

Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-*oncogene*

(immortalization of rare cell types/insulin secretion/insulinoma lines)

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ABSTRACT Three pancreatic beta-cell lines have been established from insulinomas derived from transgenic mice carrying a hybrid insulin-promoted simian virus 40 tumor antigen gene. The beta tumor cell (β TC) lines maintain the features of differentiated beta cells for about 50 passages in culture. The cells produce both proinsulin I and II and efficiently process each into mature insulin, in a manner comparable to normal beta cells in isolated islets. Electron microscopy reveals typical beta-cell type secretory granules, in which insulin is stored. Insulin secretion is inducible up to 30-fold by glucose, although with a lower threshold for maximal stimulation than that for normal beta cells. β TC lines can be repeatedly derived from primary beta-cell tumors that heritably arise in the transgenic mice. Thus, targeted expression of an oncogene with a cell-specific regulatory element can be used both to immortalize a rare cell type and to provide a selection for the maintenance of its differentiated phenotype.

Pancreatic beta cells synthesize and secrete insulin, a hormone involved in regulation of glucose homeostasis. In rodents there are two nonallelic insulin genes (I and II), which differ in the number of introns as well as in chromosomal location. Both genes are expressed in beta cells (1). An adult murine pancreas contains about 10^6 beta cells, clustered in the islets of Langerhans, which are dispersed throughout the exocrine tissue. As a consequence, molecular analyses of beta-cell function has in large part depended on *in vitro* cultures. Cells from isolated islets do not grow well in culture, although they maintain viability for a few weeks (2). In recent years, several lines of transformed beta cells have been generated (3-6). Two of these, RIN-m 5F, derived from an x-ray-induced rat insulinoma, and HIT, from hamster islets transformed by simian virus 40, have been used extensively for characterization of insulin gene expression (4, 5, 7, 8). However, it is unclear to what extent they represent normal beta cells, given that the levels of insulin secreted are considerably lower than those of beta cells *in vivo*.

The ability to target expression of oncogenes to particular cells in transgenic mice, by using cell-specific regulatory elements, presents a method for immortalization of rare cell types. We have reported that transgenic mice harboring insulin-simian virus 40 tumor (T) antigen (RIP-Tag) hybrid genes heritably develop beta-cell tumors (9-11). Here we describe the characterization of several beta tumor cell (β TC) lines obtained from transgenic mouse tumors and propagated in culture for over 60 passages. These cells provide a useful tool for studies of beta-cell regulation and gene expression.

METHODS

Cell Cultures. Pancreatic insulinomas were excised from transgenic mice and disrupted in Dulbecco's modified Eagle's medium (DMEM). To minimize contamination by fibroblasts and other nontransformed cells, the tumors were not trypsinized. Rather, the tumor capsule was gently removed, and the tumor cells were mechanically dispersed. After one wash, they were plated in 12-well plates (Corning) at about 10^6 cells per well, in DMEM containing 25 mM glucose and supplemented with 15% horse serum (GIBCO), 2.5% fetal bovine serum (Armour, Kankakee, IL), penicillin (100 units/ml), and streptomycin (0.1 mg/ml) and incubated in humidified 5% CO₂/95% air at 37°C. When cells reached about 50% confluency, they were transferred to 100-mm plates (Falcon) by trypsinization with 0.05% trypsin/0.5 mM EDTA. The cells were then subcultured approximately every 7 days and refed twice a week. The cells can be frozen in 90% fetal bovine serum/10% dimethyl sulfoxide and thawed with good viability after storage in liquid nitrogen.

HIT cells (6) (clone T15) were grown in DMEM containing 25 mM glucose and supplemented with 15% horse serum and 2.5% fetal bovine serum. RINr cells (clone 1046-38) were obtained from W. L. Chick (University of Massachusetts Medical School, Worcester) and were grown in either medium 199 (GIBCO) supplemented with 5% fetal bovine serum or in a serum-free RPMI 1640 medium supplemented with hormones and growth factors as described (12).

HPLC Analysis of Insulin Peptides. Cells were incubated at $3-5 \times 10^5$ cells per ml for 30 min in leucine- and methionine-free RPMI 1640 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and 11 mM glucose or in Krebs-Ringer bicarbonate medium supplemented with 20 mM Hepes, 5 mM NaHCO₃, 0.2% bovine serum albumin, penicillin (200 units/ml), streptomycin (0.2 mg/ml), and 2 mM L-glutamic acid (KRB medium) containing either 0.5 or 25 mM glucose. The cells were labeled with [³H]leucine (1 mCi/ml; 1 Ci = 37 GBq) and [³⁵S]methionine (1 mCi/ml) (Radiochemical Center, Amersham), followed by a chase period of 30 min in nonradioactive complete RPMI. Islets from both normal B6D2F1/J mice and from transgenic mice of the RIP-Tag lineages were isolated (13) and pulse-chase labeled as above. Tumors were similarly labeled. The cells were homogenized by sonication and fractionated by reversed-phase HPLC by using a LiChrosorb RP-18 (5 μ m), 250- \times 4-mm column and eluted at 1 ml/min with a linear gradient of acetonitrile (25-30%) in 0.125 M triethylammonium phosphate at pH 4.0. The column eluate was collected in 0.3-ml fractions and monitored for ³H and ³⁵S radioactivity as well as for absorbance at 210 nm. Positive

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Abbreviation: T antigen, tumor antigen.

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identification of the peaks was based both upon amino acid sequencing and RIA (S.L., J. H. Nielsen, B. Hansen, and B. S. Welinder, unpublished results).

Insulin RIA. Insulin was assayed by using guinea pig anti-insulin serum (GP12), with monoiodinated porcine insulin as tracer (14) and rat insulin (NOVO Industries, Bagsvaerd, Denmark) as a standard. Bound and free insulin were separated by using ethanol as described (15). The inter- and intraassay coefficients of variation between duplicate samples were <10%.

Insulin Secretion: Perfusion. The column perfusion system has been described (16). Cells at 30–50% confluency were cultured in RPMI 1640 containing either 0.5 or 5 mM glucose for 16 hr prior to perfusion. Preincubation in 0.5 mM glucose resulted in 2- to 3-fold higher insulin peaks and was therefore used in all the following experiments. Cells were detached from the plate by a rubber policeman, mixed with Bio-Gel P-2 (200–400 mesh, Bio-Rad), placed in the columns, and perfused at 0.25 ml/min with KRB medium supplemented with different concentrations of glucose. Fractions were collected over 5- to 10-min periods and analyzed by an insulin RIA.

Insulin Secretion and Insulin Content: Static Incubation. Cells were passaged in 12-well plates 4–7 days prior to each experiment and allowed to reach 30–40% confluency. The cells were then washed twice in medium (RPMI 1640 or DMEM) containing dialyzed serum and 0.5 mM glucose and cultured for 16 hr. The medium was replaced with fresh medium of the same type containing 5 mM glucose. Samples were collected 2, 4, and 12 hr later, centrifuged briefly, diluted 1:10 in NaFAM buffer [40 mM sodium phosphate, pH 7.4/0.1 M NaCl/bovine serum albumin (5 mg/ml)], and stored at -20°C . The cells were detached with a rubber policeman, centrifuged, homogenized in a small volume of H_2O by sonication, and then analyzed for DNA and insulin. Samples for insulin analysis were extracted with 3 M acetic acid, followed by freeze-drying and solubilization in NaFAM buffer. DNA was determined fluorimetrically as described (17).

Immunohistochemistry. Plates of βTC cells were washed in phosphate-buffered saline, fixed for 10 min at -20°C with ice-cold acetone/methanol, 1:1 (vol/vol), and immunostained essentially as described (10). Antibodies were used at the following dilutions: guinea pig anti-insulin (Linco, Eureka, MO), 1:100; rabbit-anti-T antigen (10), 1:5000; horseradish peroxidase-conjugated goat anti-guinea-pig IgG and goat anti-rabbit IgG (Accurate Chemicals, Westbury, NY), 1:200.

RNA Analysis. Isolation of RNA and Northern blotting analysis have been described (10).

Electron Microscopy. Cells were trypsinized, fixed in 2% glutaraldehyde, postfixed in 2% osmium tetroxide, solidified in 2% agar, dehydrated, and embedded in PolyBed 812 (Polysciences, Warrington, PA). Thin sections (100 nm) were poststained with uranyl acetate and lead citrate. For immunogold staining, cells were fixed in 0.5% glutaraldehyde and washed with sodium borohydride at 0.5 mg/ml. The cells were pelleted in 2% agar, dehydrated, embedded, and sectioned. Grids were incubated with 10% H_2O_2 followed by 10% bovine serum albumin for 1 hr each. They were then incubated with guinea pig anti-insulin antibody at a dilution of 1:350 for 1 hr, washed, incubated with protein A-gold particles [E-Y Laboratories (San Mateo, CA), 15 nm], diluted 1:20, poststained, and analyzed by EM.

RESULTS

Adaptation of Beta Tumor Cells to Growth in Culture. Cells were initially dispersed from a tumor and plated at a relatively high density in medium containing high serum concentrations. These conditions have been found to be important for the cells to attach to the plate and start dividing. In addition,

the combination of mechanical release of tumor cells from carefully cleaned, encapsulated tumors with their subsequent culture at high density in high concentrations of serum has been found to reduce contamination by fibroblasts and other nontransformed cells. The characterization of βTC lines in this report was performed with nonclonal cell cultures, of which the best characterized thus far is βTC1 , which was derived from a mouse in the RIP-Tag4 lineage. The cells grow in clusters, which are relatively flat and well-attached initially (Fig. 1) but tend to round and loosen their attachment to the plate as cell mass in the cluster increases. After the first few passages, the doubling time for the βTC1 line stabilized at ≈ 58 hr. The cells can be grown to about 50% confluency, after which increased cell mortality is observed. In addition, the cells do not proliferate well below 10% confluency. Consequently, the cells cannot be easily cloned. βTC1 cells analyzed for tumorigenicity in nude and histocompatible B6D2F1/J mice after more than 30 passages have generated subcutaneous beta-cell tumors, although at a markedly decreased incidence when compared to cell suspensions prepared directly from tumors (data not shown).

The conditions developed during the establishment of the βTC1 line allow for reproducible derivation of new cell lines

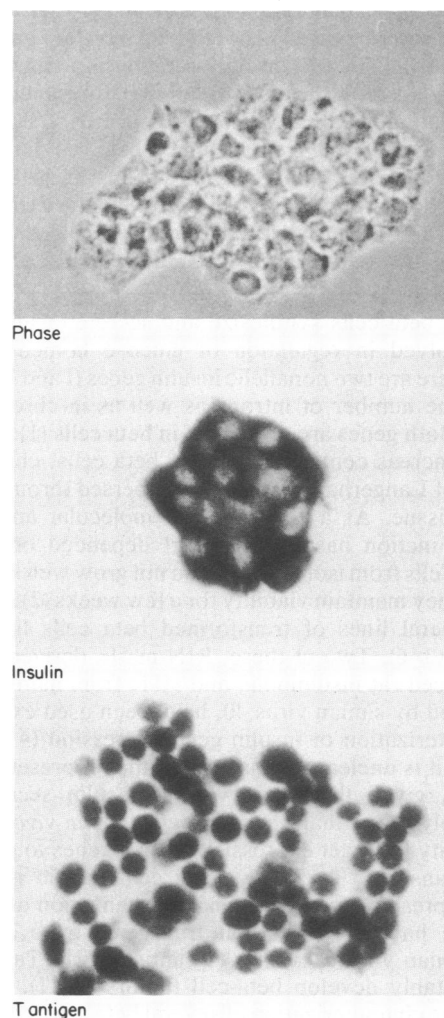


FIG. 1. Immunohistochemical analysis of insulin and large T antigen in βTC1 cells. Cells grown on tissue culture plates were photographed at passage 7 with a research microscope under phase-contrast (Top) or under bright-field illumination after immunohistochemical staining with antibodies directed against insulin (Middle) or large T antigen (Bottom). A different cluster of cells is shown in each panel. ($\times 450$.)

from primary tumors. To date, about 10 cell lines have been established, of which 3 are described in this report.

Immunohistochemical Analysis of β TC1 Cells. The expression of insulin and the insulin-promoted T-antigen transgene in β TC1 cells was analyzed by using immunohistochemical techniques. As shown in Fig. 1, all the cells express both the cell-specific marker (insulin) and the hybrid oncogene product (T antigen). This indicates that other cell types do not contaminate the cell population and therefore that the conditions of establishing the cell cultures do not select for sporadic activation of the hybrid oncogene in non-beta cells. Some decrease in the intensity of the signal for both proteins is observed after a large number of passages (>50). Two other cell lines derived from the insulin-T-antigen transgenic mice, denoted β TC2 and β TC3, show a similar pattern of immunostaining (data not shown). The β TC2 line is derived from a tumor that developed in a mouse of the RIR-Tag2 lineage (10). The β TC3 line originated from a tumor that arose in a mouse from a third independent lineage (RIP-Tag2).

A weak glucagon immunoreactivity appears in all the cells of these three lines after a few passages in culture (data not shown). Moreover, they secrete glucagon, which amounts to 1% (molar ratio) of the insulin secretion (J. Habener, personal communication).

Analysis of Insulin Biosynthesis in β TC1 Cells. Synthesis and processing of insulin I and II in β TC1 cells has been compared to that of normal mouse islets by using reversed-phase HPLC analysis. This method allows the separation of proinsulins, C-peptides, and mature insulins I and II. The ratio between newly synthesized proinsulin I and II was \approx 1:2. However, the ratio between I and II was reversed for both newly synthesized mature insulins and C-peptides, which indicates a slower conversion of proinsulin II (Fig. 2). Analysis of normal mouse islets (Fig. 2 *Inset*) and transgenic

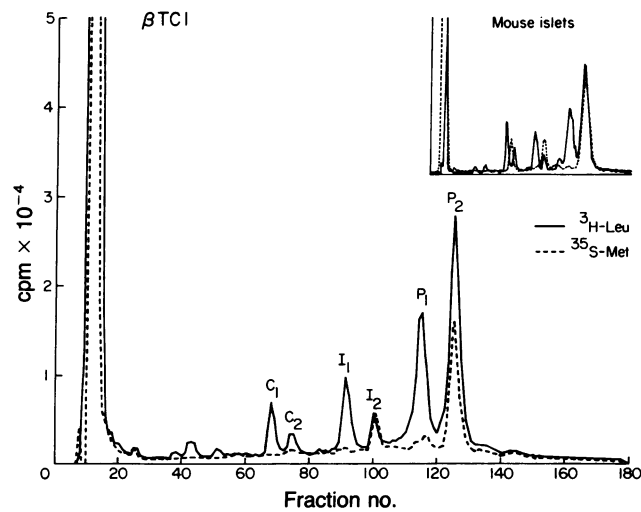


FIG. 2. Biosynthesis and conversion of insulin in β TC1 cells. Cells between passages 39 and 44 were pulse-chase labeled (30-min pulse, 30-min chase) with [35 S]methionine and [3 H]leucine in KRB medium containing 25 mM glucose, and cell homogenates were fractionated by reversed-phase HPLC. The fractions were analyzed for 3 H and 35 S radioactivity. The peaks are labeled as follows: C₁, C-peptide I; C₂, C-peptide II; I₁, insulin I; I₂, insulin II; P₁, proinsulin I; P₂, proinsulin II. Positive identification of the peaks was based both upon amino acid sequencing and RIA. Insulins I and II have identical numbers of leucine residues. Only proinsulin II and insulin II contain methionine. Comparable results were obtained with cells labeled in either RPMI 1640 medium with 11 mM glucose or KRB medium containing 0.5 mM glucose. (*Inset*) Analyses of normal mouse islets. Islets have a major unidentified peptide (containing methionine but no leucine) that migrates between C-peptide I and C-peptide II. This peptide is not present in β TC1 cells.

islets and tumors (data not shown) revealed the same conversion rate and relative proportions between newly synthesized proinsulins, mature insulins, and C-peptides I and II as those in β TC1 cells.

The HPLC fractions were also analyzed for absorption at 210 nm, to assess the relative amounts of peptides stored in the cells (data not shown). In this analysis, only the mature insulin I and II could be detected. The proinsulins were not detected, which indicates that the majority of insulin stored in the cells is present in the mature form. The failure to detect stored C-peptides is consistent with previous studies (ref. 18; S.L., J. H. Nielsen, B. Hansen, and B. S. Welinder, unpublished results). The ratio between stored insulins I and II was about 1:2, which is similar to that of the newly synthesized proinsulins and comparable to the pattern of stored insulin observed in normal mouse islets. Therefore, it appears that β TC1 cells have a normal pattern of insulin biosynthesis, conversion, and storage.

Glucose Induction of Insulin Secretion from β TC1 Cells. The short-term response of isolated islets to an increase in glucose levels in the culture medium is the release from the cells of insulin stored in secretory granules. To evaluate the response of β TC1 cells to glucose, the cells were mixed with a gel matrix and placed in a column for perfusion analyses. The cells were first perfused for 40 min without glucose to establish a basal level of insulin release (\approx 0.2 microunits per μ g of DNA per min). Stimulation by 5 mM glucose resulted in a 27.5 (\pm 3.7)-fold (mean \pm SD) increase in insulin release in the first fraction sampled over a 5-min period (Fig. 3). Insulin secretion gradually decreased thereafter. Perfusion experiments at 1.25, 2.5, and 3.75 mM glucose showed that a maximum stimulation of insulin secretion was reached at 1.25 mM glucose. The extent of induction and the secretion profile closely resemble those of cultured normal mouse islets (16). However, the latter require 15 mM glucose for optimal

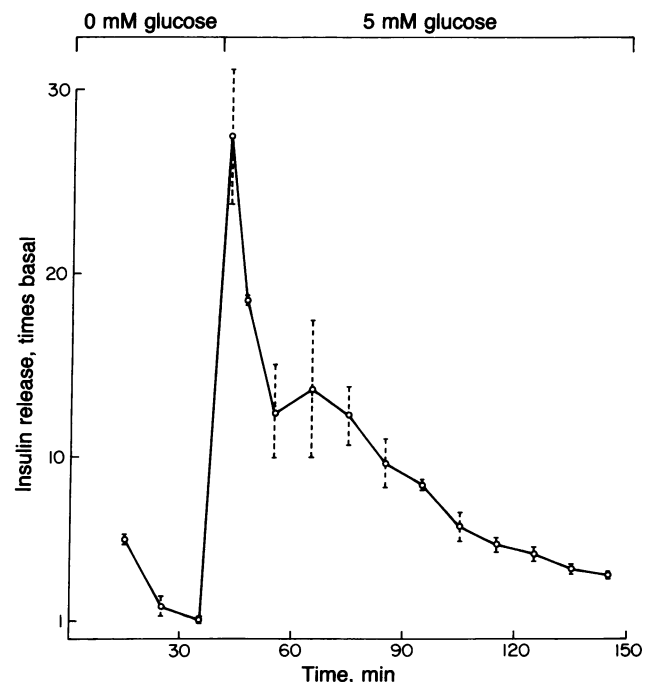


FIG. 3. Induction of insulin release from β TC1 cells by glucose. Cells between passages 42 and 50 were cultured for 16 hr in RPMI 1640 medium containing 0.5 mM glucose and then perfused in a gel matrix with KRB medium containing the indicated glucose concentrations. Fractions (5 and 10 min) were collected for insulin RIA. The data shown are the mean \pm SD of three experiments.

stimulation (2, 19), which is at least 12-fold higher than the levels required by β TC1 cells.

Insulin Release and Insulin Content of β TC Cells in Static Incubations. Table 1 shows the insulin release per day (24 hr) and insulin content after culture in 5 mM glucose. The levels of secreted insulin differ little between DMEM and RPMI 1640. However, insulin content is somewhat higher in the latter. Glucose concentrations up to 25 mM induce similar levels of both secreted and stored insulin (data not shown).

The analyses indicate that insulin protein synthesis and secretion vary as a result of both passage number and the tumor of origin. For example, a decrease in insulin secretion by a factor of 10 was observed in β TC1 between passages 50 and 63. Moreover, β TC2 cells secrete 6 times less insulin than β TC3 at similar passage numbers. Yet the steady-state levels of insulin mRNA are similar in all three β TC lines at the passages analyzed in Table 1 (data not shown). The highest figures obtained for insulin secretion and content (with β TC3 cells) are still several times lower than those of beta cells in cultured normal mouse islets, which release 420 milliunits per 10^6 cells per day and contain 225 milliunits per 10^6 cells at optimal glucose stimulation (15 mM) (19).

Analysis of Insulin and T-Antigen mRNAs in β TC1 Cells. The abundance of insulin and T-antigen mRNAs in β TC1 cells is very similar to that observed in the tumor tissue obtained directly from transgenic mice (Fig. 4), which indicates that growth in culture has not affected transcription of either gene. The levels of these transcripts do not change appreciably between 5 and 25 mM glucose (Fig. 4) and remain the same even following a 2-day incubation at 0.5 mM glucose (data not shown).

β TC1 cells contain much higher steady-state levels of insulin mRNA than either HIT or RIN cells, as shown in Fig. 4. This corresponds to the higher amounts of insulin secreted from β TC1 cells compared to HIT cells (Table 1). These results could be explained either by increased transcription of the insulin genes or by greater stability of insulin mRNA in β TC1 cells.

Electron Microscopic Analysis of β TC Cells. β TC cells were subjected to electron microscopic analysis to compare their ultrastructure to that of normal beta cells (Fig. 5). In pancreatic tissue, beta cells are characterized by a high density of insulin secretory granules (20), in which the processing of proinsulin takes place. Analysis of transformed beta cells in these transgenic mice (21) has shown that *in vivo* the tumor cells have a characteristic beta-cell morphology, with a normal number and distribution of insulin granules. In β TC cells,

Table 1. Insulin secretion and content in β TC lines

Cell line	Passage no.	Medium	Insulin secretion, milliunits per 10^6 cells per day	Insulin content, milliunits per 10^6 cells
β TC1	50	DMEM	43.7 ± 7.9	4.0 ± 0.6 (9)
	63	DMEM	5.2 ± 0.5	4.1 ± 1.0 (79)
	63	RPMI	4.0 ± 0.0	7.5 ± 0.7 (187)
β TC2	15	DMEM	9.6 ± 0.0	16.9 ± 1.1 (176)
	15	RPMI	9.4 ± 0.3	26.2 ± 0.6 (279)
β TC3	10	DMEM	53.4 ± 1.4	15.8 ± 3.0 (30)
	10	RPMI	57.6 ± 5.2	46.7 ± 3.9 (81)
HIT	?	DMEM	0.2 ± 0.0	<0.04
	?	RPMI	0.2 ± 0.1	<0.04

Cells were transferred from medium containing 0.5 mM glucose to medium with 5 mM glucose. Two, 4, and 12 hr later aliquots of the medium were collected and assayed for insulin. Following the 12-hr incubation, cells were assayed for both insulin and DNA content. Mean values \pm SD ($n = 6$ for secretion and $n = 2$ for content) are shown. Cell number is calculated assuming 1 cell = 7 pg of DNA. Numbers in parentheses represent insulin content as a percent of insulin secretion. The precise passage number of the HIT cells is unknown.

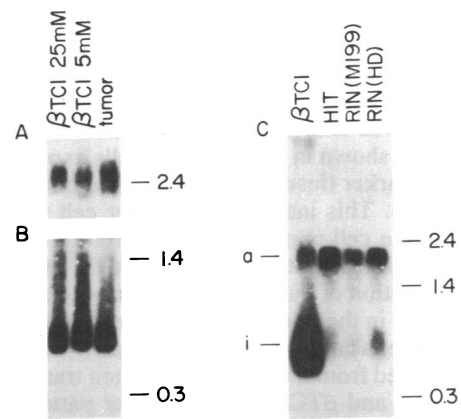


FIG. 4. Northern blot analysis of RNA from β TC1 cells. RNA was extracted from the following: β TC1 cells at passage 31 grown in DMEM containing 25 mM glucose or after 48 hr in DMEM containing 5 mM glucose, tumor tissue obtained from a RIP-Tag mouse, HIT cells, and RIN cells grown either in medium 199 (M199) or in hormonally defined (HD) medium. Twenty micrograms of total RNA was separated on a 1.1% formaldehyde/agarose gel, transferred to nitrocellulose, and hybridized with nick-translated simian virus 40 DNA (A), mouse insulin I probe (B), or with both the insulin (i) probe and a chicken β -actin (a) plasmid (C). The actin band serves to control for relative RNA levels and quality. RNA size markers are indicated in kilobases.

typical insulin granules are observed (Fig. 5). Most of them are of the mature type, characterized by a dense core, although a few immature granules, appearing uniformly pale, are also observed. The number of granules is low, when compared to normal and transformed beta cells in the pancreas (21). Immunoelectron microscopy was used to demonstrate that the observed granules actually contain insulin. Cell sections incubated with anti-insulin serum and labeled with protein A-colloidal gold show specific immunoreactivity in the granules (Fig. 6), which confirms their insulin content.

DISCUSSION

The results presented here demonstrate that the β TC lines possess many characteristics of differentiated beta cells in normal mice. The cells synthesize and secrete considerable

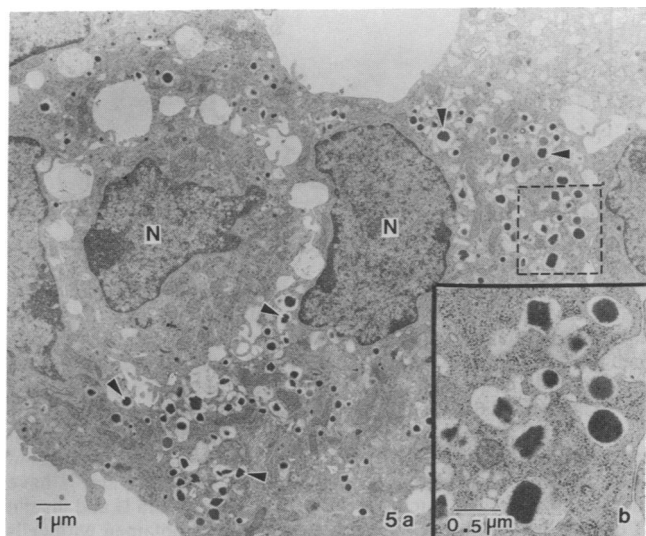


FIG. 5. Electron microscopic analysis of β TC3 cells. (a) Cells at passage 12 were cultured overnight in DMEM containing 0.5 mM glucose. Arrows indicate insulin secretory granules. N, nucleus. (b) Magnification of the area in a marked by dashed lines.

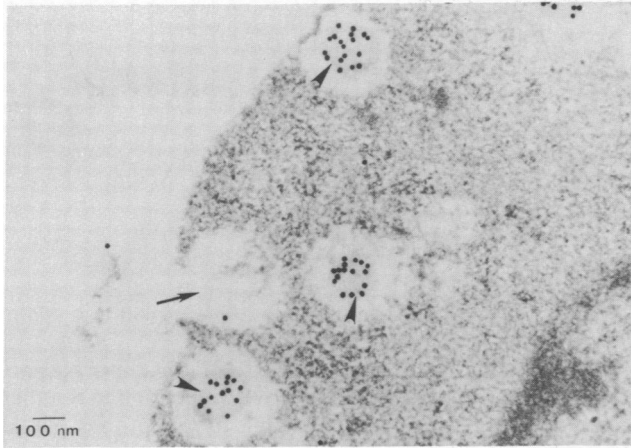


FIG. 6. Immunoelectron microscopy of β TC1 cells. Cells at passage 36 were cultured for 48 hr in DMEM containing 5 mM glucose. They were then incubated with anti-insulin serum, followed by protein A-gold particles. Specific signal is observed over insulin granules (arrowheads). One insulin granule appears to have secreted its contents (arrow). Control sections incubated with phosphate-buffered saline instead of the primary antibody showed no reactivity.

amounts of insulin, as measured by RIA and by insulin mRNA analysis. The secretion of insulin is regulated by glucose, as revealed in column perfusion experiments. Induction levels of up to 30-fold were obtained within minutes after shifting the cells from no glucose to 5 mM glucose. β TC1 cells are sensitive to much lower levels of glucose, when compared to normal beta cells. They secrete appreciable amounts of insulin at 0.5 mM and reach maximal stimulation at 1.25 mM, in contrast to the 15-mM level required for maximal stimulation of secretion from normal mouse beta cells in intact cultured islets. This difference may result from the presence of other cell types in intact islets, which normally influence insulin secretion by beta cells. Alternatively, the continuous proliferation may constrain the cells to metabolize high levels of glucose and thereby abrogate their normal sensitivity and response to glucose. The steady-state levels of insulin mRNA are not affected by glucose concentrations between 0.5 and 25 mM. Although lower levels (<0.5 mM) might reveal a potential regulation of insulin biosynthesis by glucose, such levels are incompatible with viability of β TC cells.

β TC cells produce low amounts of glucagon, as judged by immunohistochemical and biochemical analysis, in contrast to the tumors *in vivo* (9). In the case of RIN cells, the initial radiation-induced insulinoma has given rise to clones producing insulin, glucagon, and somatostatin in various combinations (22, 23). The appearance of glucagon immunoreactivity in β TC cells indicates that they have undergone a change in gene regulation during propagation *in vitro*, which might be considered dedifferentiation, given that insulin and glucagon are normally coexpressed during islet cell development (24).

The features of β TC cells are likely to render them useful for studies on regulation of the insulin genes and on beta-cell physiology, for the isolation of proteins that control insulin gene expression, and perhaps for characterization of beta-cell molecules involved in the autoimmune response in diabetes. In contrast to the other insulinoma cell lines, the availability of lineages of transgenic mice that heritably develop beta-cell tumors allows for repeated derivation of cell lines from the primary tumors. Thus adaptation and deviation from the observed beta-cell phenotype can be regularly cross-checked *in vivo*, and new cell lines can be established if the old lines become anaplastic.

These results demonstrate that targeted expression of an oncogene in transgenic mice, by using a cell-specific regulatory element, can be applied for immortalization and establishment in cell culture of a rare cell type. The use of cell-specific control regions not only provides access to dispersed, rare cells but also provides a selection for the differentiated phenotype of those cells in culture. Thus, the hybrid oncogene represents a type of feedback loop that on the one hand induces continuous cell proliferation and on the other requires that high level expression of a cell-specific gene be maintained.

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