucagon (Ghatei et al. 1983), glucagon-like polypeptide I 7–36 NH2 (Kreiman et al. 1978) and enteroglucagon (Ghatei et al. 1983). None of these assays showed any cross-reaction, except the enteroglucagon assay with glucagon assay. Enteroglucagon (proglucagon residues 1–69) concentrations were calculated by subtracting specifically measured pancreatic-glucagon (C-terminal immunoreactivity measured with antiserum RCS-5) from total N-terminal glucagon immunoreactivity (measured with antiserum R-59) (Ghatei et al. 1983; Holst 1988). The plasma samples were thawed and different aliquots were assayed in duplicate. All assays were performed in a total volume of 0.8 ml of 0.06 mol/l phosphate buffer, pH 7.4, containing 10 mM/l EDTA, 45 μmol/l bovine serum albumin and 20 kIU/ml aprotinin and were incubated at 4° C for 5 days. Antibody-bound label was separated from free label by adding to each tube 250 μl of a suspension containing 4–8 mg of charcoal (Norit GSX, Hopkins and Williams, Chadwell Heath, UK) coated with clinical grade dextran (1:10 g of charcoal, average molecular weight 70000; Sigma, Poole, UK). The tubes were centrifuged at 1600 *g* for 20 min at 4° C, followed by immediate separation of the supernatant.

Results

Glu2-Tag transgenic mice were analysed in two age groups consisting of younger (transgenic mice nos. 5, 6 and 7; 6, 7 and 7 months of age respectively) and older animals (transgenic mice nos. 1, 2, 3 and 4; 9, 18, 18 and 18 months of age respectively). Ages were chosen on the basis of tumour development timing as described by Efrat et al. (1988), expecting fully developed neoplasms in older mice and early lesions in younger mice. The wild-type controls (C57B1/6J) showed no lesion of any kind and were used throughout as normal standards for comparison.

At autopsy, no gross abnormalities were detected in younger mice. One single round, highly vascularized, reddish mass (maximum diameter 0.5 cm) was detected in the pancreas of each of the four older mice. Additionally, transgenic mouse no. 4 showed a large, whitish pancreatic mass intimately connected with the above vascularized lesion and infiltrating the adjacent intestinal wall. Whitish enlargements of both periadrenal spaces and lymph nodes in the upper part of the retroperitoneum

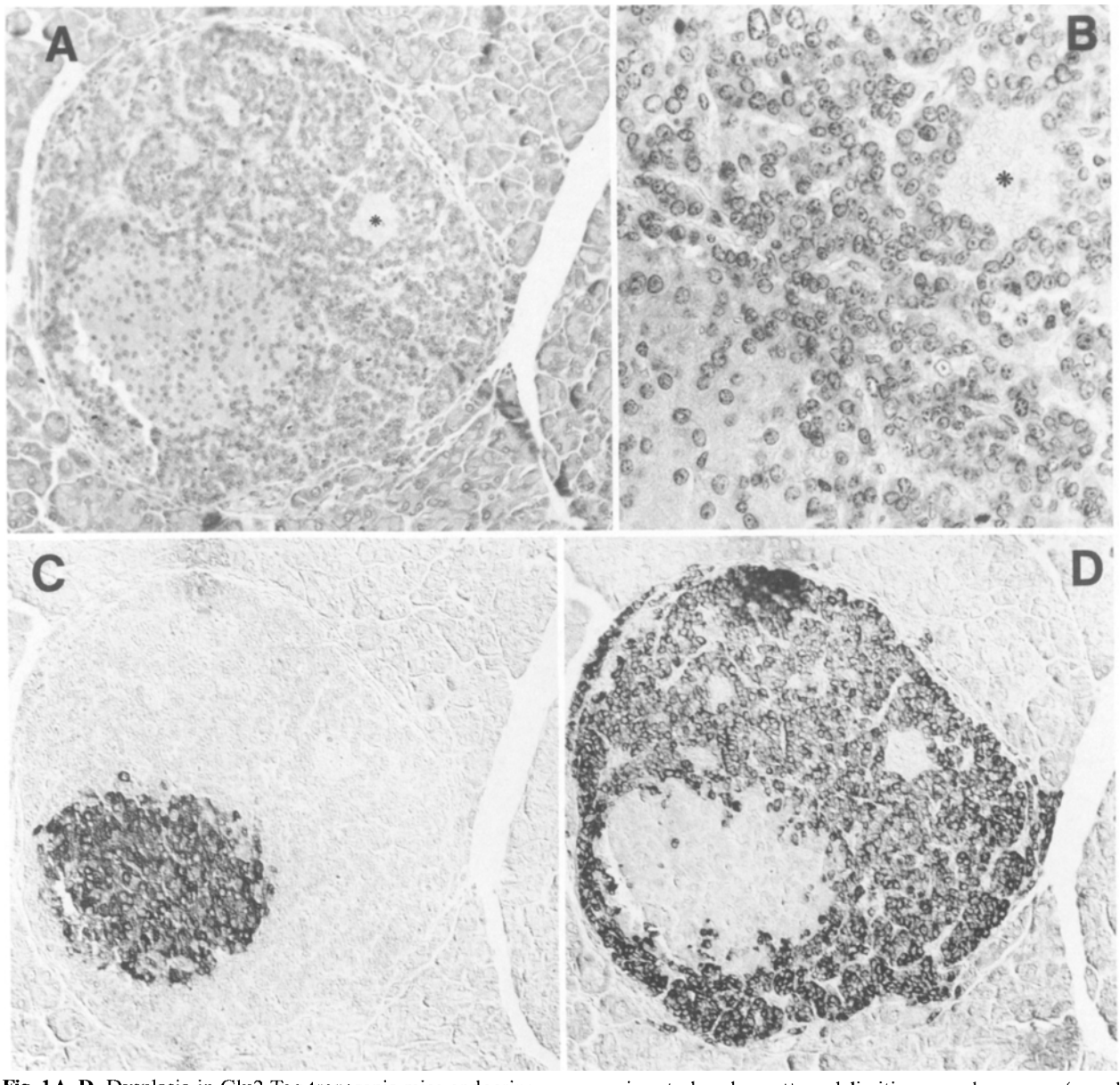


Fig. 1 A–D. Dysplasia in Glu2-Tag transgenic mice endocrine pancreas. Transgenic mouse no. 5 pancreas. A rare dysplastic islet showing a completely deranged architecture: a large mantle of atypical cells surrounds a group of histologically normal cells (**A**). At higher magnification (**B**), the cells crowding the periphery of the islet reveal severe cytological atypia and grow disordinately in a trabecular pattern delimiting vascular spaces (pseudorosettes) (*asterisk*). In the following serial sections, the group of normal cells are positive for insulin (**C**), while the proliferating mantle cells are immunoreactive for glucagon (**D**). Haematoxylin and eosin, ×84 (**A**), ×210 (**B**); PAP method (**C**), ABC method (**D**), Nomarski optics, ×84 (**C, D**)

and along the posterior chest wall up to the neck were also detected. All the remaining organs and the central nervous system appeared to be normal in all transgenic mice.

Light microscopy

No histological abnormality was detected in any visceral organ, except in the endocrine pancreas. In particular, no significant lesion such as epithelial hyperplasia or dysplasia was found in the gut mucosa. For the morphological evaluation of the endocrine lesions in the pancreas, we used histological criteria as previously defined (Rindi et al. 1990): presence of cell atypia, loss of internal islet structure and increased islet size. True tumour growths showed severe cellular atypia, complete loss of internal structure and a minimum diameter of 0.5 mm. Atypical lesions not presenting all these changes were defined as dysplastic islets. The endocrine compartment of Glu2-Tag transgenic mice pancreata presented normal islet density, comparable to that seen in controls. In younger transgenic mice, no histological change was observed except for rare hyperplastic-dysplastic islets

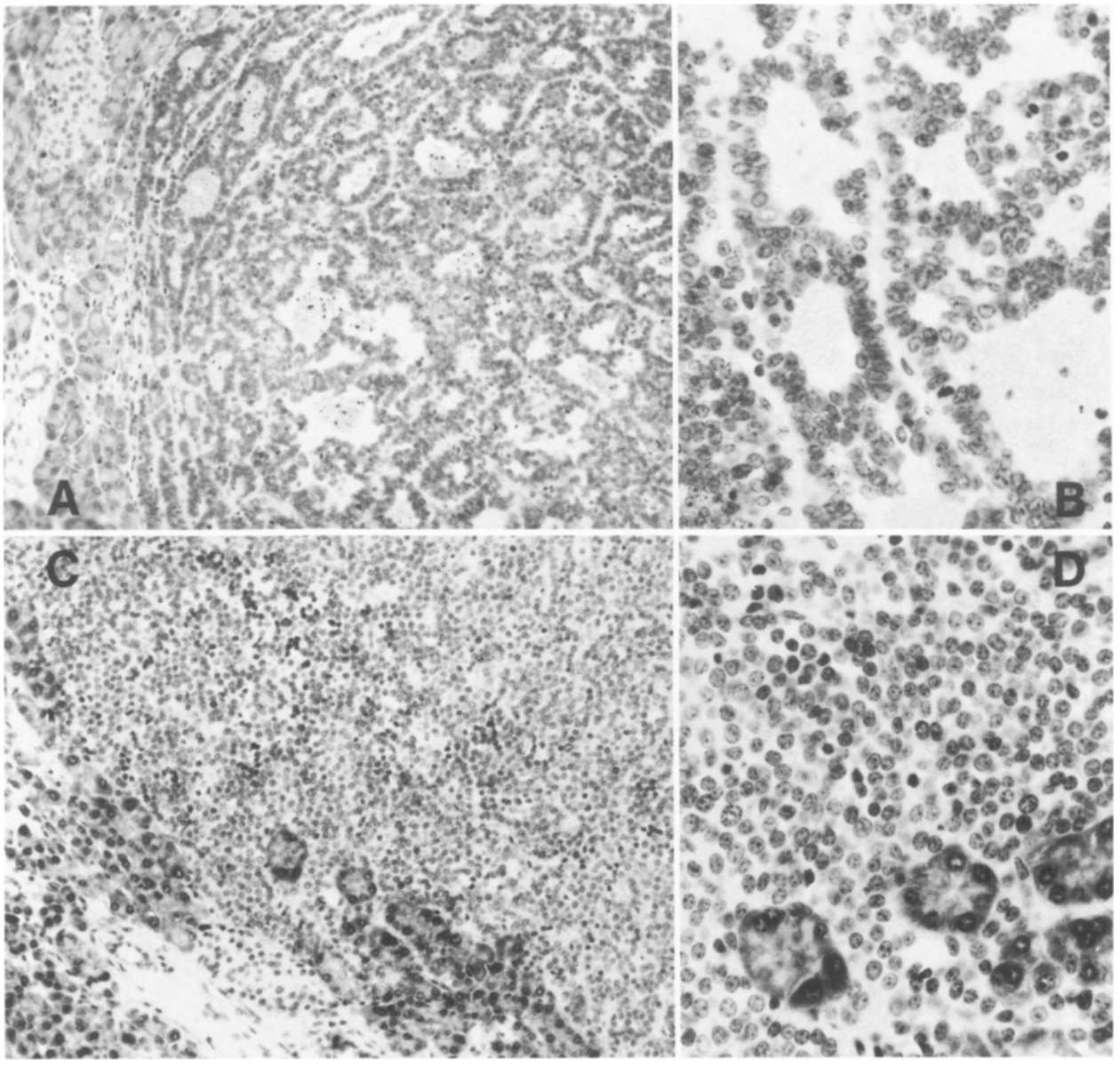


Fig. 2A–D. Histology of tumours. **A, B** Transgenic mouse no. 1 pancreas. Typical trabecular tumour showing an expansive pattern of growth with margins pushing off the exocrine parenchima. Compare with the normal islet architecture and cytology (*upper left corner*) (**A**). Ribbons of atypical cells are delimiting vascular spaces forming pseudorosettes: note the frequent mitoses (**B**). Haematoxylin and eosin, ×84 (**A**), ×210 (**B**). **C, D** Transgenic mouse no. 3 pancreas. Diffuse tumour with microinfiltrative pattern of growth. Tumour cells are crowded and appear to erode the exocrine parenchyma surrounding some glandular acini: note the cytological atypia (**D**). Haematoxylin and eosin, ×84 (**C**), ×210 (**D**)

found in transgenic mice nos. 5 and 6. Dysplastic islets presented as groups of endocrine cells with variable degrees of cytological atypia crowding the periphery of the islet (Fig. 1A, B). In older transgenic mice one tumour was observed per organ and no accompanying lesion, such as dysplastic islets, was seen. Tumour growth was either expansive, with margins pushing off the exocrine parenchyma (transgenic mice nos. 1 and 2) (Fig. 2A, B), or infiltrative with neoplastic cells penetrating between the acinar cells (transgenic mouse nos. 4) (Fig. 2C, D). Tumour structure was either trabecular (i.e. ribbon-like or gyriform) (transgenic mice nos. 1 and 2), or diffuse (transgenic mouse no 4). Transgenic mouse no. 3 showed a mixture of both structures and patterns of growth. The diffuse structure was associated with an infiltrate growth pattern (Fig. 2C, D). Tumour stroma varied in amount. Trabecular tumours contained abundant and highly vascularized stroma with formation of lacunar spaces filled with erythrocytes or plasma (pseudorosette), while stroma was scanty in diffuse tumours. Tumour cells were fairly monomorphous with a variable amount of eosinophilic cytoplasm and increased nuclear/cytoplasmic ratio. Nuclei were oval, polymorphic in size, sometimes large and bizzare with prominent nucleoli.

Mitoses were frequent. Anaplastic changes were more evident in diffuse tumours and in the malignant case under study. The whitish pancreatic mass of transgenic mouse no. 4 showed a diffuse structure and was composed of anaplastic undifferentiated cells. Similar histological features were observed in tumour cells invading the intestinal wall, the retroperitoneal spaces and colonizing some retroperitoneal and mediastinal lymph nodes. Therefore, retroperitoneal and lymph node tumours were interpreted as metastases deriving from the pancreatic mass.

Histochemistry

In both the histologically normal and dysplastic islets, Grimelius' silver impregnation method revealed the expected deep brown argyrophilia of peripheral cells (Grimelius 1968). Tumours were composed of variably argyrophilic cells, although the typical dark brown staining of A-cells was detected only in a proportion of the tumour population. In the large pancreatic mass of transgenic mouse no. 4, argyrophilia was detected only in the area corresponding to the more vascularized part of the tumour (approximately 15% of tumour area in sections). No argyrophilia was detected in cells invading the intestinal wall, the retroperitoneum or lymph nodes.

Immunohistochemistry

Serial sections from pancreases of transgenic mice and controls were stained with a panel of 12 specific antisera (Table 1). The estimated percentages of positive cells per tumour section are reported (Table 2). In addition, serial sections from small and large intestine of transgenic mice and controls were immunostained for pancreatic-polypeptide, glicentin and SV40 large T antigen (TAG) (Table 1) to determine any possible abnormality not detectable on conventional light microscopy. The profiles of all antigens immunostained were entirely normal in these specimens (not shown). Notably, no TAG-immunoreactive cell was detected in the gut, as has been reported previously (Efrat et al. 1988).

General neuroendocrine markers

Normal and dysplastic islets were immunostained with antibodies to all the general neuroendocrine markers (see Table 1) (Bishop et al. 1988). All islet cells showed strong immunoreactivity for the cytosol marker neurone-specific enolase (NSE) (Schmechel et al. 1978; Polak and Marangos 1984) and the granular markers chromogranin A (Lloyd and Wilson 1983; Lloyd et al. 1984) and synaptophysin-P38 (Jahn et al. 1985; Chejfec et al. 1987). Only cells at the islet periphery were faintly stained by sera against the cytosol marker protein-gene product 9.5 (PGP 9.5) (Thompson et al. 1983; Rode et al. 1985; Wilkinson et al. 1989) and the granular marker 7B2 (Hsi et al. 1982; Suzuki et al. 1987). The same sera revealed a multi-layered ring of positive cells in the rare dysplastic islets. Additionally, in all pancreases, ganglion cells and nerve fibres were positive for NSE, PGP 9.5, and synaptophysin (Fig. 3A, B, D). Transgenic mice nos. 1–3 tumours were composed of cells strongly immunoreactive for NSE, PGP 9.5, P38 and weakly positive for 7B2 (Fig. 3A–D) (Table 2). Strong chromogranin-A immunoreactivity was also detected in a variable percentage of tumour cells (Fig. 3A, inset). In transgenic mouse no. 4 the large pancreatic mass showed this pattern of immunoreactivity only in the most vascularized region. The remaining large, whitish part, as well as the retroperitoneal tumour mass and the lymph node metastases were composed of unreactive cells. Additionally, few scattered cells positive for NSE, PGP 9.5 and/or P38 were detected among the vast majority of unreactive elements.

Bioactive peptides

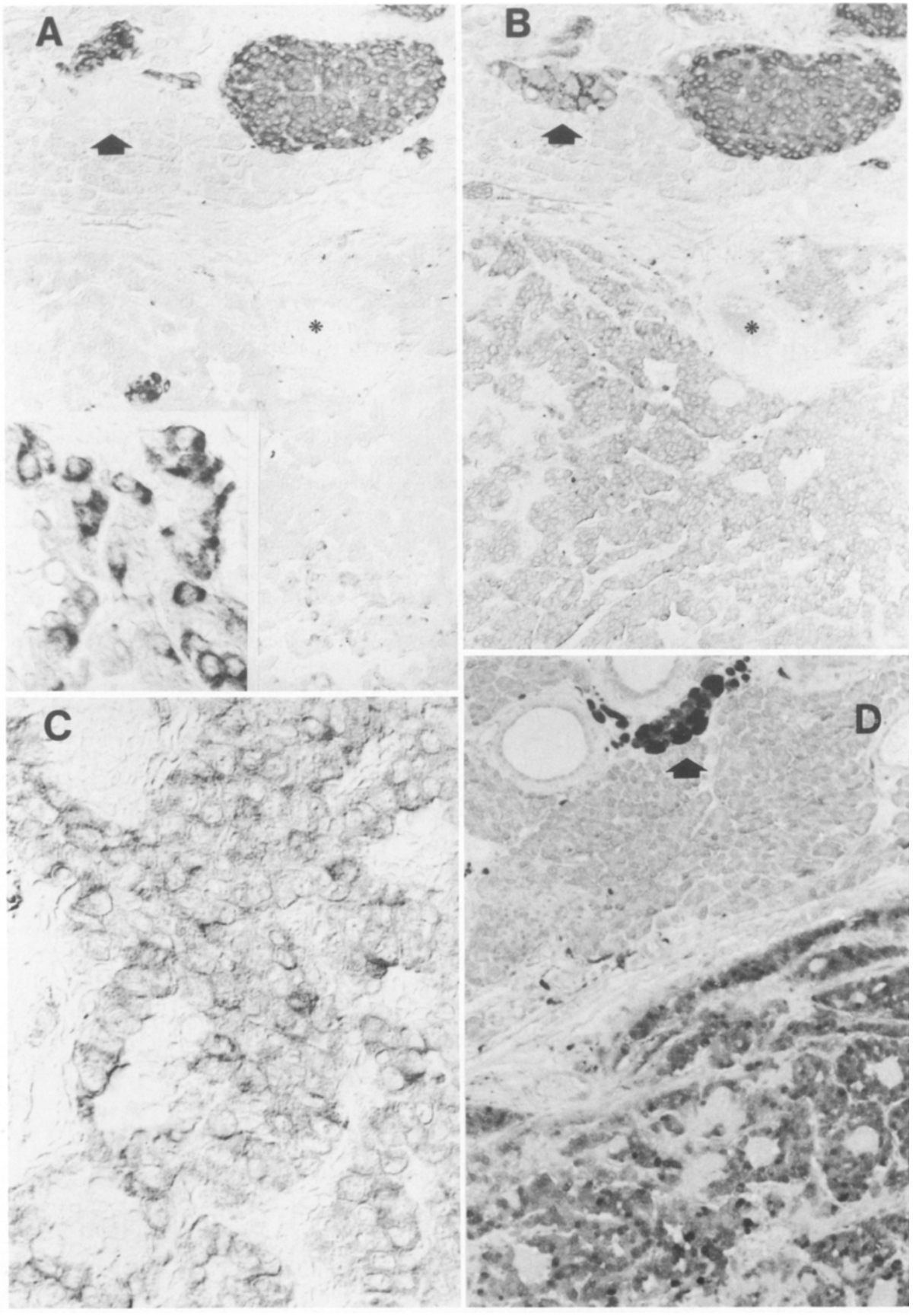
Histologically normal islets revealed a balanced proportion of A (glucagon-immunoreactive), B (insulin-immu-

Table 2. Immunoreactivities of Glu2-Tag tumours.

Glu2-Tag tumours	General neuroendocrine markers					Pro-glucagon derivatives				Other peptides		
	CgA	7B2	P38	NSE	PGP	GLU	GLP1	GLP2	GLI	INS	PP	SOM
Mouse no. 1 tumour	+	+	+	+	+	+	+	+	+	+	−	−
cells %	30	90	90	90	90	90	60	80	80	Rare		
Mouse no. 2 tumour	+	+	+	+	+	+	+	+	+	+	+	+
cells %	20	90	90	90	80	90	15	60	80	Rare	Rare	Rare
Mouse no. 3 tumour	+	+	+	+	+	+	+	+	+	−	+	−
cells %	90	90	90	90	90	80	60	10	80		Rare	
Mouse no. 4[a] tumour	+	+	+	+	+	+	+	+	+	+	+	+
cells %	15	10	70	90	70	70	40	10	60	Rare	Rare	Rare

CgA, chromogranin A; P38, synaptophysin; NSE, neuron-specific enolase; PGP, protein-gene product 9.5; GLU, glucagon; GLP 1 and 2, glucagon-like polypeptides 1 and 2; GLI, glicentin; INS, insulin; PP, pancreatic polypeptide; SOM, somatostatin; +, positive test; −, negative test; cells %, estimated % of positive cells; Rare, 1–10% of positive cells

[a] Data referred to the highly vascularized part only (see text)


A
B
C
D

noreactive), D (somatostatin-immunoreactive) and PP (pancreatic polypeptide-immunoreactive) cells (Orci 1981). Dysplastic islets showed a large peripheral ring of glucagon-immunoreactive cells with reduction of the other elements (Fig. 1C, D). Immunoreactivity for different fragments of proglucagon (glicentin, glucagon-like polypeptides (GLP) 1 and 2) (Conlon 1988) was also localized to A cells. Transgenic mice nos. 1, 2 and 3 tumours were composed of cells variably reactive to the sera against proglucagon fragments. The majority of cells showed strong immunoreactivity for glucagon, glicentin and/or GLP1 and GLP 2 (Fig. 4A, B) (Table 2). Few cells positive for insulin, somatostatin or PP were also detected in all tumours (Fig. 4C–E). The pancreatic mass in transgenic mouse no. 4 showed similar distribution of peptide immunoreactivity in the most vascularized area only. The remaining part of this tumour, as well as retroperitoneal and lymph node metastases, presented rare, scattered cells positive for sera against the different proglucagon fragments among the vast majority of unreactive elements.

SV40 oncoprotein

Variable immunoreactivity for the transgene-encoded oncoprotein TAG was occasionally observed in the nuclei of some glucagon-immunoreactive cells of apparently normal islets and, consistently, in nuclei of most cells at the periphery of dysplastic islets. All tumour cells of transgenic mice nos. 1, 2 and 3 also expressed TAG. In transgenic mouse no. 4 TAG-immunoreactivity was observed only in the tumour area coexpressing proglucagon fragments. Cells composing the remaining large pancreatic mass were negative, as well as cells invading the intestinal wall, the retroperitoneum and lymph nodes (not shown).

Electron microscopy

Ultrastructural analysis was performed on samples from transgenic mice nos. 1, 2, 4 pancreatic tumours and transgenic mouse no. 4 retroperitoneal and lymph nodes masses. Transgenic mice nos. 1 and 2 tumour cells were characterized by a variable number of granules, showing a highly electron-dense core surrounded by a clear halo typical of alpha granules (Lacy 1962) (mean diameter 152 ± 34 nm) (Fig. 5A–C). Occasional granules showed an inner core of low electron density with a surrounding dense mantle, as observed in some human fetal alpha granules (Like and Orci 1972). Some cells had few, if any, electron-dense granules (Fig. 5A, B). Other cells contained large, round mitochondria engulfing a region of the cytoplasm (oncocytoid pattern). The electron-dense granules were either scattered in the cytoplasm or distributed along the plasma membrane, sometimes polarized at one edge of the cell near the vascular spaces (Fig. 5B). The granular endoplasmic reticulum was prominent. Lipid droplets and multilamellar bodies were frequently observed. Junctional structures were detected in tumour cells delimiting true glandular spaces. The vascularized area of transgenic mouse no. 4 tumour was composed of cells showing a variable number of atypical, small, electron-dense granules (mean diameter 124 ± 42 nm). Typical and atypical granules of all tumours reacted positively with sera against glucagon, GLP1 and GLP2 and glicentin. Single and double immunogold labelling (see Fig. 5B–E) showed glucagon, glicentin, GLP1 and GLP2 immunoreactivity to be mainly concentrated in the electron-dense core of alpha granules. Only in a minority of granules, glicentin immunoreactivity was segregated in the peripheral halo (Fig. 5E, inset). These features, together with positive immunohistochemical tests, suggest that these tumours are composed of A type cells.

The large pancreatic mass of transgenic mouse no. 4 and the retroperitoneal and lymph node tumours were mainly composed of irregular cells with a thin rim of cytoplasm and few dispersed organelles, often represented by scattered ribosomes (Fig. 6A). No junctional structure was detected. These features suggest that the majority of tumour cells are undifferentiated. Additionally, rare scattered cells showed prominent rough endoplasmic reticulum revealing abundant synthetic activity (Fig. 6A). Some cells also showed few round, medium size, endocrine-like granules, or large, zymogen-like granules. Sometimes both types of electron-dense granules were detected in the same cell (Fig. 6A, B). These features are ultrastructural markers of endocrine or exocrine differentiation. All the described cell types (either undifferentiated or differentiated) revealed a variable number of round, small, slightly electron-dense particles (mean diameter 78 ± 8 nm) inside some cisternae of the loose endoplasmic reticulum, or free in the cytosol (Fig. 6A, B). These particles were frequently seen budding off the plasma membrane and outside tumour cells, either along the plasma membrane or at distance in the extracellular space (Fig. 7A). The structure of these particles was characterized by a double outer membrane and by a slightly electron-dense, inner core which was sometimes geometrical in shape (Fig. 7B). These particles were interpreted as being C-type retrovirus structures (Bernhard 1960; Teich 1982). All these features suggest the presence of an unidentified retrovirus active-

Fig. 3A–D. General neuroendocrine marker immunohistochemistry. Transgenic mouse no. 2 pancreas. Granular markers immunohistochemistry (**A–C**): chromogranin A (**A**), synaptophysin (**B**) and 7B2 (**C**). Cytosolic marker: PGP 9.5 (**D**). Chromogranin A immunoreactivity (**A**) is present in normal islets (*upper part* of the micrograph) and in a percentage of tumour cells (*lower part*), with variable intensity (*inset*). In a consecutive section, synaptophysin-immunoreactivity (**B**) is found in normal islets, in nerve varicosities and ganglion cells (*upper part*) and in all tumour cells with apparent uniform intensity. In **A** and **B** the *arrows* indicate the same ganglion structure: note the intimate contact with some endocrine cell (neuroendocrine complex). The *asterisk* in the lumen of a vessel serves as a referral point. Weak 7B2 immunoreactivity is found in the majority of tumour cells (**C**). The cytosolic general neuroendocrine marker PGP 9.5 (**D**) is positive in ganglion cells (*arrow*), nerves and apparently in all tumour cells. ABC method, Nomarski optics (**A–C**), haematoxylin counterstain (**D**), ×105 (**A, B**), ×415 (*inset* of **A, C**), ×50 (**D**)

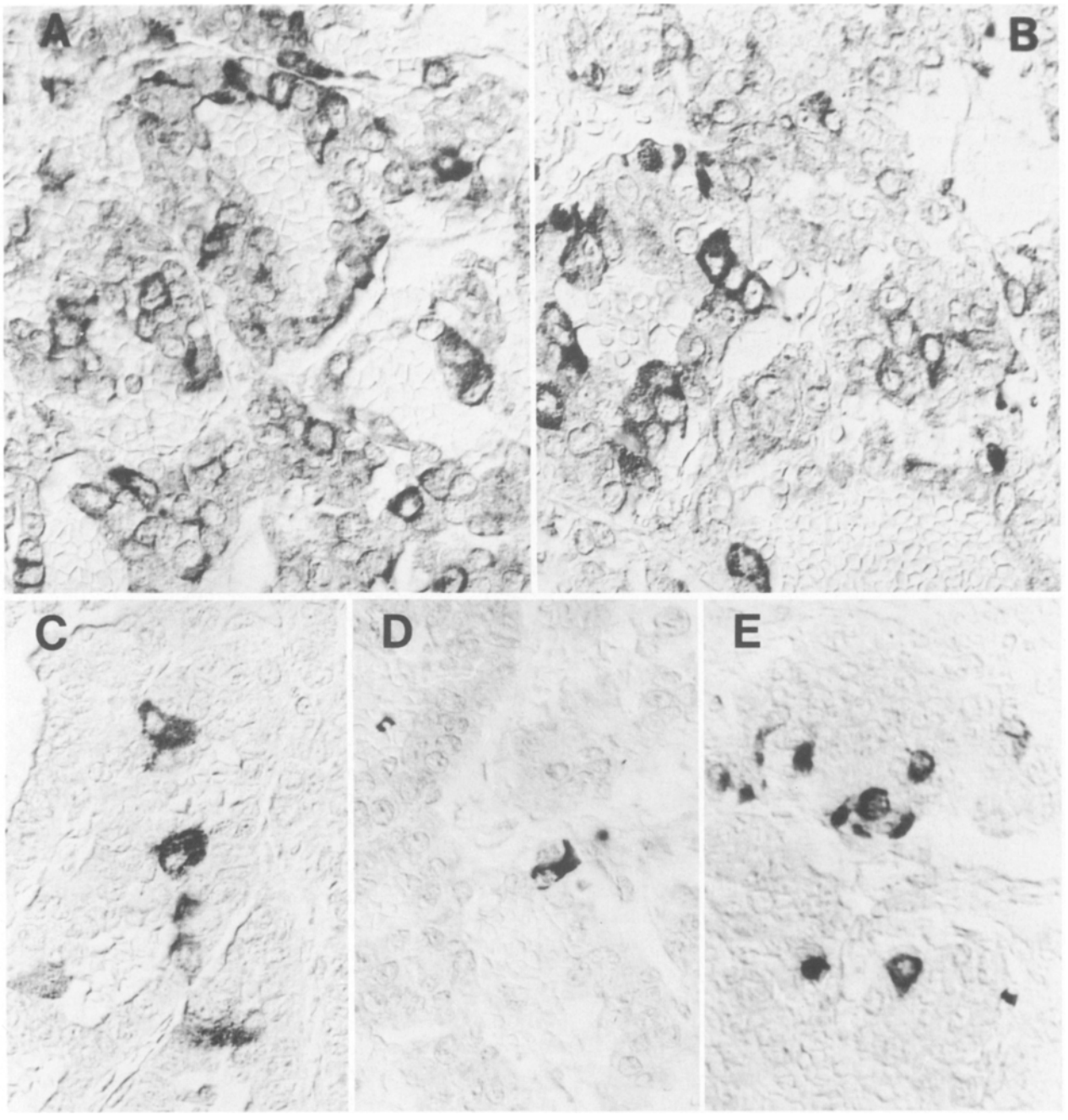
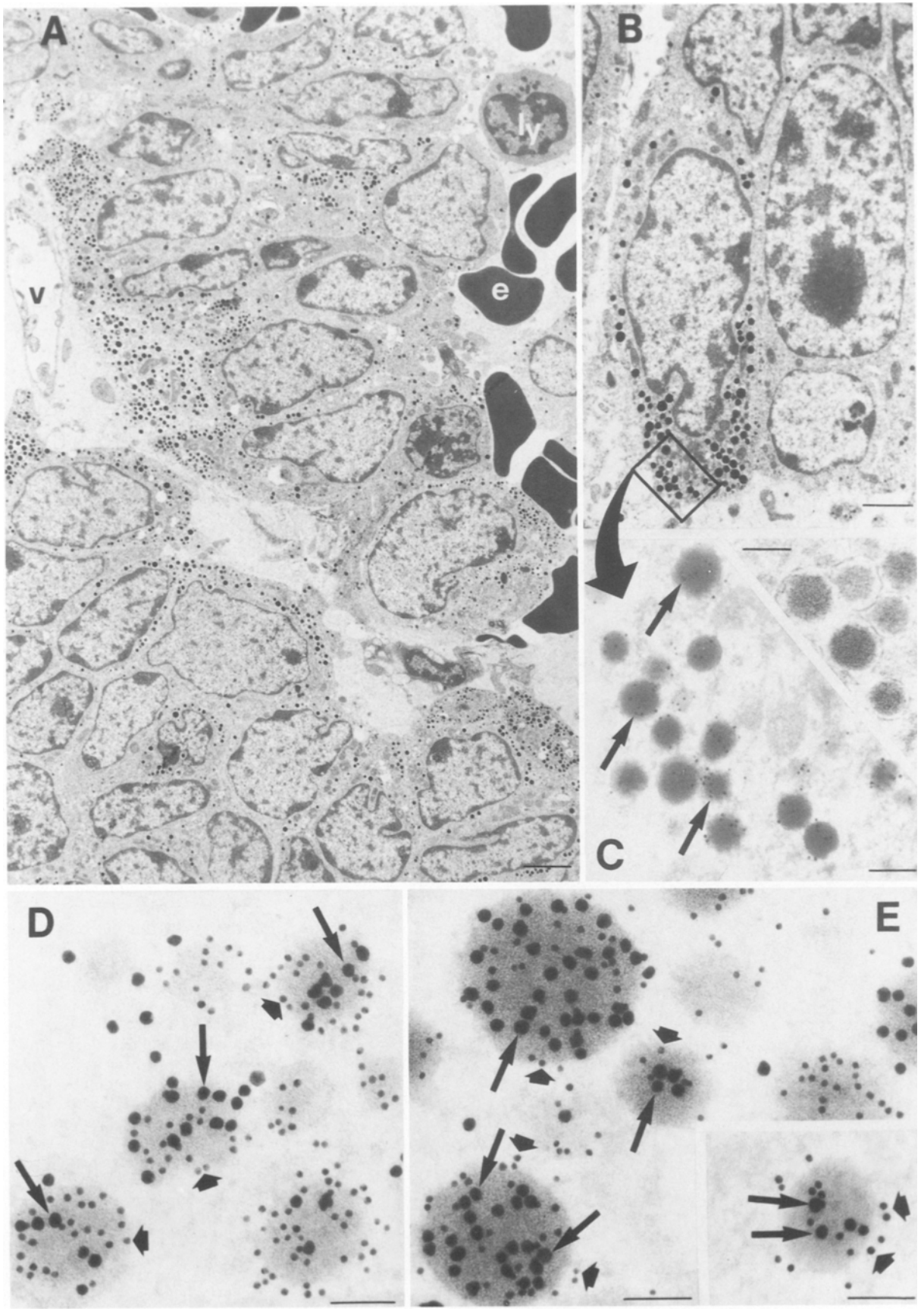


Fig. 4 A–E. Peptide immunohistochemistry. Transgenic mouse no. 2 tumour. Immunoreactivity for GLP 2 (**A**), glicentin (**B**), insulin (**C**), somatostatin (**D**) and pancreatic polypeptide (**E**). The majority of tumour cells are variably positive with sera against different parts of the proglucagon molecule (**A, B**). Few tumour cells, either isolated (**D**) or grouped (**C, E**), expressed the other bioactive islet peptides. ABC method, Nomarski optics, ×415 (**A–E**)

Fig. 5 A–E. Electron microscopy of well differentiated A cell tumours. Transgenic mouse no. 1 tumour. **A** General view of the tumour. Tumour cells of different sizes and shapes are organized in large multilayered ribbons interlacing with bundles of loose extracellular matrix and vascular spaces. Note the erythrocytes (*e*), one lymphocyte (*ly*) (*right part* of the micrograph) and a true vessel (*v*) (*left part*). Tumour cells show a variable number of electron-dense endocrine granules sometimes amassed at the cell pole overlooking the extracelluar matrix or vessels. Non osmium postfixed specimen, uranyl acetate/lead citrate counterstain, ×4600. *Bar* = 2.1 μm. **B** Ultrastructure of tumour cells. A group of variably granulated tumour cells: one cell shows a large number of electron-dense, endocrine granules fairly polarized at one edge. The other tumour cells are largerly devoid of granules. No osmium postfixed sample, indirect immunogold labelling (see **C**), uranyl acetate/lead citrate counterstain, ×8900. *Bar* = 1.12 μm. **C** Morphology of the electron-dense granules. A detail of **B** showing the electron-dense endocrine granules heavily immunolabelled with anti GLP 1 serum (*long arrows* indicate the 10 nm gold particles). Compare with the osmium postfixed preparation of the same specimen (*upper right corner*). The granules show an electron-dense core and an electron-lucent halo delimited by a membrane, the typical structure of glucagon-storing alpha granules. ×58000. *Bar* = 0.2 μm. **D, E** Double immunogold labelling of alpha granules in tumour cells. Glucagon-immunoreactivity (20 nm gold particles, *long arrows*) is colocalized with GLP 2 immunoreactivity (10 nm gold particles, *short arrows*) (**D**) and glicentin immunoreactivity (10 nm gold particles, *short arrows*) (**E**). Glicentin immunoreactivity was sometimes identified in the electron-lucent halo (*lower right* corner of **E**). Indirect immunogold labelling, non osmium postfixed samples, uranyl acetate/lead citrate counterstain, ×145000. *Bar* = 0.1 μm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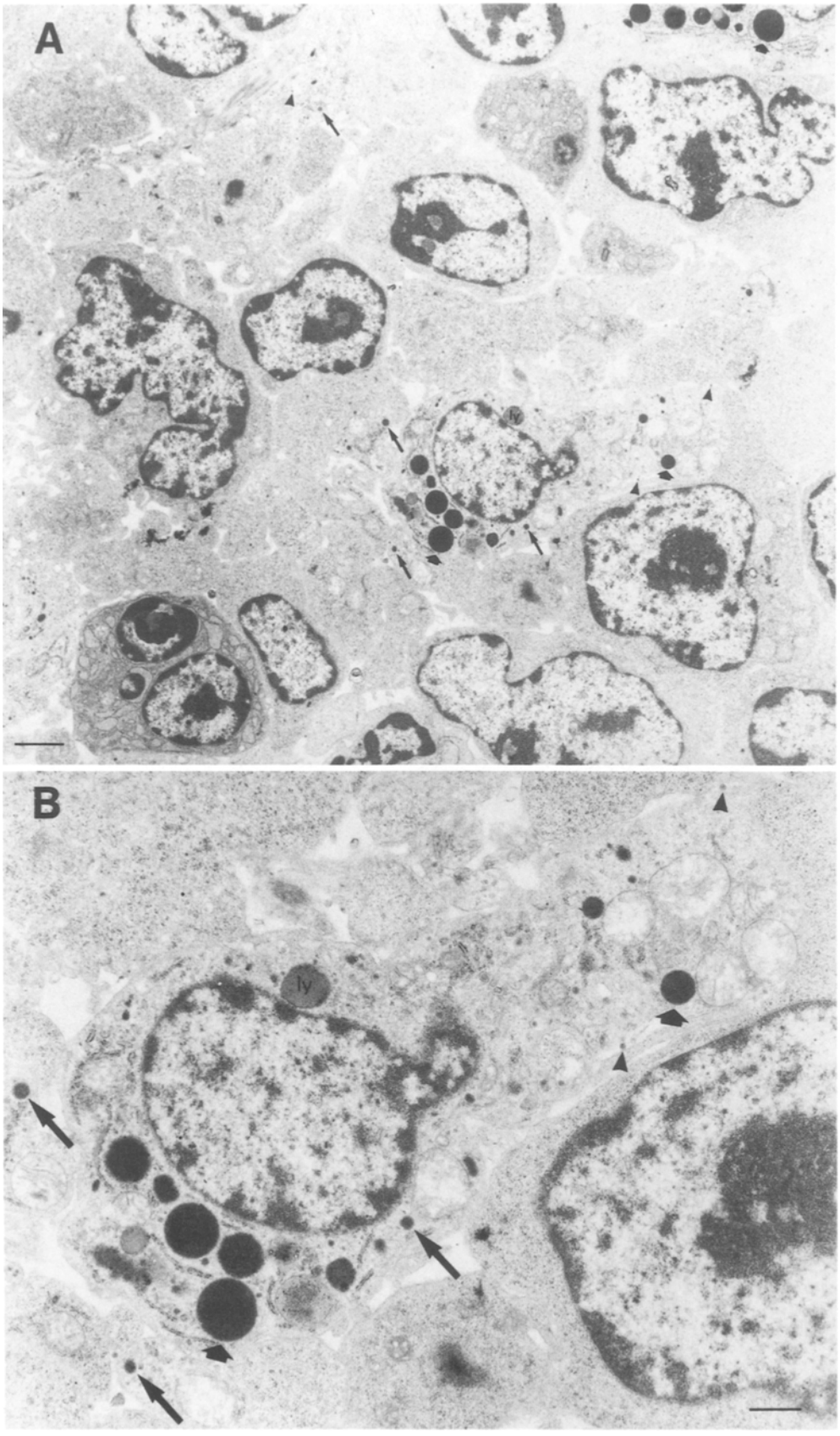

Fig. 6A, B. Electron microscopy of transgenic mouse no. 4 malignant tumour. **A** General view of the tumour. The majority of the cells have an undifferentiated appearence with few organelles and dispersed ribosomes in their cytoplasms. Note the presence of small electron-dense particles either inside the cytoplasm or along the plasma membrane. Some differentiated tumour cells (*centre* and *top right* of the micrograph) show large, zymogen-like granules (*short arrows*), medium-sized, endocrine-like granules (*long arrows*) in addition to small, retroviral particles (*arrowheads*) (see **B**). Other tumour cells show a prominent rough endoplasmic reticulum suggesting an intense protein synthesis activity (*bottom left*). *Ly*, Lysosome. Osmium postfixed sample, uranyl acetate-lead citrate counterstain, ×6250. *Bar* = 1.6 μm. **B** Morphology of the electron-dense granules. The tumour cell depicted in the centre of **A** is enlarged to illustrate the ultrastructure of zymogen-like granules (*large arrows*), endocrine-like granules (*long arrows*) and retroviral particles (*arrowheads*). The retroviral particles are also dispersed in the cytosol or inside endoplasmic cysternae. *Ly*, Lysosome. ×14175. *Bar* = 0.7 μm

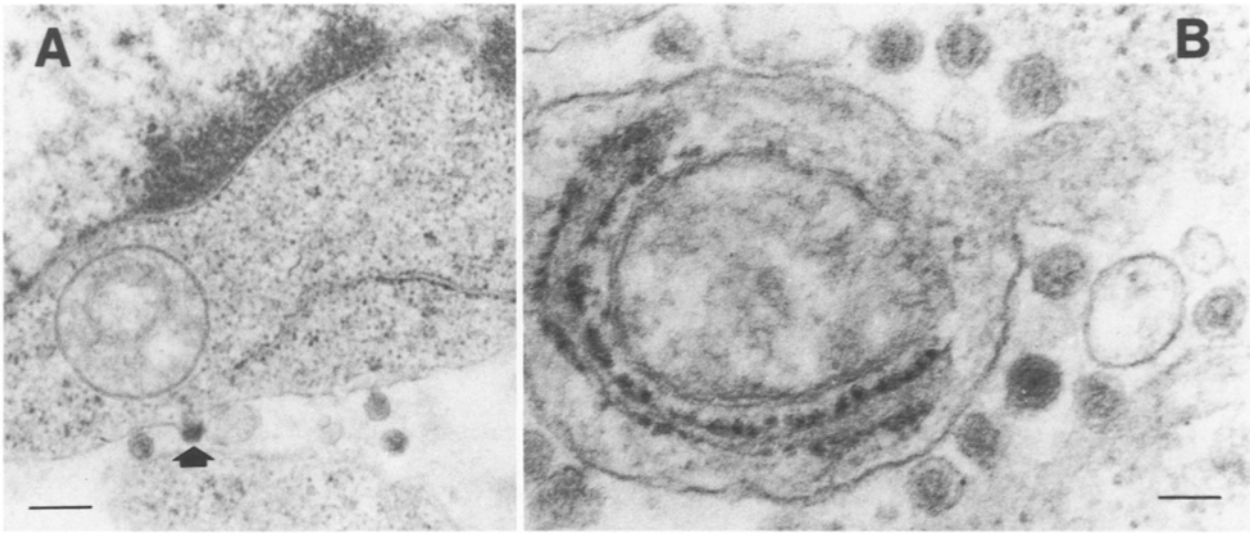

Fig. 7A, B. Retroviral particles. **A** Small, electron-dense, virus particles budding off the plasma membrane (*large arrows*) and outside the cell. × 50000. *Bar* = 0.2 μm. **B** Intracytoplasmic particles showing an outer membrane and an inner, electron-dense core, sometimes geometrical in shape. Note the close relationship with the rough endoplasmic reticulum and a large cytoplasmic cysterna (*centre* of the micrograph). Osmium postfixed samples, uranyl acetate/lead citrate counterstain, × 108000. *Bar* = 0.092 μm

ly replicating in the tumour cells of transgenic mouse no. 4 large pancreatic mass and retroperitoneal and lymph nodes metastases.

Radioimmunoassay

All radioimmunological data are detailed in Table 3. Plasma samples from 7 transgenic mice and 5 controls were assayed for total N-terminal glucagon, C-terminal pancreatic glucagon, GLP 1 7–36 NH_2 and insulin. Enteroglucagon concentrations were calculated by subtracting the specifically measured pancreatic glucagon from total N-terminal glucagon immunoreactivity (see Materials and methods) (Ghatei et al. 1983; Holst 1988). In younger transgenic mice, all circulating glucagon-related immunoreactants were in the range of control values (Table 3). In older transgenic mice, pancreatic glucagon-like immunoreactant was elevated approximately 8-fold (control, mean value = 10.8 pmol/l; transgenic mice, mean value = 85.5 pmol/l), total N-terminal glucagon-like immunoreactant 9-fold (control, mean value = 63.5 pmol/l; transgenic mice, mean value = 572 pmol/l) and GLP 1-like immunoreactant 25-fold (control, mean value = 7; transgenic mice, mean value = 175 pmol/l). In addition, by subtracting specifically measured pancreatic glucagon-like immunoreactant from total N-terminal glucagon-like, circulating enteroglucagon was estimated to be elevated 9-fold in transgenic mice (control, mean value = 52.7 pmol/l; transgenic mice, mean value = 486.5 pmol/l). Moreover, in all groups of mice, enteroglucagon percentage of total N-terminal glucagon was in the same range of values (control, mean value = 82.9%; younger, mean value = 83%; older, mean value = 85%).

Table 3. Plasma radioimmunoassay data

Sample	Lineage	PG	TG	GLP 1	INS
Mice (9–18 months old)					
Mouse no. 1	Glu2-Tag5	70	340	140	72
Mouse no. 2	Glu2-Tag6	42	328	15	52
Mouse no. 3	Glu2-Tag3	20	140	45	28
Mouse no. 4	Glu2-Tag3	210	1480	500	40
	Mean	85.5	572	175	56
Mice (6–7 months old)					
Mouse no. 5	Glu2-Tag3	10	50	–	48
Mouse no. 6	Glu2-Tag5	10	–	–	24
Mouse no. 7	Glu2-Tag6	10	68	5	96
	Mean	10	59	5	56
Controls					
C1	C57B1/6J	10	50	–	32
C2	C57B1/6J	14	–	–	24
C3	C57B1/6J	10	64	10	40
C4	C57B1/6J	16	60	7	40
C5	C57B1/6J	4	80	4	100
	Mean	10.8	63.5	7	44.8

All values expressed in pmol/l; PG, pancreatic glucagon-like immunoreactivity; TG, total glucagon-like immunoreactivity; GLP 1, glucagon-like polypeptide 1-like immunoreactivity; INS, insulin-like immunoreactivity; –, plasma not sufficient to complete the assay

Circulating insulin-like immunoreactant showed no difference between wild-type and transgenic mice (control, mean value = 44.8 pmol/l; transgenic mice, mean value = 51.4 pmol/l).

Discussion

In this report, we have described the morphological and secretory features of pancreatic A cell tumours which develop in Glu2-Tag transgenic mice (Efrat et al. 1988). Our investigation was aimed at establishing this strain of trangenic mice as a possible model for human disease.

Tumour development in Glu2-Tag mice

A cell hyperplasia has been observed by Efrat et al. (1988) in Glu2-Tag transgenic mice before the onset of tumour nodes. In this study, in addition to hyperplasia, we also observed multilayered, peripheral growth of moderately atypical, TAG-positive A cells, associated with loss of internal islet structure (see Fig. 1). The atypical features of this lesion support its dysplastic nature and may be considered to be the starting point of Glu2-Tag glucagonomas. We found dysplastic islets to be rare, usually single lesions in pancreases of younger transgenic mice only. The fact that only solitary lesions are observed in pancreases of both younger (dysplastic islets) and older (tumour nodes) transgenic mice, together with the patterns of TAG expression and cellular and structural atypia, suggests that tumorigenesis is a unifocal event in Glu2-Tag transgenic mice. This is at variance with previous findings reporting heritable multifocal endocrine tumorigenesis obtained in pancreas of different transgenic mice (Hanahan 1985; Murphy et al. 1987; Rindi et al. 1990). However, morphological data reported for Glu2-Tag mice confirm those obtained in previous transgenic experiments suggesting endocrine tumours derive from differentiated islet cell types (Hanahan 1985; Murphy et al. 1987; Rindi et al. 1990). In humans, similar preliminary findings have been observed in hereditary neoplastic disease of endocrine pancreas (Rindi et al. 1989).

Glu2-Tag tumours and human glucagonomas share a number of morphological features

Of the four tumours investigated, two (transgenic mice nos. 1 and 2) had benign histology (i.e. expansive growth and trabecular structure) and behaved as slow growing adenomas, while two (transgenic mice nos. 3 and 4) showed aggressive histological features (i.e. infiltrative growth and diffuse, or mixed trabecular-diffuse structure), one of which produced distant metastases (see following paragraph). Human glucagonomas have similar histological features: metastasizing and clinically functioning glucagonomas have been reported to show mostly a mixed diffuse-trabecular architecture, while non-functioning, benign adenomas have a uniform, trabecular pattern (Ruttmann et al. 1980). In Glu2-Tag transgenic mice, differentiated tumours were composed of argyrophilic cells expressing a wide range of general neuroendocrine markers. Some of these markers are associated with cytosol functions intrinsic to neuroendocrine activities (NSE and PGP 9.5) (Schmechel et al. 1978; Thompson et al. 1983; Polak and Marangos 1984; Rode et al. 1985; Wilkinson et al. 1989) while others are related to the endocrine granule content (chromogranin A, synaptophysin and protein 7B2) (Hsi et al. 1982; Lloyd and Wilson 1983; Lloyd et al. 1984; Jahn et al. 1985; Chejfec et al. 1987; Suzuki et al. 1987). This profile of immunohistochemical neuroendocrine markers is observed consistently in normal pancreatic A cell. Therefore, we suggest that Glu2-Tag tumour cells retain the function and properties of differentiated, mature endocrine cells, possibly of the A type. In human neuroendocrine pancreatic tumours, argyrophilia has been reported to be mostly related to proliferating A and PP cells (Grimelius 1968; Ruttmann et al. 1980; Klöppel and Heitz 1988). Furthermore, the general neuroendocrine markers immunostained in the present study have been shown to be highly expressed in glucagonomas (Lloyd et al. 1984; Polak and Marangos 1984; Rode et al. 1985; Hamid et al. 1986; Chejfec et al. 1987; Suzuki et al. 1987; Bordi et al. 1988). As for specific hormone production in differentiated Glu2-Tag tumours, glucagon and glicentin immunoreactivity was consistently detected in 80% of tumour cells, while GLP1 and 2 immunoreactive cell percentage varied (see Table 2). This suggests that Glu2-Tag tumour cells possess the immunohistochemical hormone profile of mature A cells. Immunoreactivity for proglucagon fragments is also found in intestinal L-type cells and derived tumours (Fiocca et al. 1987; Holst 1988). This endocrine cell is different ultrastructurally from the pancreatic A cell type and is known to coexpress the PP-related peptide, peptide tyrosine tyrosine (PYY) (Holst 1988). Glu2-Tag tumour cells were immunoreactive for proglucagon fragments but lacked reactivity to the PP antiserum used which cross-reacts with PYY (Fiocca et al. 1987). This finding confirms the A-type nature of the majority of Glu2-Tag tumour cells, in agreement with data from Glu2-Tag-derived αTC cell line (Hamagouchi and Leiter 1990; Powers et al. 1990). Notably Glu2-Tag mice do not express TAG in the intestinal L-type cells (Efrat et al. 1988; this report). In addition to A-type peptides, rare scattered Glu2-Tag tumour cells expressed other islet hormones (insulin, PP, somatostatin). In human glucagonomas, a similar pattern of immunoreactivity for proglucagon fragments has been reported, together with the presence of scattered cells immunoreactive for other hormones (Bordi et al. 1979; Ruttmann et al. 1980; Heitz et al. 1982; Hamid et al. 1986). Electron microscopy of Glu2-Tag tumour cells revealed the presence of "typical" and "atypical" endocrine granules, both reacting with sera specific for various proglucagon fragments, the former sometimes revealing the partial intragranular segregation described for alpha granules in normal cells (Ravazzola and Orci 1980; Varndell et al. 1985) and neoplastic cells of human glucagonomas (Bordi et al. 1979; Ruttmann et al. 1980; Hamid et al. 1986).

In conclusion, the data suggest that Glu2-Tag tumours are mainly composed of typical, mature A-type cells and are morphologically comparable to human glucagonomas.

Glu2-Tag tumours are functioning glucagonomas

Our radioimmunological data demonstrated that Glu2-Tag investigated tumours had secretory properties. All transgenic mice with increased levels of circulating pro-glucagon fragments had pancreatic A cell tumours. In contrast, circulating levels of pro-glucagon fragments were normal in non tumour-bearing younger transgenic mice. This suggests that the high levels found in older transgenic mice were related to the presence of tumours. In addition, the increase in C-terminal pancreatic glucagon paralleled the increase in N-terminal glucagon. This evidence suggests that Glu2-Tag tumour cells process the proglucagon molecule in the same way as normal pancreatic A cells (Conlon 1988; Holst 1988), as has been observed in Glu2-Tag tumour-derived αTC cell line (Hamagouchi and Leiter 1990; Powers et al. 1990). In agreement with data reported in rat (Kervran et al. 1987), enteroglucagon (proglucagon residues 1–69) represented the largest fraction of circulating proglucagon fragments both in controls and transgenic mice. However, the possibility that a small amount of enteroglucagon was produced specifically and released by Glu2-Tag tumours cannot be ruled out and is suggested by the slight increase in enteroglucagon percentage of total N-terminal glucagon values reported in tumour-bearing mice (older transgenic mice, mean value = 85%, in respect to younger transgenic mice, mean value = 83% and control mean value = 82.9%; see radioimmunoassay data). This is consistent with the reported finding of small quantities of enteroglucagon produced by pancreas in rat (Yanaihara et al. 1985). Despite the absolute high levels of circulating enteroglucagon, no significant histological change was detected in the gut mucosa of tumour-bearing Glu2-Tag mice, as would be expected on the basis of the proposed (Sagor et al. 1983) and debated (Gregor et al. 1987) trophic effects of enteroglucagon on this tissue. We also report high levels of circulating GLP1-like immunoreactivity in concentrations exceeding those of corresponding immunoreactive C-terminal or N-terminal glucagon. This may reflect a longer half-life of this peptide in the circulation (Uttenthal et al. 1987). Chromatographic analyses are required to elucidate the molecular forms of pro-glucagon fragments detected in the plasma of Glu2-Tag mice. In human pathology, functioning glucagonomas may be associated with high levels of circulating proglucagon fragments and typical dysmetabolic symptoms ("glucagonoma syndrome") (Mallinson et al. 1974; Bloom and Polak 1987; Uttenthal et al. 1987). Cutaneous lesions are the most characteristic manifestation of this extremely varied, hyperfunctional syndrome (Bloom and Polak 1987). The development of symptoms apparently requires large A-cell tumour masses and long tumour history (Ruttmann et al. 1980; Heitz et al. 1982). However, no data are available concerning the minimal levels of circulating glucagon required to determine detectable clinical symptoms and consequent timing of the syndrome. In tumour-bearing, Glu2-Tag mice we failed to detect evident skin lesions. However, specific analyses have not been performed to determine other possible changes associated with the glucagonoma syndrome (stomatitis, glossitis, normochromic normocytic anaemia, mild diabetes mellitus, etc) (Bloom and Polak 1987).

In conclusion, we have documented that Glu2-Tag tumour cells retain the capacity of pancreatic A-type cells to process the proglucagon molecule and, as a consequence, Glu2-Tag tumours seem to behave like functioning human glucagonomas.

Glu2-Tag tumours may be malignant

Transgenic mouse no. 4 had the largest tumour mass and the highest levels of circulating proglucagon fragments. However, only few tumour cells retained the immunohistochemical and ultrastructural profile of proliferating A cells. Similar features were observed in retropertioneal and lymph node metastases. These findings suggest that transgenic mouse no. 4 tumour is a malignant glucagonoma (glucagon-producing neuroendocrine carcinoma). In contrast, the majority of Glu2-Tag A-cell tumours are reported to be slow growing, benign adenomas (Efrat et al. 1988; this report). Interestingly, the majority of tumour cells in transgenic mouse no. 4 tumour did not show transgene-encoded nuclear TAG immunoreactivity, which however was present in rare cells still retaining proglucagon expression. Additionally, transgenic mouse no. 4 tumour cells possess ultrastructural evidence of the presence of an unknown, actively replicating retrovirus. No evidence of virus replication has been found in the other Glu2-Tag tumours analysed in the present study. It is reasonable to conclude that this is a peculiar characteristic of transgenic mouse no. 4. The reasons for this retroviral activation and its possible role in TAG transformed A cells remains to be elucidated. Retroviruses have been reported so far in murine pancreatic B cells (Leiter 1985) and in benign B cell tumours of transgenic mice and derived cell lines (S. Efrat and D. Hanahan, unpublished data). Nevertheless, it can be hypothesized that the dramatic changes observed in the cellular profile and clinical behaviour of transgenic mouse no. 4 tumour may be somehow associated with retroviral activation. In human pathology, functioning glucagonomas are generally malignant tumours, frequently diagnosed following detection of metastases (Ruttmann et al. 1980; Heitz et al. 1982). However, no evidence of retroviral activation has ever been reported in human islet cell carcinomas.

In conclusion, this paper provides evidence that Glu2-Tag transgenic mice develop functioning pancreatic glucagonomas. These tumours present a large spectrum of morphofunctional features, paralleling those of typical human glucagonomas. For these reasons, tumour-bearing Glu2-Tag mice may be a model for the human disease and may offer unique opportunities for the study of relationships between chronic hyperglucagonaemia and onset of dysmetabolic symptoms. Glu2-Tag tumours and derived cell lines provide new tools for studying the so far poorly defined functions of the general neuroendocrine markers.

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